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Long-term survival of type XVII collagen revertant cells in an animal model of revertant cell therapy

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Sociedad Española
Terapia Génica y Celular

**The European Society for Gene and Cell Therapy
and the Spanish Society for Gene and Cell Therapy
Collaborative Congress 2013**

Invited Speakers

Inv001

Bases of gene therapy in leukemias

C. Bonini

Experimental Hematology Unit, Division of Regenerative Medicine, Gene Therapy and Stem Cells, Program of Immunology, Gene Therapy and Bio-Immunotherapy of Cancer, Leukemia Unit, San Raffaele Scientific Institute, Milan, Italy

Hematopoietic stem cell transplantation from a healthy donor (allo-HSCT) represents the most potent form of cellular adoptive immunotherapy to treat leukemias. During the past decades, allo-HSCT has developed from being an experimental therapy offered to patients with end-stage leukemia into a well-established therapeutic option for patients affected by several hematological malignancies. In allo-HSCT, donor T cells are double edge-swords, highly potent against residual tumor cells, but potentially highly toxic, and responsible of the graft versus host disease (GVHD), a major clinical complication of transplantation. Gene transfer technologies can improve the safety (ie: use of suicide genes), and the efficacy (ie: TCR gene transfer, TCR gene editing, CAR gene transfer) of adoptive T-cell therapy in the context of allo-HSCT. The encouraging preclinical and clinical results obtained in these years with genetically engineered T lymphocytes in the treatment of leukemias will be discussed.

Inv002

Recent developments in gene therapy of solid tumors

R. Hernandez

Division of Gene Therapy and Hepatology, Universidad de Navarra, Madrid, Spain

Treatment of cancer has been one of the earliest and most frequent applications of gene therapy in experimental medicine. However, this indication entails unique difficulties, especially in the case of solid tumors. Pioneering strategies were aimed to reverse the malignant phenotype or to induce the death of cancer cells by transferring tumor-suppressor genes, inhibiting oncogenes or selectively expressing toxic genes. Proof of principle has been generated in abundant pre-clinical models and in humans. However, clinical efficacy is hampered by the difficulty in delivering therapeutic genes to a significant proportion of cancer cells in solid tumors using the currently available vectors. Therefore, current work aims to extend the effect to non-transduced cancer cells. This can be achieved by local or systemic expression of secreted proteins with the ability to block key pathways involved in angiogenesis, cell proliferation and invasion. Recent advances in gene therapy vectors allow sustained expression of transgenes and make these strategies feasible in the clinic. Another attractive option is the stimulation of immune reactions against cancer cells using gene transfer.

In this case the therapeutic genes are antigens, cytokines or proteins capable of blocking the immunosuppressive micro-environment of tumors. Adaptation of replication-competent (oncolytic) viruses as vectors for these genes combines the intrinsic immunogenicity of viruses, their capacity to amplify gene expression and their direct lytic effect on cancer cells. In general, the “immunogene therapy” strategies offer the opportunity to destroy primary and distant lesions, especially if they are combined with other treatments that reduce tumor burden. More importantly, vaccination against cancer cells could prevent cancer relapse. Finally, gene and cell therapies are joining forces to improve the efficacy of adoptive cell therapy. *Ex vivo* gene transfer of natural or chimeric tumor-specific receptors in T lymphocytes enhances the cytotoxic potency of the cells and is expanding the applicability of this promising approach to different tumor types.

Inv003

Neural Stem Cells

J. Canals

Inv004

Hepatic progenitor cells in liver regeneration

S.J. Forbes

MRC Centre for Regenerative Medicine, The University of Edinburgh, Edinburgh, Scotland, UK

Following acute liver injury or partial hepatectomy the liver rapidly regenerates through the proliferation of normally quiescent hepatocytes. However, during chronic injury hepatocyte regeneration becomes impaired and a population of bipotential hepatic progenitor cells (HPCs) become activated to regenerate both cholangiocytes and hepatocytes, although their functional significance is not well defined. We have analysed their function in an inducible genetic model of liver injury: p450 dependent MDM2 deletion from differentiated hepatocytes which induces hepatocyte senescence and death. This results in massive HPC activation; these HPCs are genetically normal, bipotential, repopulate the liver and are transplantable making them a suitable therapeutic target. Understand the controls of HPC mediated regeneration are therefore important. In human diseased liver and mouse models Notch and Wnt pathways are activated in biliary and hepatocellular injury respectively. Macrophages activate HPCs in a TWEAK/fn14 dependent manner and promote their hepatocyte differentiation. When macrophages phagocytose hepatocyte debris they upregulate Wnt secretion at the HPC niche. In models of chronic liver injury macrophage injections result in hepatic chemokine up-regulation, recruitment of endogenous macrophages, increased MMP –13

and α 9 expression in the liver, reducing fibrosis and improving liver function. We are therefore developing autologous macrophage therapy in man for the treatment of liver cirrhosis using apheresis of peripheral monocytes from patients with cirrhosis as a source of cells that are differentiated *ex vivo* into "regenerative macrophages".

Inv005

Planning an academic trial - the sponsor's perspective

K.M. Champion

University College, London, UK

By sponsoring a Clinical Trial (CT) the sponsor becomes responsible for ensuring the trial complies with national legislation and Good Clinical Practice (GCP). This requires implementation of an effective quality system to provide oversight of the sponsorship functions and ensures arrangements are in place for adequate management, monitoring and reporting to required standards. Although any or all of the sponsor's functions may be formally delegated to an individual, a company or an institution, the sponsor ultimately remains responsible. A risk assessment should be undertaken at the protocol development stage to help inform the sponsor's decision whether to proceed or not with sponsorship and to help identify and mitigate against potential risks associated with the CT. Potential risks to be considered include: knowledge and experience with the investigational medicinal product (IMP) in the target population, risks from other interventions in the trial and the financial and legal risks to the sponsor. The design of early-phase CTs of cell and gene therapy products often involves aspects of clinical safety issues, manufacturing, product characteristics, complex administration procedures and/or logistical issues that are encountered less commonly or not at all in the development of other pharmaceuticals. A feasibility assessment must take place to ensure the CT only takes place in appropriate clinical facilities and is conducted by investigators and support staff who are suitably trained and have the necessary expertise and experience to perform their delegated responsibilities. The safety of the subject participating in the trial is the paramount consideration. There should be a specific plan to monitor and manage anticipated or unexpected adverse events and adverse reactions. The protocol should describe procedures and responsibilities for safety reporting and for modifying or stopping the trial, if necessary. The duration of monitoring for adverse events is generally calculated to cover the time during which the product is reasonably thought to present safety concerns. All sponsors, whether academic or commercial, work under the same legal framework. However, CTs with Advanced Therapies, as defined in Regulation 1394/2007, must consider also the additional regulatory requirements and GCP guidelines supplementing those set out in Directives 2001/20/EC and 2005/28/EC.

Inv006

Production of vector and genetically modified stem cells

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Hematopoietic gene therapy is currently used to treat a variety of genetic disorders of the blood and immune systems, or

metabolic diseases, with promising results. The approach currently relies on the infusion of patient-autologous hematopoietic stem cells that have been subjected to gene-transfer *ex vivo* with a viral vector of clinical grade, during a short period of culture. The manufacture of such advanced therapy medicinal products for clinical trials should comply with the clinical trials EC directive. Requirements for gene and cell-based medicinal products both apply, therefore a high level of complexity is involved in the development of such products. Hematopoietic cell and gene therapy has many potential indications based on encouraging preclinical and early-phase clinical results. However, somatic cell and gene therapy medicinal products are still in early phases of development and no such product has been registered yet. The standardization of the manufacturing process and characterization of the drug product (i.e. genetically-modified cells) are important but present challenges. Many aspects, and in particular limited available patient material, complicate a precise characterization of the drug product. On the other hand, clinical-grade gene transfer retroviral vectors are well-characterized starting materials that are described in a pharmacopeia monograph and can be robustly manufactured in successive campaigns of production under GMP conditions. Examples obtained in preclinical and ongoing clinical studies to treat Wiskott Aldrich Syndrome illustrate the vast differences in the level of characterization between the viral vector starting material and the drug product used in hematopoietic gene therapy. Characterization of the products and standardization/validation of the manufacturing process are the next challenges in the field.

Inv007

The role of the QP in assessing ATMPs

E. Berrie

Clinical BioManufacturing Facility, University of Oxford, Oxford, UK

In this presentation, I will briefly look at the requirements for QP certification of investigational medicinal products, as referred to in Directive 2003/94/EC, i.e. GMP Certification / Statement of EU GMP Compliance and QP batch certification, i.e. the Trial Specific Certification as referred to in Art. 13(3) of Directive 2001/20/EC. I will be making specific reference to Advanced Therapy Medicinal Products (ATMPs).

I will discuss the specifics of manufacturing ATMPs, starting materials, the Product Specification File, written manufacturing instructions, labelling and certification. I will briefly look at QP importation of IMPs manufactured outside of the EU and what a QP looks for before completing the QP Declaration and subsequent QP Certification.

Inv008

AAV gene therapy for haemophilia

A. Nathwani

Inv009

Lentiviral vector GT for beta-thalassemia

G. Ferrari

Ino010**Gammaretro and lentiviral vectors for the gene therapy of X-linked chronic Granulomatous disease**

M. Grez

Institute for Biomedical Research, Georg-Speyer-Haus, Frankfurt, Germany

Gene therapy of inherited diseases has provided convincing evidence of therapeutic benefits for many treated patients. In particular, treatment of primary severe congenital immunodeficiencies by gene transfer into hematopoietic stem cells (HSCs) has proven in some cases to be as beneficial as allogeneic stem cell transplantation, the treatment of choice for these diseases if HLA-matched donors are available. We conducted a Phase I clinical trial aimed at the correction of X-CGD, a rare inherited immunodeficiency characterized by severe and life threatening bacterial and fungal infections as well as widespread tissue granuloma formation. Phagocytic cells of CGD patients fail to kill ingested microbes due to a defect in the nicotinamide dinucleotide phosphate (NADPH) oxidase complex resulting in compromised antimicrobial activity. In this clinical trial we used a gammaretroviral vector with strong enhancer-promoter sequences in the long terminal repeats (LTRs) to genetically modify CD34⁺ cells in two X-CGD patients. After successful reconstitution of phagocytic functions, both patients experienced a clonal outgrowth of gene marked cells caused by vector-mediated insertional activation of proto-oncogenes leading to the development of myeloid malignancies. Moreover, functional correction of gene transduced cells decreased with time, due to epigenetic inactivation of the vector promoter within the LTR, resulting in the accumulation of non-functional gene transduced cells. The understanding of the molecular basis of insertional mutagenesis has motivated the development of advanced integrating vectors with equal therapeutic potency but reduced genotoxicity. In particular, the deletion of the enhancer elements within the viral LTR U3 regions has significantly contributed to the reduction of genotoxic effects associated with LTR-driven gammaretroviral vectors. Moreover, the use of tissue specific promoters, which are inactive in stem/progenitor cells but active in terminally differentiated cells, should further increase the safety level of SIN vectors. Based on the aforementioned advancements, we developed SIN gammaretroviral and lentiviral vectors for the safe and effective gene therapy of X-linked CGD. We combined the SIN configuration with an internal promoter, with preferential expression in myeloid cells. However, the introduction of a new vector into the clinic demands a series of sophisticated pre-clinical studies, which are quite challenging in particular within an academic environment. In this presentation we will report on the comprehensive and thorough preclinical efficacy and safety testing of both SIN vectors assessing dosage requirements, therapeutic efficacy, resistance to transgene silencing and genotoxic potential.

Ino011**Translational research in the *ex vivo* gene therapy of monogenic diseases**

B. Gaspar

Professor of Paediatrics and Immunology, Centre for Immunodeficiency, UCL Institute of Child Health/Great Ormond Street NHS Trust, London, UK

Primary immunodeficiencies have played a major role in the development of gene therapy for monogenic diseases of the bone marrow. The last decade has seen convincing evidence of long term disease correction as a result of *ex vivo* viral vector mediated gene transfer into autologous haematopoietic stem cells. The success of these early studies has been balanced by the development of vector related insertional mutagenic events. More recently the use of alternative vector designs with self inactivating (SIN) designs which have an improved safety profile has led to the initiation of a wave of new studies which are showing early signs of efficacy. These studies in SCID-X1, ADA-SCID, Wiskott-Aldrich syndrome and Chronic Granulomatous disease are all multi-centre studies using lentiviral vectors and have the potential to recruit patients rapidly and to show efficacy and safety. The ongoing development of safer vector platforms and gene editing technologies together with improvements in cell transduction techniques and optimised conditioning regimes is likely to make gene therapy amenable for a greater number of PIDs. If long term efficacy and safety are shown, gene therapy will become a standard treatment option for specific forms of PID. These technologies may also be important for other monogenic disorders of the haematopoietic system including metabolic diseases and haemoglobinopathies.

Ino012**Progress and challenges of *in vivo* gene transfer with AAV vectors**F. Mingozzi^{1,2}*¹Genethon, Evry, France; ²University Pierre and Marie Curie, Paris, France*

In vivo gene replacement for the treatment of an inherited disease is one of the most compelling concepts in modern medicine. Adeno-associated virus (AAV) vectors have been extensively used for this purpose and have shown therapeutic efficacy in a range of animal models. The translation of preclinical results to the clinic was initially slow, but early studies in humans helped defining the roadblocks to successful therapeutic gene transfer *in vivo*, which are highly depending on the target tissue, the route of vector delivery, and the specific disease. The development of strategies to overcome these limitations allowed achieving long-term expression of donated genes at therapeutic levels in patients with inherited retinal disorders, hemophilia B and other diseases. The recent market approval of Glybera, an AAV vector-based gene therapy product for lipoprotein lipase deficiency, further confirmed the potential of AAV vectors as a therapeutic platform, raising hopes for the development of *in vivo* gene transfer treatments for many additional inherited and acquired diseases.

Ino013**Clinical trials with mesenchymal stem cells in wound healing**

D. García-Olmo

Professor of Surgery, Director of Cell Therapy Unit and Head of Colorectal Surgery Unit, La Paz University Hospital, Universidad Autónoma de Madrid, Spain

Mesenchymal stem cell (MSC) research has developed rapidly during the last decade and the promising results obtained from *in vitro* and *in vivo* studies have generated growing optimism. Although bone marrow is the most often used source, MSCs with

similar biological properties have also been isolated from other tissues including adipose tissue, skeletal muscle and cord blood. Of special interest is adipose tissue since it represents an abundant and accessible source of MSCs. These cells are denominated adipose-derived stem cells (ASCs) and have been widely studied since they were first described in 2001. In recent years, substantial knowledge of ASCs interaction with the immune system has been acquired. The mechanisms underlying the immunosuppressive effects of ASCs have not been clearly defined but it seems that ASCs modulate the function of different cells involved in the immune response. Recent studies address the potential benefit of application of MSCs in systemic acute inflammatory response in septic shock. However, little is known of the impact these cells on the local acute inflammation. In this first line of defence, the immune response is nonspecific and characterized by changes in microcirculation: exudation of fluid and migration of leukocytes from blood vessels to the area of injury. Neutrophils are the main effectors and the most predominant cell type during the first 24 hours. Macrophages migrate to the damaged tissues after the arrival of neutrophils and their number slowly increases facilitating the elimination of dead material after 2–3 days of neutrophil activity. Other cell types (lymphocytes, plasma cells, etc) are also involved in the initial inflammatory reaction and, together with the increasing number of macrophages, represent the most prominent cells in chronic inflammation. The acute inflammatory reaction is a double-edged sword. It plays a key role in the initial host defence response, particularly against many infections, but its aim is imprecise and, as a consequence, when it is drawn into battle, it can cause collateral damage in tissues and impair wound healing. Healing is the basis of Surgery and then, we hypothesized that some of the above mentioned properties of ASCs could be involved in the earlier stages of the immune response and modulate the local acute inflammation and hence could improve healing. In order to probe this hypothesis we used an animal model of colon anastomosis, and compared the application of biosutures (sutures enriched with ASCs) with conventional sutures. Also we have performed several experiments in Colorectal Diseases. Colorectal tracts are not a sterile medium and are exposed to external environment aggressions. As a result its diseases are complex and encompass a broad spectrum of pathogenic mechanisms and their treatment is often challenging and frequently incurable or chronicity-tending processes are found. In this scenario, stem cells with their potential and encouraging properties could be helpful. Nowadays stem cells have yielded promising results in preclinical studies, some approaches have started clinical development and registered clinical trials are exponentially increasing every year from 2001. In 2013, the most advanced programmes, involving Crohn's disease and fistulous disease, have reached phase III of development. Other tested diseases include ulcerative colitis or faecal incontinence. If the preliminary results are confirmed, we think that this therapy may become a clinical reality for surgeons in the near future.

Inv014**Ethic issues in cell and gene therapy**

O. Cohen-Haguenauer

Inv015**EU regulations for ATMP and clinical trials**

L. D'Apote

Inv016**Ensuring GCP compliance, patient safety and data integrity**

K.M. Champion

University College London, UK

Clinical trials with investigational medicinal products must be conducted to Good Clinical Practice (GCP) standards. GCP is a set of ethical and scientific quality standards for designing, conducting, recording and reporting clinical trials. Complying with these standards and its 13 core principles provides assurance that the rights, safety and wellbeing of trial subjects are protected and that the CT data are credible. The Commission Directives 2001/20/EC and 2005/28/EC provide the legal framework for GCP, which EU member states have implemented into national regulations and against which the National Competent Authorities perform inspections. Every party involved in a clinical trial has a responsibility to comply with GCP, including sponsors, investigators and site staff, contract research organisations, ethics committees and regulatory authorities. As such, each individual involved in a trial should be qualified by education, training and experience to perform their respective task(s). The sponsor's responsibilities include implementing quality systems and arrangements to ensure the trial and related documents and procedures follow and adhere to the principles of GCP. All clinical trial information should be recorded, handled, and stored in a way that allows its accurate reporting, interpretation and verification. To ensure data integrity investigator sites must ensure the data reported are complete, accurate and verifiable from source documents which have not been altered or falsified. In a clinical trial patient safety is always the priority. Pharmacovigilance, the collection, detection, assessment, monitoring and prevention of adverse effects with investigational medicinal products (IMP) is therefore of foremost importance. Identifying hazards associated with IMPs and minimising the risk of any harm that may come to trial subjects is an important principle of GCP and the backbone of optimising and promoting safe development and use of medicinal products.

Inv017**Glybera[®] approval: a road map for advanced therapies in the orphan space**

H. Petry

uniQure, Amsterdam, Netherlands

Glybera[®], is a gene therapy product based on the use of recombinant adeno-associated virus for gene delivery. It is designed for patients with Lipoprotein Lipase Deficiency (LPLD). On November 2, 2012, the European Commission approved the marketing authorisation for Glybera[®] as a treatment for LPLD, under exceptional circumstances, in all 27 EU member states. Glybera[®] is intended to treat patients with lipoprotein lipase deficiency. LPLD is caused by errors in the gene that codes for the protein lipoprotein lipase (LPL). LPL has a central role in fat metabolism. Non-functional LPL can lead to pancreatitis attacks, the most severe phenotype of this disease. The presentation will cover a summary of the clinical development, as well as a summary of the regulatory process. In addition post approval commitments will be discussed and their importance to follow up on the long term safety and efficacy of this gene therapy product.

Inv018**Phase Ib/IIa, escalating dose, single blind, clinical trial to assess the safety of the intravenous administration of expanded allogeneic adipose-derived mesenchymal stem cells (eASCs) to refractory rheumatoid arthritis (RA) patients**

L. Dorrego

Tigenix, Madrid, Spain

Advanced therapies are emerging and fast-growing biotechnology sector paves the way for new, highly promising treatment opportunities for European patients.

Tigenix is a leading European cell therapy company a marketed product for cartilage repair, and a strong pipeline with advanced clinical stage allogeneic adult stem cell programs for the treatment of autoimmune and inflammatory diseases.

Tigenix has developed an innovative trial design in the stem cell area for treating refractory rheumatoid arthritis (RA) using expanded allogeneic adipose-derived mesenchymal stem cells (eASCs). The multicenter, randomized, double blind, placebo-controlled Phase IIa trial enrolled 53 patients with active refractory rheumatoid arthritis (mean time since diagnosis 15 years), who failed to respond to at least two biologics (mean previous treatment with 3 or more disease-modifying antirheumatic drugs and 3 or more biologics). The study design was based on a three-cohort dose-escalating protocol. For both the low and medium dose regimens 20 patients received active treatment versus 3 patients on placebo; for the high dose regimen 6 patients received active treatment versus 1 on placebo. Patients were dosed at day 1, 8, and 15 and were followed up monthly over a six-month period. Follow-up consisted of a detailed monthly workup of all patients measuring all pre-defined parameters. The aim was to evaluate the safety, tolerability and optimal dosing over the full 6 months of the trial, as well as exploring therapeutic activity.

Twenty five Spanish sites participated in this clinical trial. Coordinating Investigator: Dr. José María Alvaro-Gracia

Inv019**Regulatory challenges in development of lentiviral *ex vivo* gene therapy products**

A-V. Eggimann

Bluebird Bio, Inc., Cambridge, United States

Developing autologous *ex vivo* gene therapy products in a GMP, GLP and GCP environment faces significant challenges. These challenges include the design of the appropriate toxicology studies, the scale up of manufacturing processes and having appropriately located drug product manufacturing facilities, and the conduct of clinical studies that must take into account GMP constraints and traceability requirements in addition to GCP. Additional hurdles to obtain clinical trial approval from the regulatory perspective come from the Genetically Modified Organisms (GMOs) specific regulations in the EU and the overall inadequacy of the CTD for gene therapy products. Expected changes in the EU regulatory landscape for gene therapy will be discussed briefly (revision of Directive 2001/20/EC, adaptive licensing pilot EMA program and how it could apply to gene therapy), as well as the anticipated value of certification procedures at EMA. Differences between the EU approach and the US will be discussed. Case studies will be used to illustrate the main points. Beyond the appeal of gene therapy's "cutting edge", there is a real need to understand the underlying science to drive the

development of these products. Close collaboration between scientists and development teams should be encouraged to increase the quality of these products and of their development ultimately leading to approvals - as seen recently in the EU. Regular and pro-active communication with the regulators is advisable and will be illustrated with examples, as well as recommendations for improving the regulatory paradigm for gene therapy products, including increasing regulatory interactions, creating customized application requirements, increasing harmonization within the EU and between the EU and the US, and clarifying expectations for approval.

Topics to be covered:

- Update ICH CTD with specific to gene therapy products information and guidance
- Description/definition of Drug Substance and Drug Product; Product Definition; EU vs US
- CMC: Harmonization of titer assays, development of new RCL assay suitable for Phase 3 and registration with attenuated control
- Harmonization of expectations for nonclinical toxicology studies; positive control, DART, biodistribution
- CTAs vs INDs; content and contrasts
- Pediatric Investigational Plan (PIP)
- New trends in regulatory that are likely to impact gene therapy products (in the EU pilot adoptive licensing program, revision of clinical trial Directive 2001/20/EC to shorten timelines to CTA approvals, in the US Breakthrough therapy designation and recent FDA nonclinical and clinical guidelines) – unclear impact of joint regulatory/health technology assessments meetings
- Recommendation to develop vector specific guidelines
- Increase intra-industry communication

Inv020**ATMP in the EU: the long and winding road**

S. Ruiz, M. Timón, P. de Felipe, S. Rojo, S. Moreno

Spanish Medicines Agency (AEMPS) Madrid, Spain

Advanced therapy medicinal products (ATMP) in the European Union (EU) include those based on gene therapy, somatic cell therapy and tissue engineering. European legislation requires a centralized marketing authorization procedure for ATMP (although exceptions are allowed). In accordance with the regulation, a new and multidisciplinary committee (Committee for Advanced Therapies, CAT) was established in January 2009 within the European Medicines Agency (EMA). The main responsibility of the CAT is to prepare a draft opinion on each ATMP application submitted to the EMA, before the Committee for Medicinal Products for Human Use (CHMP) adopts a final opinion. So far, four different ATMP have received approval for marketing authorization through the centralized procedure; three somatic cell therapy products: ChondroCelect, MACI, and Provenge and one gene therapy medicinal product, Glybera. Due to the peculiarities of these products, the evaluation process and final opinion has been controversial. Several other applications for ATMP have been presented but either received a negative opinion or were withdrawn by the applicant before a negative opinion by the CHMP was given. Some of these products were already on the market in some EU countries but quality and/or clinical studies were not considered to meet the current standards or provide enough reassurance for a positive benefit/risk ratio. Details on the evaluation process, challenges, and conclusions for the four approved ATMP will be provided during the presentation.

Inv021

J. Samulski

Inv022**Induction of multi-, pluri- and totipotency**

H.R. Schöler

Department Cell and Developmental Biology, Max Planck Institute for Molecular Biomedicine, Muenster, 48149, Germany

The pluripotent and multipotent states of stem cells are governed by the expression of few, specific transcription factors forming a highly interconnected regulatory network with more numerous, widely expressed transcription factors. When the set of master transcription factors comprising Oct4, Sox2, Klf4, and Myc is expressed ectopically in somatic cells, this network organizes itself to support a pluripotent cell state. But when Oct4 is replaced by Brn4, another POU transcription factor, fibroblasts are converted into multipotent neural stem cells. These two transcription factors appear to play distinct but interdependent roles in remodelling gene expression by influencing the local chromatin status during reprogramming. Furthermore, structural analysis of Oct4 bound to DNA shows that the Oct4 linker—a region connecting the two POU domains of Oct4—is exposed to the surface, and we therefore postulate that it recruits key epigenetic players onto Oct4 target genes during reprogramming. The role of Oct4 in defining totipotency and inducing pluripotency during embryonic development remains unclear, however. We genetically eliminated maternal Oct4 using a Cre/lox approach and found no effect on the establishment of totipotency, as shown by the generation of live pups. After complete inactivation of both maternal and zygotic Oct4 expression, the embryos still formed Oct4-GFP- and Nanog-expressing inner cell masses, albeit nonpluripotent, indicating that Oct4 is not a determinant for the pluripotent cell lineage separation. Interestingly, Oct4-deficient oocytes were able to reprogram fibroblasts into pluripotent cells. Our results indicate that, in contrast to its crucial role in the maintenance of pluripotency, maternal Oct4 is crucial for neither the establishment of totipotency in embryos, nor the induction of pluripotency in somatic cells using oocytes.

Inv023**Reprogramming *in vivo* is possible and generates a new type of iPSC**

M. Serrano

Spanish National Cancer Research Center (CNIO), Madrid, Spain

Reprogramming into induced pluripotent stem cells (iPSCs) has opened new therapeutic opportunities, however, little is known about the possibility of *in vivo* reprogramming within tissues. We have generated transgenic mice with inducible expression of the four Yamanaka factors. Interestingly, transitory induction of the reprogramming factors results in teratomas emerging from multiple organs, thereby, implying that full reprogramming can occur *in vivo*. Analyses of the stomach, intestine, pancreas and kidney reveal groups of dedifferentiated cells that express the pluripotency marker NANOG, indicative of *in situ* reprogramming. Also, by bone marrow transplantation, we demonstrate that hematopoietic cells can also be reprogrammed *in vivo*. Remarkably, induced reprogrammable mice

also present circulating iPSCs in the blood. These *in vivo*-generated iPSCs can be purified and grown (in the absence of further induction of the reprogramming factors). Strikingly, at the transcriptome level, the *in vivo*-generated iPSCs are closer to embryonic stem cells (ESCs) than to standard *in vitro*-generated iPSCs. Moreover, *in vivo*-iPSCs efficiently contribute to the trophoderm lineage, suggesting that they achieve a more plastic or primitive state than ESCs. Finally, *in vivo*-iPSCs show an unprecedented capacity to form embryo-like structures upon intraperitoneal injection, including the three germ layers of the proper embryo and extraembryonic tissues, such as extraembryonic ectoderm and yolk sac-like with associated embryonic erythropoiesis. These capacities are absent in ESCs or in standard *in vitro*-iPSCs. In summary, *in vivo*-iPSCs represent a more primitive or plastic state than ESCs or *in vitro*-iPSCs. These discoveries could be relevant for future applications of reprogramming in regenerative medicine.

Inv024**Sleeping Beauty transposons for molecular medicine**

J.C. Izpisua Belmonte

Salk Institute for Biological Studies, La Jolla, CA, USA

The development of gene-editing technologies in combination with the generation of patient-specific induced pluripotent stem cells (iPSCs) represents the merge of both the stem cell and gene therapy fields. Novel gene-editing technologies in combination with iPSCs derivation methodologies open the possibility not only for direct gene therapy but also for the replenishment of loss and/or defective cell populations with gene-corrected cells. We will present recent examples developed in our laboratory to illustrate some of the different approaches being undertaken in these fields.

Inv025**The Sleeping Beauty transposon system for molecular medicine**

Z. Ivics

Paul Ehrlich Institute, Langen, Germany

Non-viral gene transfer approaches typically result in only short-lived transgene expression in primary cells, due to the lack of nuclear maintenance of the vector over time and cell division. The development of efficient and safe non-viral vectors armed with an integrating feature would thus greatly facilitate clinical gene therapy studies. The latest generation transposon technology based on the Sleeping Beauty (SB) transposon may potentially overcome some of these limitations. SB was recently shown to provide efficient stable gene transfer and sustained transgene expression in primary cell types, including human hematopoietic progenitors, mesenchymal stem cells, muscle stem/progenitor cells (myoblasts), iPSCs and T cells. The first-in-man clinical trial has been launched to use redirected T cells engineered with SB for gene therapy of B cell lymphoma. In addition, an EU FP7 project was recently initiated with the aim of replacing degenerated retinal pigment epithelial cells with cells that have been genetically modified by SB gene vectors *ex vivo* to produce an anti-angiogenic and neuroprotective factor for the potential treatment of patients suffering from age-related macular degeneration. I discuss aspects of

cellular delivery of the SB transposon system, transgene expression provided by integrated transposon vectors, and target site selection of the transposon vectors.

Inv026

Non-viral CFTR gene delivery to the lungs of Cystic Fibrosis patients

S.C. Hyde

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Cystic fibrosis (CF) is the most common lethal inherited disease of Caucasian populations, affecting ~80,000 individuals worldwide. CF individuals, homozygous for mutations in the CFTR gene, suffer from repeated bacterial infections of the conducting airways that ultimately lead to lung failure and death. The UK Cystic Fibrosis Gene Therapy Consortium (UKCFGTC) have previously demonstrated proof-of-principle for CFTR gene replacement therapy—correcting the CF chloride channel defect for ~1 week in patients receiving non-viral vectors. Our most advanced vector system is a non-viral gene transfer vector termed pGM169/GL67A. This system comprises an entirely CG dinucleotide-free plasmid DNA (pGM169) that directs sustained CFTR lung expression in animal models, and a cationic liposome mixture (GL67A) the most effective non-viral lung gene transfer agent we identified from a large *in vivo* screen. Following successful nonclinical toxicology studies, two clinical studies have been initiated. In an open-label Phase I/IIa study we have shown that a single dose to the nose and lungs of CF patients was safe and corrected the CF chloride channel defect for up to 3 months. We have subsequently initiated a double-blinded, placebo controlled, Phase IIb study enrolling 130 CF patients, randomised 1:1 active:placebo, in which pGM169/GL67A is repeatedly (once a month for 1 year) delivered by aerosol to the lungs of CF patients.

Inv027

Clinical application of *Sleeping Beauty* system to engineer T-cell specificity

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T cells can be genetically modified to prevent and treat malignancies. However, immune tolerance typically prevents the identification and thus human application of unmanipulated autologous T cells with desired specificity and potency for desired tumor-associated antigens (TAAs). The enforced expression of TAA-specific immunoreceptors can redirect the specificity of T cells and early-phase human trials have demonstrated the safety, feasibility, and anti-tumor activity of these adoptively-transferred biological products. These human trials are supported by several clinically-appealing approaches to genetically modify T cells using viral- and nonviral-based technologies. Regarding the latter, we have recently adapted DNA vectors derived from the *Sleeping Beauty* (SB) system for human application. The electro-transfer of DNA plasmids coding for SB transposase and transposon can stably insert desired transgenes, such as a chimeric antigen receptor (CAR), to redirect T-cell specificity independent of human leukocyte antigen. Following electroporation, clinical-grade T cells expressing 2nd generation CAR (designated CD19RCD28 that activates via CD3z/CD28) can be

retrieved and numerically expanded on designer artificial antigen presenting cells (aAPC) derived from K-562 (clone #4). The dual platforms of electroporation of DNA plasmids and aAPC-mediated propagation are undertaken in compliance with current good manufacturing practice to support ongoing trials infusing of CD19-specific CAR⁺ T cells after hematopoietic stem-cell transplantation (HSCT). To date we have enrolled and manufactured product for 25 patients with multiply-relapsed ALL (n=12) or B-cell lymphoma (n=13) on three investigator-initiated trials at MD Anderson Cancer Center (MDACC) to administer thawed patient- and donor-derived CD19-specific T cells as planned infusions in the adjuvant setting after autologous (n=7), allogeneic adult (n=14) or umbilical cord (n=4) HSCT. Each clinical-grade T-cell product was subjected to a battery of in-process testing to complement release testing under CLIA. The clinical data will be updated at the meeting. Next-generation trials are being planned based on refining the CAR design, CAR-dependent targeting ROR1 on malignant B cells to spare concomitant damage to human immune system, infusing CAR⁺ T cells that recognize carbohydrate antigens, and undertaking infusion of CAR⁺ T cells to as investigational treatment of solid tumors. In addition, we have combined the insertion of CAR with the elimination of endogenous genes using artificial nucleases to further broaden the adoptive transfer of genetically modified T cells. This was achieved by the electro-transfer of *in vitro*-transcribed mRNA species coding for engineered zinc finger nucleases to preclude expression of the T-cell receptor (TCR). This results in the generation of 3rd party “universal” T cells that lack endogenous expression and specificity defined by TCR, but have desired specificity for TAA through an introduced CAR. We plan (currently under federal review) a clinical trial administering CD19-specific universal T cells as an approach to “off-the-shelf” immunotherapy so that one donor’s T cells can be pre-prepared and immediately infused on demand into multiple recipients. In summary, the SB system is a nimble and cost-effective approach to manufacturing that as combined with aAPC (and artificial nucleases) can be employed to improve the availability, persistence, and therapeutic potential of genetically modified clinical-grade T cells.

Inv028

Stemness control by cell cycle regulators

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Relative quiescence and self-renewal are defining features of adult stem cells, but their potential coordination remains unclear. Cells initiate progression through the cell-division cycle in response to growth-promoting signals when a threshold of cyclin-dependent kinase (CDK) activity is reached during the G1 phase. In turn, reversible or permanent growth arrests are maintained by CDK inhibitors (CKI) which regulate or prevent progression into the S-phase through inhibition of G1-specific CDKs. Subependymal neural stem cells (NSC) lacking the CKI p21^{WAF1/Cip1} exhibit rapid expansion that is followed by their permanent loss later in life. Because p21 appears to be required for both stem cell maintenance and quiescence, it constitutes a promising candidate to explain how the loss of a proliferation break leads to exhaustion of stem cell potential in endogenous adult niches. We will present data indicating that p21 can act as a transcriptional negative regulator in a cell cycle-independent manner and that

p21 action at specific promoters underlies its essential role in the maintenance of NSCs in the adult brain. Specifically, p21 is required in NSCs to repress both *Bmp2* and *Sox2* genes: BMP2 repression is essential to maintain stemness/undifferentiation and can be modulated by Noggin, whereas *Sox2* acts as an oncogene, generating replicative senescence and cell cycle withdrawal independently of Noggin. This dual function provides a physiological example of combined cell autonomous and non-autonomous functions of p21 with implications in self-renewal, linking relative quiescence of adult stem cells to their longevity and potentiality.

Inv029

X-reactivation impacts human iPSC differentiation potential towards blood

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To determine novel key regulators that direct ES/iPS cell differentiation to hematopoietic lineages, we compared the gene expression profiles of multiple iPS cell lines with differential blood forming capacity. We generated multiple iPS cell lines from amniotic fluid derived mesenchymal stromal cells (AF-iPS) which differentiated towards hematopoietic lineages using our standardized and highly reproducible differentiation protocol. Of the 9 AF-iPS cell lines derived from an individual female patient, the average efficiency of CD45+ hematopoietic cells was 14.2 + / - 9% (range 1.6 to 26.3%). To elucidate the possible reasons for this diversity in efficiency, we grouped the AF-iPS cell lines on the basis of lowest and highest blood differentiation capacity and compared their gene expression profiles by microarray. We found very few changes above 1.5-fold, but interestingly, among the 11 genes that were over-expressed in the AF-iPSC lines with poor blood differentiation efficiency, 10 were located on X chromosome, and the remaining one reported to be involved in Notch signalling. A combination of cumulative sum analysis and the location of differentially expressed genes on the X chromosome identified putative regions of reactivation at multiple, but distinct locations. The possibility of X-reactivation in these female lines was reinforced further where lower levels of XIST were seen in AF-iPSC lines shown to have low blood forming potential, however only half of the iPS cell lines with high blood differentiation capacity showed normal XIST expression when compared to the amniotic fluid mesenchymal starting cell material. To determine whether the block in differentiation was tissue specific we tested the differentiation capacity of the AF-iPSC lines towards neuronal lineages. Intriguingly, we found neural cell differentiation was not hampered within all lines with poor blood potential suggesting that the over-expression of genes as a consequence of X-reactivation can impart a specific negative effect on differentiation towards the blood lineages from pluripotency stage, while not having an effect on neuronal cell development. To further define the source of this block, we have begun working knocking down the overexpressed genes on X chromosome in lines with poor blood differentiation potential to determine whether the efficiency can be increased (or fully rescued) with one, or a combination of these 11 candidate genes. These results have implications for the identification and selection of female iPS lines suitable for therapeutic purposes. I will also discuss the identification of three new factors for improving blood lineage potential of iPS cells lines.

Inv030

DLL4/Notch1 signaling is required for endothelial-to-hematopoietic transition in a hESC model of human embryonic hematopoiesis

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Notch signaling is essential for definitive embryonic hematopoiesis, but little is known on how Notch regulates hematopoiesis in early human embryonic development. Here we analyzed the contribution of Notch signaling to human embryonic hematopoietic differentiation using hESCs. We determined the expression of Notch receptors and ligands during hematopoietic differentiation of hESCs and found that expression of the Notch ligand DLL4 strongly parallels the emergence of bipotent hematoendothelial progenitors (HEPs). Co-cultures of hESCs with OP9-DLL4 cells demonstrated that DLL4 has a dual role in hematopoietic differentiation: during HEPs specification untimely DLL4-mediated Notch activation is detrimental for HEPs generation; however, once HEPs are specified, activation of Notch by DLL4 enhances hematopoietic commitment of these HEPs. We determined by flow cytometry that in hESCs differentiation, DLL4 is only expressed in a subpopulation of HEPs. Gene expression profiling of DLL4^{high} and DLL4^{low/-} HEPs showed that these two subpopulations already exhibit a distinct transcriptome program which determines their differentiation commitment: DLL4^{high} HEPs are highly enriched in endothelial genes, while DLL4^{low/-} HEPs display a clear hematopoietic transcriptional signature. Single cell cloning analysis of these two populations confirmed that DLL4^{high} HEPs are enriched in committed endothelial precursors, while DLL4^{low/-} HEPs contain committed hematopoietic progenitors. Confocal microscopy analysis of whole embryoid bodies revealed that DLL4^{high} HEPs are located in close proximity to DLL4^{low/-} HEPs, and at the base of clusters of CD45+ cells forming structures that resemble AGM hematopoietic clusters found in mouse embryos. Moreover, we found active Notch1 in clusters of emerging CD45+ cells. Overall, our data indicate that DLL4 regulates blood formation from hESCs, with DLL4^{high} HEPs enriched in endothelial potential, whereas DLL4^{low/-} HEPs are transcriptional and functionally committed to hematopoietic development. We propose a model for human embryonic hematopoiesis in which DLL4^{low/-} HEPs receive a signal from DLL4^{high} HEPs to activate Notch1, to undergo an endothelial-to-hematopoietic transition and differentiate into CD45+ hematopoietic cells, resembling what occurs in mouse AGM hematopoietic clusters.

Inv031

Immune responses to AAV vectors in human trials

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Several gene therapeutics based on the adeno-associated viral (AAV) vector platform for *in vivo* gene transfer are currently being tested in the clinic for a variety of indications. The recent

market approval by the European Medicine Agency of an AAV-based gene therapy for the treatment of lipoprotein lipase deficiency further confirms the trend of the gene therapy field towards the clinic. Because AAV vectors are administered directly to patients, they interact with the host immune response at many levels, as shown in preclinical and clinical studies. In particular, immunomonitoring studies in human gene transfer trials showed that immune responses to the virus capsid, or to the transgene product encoded by the vector, can result in lack or loss of therapeutic efficacy. Studies conducted so far enabled investigators to monitor these responses and to develop strategies to manage them to achieve long-term expression of the donated gene. However, a comprehensive understanding of the determinants of immunogenicity of AAV vectors, and of potential associated toxicities, is still lacking. Careful immunosurveillance conducted as part of ongoing clinical studies, together with the use of animal models predictive of the outcome of gene transfer in humans, will be crucial to the development of safe and effective *in vivo* gene therapies for genetic diseases.

Inv032

***In vitro* and *in vivo* use of lentiviral vectors to induce Ag-specific immune tolerance**

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Establishing immune tolerance is critical to prevent clearance of gene corrected cells and to develop successful immunotherapies for immune-mediated disorders. Regulatory T cells (Tregs) play a pivotal role in promoting and maintaining tolerance. Several types of Tregs have been identified including the CD4⁺ Foxp3⁺ Tregs and the CD4⁺ IL-10-producing type 1 regulatory T (Tr1) cells. Much effort has been dedicated to develop suitable methods for the induction/expansion of Foxp3⁺ Tregs and Tr1 cells *in vivo* or *in vitro* for Treg-based cell therapy. We developed protocols to transduce polyclonal and Ag-specific CD4⁺ T cells with lentiviral vectors (LVs) encoding for IL-10 or Foxp3 genes. Enforced LV-mediated IL-10 expression confers Tr1 cell phenotype and functions to CD4⁺ T cells. IL-10 engineered CD4⁺ T cells efficiently suppress *in vitro* and *in vivo* in a model of xenogeneic graft-versus-host-disease. In addition, LV-mediated gene transfer of Foxp3 in CD4⁺ T cells converts effector T cells into CD4⁺ FOXP3⁺ Tregs (CD4⁺FOXP3⁺) in normal donors and in IPEX patients. Patients-derived CD4⁺FOXP3⁺ cells expressed Treg markers, were anergic, and displayed potent suppressive activity, both *in vitro* and *in vivo*. We recently developed a lentiviral vector platform, using microRNA142-regulated (miR142T) LV, which selectively targets expression of the transgene in hepatocytes *in vivo* inducing tolerance to self or exogenous Ag. A single systemic administration of LV.miR142T encoding for the gene of interest leads to the induction of Ag-specific Foxp3⁺ Tregs, which mediates Ag-specific tolerance in several preclinical models of immune mediated pathology. In Hemophilia B mice gene therapy with FIX-encoding LV.miR142T efficiently provides therapeutic FIX levels and induces FIX-specific tolerance in both naïve mice and in mice with pre-existing neutralizing response to FIX. In Type 1 diabetes (T1D) models a single insulinB9-23-encoding LV.miR142T injection in 10 weeks old NOD mice protected from T1D development, maintaining normal blood glucose levels. These findings prove the efficacy of adoptive immune therapy with

regulatory T cells generated *in vitro* by LV-10 or LV-Foxp3 gene transfer and of the LV.miR142T platform to mediate and restore immune tolerance to transgenes.

Inv033

Detection of nucleic acids by the innate immune system

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A central function of our innate immune system is to sense microbial pathogens by the presence of their nucleic acid genomes or their transcriptional or replicative activity. In mammals, a receptor-based system is mainly responsible for the detection of “non-self” nucleic acids. In the past years tremendous progress has been made to identify host constituents that are required for this intricate task. With regard to the detection of RNA species, a picture is emerging that certain families of the toll-like receptor family (TLR-7, -8 and -13) and the RIG-I like helicases (RIG-I and MDA5) respond to microbial RNA molecules. Moreover, TLR9 detects microbial DNA within the endolysosomal compartment. At the same time, a cytosolic surveillance system detects the presence of cytoplasmic DNA. This system relies on the recently discovered nucleotidyltransferase cGAS, which upon DNA sensing, produces the second messenger cGAMP(2'-5') that binds to and activates the ER-resident protein STING. Activation of these nucleic acid sensing pathways can lead to potent innate immune responses, e.g. the induction of antiviral immunity. Of note, all of these sensing pathways can also be activated in response to DNA or RNA based vector systems and as such interfere with the outcome of the primary objective (e.g., transgene expression). In this talk an overview will be given of nucleic acid sensing pathways with a special focus on intracellular DNA recognition.

Inv034

Multi-modal imaging of gene expression *in vivo*

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For gene and cell-based therapies, it is important (i) to understand the molecular changes within the diseased target tissue and (ii) to follow the effects of transduced gene and transplanted cell activity *in vivo*. In the past years we have extensively worked on herpes- and lenti-viral based vector systems to follow gene expression *in vivo*. We have used various imaging technologies including positron emission tomography (PET), magnetic resonance imaging (MRI), and optical imaging (OI) to follow gene expression with relation to the induced therapeutic effect. Most of the work was performed in animal models of gliomas, however, recent work also concentrates on model systems for neurodegenerative diseases, such as Alzheimer's and Parkinson's disease.

The presentation will give an overview of how modern molecular imaging technologies can be implemented in modern gene and cell therapy based approaches to enhance our knowledge of the efficiencies of these therapies in the *in vivo* setting.

References

Jacobs *et al.* *Neoplasia* 1999; Jacobs *et al.* *Cancer Res* 2001; Jacobs *et al.* *Lancet* 2001; Jacobs *et al.* *Human Gene Therapy* 2003; Rüger *et al.* *Gene Therapy* 2005; Jacobs *et al.* *Cancer Res* 2007; Winkeler *et al.* *PLoSOne* 2007; Miletic *et al.* *Mol Therapy* 2007; Miletic *et al.* *Clin Cancer Res* 2007; Monfared *et al.* *Cancer Res* 2008; Waerzeggers *et al.* *Mol Imaging* 2008; Kaestle *et al.* *Mol Imaging* 2011; Rüger *et al.* *MIB* 2011; Klose *et al.* *Neoplasia* 2011; Viel *et al.* *JNM* 2012; Viel *et al.* *Mol Therapy* 2013; Viel *et al.* *PLoSOne* 2013

Inv035

Development of non viral gene delivery vectors through SPECT/CT imaging

G. Vassaux

Inv036

Clinical applications of reporter gene technology imaging

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Real-time imaging of gene expression in living subjects at high spatial resolutions has long been pursued in biomedical research. Molecular imaging has increasingly been used to successfully image gene expression in living animals, thus making significant contributions to the field of gene and cellular gene therapy. However, there has been slow progress in translating these technologies into clinical application, even though there is a real need to develop, test, and validate sensitive and reproducible noninvasive imaging methods that could be repeatedly and safely performed in patients undergoing gene therapy.

If adequate techniques were available, both endogenous and exogenous gene expressions could be studied in live animals and potentially in the clinical arena. In this sense, molecular imaging has emerged as a non-invasive technology for *in vivo* mapping of gene expression and provides promising tools for rational progress of molecular medicine and gene therapy.

The uncertainties in clinical gene therapy are related not only to problems of gene delivery and gene expression but also to the lack of objective clinical endpoints to evaluate therapy response. In many cases the clinical benefit of the therapeutic procedure is hardly measurable or requires complex invasive procedures that give only a partial picture of the situation, as it is mostly based on molecular and histopathological analysis of biopsies. Hence, there is a real need for a technology that allows routine clinical use for a quantitative, whole-body spatiotemporal evaluation of gene delivery and expression as a way to determine and even predict the clinical outcome.

Noninvasive molecular imaging in gene therapy can be used in several different ways. These technologies can be used to monitor gene expression both locally and for the whole-body, evaluate the treatment by imaging the functional effects of gene therapy at the biochemical level and determine prognosis. Furthermore, they would help to improve the design of more efficient, specific and safer vectors, and to determine the appropriate dose to be used in each case and in consequence facilitate their approval for human use, thus speeding up all the process.

Similar principles to those developed for gene therapy imaging can also be applied to cell-based therapies. Monitoring of cell

trafficking with genetically modified cells will permit grafting experiments and long-term follow-up of the cell fate. For imaging of cell trafficking using reporter genes one must keep in mind the minimum number of cells that need to accumulate at one site to be detectable as well as the possibility that the cells may shut down the expression of the reporter gene.

The use of multimodality imaging would undoubtedly facilitate translational research by illuminating with different strategies the underlying molecular events. It is evident that no single technique alone will provide the full spectrum of specifications needed to image all aspects of gene therapy from basic research to clinical application. Developing a molecular imaging paradigm for a particular disease requires finding a molecular target relevant to the disease and finding a high-affinity ligand for such a target. If this basic principle is possible, a molecular imaging agent has to be found that can be used to detect the target *in vivo*. Unfortunately, in many cases such molecules might not be amenable for use in humans.

In the case of reporter gene imaging, multimodality imaging vectors would probably be the best way to validate a procedure and even get different molecular information from different imaging technologies. For example, a vector comprising a triple-fusion imaging reporter gene amenable for bioluminescence, fluorescence, and PET imaging would have many advantages. Bioluminescence imaging would permit fast and affordable testing of the construct in laboratory animals with high throughput but would lack tomographic information. The use of fluorescence, although less sensitive than bioluminescence for most applications, might permit fast kinetic analysis not possible with bioluminescence and would enable not only *in vivo* imaging in animals but also detailed histological examination of explanted tissues if required. However, none of the above technologies could be used in humans *in vivo*, but the radionuclide-based or MRI reporter might permit tomographic transgene expression imaging in small animals and definitely transgene expression monitoring in humans in the clinical setting.

Future studies will be influenced by the development of improved gene therapy vectors with higher transduction or transfection efficiencies and specificities. Development of new tracers with higher affinities for reporter proteins (enzymes, receptors or transporters) will improve the sensitivity of imaging.

Inv037

Neural crest contributions to the haematopoietic stem-cell niche

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Stem cell niches in adult tissues share some similarities, including a physical and functional association with blood vessels. However, the structural organization of these niches remains largely unknown. We have shown that perivascular mesenchymal stem cells (MSCs) are required to maintain haematopoietic stem cells (HSCs) in the bone marrow. Adult MSCs express nestin and regulate HSC traffic in and out the bone marrow. We have shown that the brain can control HSC migration through sympathetic regulation of nestin⁺ MSCs, directly innervated by sympathetic nerve fibres in the bone marrow. Other groups have shown that Schwann cells covering these sympathetic axons regulate HSC proliferation. Like peripheral sympathetic neurons, Schwann cells derive from the neural crest, but their ontogenic

relationships to other cells in the bone marrow microenvironment remain unknown. I will discuss the contributions of the neural crest to the establishment, maintenance and regulation of HSCs in the bone marrow, as well as the potential implications of this regulatory network in pathophysiology.

Inv038

Hematopoiesis under the stress

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Hematopoietic stem cells (HSCs) are sustained in a specific microenvironment known as the stem cell niche. Adult HSCs are kept quiescent during the cell cycle in the endosteal niche of the bone marrow (BM). The quiescent state is thought to be a characteristic property for the maintenance of HSCs. Normal HSCs maintain intracellular hypoxia, stabilize the hypoxia-inducible factor-1 α (HIF-1 α) protein and generate ATP by anaerobic metabolism. In HIF-1 α -deficiency, HSCs become metabolically aerobic, lose cell cycle quiescence, and are finally exhausted. An increased dose of HIF-1 α protein in VHL mutated HSCs and their progenitors induced cell cycle quiescence and accumulation of HSCs in the BM. Restored glycolysis by pyruvate dehydrogenase kinases (PDK) ameliorated cell cycle quiescence and stem cell capacity. Taken together, HSCs directly utilize the hypoxic microenvironment to maintain their cell cycle by HIF-1 α -dependent metabolism.

On the basis of the physiological nature of HSCs, I would like to discuss the abnormal HSCs and niches under the stress such as inflammation and hematological malignancies such as chronic myelogenous leukemia (CML). Comparison between normal and abnormal HSCs and niches will be important to development of the new treatment for diseases.

Inv039

Regulation of normal and leukemic human stem cells: dynamic stem cell interactions with the bone marrow microenvironment

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Hematopoietic stem cells (HSC) mostly reside in the bone marrow in a quiescent, non-motile mode via adhesion interactions with stromal cells and macrophages. Bone marrow (BM) endothelial cells (BMECs) serve as 'niche' cells for both hematopoietic and mesenchymal stem and progenitor cells (HSPC/MSPC). Yet BMECs control of HSPC bi-directional trafficking between the BM and peripheral blood (PB) through the blood-bone marrow-barrier (BBMB) is poorly understood. *In vivo* treatment with the angiogenic cytokine FGF-2 resulted in increased BM HSPC/MSPC numbers and repopulation potential, while reducing peripheral blood (PB) HSPC numbers and repopulation potential. FGF-2 treated mice had reduced BBMB permeability evaluated by dye penetration and incorporation, and reduced HSPC BM homing. We have generated endothelial specific inducible FGF receptor knock-out (eFGFR1/2 KO), using VE-Cadherin-CREERT2 and FGFR1flx/flx/FGFR2flx/flx transgenic mice). Importantly, eFGFR1/2 KO mice exhibited reduced BM stem cell levels. FGF-2 treatment was able to increase stem cell levels only

in WT BM, but not in the BM of eFGFR1/2 KO mice. FGF-2 treated eFGFR1/2 KO mice failed to exhibit increased HSPC retention and reduced BM homing like in WT treated mice. These results suggest that endothelial specific activation of FGF signaling regulates the BBMB control of HSPC bi-directional trafficking. Thus, hampering endothelial FGF signaling interferes with stem cell maintenance and expansion, while increasing BBMB permeability. In addition, we have observed Nestin-GFP+ BMEC expressed by non-permeable arteriolar blood vessels, suggesting that these vessels serve as stem cell maintenance and expansion sites. Mechanistically, FGF-2 treatment resulted in decreased BM MMP-9 activity combined with upregulated Timp-1 (MMP-9 inhibitor) mRNA levels. Furthermore, FGF-2 treatment activated AKT, and reduced NO and ROS content in BMEC. Importantly, permeability regulating endothelial junction molecules such as VE-Cadherin and ZO-1 were upregulated following FGF-2 treatment. Administration of neutralizing VE-Cadherin antibodies or the mobilizing agent AMD3100, efficiently increased HSPC egress by increasing BBMB permeability. These results suggest that interfering with endothelial adhesion interactions alone, or CXCR4 activation, increase HSPC trafficking. Examination of eNOS KO and Timp-1 KO mice revealed that HSPC bi-directional trafficking was decreased in eNOS KO mice and increased in Timp-1 KO mice. In the BM, primitive, repopulating HSPC numbers were increased in eNOS KO mice and decreased in Timp-1 KO mice. Of interest, we observed that chimeric immunodeficient mice transplanted with malignant human pre-B ALL cells, exhibited reduction in BBMB permeability. We suggest that leukemic cells stimulate BMEC via FGF-2 secretion, in order to support their own maintenance, expansion and chemoresistance by reducing BBMB permeability, and CXCL12 while increasing membrane bound SCF levels. This suggests that human leukemias can modify the BBMB to a malignant-supportive microenvironment and that BBMB targeting could hamper leukemia expansion and protection, to improve clinical chemotherapeutic protocols. In conclusion, our findings reveal that normal and malignant stem cell migration and development are dynamically regulated by BMEC. We suggest that BMEC balance their dual roles as stem cell niches, and as an anatomical barrier synchronizing HSPC bi-directional trafficking. Thus, unbalanced BBMB function towards either stem cell trafficking or maintenance will come at the expense of the other. The role of the coagulation system in regulation of stem cell migration and development via thrombin/PAR-1 and EPCR shedding, or in maintaining stem cell adhesion to BM stromal cells via APC/EPCR and PAR-1 will also be presented. Finally we will also present preliminary results with the bioactive lipid S1P which regulates an inverse HSPC and MSPC metabolic state during quiescence and proliferation via ROS transfer between the two populations.

Inv040

Preclinical and early clinical development of an encolytic group B adenovirus, ColoAd1

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ColoAd1 is a chimeric Ad11p/Ad3 group B adenovirus, discovered by bio-selection from a library of chimeric adenoviruses for the ability to replicate and exit rapidly from human colorectal carcinoma cells (Kuhn, Harden *et al.* 2008). The virus replicates rapidly within and kills carcinoma cells derived from many

different tissue types, whilst sparing normal cells. It also mediates good anticancer efficacy when administered i.v. to mice bearing metastatic HT29 tumours. ColoAd1 kills cells more quickly than Ad5 and Ad11p, and the death pathway is independent of P53 and caspases. Matched chemo-sensitive and -resistant cells were killed with equal efficiency, compatible with an apoptosis-independent death pathway. Whereas Ad5-based adenoviruses are efficiently neutralised upon coming into contact with human blood, ColoAd1 shows good blood stability and can infect tumour cells in the presence of whole human blood. This makes it an attractive candidate for intravenous delivery in the clinic. The EVOLVE study is an international, multi-centre i.v. phase I/II clinical study of ColoAd1. The study design allows for an initial dose escalation stage, followed by a dose expansion stage and ultimately a phase II randomized stage. Preliminary pharmacokinetics and safety data from this ongoing trial will be discussed.

Reference

Kuhn, I., P. Harden, *et al.* (2008). "Directed evolution generates a novel oncolytic virus for the treatment of colon cancer." *PLoS One* 3(6): e2409.

Inv041

Parvovirus infections: prospects for cancer treatment

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Rodent parvoviruses (PV) are recognized for their intrinsic oncotropism and oncolytic activity. These features contribute to the natural capacity of PV for tumor suppression, for which human cancer cells can be targets in animal models. Although PV uptake occurs in most host cells, some of the subsequent steps leading to expression and amplification of the viral genome and production of progeny particles are upregulated in malignantly transformed cells. By usurping cellular processes such as DNA replication, DNA damage response, and gene expression, and/or by interfering with cellular signaling cascades involved in cytoskeleton dynamics and cell integrity, PVs can induce cytostasis and cytotoxicity. Furthermore, there is growing evidence that parvoviral oncosuppression involves an immune component. Besides exerting direct oncolytic effects, PV can indeed serve as adjuvants to hand further tumor destruction to the immune system in animal models. Current and planned parvovirotherapy clinical trials should indicate whether PV oncolysis can be translated into long-term protection by the immune system with improved tumor destruction and patient survival.

Inv042

K. Harrington

Inv043

Telomerase-targeting oncolytic adenovirus as a therapeutic and diagnostic agent

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Telomelysin is a genetically engineered adenovirus to selectively replicate in malignant cells, by replacing adenoviral E1 gene promoter with hTERT promoter. Preclinical studies demonstrated that Telomelysin has anticancer activity in various cancer cell lines either singly or in combination with chemotherapy and radiotherapy. A phase I study of Telomelysin conducted in the US confirmed the safety, tolerability and feasibility of this agent. Then, we have planned a Phase I/II study to assess the safety of intratumoral injection in combination with local ionizing radiation for head and neck and thoracic malignant tumors, and are now recruiting patients in Japan. We further developed TelomeScan by inserting the green fluorescent protein (GFP) gene into the E3 region of the Telomelysin genome, which allows cancer specific GFP expression. TelomeScan is applicable to detect circulating tumor cells (CTC) in the peripheral blood and to visualize metastatic or disseminated tumors *in vivo*. We hope that these therapeutic and diagnostic applications of Telomerase-targeting adenovirus will aid treatment strategies in clinical practice in near future.

Inv044

Lentiviral gene therapy for primary immune deficiencies: preclinical evaluation of efficacy, safety and conditioning modalities

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In this review, a comparative efficacy and safety analysis is provided for self-inactivating lentiviral (SIN-LV) hematopoietic stem cell (HSC) gene therapy using codon-optimized human therapeutic transgenes driven by either viral or cellular promoters in mouse models for recombination activating gene 1 (RAG1) and 2 (RAG2) deficiencies and X-linked SCID (*Il2rg*^{-/-} mice). The methodology, including an efficient overnight transduction protocol, proved highly efficacious with restoration of immune functions for RAG2 deficiency and X-linked SCID using cellular promoters at a relatively low vector copy number per cell and pre-transplant low-dose cyto-reductive conditioning by sublethal total body irradiation (TBI). However, in RAG1 deficiency, only the strong spleen focus forming virus (SF) promoter yielded some efficacy, while a vast number of treated mice displayed an Omenn-like syndrome, underlining that further development is required for improved expression to successfully treat RAG1 patients. As cyto-reductive conditioning of SCID patients is undesirable, in *Il2rg*^{-/-} mice the effect of pre-transplant G-CSF mobilization of stem cells to create open bone marrow niches was investigated. The results demonstrated efficacy similar to low-dose TBI, encouraging further development with (combinations) of other mobilizing agents, in progress. Safety evaluation demonstrated, as expected, no propensity of cellular promoters for integration near proto-oncogenes. In *Il2rg*^{-/-} mice, leukemia occurred in 14 of 212 mice monitored. Similar leukemia frequencies were seen in mice given IL2RG vectors, control green fluorescent protein (GFP) vectors or untransduced cells and no increased risk was apparent for the SF promoter relative to eukaryotic promoters. Considering the tumor-prone background of the *Il2rg*^{-/-} mice used, these observations indicate that the insertional oncogenesis risk when using SIN-LV HSC gene therapy is very low and clinically acceptable.

Inv045**Thymic stromal pathologies: A target for gene replacement therapy?**

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The thymic microenvironment is unique in its ability to promote the development of naïve T cells with a repertoire purged of vital "Self" specificities and poised to react to injurious "Non-Self". Thymic epithelial cells (TECs) constitute the major component of the thymic stroma and can be categorized into separate cortical (c) and medullary (m) lineages based on their specific molecular, structural and functional characteristics. cTEC induce the commitment of blood-borne precursor cells to a T cell fate, foster the subsequent maturation and control the positive selection of antigen receptor bearing thymocytes. In contrast, mTEC promote the terminal differentiation of thymocytes, which includes the establishment of immunological tolerance to self-antigens via a deletional mechanism and the generation of natural regulatory T cells. In this way, mTEC generate the self-tolerant T cell repertoire in a direct instructive fashion. This essential capacity depends on the mTEC's promiscuous expression of a large programme of transcripts that encode proteins which are normally only detected in differentiated organs residing in the periphery (a.k.a. tissue restricted self antigens). Several primary immunopathologies have been identified at the molecular level that affect thymic epithelial cell differentiation and, consequently, the maturation of functionally competent T cells with a regular antigen receptor repertoire. These pathologies are discussed and the suitability for therapeutic gene correction will be examined.

Inv046**Current status and perspectives from international trials of gene therapy for primary immune deficiencies**

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A multi-center international strategy was developed to rapidly assess the safety and efficacy of gene therapy in very rare primary immune deficiencies such as Wiskott Aldrich Syndrome (WAS) or X-linked chronic granulomatous disease (each with an estimated incidence of 4 to 10 cases per million live births). In the case of WAS, 3 clinical centers are open in Europe and in the US. In the case of X-CGD, 1 center is open and 6 additional centers are scheduled to open in the next year in Europe and in the US. All of these protocols concern severely-affected patients lacking a matched bone marrow donor. Patients are transplanted with gene-modified autologous hematopoietic stem cells following disease-adapted conditioning regimens. Gene transfer vectors used in these studies are SIN lentiviral vectors (LV) that express the therapeutic cDNA at physiological levels in target cells. Robust manufacturing capacity of clinical-grade LV at Genethon (50 L per batch) enables the supply of multiple clinical centers with high titer purified vector lots in quantities sufficient to treat infants and young adults. Encouraging results have been obtained in the WAS trials as 7 patients have now been treated without safety issues.

Ongoing efforts will help to establish models to expedite the development of new treatments for primary immune deficits or blood disorders.

Inv047**Forelimb treatment in a large cohort of dystrophic dogs supports delivery of a recombinant AAV for exon skipping in Duchenne patients**

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Duchenne Muscular Dystrophy (DMD) is the most frequent muscular dystrophy caused by mutations in the dystrophin gene. No curative treatment is yet available. We evaluated a gene therapy product using a recombinant adeno-associated virus vector from serotype 8 (rAAV8) carrying a modified U7snRNA sequence promoting exon skipping to restore a shorter albeit functional dystrophin transcript. For the transduction of large muscular territories, the mode of delivery intended for the first time in patients is the locoregional transvenous perfusion of the forearm. Using the Golden Retriever Muscular Dystrophy (GRMD) dog, we established the safety profile and the therapeutic index of the recombinant vector after we had validated functional read-outs, including electrophysiology and Nuclear Magnetic Resonance (NMR) imaging and spectroscopy. Eighteen GRMD dogs received increasing doses of a GMP-like rAAV8-U7snRNA vector ranging from 2.5E12 to 2.5E13vg/kg. In all cases, the treatment was well tolerated and no acute or delayed adverse effects including systemic and immune toxicity were detected. There was a dose relationship with the amount of skipping with up to 80% of myofibers expressing dystrophin at the highest dose and ~35% and ~10% in animals receiving the intermediate and the low doses, respectively. Similarly, histological and NMR pathological indices responded in a dose dependant manner. Importantly, strength was improved in muscles with more than 40% dystrophin expression. Altogether, the reassuring safety and biodistribution/shedding profiles combined with robust clinical efficacy in juvenile dystrophic dogs support the concept of a Phase I/II trial of locoregional delivery into upper limbs of non-ambulatory patients.

Inv048**Optimised AAV-microdystrophin gene therapy for Duchenne muscular dystrophy**

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Muscular dystrophies refer to a group of inherited disorders characterized by progressive muscle weakness, wasting and degeneration. So far, there are no strongly effective treatments but new gene-based therapies are currently being developed

with particular advances in using exon skipping and other RNA-based approaches, conventional gene replacement strategies, and cell-based gene therapy. In the case of DMD, putting aside exon skipping therapy, a number of groups are testing gene therapy with adeno-associated virus vectors expressing engineered micro-dystrophins (AAV-MDs). In our hands, highly sequenced optimised AAV-MDs are available for use in mouse, dog and ultimately humans, expressed using a strong synthetic promoter specific for skeletal and cardiac muscle cells have been tested in detail in mdx mice, and in the GRMD dog. Our studies strongly supports the hypothesis that the current optimised microdystrophins are highly functional, not only in mice, but also in a large animal model, and that AAV2/8 vector delivery results in sustained expression of microdystrophin without adverse immune responses. The current optimised AAV-microdystrophin configuration thus lays a sound basis for a translation programme towards clinical trials in DMD patients.

Inv049

Gene therapy of myotubular myopathy

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Loss-of-function mutations in the myotubularin gene (MTM1) cause X-linked myotubular myopathy (XLMTM), a severe congenital disease that affects the entire skeletal musculature. Previous local studies in Mtm1-mutant mice demonstrated potential efficacy of gene therapy to treat the disease. Here we report long-term survival data in mice and dogs following intravenous delivery of recombinant adeno-associated virus serotype 8 (AAV8) vectors expressing myotubularin under the muscle-specific desmin promoter. Injection of an AAV8-Mtm1 vector into the tail vein of myotubularin knockout mice at the onset or at late stages of the disease resulted in robust improvement in muscle strength, corrected the pathology and prolonged survival up to the end of a 6-month study. In addition, intravascular delivery of an AAV8 vector carrying the canine MTM1 cDNA in XLMTM dogs ameliorated motor and respiratory functions, and prolonged lifespan to more than one year in the absence of immune response against the transgene. Altogether, our results demonstrate that intravenous AAV8-mediated gene therapy leads to long term systemic correction of myotubular myopathy in small and large animal models and pave the way to a clinical trial in patients.

Inv050

Production of lentiviral vectors by transfection of suspension cells in single use systems

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Généthon, France

Genethon develops gene therapies based on lentiviral and adeno-associated viral vectors for neuromuscular, blood, eye and metabolic genetic diseases. The production of lentivirus vectors relies on the simultaneous expression of viral proteins gag, pol, rev, a pseudotyped envelop protein and the transgene RNA to be packaged into the vector particles. The most commonly used manufacturing process consists of transfecting 4 plasmids in adherent cells grown in cell factories using calcium

phosphate. However, this method is cumbersome, prone to variability and shows limited scalability. To address those drawbacks, we have developed a transfection process in suspension cells in disposable bioreactors. Today, the efficiency of the process has been demonstrated up to 50 L scale using stirred tanks as well as wave technology. The cell culture is performed in chemically defined media to eliminate the potential variability and virus/TSE risks associated with bovine serum. Finally, a downstream process was designed to eliminate efficiently the culture derived impurities. The full process is already a robust alternative to the cell factory system and will be scaled up to 200 L scale soon.

Inv051

Bioengineering approaches for up- and down-stream processing of human stem cells for clinical application

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Stem cell (SC) transplantation has emerged as an exciting treatment for patients with acute myocardial infarction. The major challenges in this field are lack of expertise in product characterization and specialized cell biomanufacturing which are imperative to bring SC-based products to clinic^[1]. Our work has been focused on production and characterization of challenging SC-based products: cardiomyocytes derived from induced pluripotent SC (iPSC), which are capable to regenerate myocardium^[2], and adult SC (human mesenchymal and cardiac SC- hMSC and hCSC), which trigger paracrine mechanisms that activate endogenous SC to promote regeneration^[3]. Our strategy for iPSC-derived cardiomyocytes production consisted in designing an integrated bioprocess by combining expansion, differentiation and purification steps in environmentally controlled bioreactors operating in perfusion. iPSC were cultivated as aggregates and the impact of different bioprocessing parameters on differentiation/purification efficiency was evaluated. By optimizing dissolved oxygen and agitation type we were able to improve by 1000-fold the final yields of functional cardiomyocytes with high purity (>98%). Regarding hMSC and hCSC biomanufacturing, an efficient protocol for cell cultivation using microcarrier-based stirred culture systems was implemented. For cell purification, our strategy was focused in the establishment of a continuous downstream approach using membrane technology and chromatographic tools. Moreover, novel mass spectrometry methodologies have been applied to obtain a complete characterization of the Secretome and Receptome of SC. From hCSC Receptome analysis, more than 2000 proteins were identified, including 100 receptors. The knowledge generated from our study will establish a new way to streamline the design and biomanufacturing of novel cell-based therapies.

References

- [1] Serra M *et al.*, 2012, Trends in Biotechnol, 30: 350; [2] Carpenter L *et al.*, Stem Cells Dev 2012, 21:977; [3] Torella D, *et al.*, 2007, Cell Mol Life Sci, 64: 661

Inv052**Challenges in vector and cell manufacturing for gene therapy**

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Gene therapy is significantly advancing and manufacturing to meet expected demands and quality-control standards are great challenges, especially for more prevalent disease, such as hemophilia. A GMP process for lentiviral vector (LV) production and purification has been developed in collaboration with HSR-TIGET and Généthon and is currently used for LV manufacturing for *ex vivo* gene therapy clinical trials. Besides the manufacturing process, which has been further improved in terms of both yield and reduction of contaminants, a quality control strategy has been defined and appropriate analytical methods have been developed and validated, leading to manufacturing of high quality purified vectors and testing methods largely applicable to 3rd generation LV carrying different transgenes. Improved yields allow the transduction of a larger number of patients' cells with the same vector lot, and GMP transduction processes guarantee the overall quality of products. Nevertheless, efforts to develop stable LV systems to face more prevalent diseases are warranted. We will discuss the current status of the proprietary RD-MolPack packaging system for the stable production of both Tat-dependent, Tat-independent, LTR-driven and self-inactivating (SIN) LV. In contrast to most similar available technologies, which usually rest on the regulated expression of the VSV-G envelope, RD-MolPack is ground on the constitutive expression of LV thanks to pseudotyping with the non-toxic and CD34+ -selective RD114-TR envelope. Another unique feature of MolMed developed technology consists in the insertion of the viral genes into HEK-293T cells by integrating vectors such as the chimeric baculo-AAV vector and SIN-LV. By comparing equal amount of physical particles of VSV-G-LV, produced transiently by current method, and RD114-TR-LV, produced by RD-MolPack system, we demonstrated similar transduction efficiency and vector copy number (VCN) integration in human CD34+ cells by using 2-log less RD114-TR-LV. This mark and the safety of the system make this technology a valid option to current large scale LV manufacturing for clinical applications.

Inv053**MuStem cells: a therapeutic candidate for cell-based therapy of Duchenne Muscular Dystrophy**

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Duchenne Muscular Dystrophy (DMD) is a genetic muscle disease resulting from the dystrophin lack and without effective treatment. Based on delayed adhesion properties, we isolated adult stem cells from healthy dog skeletal muscle, characterized them and investigated their therapeutic potential after systemic delivery in immunosuppressed dystrophic dog, the clinically relevant DMD animal model. These cells, named cMuStem cells, display a high proliferation rate and a multi-lineage differentiation potential even though they appeared to be committed to the myogenic lineage. Intra-arterial administration resulted in dystrophin expression, muscle damage course limitation and a long-term stabilization of the transplanted dog's clinical status. We isolated human cells (hMuStem) from healthy muscle biopsies

and initiated their characterization. They are defined by a clonogenic potential and a large *in vitro* expansion capacity. Myogenicity was demonstrated by Myf5 expression and *in vitro* myogenic differentiation. Also, fusion ability with host fibers was observed after implantation into scid mice muscle. hMuStem cells are positive for several perivascular cell and mesenchymal markers that could suggest a common filiation. The translational findings demonstrate that MuStem cells provide an attractive therapeutic avenue for DMD patients.

Inv054**A clonal strategy for *ex vivo* gene therapy of epidermis**

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Self-renewal and the capacity to generate a tissue for an extended period of time (theoretically a lifetime) are fundamental properties of adult tissue stem cells. Indeed, long-term reconstruction of bone marrow and skin (epidermis and hair follicles) from a single tissue stem cell has been experimentally demonstrated in animal models. Safety is critical when it comes to *ex vivo* autologous gene therapy but current stem cell technology makes it difficult to thoroughly investigate the properties of stem cells stably transduced by means of viral vectors. Hence, we have taken advantage of the unique capabilities of human epidermal stem cells (holoclones) to demonstrate the feasibility of a single cell approach for *ex vivo* gene therapy of skin using recessive dystrophic epidermolysis bullosa (RDEB) as a model system. A clonal strategy permits to thoroughly evaluate the properties of the genetically corrected stem cells (proviral insertion, absence of tumorigenicity and dissemination) before the patient is transplanted and brings safety to a level that is difficult to achieve otherwise.

Inv055**Systemic cell therapy for epidermolysis bullosa: repair of skin extracellular matrix**J. Tolar¹, J.A. McGrath², D.R. Keene³, K. Hook⁴, M.J. Osborn¹, M. Hordinsky⁴, D. Woodley⁵, M. Chen⁵, A. Hovnanian⁶, K. Tamai⁷, B.R. Blazar¹, and J.E. Wagner¹*¹Pediatric Blood and Marrow Transplantation, University of Minnesota, Minneapolis, US; ²King's College London, UK; ³Shriners Hospital, Portland, OR, US; ⁴Dermatology, University of Minnesota, Minneapolis, US; ⁵Dermatology, University of Southern California, Los Angeles, US; ⁶INSERM, Necker Hospital, Paris, France; ⁷Department of Stem Cell Science, Osaka, Japan*

RDEB results from deficiency of type VII collagen (C7), which results in lack of attachment between the epidermis and dermis, severe blistering, and a poor quality of life. Between 2007 and 2012, 18 individuals (0.7–20 y) with life-threatening, severe generalized RDEB have undergone allogeneic hematopoietic cell transplantation at the University of Minnesota. Of these, 13 were treated with a myeloablative (MA) conditioning (busulfan, fludarabine [FLU], and cyclophosphamide [CY]) and 5 were treated with non-MA conditioning (CY, FLU, anti-thymocyte globulin, and total body irradiation). RDEB individuals were transplanted with bone marrow (BM) from an HLA-matched sibling (n=10),

an HLA-matched unrelated donor (n=3), or a partially matched umbilical cord blood (UCB, n=5). Importantly, non-MA conditioning was well tolerated with a marked reduction in risk of infection and pulmonary or renal toxicity. For the entire cohort thus far, the overall probability of survival is 73% (95% CI, 49–96%) with 11 demonstrating partial to marked biochemical and clinical improvement in mucocutaneous disease on the basis of C7 expression, body surface area affected, and resistance to blistering. We conclude that BM is the preferred graft (alive and engrafted: 11 of 13 [85%]) versus UCB (1 of 5 [20%]) (p-value=0.02) and that early results with the non-MA conditioning are promising in terms of toxicity profile and engraftment. Overall, HCT has the potential of being a durable, systemic therapy for many people with different forms and severities of EB, and sets the stage for using BM cells in the treatment of a broad spectrum of extracellular matrix disorders.

Inv056

Modeling neurodegenerative disease through iPSC cell technology

Angel Raya

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A critical step in the development of effective therapeutics to treat neurodegenerative diseases is the identification of molecular pathogenic mechanisms underlying chronically progressive neurodegeneration in patients. While animal models have provided valuable information about the molecular basis of several diseases, the lack of faithful cellular and animal models that recapitulate human pathophysiology is delaying the development of new therapeutics in other diseases such as Parkinson's disease (PD). The reprogramming of somatic cells to induced pluripotent stem cells (iPSC) using defined combinations of transcription factors is a groundbreaking discovery that opens great opportunities for modeling human diseases, including PD, since iPSC can be generated from patients and differentiated into disease-relevant cell types, which would capture the patients' genetic complexity. Furthermore, human iPSC-derived neuronal models offer unprecedented access to early stages of the disease, allowing the investigation of the events that initiate the pathologic process in PD. Recently, human iPSC-derived neurons from patients with familial and sporadic PD have been generated and shown to recapitulate some PD-related cell phenotypes, including abnormal α -synuclein accumulation in vitro, and alterations in the autophagy machinery. I will present the current status of iPSC-based models for understanding PD pathogenesis and discuss the potential future research directions of this field.

Inv057

Transdifferentiation of human adult cells into endocrine pancreatic cells; cell replacement therapy for diabetic patients

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Cellular differentiation and lineage commitment were considered irreversible processes. However, recent reports have in-

dicated that differentiated adult cells can be reprogrammed to an embryonic like pluripotent state and in some cases, directly to alternate committed lineages. Dominant instructive roles for transcription factors in reprogramming somatic cells have been suggested. However, while numerous somatic cell sources give rise to pluripotent cells, it is believed that committed lineages are generated either in developmentally related tissues or by the differentiation of pluripotent cells. Here we challenge this view and suggest that adult cell reprogramming along alternate committed lineages can be a direct process, which crosses the boundaries of the distinct developmental germ layers, without the need for pre-induced pluripotency. We demonstrate that the endoderm derived pancreatic lineage and β -cell-like function are induced in adult cells which are derived from all three germ layers, by ectopic expression of non-integrating pancreatic differentiation factors. The direct reprogramming process along the alternate committed lineage is fast, specific and relatively abundant, compared to reprogramming pluripotent cells. The temporal control of transdifferentiation and the role of Epithelial-Mesenchyme transitions in the process will be discussed. Direct reprogramming of somatic cells carries important implications in developing patient specific regenerative medicine approaches which may prove safer than using ESC or iPSC cells as progenitors. The generation of insulin producing cells by adult cells reprogramming allows the diabetic patient to serve also as the donor of his own therapeutic tissue.

Inv058

AAV-mediated gene targeting approaches for the derivation of histocompatible pluripotent stem cells

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The clinical use of human pluripotent stem cells and their derivatives is limited by the rejection of transplanted cells due to differences in their HLA genes. This has led to the proposed use of histocompatible, patient-specific stem cells, however the preparation of many different stem cell lines for clinical use is a daunting task. In this presentation I will discuss genetic engineering approaches that address this problem and allow the engraftment of allogeneic cells in multiple recipients. These approaches include the derivation of HLA-homozygous stem cells with common haplotypes that would match a significant proportion of the population, the creation of HLA class I-negative "universal donor" cells by targeted disruption of the *B2M* genes, and the expression of single chain HLA fusion proteins in otherwise class I-negative cells to prevent lysis by NK cells and enable the presentation of specific, HLA-restricted antigens. In each strategy, we used adeno-associated virus vectors for efficient gene targeting in the absence of potentially genotoxic nucleases.

Inv059

Immunoregulation without immunosuppression: the promise of low-dose IL-2

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The immune system is composed of numerous interacting cell populations with distinct functions. As for any system, it is (it has to be) tightly regulated. Failure of regulation leads notably to inflammatory and autoimmune diseases. A simplistic paradigm is that the balance between effector and regulatory T cells (Teff and Treg) plays a major role in dosing/regulating the immune response. Likewise, autoimmune diseases are caused by an imbalance that favors the effector arm of the immune response, whether caused by an increase in Teff or a defect in Tregs numbers/functions. While blocking Teff with immunosuppressant has long been the only therapeutic option to correct this imbalance, it now appears that activating/expanding Tregs may achieve the same purpose without the toxicity and side effects of immunosuppression. We recently reported that interleukin-2 (IL-2), which has so far been used at high-dose and with high toxicity to stimulate Teffs in cancer, could be used at well tolerated low-doses to stimulate Tregs *in vivo* in humans^{1,2}. We now report that IL-2 can be given chronically without immunosuppression in mice. This opens broad applications for treatment of a wide range of chronic diseases, from inflammatory to autoimmune diseases, and more broadly for tolerance induction. The concepts and tools of cell and gene therapy will be useful for these developments, and conversely IL-2 induced immunoregulation may help control adverse immune reactions in cell and gene therapy.

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Inv060

MSCs generate a CD4⁺CD25⁺FOXP3⁺ regulatory T cell population

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Background: The potential of MSCs to modulate the host immune response, mainly by inhibiting the proliferation of T lymphocytes, introduced the possibility that they might be effective in inflammatory arthritis where the T cell response is prominent. Studies using the collagen-induced arthritis (CIA) experimental mouse model reported improvement of clinical and biological scores after injection of MSCs derived from bone marrow or adipose tissue. We also showed that therapeutic effect of MSCs was observed during a narrow window of MSC application suggesting that discrepancy between studies may be related to the time of injection and/or the immune status of animals at that time. Indeed, it has been well described that MSCs are able to suppress T lymphocyte proliferation through several mechanisms including their ability to generate FoxP3⁺ regulatory T-cells *in vitro* and *in vivo*. However, the effect of MSCs on T cells induced to differentiate into pro-inflammatory Th1 or Th17 cells and on the resulting mature cells still remains to determine.

Objective: The aim of this study was to determine whether MSCs are able to generate CD4⁺CD25⁺Foxp3⁺ regulatory T-cells during the differentiation process of naïve T cells towards Th1 and Th17 cells and in the mature T cells.

Methods: Murine MSCs were obtained from bone marrow and characterized according to their surface antigen expression and multilineage differentiation potential. Murine CD4⁺ T cells isolated from spleens were induced to differentiate into Th1 or Th17 cells. At D0, D2 and D4 of the differentiation process, T cells were activated with anti-CD3/CD28 beads and co-cultured with MSCs. After 6 days of co-culture, the expression of CD25 and Foxp3 was assessed by flow cytometry. For functional assays, the "conditioned" sub-population generated in the presence of MSCs was cultured with *Concanavalin A-activated CD4⁺ T cells* labeled with CFSE. After 48 hours, proliferation was measured by flow cytometry.

Results: We demonstrated that the co-cultures of MSCs and CD4⁺ T cells, induced to differentiate into either Th1 or Th17 cells, resulted in an inhibition of T cell proliferation and the generation of CD4⁺CD25⁺Foxp3⁺ T cells (Th1: 1.25% to 25% and Th17 2% to 20% among CD4⁺CD25⁺ T cells; p<0,05). This was observed both during the differentiation process of naïve T cells and on mature Th1 and Th17 cells. *In vitro*, we showed that CD4⁺CD25⁺Foxp3⁺ T-cells generated in the co-cultures significantly suppress T cell proliferation (from 45% to 21%; p<0,05). This functional suppressive effect was associated with a significant increase of IL-10 production in the co-culture supernatants.

Conclusion: This study demonstrates that MSCs contribute to the generation of an immunosuppressive environment via the induction of T cells with a regulatory phenotype and the inhibition of activated T cells. This suppressive effect was associated with the up-regulation of IL-10. This may have important clinical implications for inflammatory and autoimmune diseases.

Inv061

Stem cell therapy for RA

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Rheumatoid Arthritis (RA) is the most common inflammatory arthritis, affecting 0.5% to 1% of the general population worldwide. The current pharmacological management of RA relies on intervention with synthetic or biological disease modifying anti-rheumatic drugs. However, RA remains as an unmet clinical need since a substantial number of patients do not have an adequate response to the current treatments, which are moreover also associated with a series of safety concerns. Human adult mesenchymal stem cells (MSCs) are considered a promising tool for cell therapy in regenerative medicine as well as for treating inflammatory and autoimmune diseases. This is based on the stem cells capacities to differentiate into more specialized cells, to release trophic factors with anti-fibrotic, anti-apoptotic or pro-angiogenic properties, and on their capacity to modulate the immune response. MSCs have been shown to exert immunomodulating properties and to regulate the function of a broad number of immune cells including B lymphocytes, T lymphocytes, macrophages and NK cells. As such, MSCs can control excessive inflammation and support the body to regain homeostasis. Such therapeutic benefit has already been reported in a number of experimental models of inflammatory diseases such as allograft rejection, graft-versus-host disease, experimental autoimmune encephalomyelitis, collagen-induced arthritis, colitis, or sepsis. MSC therefore present a new potential therapeutic approach for treating RA in humans. We have used allogenic mesenchymal adult stem cells (eASC) extracted and expanded from adipose tissue to explore their capacity in treating RA. This eASC based product (Cx611) has been tested in a phase Ib/IIa clinical trial in order to evaluate the safety, tolerability, and optimal dosing, as well as exploring therapeutic

activity upon intravenous administration of eASCS in patients with RA. The Cx611 trial was a multicenter, randomized, single-blind, placebo-controlled with 53 patients having active refractory rheumatoid arthritis. At 6 months, the study met all of its safety endpoints and demonstrated a good safety profile. Moreover, preliminary results suggest Cx611 has the potential to positively impact disease in refractory patients, showing a clear improvement over placebo over three months and a sustained benefit over six months.

The research leading to these results has received funding from the European Union Seventh Framework Programme (FP7/2007-2013) under the grant agreement n° 279174 to TiGenix (REGENER-AR project).

Inv062

Dyskeratosis congenita and related syndromes

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Dyskeratosis Congenita (DC) is a heterogeneous multi-system syndrome exhibiting marked clinical and genetic heterogeneity. In its classical form it is characterised by mucocutaneous abnormalities, bone marrow (BM) failure and a predisposition to cancer. BM failure is the principal cause of mortality and patients display features of premature aging. Studies over the last 15 years have led to significant advances with ten DC genes (*DKC1*, *TERC*, *TERT*, *NOP10*, *NHP2*, *TINF2*, *C16orf57/USB1*, *TCAB1*, *CTC1* and *RTEL1*) having been characterized. Nine of these are important in telomere maintenance. DC is therefore principally a disease of defective telomere maintenance and patients usually have very short telomeres. These genetic advances have led to the unification of DC with a number of other disorders. This includes the severe multi-system disorders Hoyeraal-Hreidarsson and Revesz syndromes as well as a subset of patients with aplastic anaemia, myelodysplasia, leukaemia, liver disease and idiopathic pulmonary fibrosis. This wide spectrum of diseases ranging from classical DC to aplastic anaemia can be regarded as disorders of defective telomere maintenance - "the telomereopathies". These advances have increased our understanding of several human diseases and highlighted the importance of telomere maintenance in human biology. They are also facilitating diagnosis (especially when presentation is atypical) and management.

Inv063

Telomeres as therapeutic targets for cancer and aging

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Recently, our laboratory has continued to dissect the role of telomerase and telomere length as key molecular pathways underlying cancer and aging, as well as addressing the potential use of telomerase activation as a therapeutic strategy for telomere syndromes and age-related diseases (Blasco *et al.*, *Cell*, 1997; Tomás-Loba, *Cell*, 2008). More recently, we have developed a telomerase-based gene therapy strategy that allows telomerase activation in adult organisms and this has shown beneficial effects in a variety of age-related pathologies in mice (Bernardes de Jesus *et al.*, *EMBO Molecular Medicine*, 2012). I will present recent find-

ings, showing the efficacy of this telomerase gene therapy in mouse models of disease. Finally, I will discuss recent findings from our group in collaboration with the Spanish node of the International Cancer Genome Consortium showing the involvement of components of the telomere protection complex, also known as shelterin, in human cancer (Ramsay *et al.*, *Nature Genetics*, 2013).

Inv064

Towards the gene therapy of fanconi anemia with lentiviral vectors

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Fanconi anemia (FA) is a complex genetic disease associated with congenital abnormalities, bone marrow failure (BMF) and cancer predisposition. Allogeneic transplantation constitutes the preferential treatment of the BMF that takes place in most FA patients. We have recently proposed a gene therapy approach of FA-A patients with lentiviral vectors. In a first step, a therapeutic lentiviral vector expressing *FANCA* under the regulation of the PGK promoter was generated (PGK-*FANCA*-WPre⁺ LV). The short incubation (<36 h) of purified CD34⁺ cells from FA-A patients with a pre-GMP FA-LV produced by Genethon facilitated the transduction of 32–85% of these progenitor cells, and efficiently reverted their characteristic hypersensitivity to DNA cross-linking drugs. To test the safety of this vector, Lin⁻ BMCs from *Fanca*^{-/-} mice were transduced overnight with the FA-LV and transplanted into irradiated *Fanca*^{-/-} recipients. Insertion site analyses showed a safe and polyclonal pattern of hematopoietic repopulation both in primary and secondary recipients, consistent with the absence of any myelodysplasia or leukemia produced by insertional oncogenesis. To facilitate the collection of a significant number of CD34⁺ cells in the clinical trial, FA patients will be treated both with G-SCF and with an antagonist of the CXCR4 receptor, plerixafor. Mobilized CD34⁺ cells will then be cryopreserved until the development of the BMF in the patient, or directly transduced and re-infused in FA patients with BMF signs. A European Consortium (EUROFANCOLEN) has been recently created to demonstrate the safety and the efficacy of the proposed gene therapy approach in FA-A patients.

Inv065

Approaches to the study of reprogramming-induced genomic instability in Fanconi anemia (FA) cells and the role of the FA pathway in early hematopoietic commitment

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Fanconi anemia (FA) is a recessive syndrome characterized by progressive fatal BM failure and chromosomal instability. FA cells have inactivating mutations in a signaling pathway that is critical for maintaining genomic integrity and protecting cells from the DNA damage caused by cross-linking agents. Our previous studies have demonstrated that somatic cell reprogramming leads

to activation of the FA pathway, increased DNA double-strand breaks, and senescence of resulting induced pluripotent stem cells (iPS). We also demonstrate that defects in the FA DNA-repair pathway decrease the reprogramming efficiency of murine and human primary cells. To determine the extent to which integrating reprogramming vectors contribute to DNA damage and for the purpose of developing isogenic (corrected and uncorrected) iPS lines, we first corrected Fanconi anemia (FA) human fibroblasts by transducing with a FANCA correction vector containing LoxP sites and generated iPS lines from these corrected cells with non-integrating episomes that express reprogramming factors. We have selected multiple clones by screening for vector integration by PCR and exogenous FANCA expression by western blot. These clones were transduced with a non integrating Cre-expressing vector to delete the correction vector. We are currently analyzing clones for fidelity of deletion of correcting FANCA vector sequences and analysis of chromosomal abnormalities.

Inv066

TCR gene editing for the treatment of hematological malignancies

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The use of Zinc Finger Nucleases (ZFN) designed to permanently disrupt a predefined gene, coupled to lentiviral-mediated gene transfer, enables the precise substitution of a biological function in primary cells. We exploited this technology to completely and permanently redirect T cell specificities toward tumor antigens through editing of the TCR in primary human T cells. This approach is based on the transient transfer of ZFNs designed to target the constant regions of the endogenous TCR α and/or β chain genes, followed by the lentiviral-mediated transfer of a tumor-specific TCR. By disrupting a single TCR chain gene (TCR single editing), we completely eliminate the entire endogenous TCR repertoire, thus allowing maximal expression of the tumor-specific TCR. The elimination of the endogenous repertoire is particularly relevant in the allogeneic setting where unmodified T cells would be expected to mediate GvHD. Furthermore, by disrupting both the α and β TCR chain genes (TCR complete editing), we completely and permanently eliminate the risk of TCR mispairing (Provasi *et al.*, Nat. Med. 2012). Here, using a tumor-specific TCR targeting NY-ESO-1, a cancer-testis antigen expressed by solid tumors and hematological malignancies, we observed that both single and complete edited T cells express higher levels of the NY-ESO-1 specific TCR than unedited T cells. Single and complete edited T cells were also more efficient than unedited-TCR transferred T cells in killing NY-ESO-1-pulsed cell lines and NY-ESO-1 /HLA-A2 myeloma cell lines. Importantly, the TCR edited cells displayed no activity against non-NY-ESO-1

expressing targets. These data suggest that the combination of TCR editing and lentiviral gene transfer of NY-ESO-1 specific TCRs may generate a superior targeted cellular immunotherapy with the potential to be deployed in the allogeneic setting.

Inv067

Progress in gene therapy clinical trials

M. Cavazzana

Inv068

Gene therapy for primary immunodeficiencies

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At the start of the 1990s, the first clinical trials of gene therapy were attempted for an inherited severe combined immunodeficiency (SCID) caused by deficiency of the intracellular enzyme, adenosine deaminase. In the absence of definitive treatment, SCID of any molecular type is usually fatal within the first year of life, although patients with ADA deficiency can be supported by administration of exogenous enzyme replacement. Even so, this is often only partially effective, and is extremely expensive. The rationale for the development of gene therapy for SCID therefore derives from the severity of the illness, the inadequacy of conventional therapy, and the considerable morbidity and mortality associated with stem-cell transplantation, particularly from a mismatched donor. Over the last decade gene transfer technology and cell handling protocols have been refined sufficiently to produce real clinical benefit not only in SCID but also in other immunodeficiencies including Wiskott-Aldrich Syndrome and Chronic Granulomatous Disease. Bearing in mind the outcome and adverse effects of conventional therapy, these are remarkable results and the first clear indication that gene therapy can offer a cure for some human diseases. In a few patients, the treatment has failed, indicating that there is more to learn about the effective dose of corrected cells and the potential for host factors to influence immune cell development. Although retroviruses are highly effective for gene transfer to haematopoietic cells, their dependence on chromosomal integration brings with it, the risk of inadvertent gene activation or inactivation. Having initially achieved successful immunological reconstitution, several patients treated using LTR-intact gammaretroviral vectors have developed malignant disease. Recent modifications to vector design including use of SIN-configurations of both gammaretroviral and lentiviral vectors, and more physiological regulatory elements are now in clinical trials for a number of PIDs. Although follow up is relatively short, these studies and trials are showing excellent efficacy, without toxicity or evidence for clonal outgrowth to date. The latest results from some of these trials will be discussed.

Inv069

A. Aiuti

Inv070

Phase I/II clinical trial of HSC gene therapy for the treatment of metachromatic leukodystrophy

A. Biffi

Inv071

Hematopoietic stem cell gene therapy with lentiviral vector in 4 patients with cerebral X-linked adrenoleukodystrophy: long-term outcome and comparison of efficacy with allogeneic hematopoietic stem cell transplantation

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The most severe form of X-linked adrenoleukodystrophy (ALD) is characterized by rapidly progressive and lethal cerebral demyelination in childhood. The progression of cerebral demyelination of ALD can be arrested by allogeneic hematopoietic stem cell (HSC) transplantation (HCT), provided the procedure is performed at an early stage of the disease. The long term beneficial effects of HCT in ALD are likely due to the progressive replacement of brain microglia that are derived from early myeloid progenitors or HSCs. Despite the increased availability of cord blood, not all boys with cerebral ALD who are candidate for allogeneic HCT have a suitable HLA-matched donor. In addition, allogeneic HCT remains associated with significant mortality and morbidity risks. Herein we report the long-term efficacy of HSC gene therapy with lentiviral vectors in 4 boys with cerebral ALD. Clinical efficacy as well as serious adverse events of gene therapy will be compared to that observed in a serie of 33 ALD boys with cerebral ALD who had been transplanted since 2000 with the same inclusion criteria as for the the 4 ALD boys treated with gene therapy. Data on hematopoiesis clonality and gene marking in long-term will be presented.

Inv072

Gene therapy for thalassemia: the challenge of a new cure

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Beta-thalassemia is a severe congenital anemia caused by reduced or absent beta-globin chain production of the adult hemoglobin tetramer. More than 300 mutations leading to the disease have been described, affecting all the steps related to the expression of the β -globin gene. It represents the most common autosomal recessive syndrome to cause a major health problem worldwide with an estimated annual birth incidence of 40.000/year. Profound anemia, rapid expansion of erythroid bone marrow mass and

secondary organ damage due to iron overload are the most frequent clinical manifestation of the severe form of beta-thalassemia major. Treatment of beta-thalassemia is essentially supportive. Patients require a lifelong transfusion regimen combined with iron chelation therapy to reduce hemosiderosis that is ultimately fatal, if not continuously treated. At present, the only curative approach is represented by allogeneic hematopoietic stem cell transplantation (HSCT), however this is limited by HLA compatibility and toxicity, due to graft versus host disease, graft rejection and immunosuppressive regimens required. Medical management and HSCT are both therapeutic options that could improve the quality of life and survival of thalassemic patients. Nevertheless, they are both burdened by complications and limitations, outlining the need for testing innovative curative approaches. For these reasons, beta-thalassemia has been the first candidate disease for treatment by gene therapy. However, only the development of lentiviral vectors (LV) and the optimization of hematopoietic stem/progenitor cells (HSPC) transduction have progressed in pioneering the treatment on the first patient in France, who will benefit from the production of hemoglobin, partially contributed by the vector. Our studies are aimed to the clinical development of an innovative and safe gene therapy approach, relying on high-titer globin vectors, new sources of HSPCs and a transplantation regimen favouring efficient engraftment of genetically modified cells in the absence of toxicity. Our major contributions to the field, from development and production of beta-globin vectors to disease correction and proof of safety in preclinical models, will be presented. Recently, results from the molecular and functional characterization of a new source of mobilized HSPCs, which are efficiently transduced by globin LV, shows that these cells are endowed with a primitive phenotype and very high *in vivo* reconstitution potential, representing the optimal target for gene transfer. Overall, these results provide the fundamental basis to the proposal of our clinical trial for beta-thalassemia.

Inv073

Advanced therapies for the treatment of erythroid metabolic diseases: The pyruvate kinase deficiency

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Pyruvate Kinase Deficiency (PKD) is a monogenic metabolic disease caused by mutations in the PKLR gene that impairs

energetic balance in erythrocytes causing hemolytic anemia in a very variable range and can be even fatal during neonatal period. PKD recessive inheritance trait and its curative treatment by allogeneic bone marrow transplantation, provide an ideal scenario for developing gene therapy approaches. With this aim, we have designed a preclinical protocol based on hematopoietic stem cell (HSC) genetic modification and transplantation into PKD mice. The therapeutic lentiviral vector developed included the hPGK eukaryotic promoter driving the expression of the PKLR cDNA, achieving clinically relevant levels of the protein and an efficient and safe correction of PKD phenotype. Although successful addition gene therapy trials are currently being applied in the clinics, ideal gene therapy will be based on gene correction strategies based on homologous recombination (HR). Induced pluripotent stem cells (iPSC) were first generated from peripheral blood mononuclear cells (PB-MNC) of PKD patients (PKDiPSCs) using Sendai vectors. Thereafter, the genetic defect of these cells was corrected by TALEN mediated HR in the PKLR locus. As deduced from the normal erythroid maturation profile of corrected PKDiPSCs, the compromised erythroid differentiation of these cells was restored. All together we have developed advanced tools for the gene correction of PKD that could be used in the near future either for addition gene therapy or in the long term for site specific gene therapy, serving as model for other erythroid metabolic diseases.

Inv074

Gene therapy for sickle cell disease

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Sickle cell disease (SCD) is the first human disease to be defined at the molecular level. The canonical E6V mutation in the beta-globin chain of adult hemoglobin leads to polymerization of the HbS under low oxygen conditions, causing RBC deformation and rigidity; the clinical complications that result include severe vaso-occlusive episodes and hemolytic anemia, with chronic progressive organ damage. SCD can be improved by transplantation of normal hematopoietic stem cells (HSC) from a well-matched donor, but most patients do not have a suitable donor. Transplantation of autologous HSC that have been gene-engineered to prevent sickling of the RBC they produce could provide similar clinical benefits to a wider range of patients, without the immunological complications of allogeneic HSC transplantation. Under a CIRM Disease Team Award (DR1-01452), we have performed pre-clinical studies on the activity and safety of a lentiviral vector with a modified human beta-globin gene (Lent/ β^{AS3} -FB) to transduce HSC from SCD patients and prevent RBC sickling. These studies demonstrate sufficient gene transfer and expression of the β^{AS3} -globin gene to prevent sickling in RBC produced *in vitro* from CD34+ cells from the bone marrow of SCD donors. A clinical trial is planned to evaluate the safety and efficacy of this approach. An alternative approach involves direct correction of the E6V β^S -globin mutation in HSC by site-directed gene modification. We have used zinc finger nucleases targeted to the region of the sickle mutation to augment homologous recombination-mediated gene repair. Using electroporation of *in vitro* transcribed mRNA encoding the ZFN pair and co-delivery of corrective donor constructs by either integration-defective lentiviral vectors or as an oligonucleotide, we can routinely specifically reverse the sickle

mutation in 5–10% of primary human bone marrow CD34+ cells. Scale-up studies are underway to translate this approach to the clinic. These new methods of gene addition and gene correction may provide novel, safe and effective approaches to treating SCD by autologous stem cell gene therapy.

Inv075

VEGF gene therapy in ischemic myocardium

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Therapeutic angiogenesis is a potentially useful treatment strategy for ischemic heart disease and peripheral arterial occlusive disease. Arteriogenesis is a process caused by increased shear stress at the arteriolar level resulting in the formation of large conduit vessels from preexisting small vessels. Angiogenesis involves growth and sprouting of capillaries and smaller vessels in ischemic tissues. Most commonly used growth factors for therapeutic angiogenesis are vascular endothelial growth factors (VEGF) and fibroblast growth factors (FGF). Some other cytokines and growth factors may also have angiogenic effects *in vivo*. Improved perfusion can be achieved by angiogenesis and arteriogenesis. However, best delivery methods and growth factors causing potentially useful effects in man remain to be determined.

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Inv076

Drug and cell delivery systems for cardiac repair

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Myocardial infarction is the leading cause of death worldwide. Since classical therapies produce more palliative than regenerative effects, extensive research has been performed to find an effective cure. New therapies, like growth factor and cell therapies, are arousing great interest. However, the clinical trials performed until now have demonstrated that although they are promising, their efficiency is limited due to some drawbacks, such as protein short half-life or low cell survival rate. With a view to reducing or eliminating their limitations, the interest in combining these therapies with drug delivery systems (DDS) has increased over the last few decades. The development of new materials have facilitated a number of studies over the last ten years using DDS in animal models of myocardial infarction suggesting that some of the current limitations may be overcome.

In my presentation I will review some of the current results we have obtained using PLGA particles loaded with different growth factors in small and large animal models of myocardial infarction in order to assess the possible benefits produced by the combination of DDS with protein and/or cell therapies in regeneration after myocardial infarction.

Inv077

Adult cardiac stem cells in mammalian heart homeostasis and repair are defined by Bmi1 expression

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The mammalian adult heart has a limited capacity to respond to acute damage, but accumulated evidence nonetheless indicates a continuous low-rate turnover throughout life. This turnover is thought to reflect mainly the activity of a reservoir of cardiac stem cells (CSCs), although recently, it has been also proposed the contribution of adult cardiomyocytes to adult heart homeostasis in mouse. One of the main gaps in CSCs field has been the absence of conclusive lineage tracing analyses that would define the localization and evolution of adult CSCs and trace their progeny. The Polycomb member Bmi1 has been established as a key transcription factor to identify and define adult stem cell compartments in some tissues, being a master regulator of the self-renewal of hematopoietic and neural systems, among others. Therefore, we have developed, for the first time, a lineage tracing strategy taking advantage of the Bmi1IresCreER/Rosa26YFP mice model. The adult mouse heart contains a resident stem cell population associated by the expression of Bmi1 (B-CSCs). B-CSCs constitute a subpopulation of the Sca-1+ fraction and are negative for the expression of the hematopoietic marker CD45 and c-Kit. The study of the whole transcriptome of B-CSCs revealed the expression of multipotent genes such as Oct-4, CD133 and Suz12 as well as transcripts related to the three lineages of the heart, supporting their tripotency. *In vivo*, B-CSCs are detected at low levels, localized in cramped cell structures (niche-like) spread throughout the heart. We have also traced them in homeostasis and demonstrated that B-CSCs contribute to the formation of new cardiomyocytes (YFP+), whose proportion increases along lifespan (2% at two months after the tamoxifen induction and up to 10% after two years). Furthermore, B-CSCs activate after an experimental myocardial infarction and respond to injury contributing to the creation of the *novoo* cardiomyocytes rising up to 21% four months after the infarction. The new cardiomyocytes are fully differentiated; they beat and exhibit the same calcium transient profile that the YFP- cardiomyocytes. In conclusion, B-CSC cells represent an undifferentiated resident subpopulation capable to contribute to the main heart cellular lineages, *in vitro* and *in vivo*. B-CSCs also respond to an acute injury, and their progeny contribute to the repair tissue. All these

features make B-CSCs a strong candidate to contain the stem reservoir in the adult heart.

Inv078

GDNF-based cell therapy in Parkinson's Disease

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The Glial cell line-Derived Neurotrophic Factor (GDNF) exerts a powerful neuroprotective effect on dopaminergic (DA) nigrostriatal neurons both *in vitro* and *in vivo*. We have investigated whether intrastriatal grafting of GDNF-producing cells has beneficial effects on preclinical and clinical models of Parkinson's disease (PD). We used cells from the carotid body (CB), a bilateral arterial chemoreceptor located in the bifurcation of the carotid artery, which contains cells that are highly dopaminergic and secrete large amounts of GDNF. Intrastriatal transplantation of CB tissue had clear beneficial effects on rodent and monkey models of PD. Autotransplantation of CB cell aggregates has also proved to be a safe and efficacious procedure in PD patients, although it showed large variability among the cases studied. As the amount of GDNF-producing cells provided by a single CB is probably too small, we have explored the possibility of expanding the CB tissue *in vitro* prior transplantation. We have identified a population of adult neural crest-derived stem cells in the rodent CB that sustains the growth of this organ during acclimatization to chronic hypoxia. In floating cultures, CB stem cells can form clonal colonies consisting of a core of neural progenitors and peripheral blebs of differentiated dopamine- and GDNF-producing neuron-like glomus cells. Intrastriatal grafting of CB-derived neurospheres induces amelioration of parkinsonian rodents. CB cells from human donors have the same physiological and neurochemical properties as CB from rodents. The potential therapeutic use of human CB-derived neurospheres is currently under evaluation.

Inv079

N. Cartier

Inv080

Intra cerebral administration of AAVrh.10 carrying human SGSH and SUMF1 cDNAs in children with MPSIIIA disease: result of a phase I/II trial

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Mucopolysaccharidosis type IIIA is a severe degenerative disease due to an autosomal recessive genetic defect in the gene coding for the lysosomal N-sulfoglycosamine sulfohydrolase (SGSH) whose catalytic site is activated by a sulfatase modifying factor (SUMF1). Four children (patient 1–3 close to 6 years of age,

patient 4 was 2ye8m) received intra-cerebral injection of an AAV-2/rh.10-SGSH-IRES-SUMF1 vector in a phase I/II clinical trial. All children were ambulatory but had already decreased cognitive abilities (marked in P1-3, moderate in P4) and for P1-3, brain atrophy detectable on MRIs. The therapeutic vector was delivered at a dose of 7.2×10^{11} viral genomes/patient in 12 preplanned simultaneous frameless stereotaxic deposits (60 μ l) in 2 hours, bilaterally within white matter anterior, medial and posterior to basal ganglia. In order to prevent elimination of transduced cells, an immunosuppressive treatment (Mycophenolate mofetil, Tacrolimus) was initiated 15 days before surgery and maintained either 8 weeks (Mycophenolate-mofetil) or throughout the follow-up (Tacrolimus, with progressive dose reduction). Safety data collected from inclusion, during the neurosurgery period and over the year of follow-up showed a good tolerance, a lack of adverse events related to AAV vector, no increase of infectious events and no biological sign of toxicity related to immunosuppressive drugs. Efficacy analysis can only be preliminary in a Phase I/II trial on 4 children and in absence of validated surrogate markers. However, neuropsychological evaluation suggested a possible improvement of behaviour, attention and sleep in P1-3 who showed cognitive decline before treatment. Neurocognitive benefit (motricity, language) was more likely present in the youngest patient.

Inv081

Reduction sensitive nanogels from side chain functional polyglycidols as non-viral transfection systems

J. Groll

Inv082

Designing gold nanoparticles for *in vivo* gene silencing as a new therapeutic tool

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Small interfering-RNAs (siRNA) show significant potential in new molecular approaches to down-regulate gene expression in cancerous cells. However, there are still significant obstacles to be overcome such as its short half-lives and degradation by RNases. We have developed effective conjugation strategies to combine biomolecules to the surface of AuNPs with specific functions such as cell penetrating peptides to overcome the cellular membrane barrier, quaternary ammonium to introduce stable positively charged in AuNPs surface, tumoral markers and siRNA complementary to a master regulator gene, the proto-oncogene c-Myc. Two approaches were designed for the binding of all these molecules to the nanoparticles, the use of a thiolated siRNA for binding covalently to the surface of the nanoparticles; and by ionic interactions incorporation positive charge to the nanoparticles. These library of novel gold nanoparticles have been tested using a hierarchical approach including three biological systems of in-

creasing complexity: *in vitro* cultured human cells, *in vivo* invertebrate (freshwater polyp, Hydra) and *in vivo* vertebrate (mouse) model. Selection of the most active functionalities was assisted step by step through functional testing adopting this hierarchical strategy. Merging these chemical and biological approaches lead to a siRNA/RGD gold nanoparticle capable of targeting tumor cells in lung cancer xenograft mouse model, resulting in successful and significant c-myc oncogene downregulation followed by tumor growth inhibition and prolonged survival of the animals.

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Inv083

Advanced technologies for engineering functional cardiac tissues

T. Dvir

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The heart is a non-regenerating organ. Consequently, the loss of cardiac cells and formation of scar tissue after extensive myocardial infarction frequently leads to congestive heart failure. Given the scarcity of cardiac donors, a potential approach to treat the infarcted heart is to repopulate the 'dead zone' with cells capable of spontaneous contraction. Cellular therapy evolved to introduce cells into diseased areas and regain function. However, two main drawbacks of this approach are the lack of control of cell accumulation site after injection, and cell death before forming cell-cell or cell-matrix interactions. These shortfalls motivated the development of the tissue engineering concept, where 3-dimensional (3D) biomaterials serve as extracellular matrix-like scaffolds to the cells, enabling the cells to assemble into effective tissue substitutes, that may restore tissue or organ function. After transplantation the scaffolds either degrade or metabolize, eventually leaving a vital tissue instead of the defected tissue. In this talk I will describe cutting-edge technologies for engineering functional cardiac tissues, focusing on synthetic and natural biomaterials and the use of inorganic nanostructures for improving tissue function. In addition, I will present our engineered cyborg tissues, involving fusion of digital world with biomaterials and cells for controlling and monitoring the performances of engineered tissues.

Inv084

Engineered T cells for cancer therapy

C. June

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Adoptive T cell transfer for cancer and chronic infection is an emerging field that shows promise in recent trials. Synthetic-biology-based engineering of T lymphocytes to express high-affinity antigen receptors can overcome immune tolerance, which has been a major limitation of immunotherapy-based strategies. Advances in cell engineering and culture approaches to enable efficient gene transfer and *ex vivo* cell expansion have facilitated broader evaluation of this technology, moving adoptive transfer from a "boutique" application to the cusp of a

mainstream technology. Recent observations from ongoing clinical trials in patients with leukemia and carcinomas will be discussed. As the field of adoptive transfer technology matures, the major engineering challenge is the development of automated cell culture systems, so that the approach can extend beyond specialized academic centers and become widely available.

Inv085

Of CARs and TRUCKs: Chimeric antigen receptor (CAR) redirected T cells with inducible IL-12

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Adoptive therapy with antigen-specific T cells is achieving impressive efficacy in early phase trials, in particular in hematologic malignancies, strongly supporting the notion that the immune system can control cancer. A current strategy of favor is based on *ex vivo* engineered T cells which are redirected by a chimeric antigen receptor (CAR) and recognize target cells in a pre-defined fashion by an antibody-derived binding domain. Such CAR T cells can substantially reduce the tumor burden as long as the targeted antigen is present on the cancer cells, however the loss of MHC or immunogenic antigens make them invisible to cytotoxic T cells and may contribute to deadly tumor relapses. We present an effective cell-based strategy to attack antigen-loss cancer cell variants by combining antigen-directed T cell therapy with the inducible release of a cytokine. Cytotoxic T cells were engineered to release inducible IL-12 upon CAR engagement in the targeted tumor lesion, which in turn attracts an innate immune cell response towards those cancer cells that are invisible to CAR T cells. Such TRUCKs, T cells redirected for universal cytokine-mediated killing, exhibited remarkable efficacy against solid tumors with diverse cancer cell phenotypes. CAR IL-12 T cells produced accumulation of activated macrophages in the targeted lesion that was critical to the anti-tumor response. Inducible cytokine supplementation by CAR redirected T cells allows to target otherwise inaccessible tumor lesions, in a manner associated with reduced systemic cytokine toxicity, by recruiting and activating innate immune cells for a pro-inflammatory response.

Inv086

Adoptive immunotherapy with CAR-modified T cells

G. Dotti

Baylor College of Medicine, Houston, Texas, USA

Chimeric antigen receptors (CARs) combine the antigen binding property of monoclonal antibodies with the cytolytic capacity of T-lymphocytes. There has been interest in generating T cells expressing CARs targeting antigens expressed in a broad array of hematological malignancies and solid tumors. Anti-tumor activity of redirected T cells both *in vitro* and *in vivo* are independent of MHC restriction and can be increased by co-expression of different costimulatory molecules within the CAR or by expressing CARs on T cells with a well defined antigen specificity, such as Epstein-Barr specific cytotoxic T cells (CTLs) or CMV-specific CTLs. Dr Dotti will report some of the preclinical and clinical results of CAR-based T cell therapies currently ongoing at Baylor College of Medicine.

Inv087

A Phase I safety study in subjects with severe hemophilia B using a single-stranded AAV8 vector to deliver the gene for factor IX

K.A. High, M. Ragni, P. Fogarty, L. Raffini, F. Mingozzi, J. Wellman, A. Lange, V. Arruda, X. Anguela, F. Wright, B. Hauck, E. Basner-Tschkarajan

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Previous work has shown the safety and efficacy of infusion of an AAV8 vector carrying a self-complementary construct encoding human Factor IX in men with severe hemophilia B, with long-term expression of Factor IX levels in the range of 5%, sufficient to convert severe hemophilia to mild disease. We wish to determine whether the self-complementary conformation is critical to the success of the approach, or whether a single-stranded construct could also drive long-term expression at therapeutic levels. The rationale for this study is that production of single-stranded AAV (ssAAV) vectors in our manufacturing process is more efficient, with yields typically 2–4 times higher than similar self-complementary constructs. In addition, Martino and colleagues (Blood 2011) have presented evidence in mouse models that self-complementary constructs increase TLR-9-dependent innate immune responses in the liver compared to ssAAV. We constructed a single-stranded codon-optimized Factor IX construct under the control of a liver-specific promoter, and produced vector in an AAV8 capsid. Direct comparison of this vector to a self-complementary F.IX-encoding vector in an AAV8 capsid in mice suggested similar levels of efficacy in this species. Based on studies in mice and non-human primates (Mingozzi *et al.*, Science Translational Medicine 2013), we also added empty capsids in a defined ratio to the final formulation of the clinical grade vector, to act as decoys to adsorb circulating antibodies to AAV. We screened subjects with severe hemophilia B and identified two subjects with low-titer antibodies to AAV8. After informed consent, infusion of AAV8-ssFIX at a dose of 1×10^{12} vg/kg occurred uneventfully. Kinetics of gene expression and immune responses will be discussed.

Inv088

AAV-mediated gene transfer in patients with severe hemophilia B: Longer follow-up and expansion of the high dose cohort

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We have previously reported the preliminary safety and efficacy data of our novel gene transfer approach in six subjects with

severe haemophilia B (Nathwani *et al*, NEJM 365:2357–65, 2011). In brief, this entailed peripheral vein infusion of a serotype-8 pseudotyped self-complementary adeno-associated viral (scAAV) vector expressing a codon-optimised coagulation factor IX (FIX) transgene (scAAV2/8-LP1-hFIXco) at one of three vector doses: 2×10^{11} (low); 6×10^{11} (intermediate) and 2×10^{12} (high) vector genomes (vg)/kg. A dose dependent increase in AAV-mediated expression of FIX at 1–6% of normal was observed in these individuals over an initial follow-up of between 6–14 months. Extended follow-up of these individuals shows that FIX levels have remained stable in each of the initial participants over a period which is now greater than 2½ years following vector infusion. In the subsequent phase of this study, we recruited 4 additional severe haemophilia B subjects to the high dose cohort in order to better understand the safety and efficacy profile of scAAV2/8-LP1-hFIXco. As before, scAAV2/8-LP1-hFIXco was administered without upfront immunosuppression at the time of vector administration. The infusion of vector was well tolerated with no acute toxicity. Peak FIX activity in all four participants was >5%, which is in keeping with what was observed in two subjects previously recruited at this dose level. The mean steady state level for the entire high dose cohort (N=6) is 5% of normal levels, which is sufficient to convert the bleeding phenotype from severe to mild. Consistent with this, 5/6 subjects treated at the high dose level have not reported spontaneous bleeding episodes despite not being on regular FIX prophylaxis. Transient asymptomatic increase in alanine transaminase over baseline values was observed in two of the four subjects recruited, occurring during weeks 7–9 post infusion. The transaminitis resolved promptly and completely in both cases in response to a short course of oral prednisolone while FIX expression was maintained. Thus, these results demonstrate that FIX expression is stably maintained at therapeutic levels for at least 2.5 years following AAV-mediated gene transfer to the liver. Immune-mediated destruction of transduced hepatocytes appears to have occurred between 7–9 weeks after gene transfer in approximately two-thirds of the patients recruited at the high dose but was controlled with transient immunosuppressive treatment with oral prednisolone. These data are highly encouraging and suggest that AAV mediated gene transfer has the potential to change a severe bleeding phenotype of haemophilia B into a milder form for a prolonged period of time.

Inv089

Overcoming the challenges in gene therapy for haemophilia

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The advancement in gene transfer technologies has greatly benefited the field of gene therapy of hemophilia. The main challenges associated with the development of an effective and safe gene therapy for hemophilia relates to (i) obtaining high and sustained clotting factor expression and (ii) minimizing immune

responses to the clotting factors and/or the gene-modified cells. To improve FVIII and FIX expression for liver-directed gene therapy, we validated a data-mining algorithm that led to the identification of combinations of regulatory elements, associated with high tissue-specific expression. Incorporation of these *de novo* designed, synthetic regulatory elements in viral and non-viral vectors enhanced FVIII and FIX expression at the transcriptional level in liver. Moreover, vector performance could be further enhanced by using synthetic codon-optimized and/or hyperactive transgenes that outperformed their wild-type counterparts. This was validated in normal or hemophilic mouse models using AAV, integration-competent LV, integration-defective LV and hyperactive transposons. The hepatocyte-restricted expression contributed to the induction of immune tolerance against the wild-type or hyperactive clotting factors. We extended some of these studies in rhesus monkeys and showed that therapeutic human FIX levels (up to 30%) could be obtained with no adverse side effects. One of the main limitations of using AAV-based vectors is that they can evoke an AAV capsid-specific, T cell-mediated immune response that eliminate transduced hepatocytes and curtail gene expression. To reduce the risk of T cell-mediated immune rejection of AAV-transduced cells, we have now generated and characterized a novel "immune stealth" AAV that was specifically designed to inhibit antigen presentation. We demonstrated that T-cell activation and T-cell mediated recognition of liver cells transduced with the AAV2 "immune stealth" vector was substantially reduced, while preserving transduction efficiency *in vivo*. This novel AAV platform may have broad implications for AAV-based gene therapy in general beyond hemophilia *per se*.

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Inv090

Fucosylated mesenchymal stem cells to treat systemic bone diseases

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Mesenchymal stem cells (MSC) are used for human cellular therapies. Local infusion is unsatisfactory in systemic diseases and intravenous administration fails because few cells reach the target organ due to poor homing capacity. Osteoporosis is a major cause of morbidity and mortality. MSC can differentiate into osteoblasts and, therefore, could positively alter the equilibrium of bone formation/destruction.

We hypothesized that adding a fucose residue in α 1-3 position of the MSC CD44 antigen using fucosyltransferase VI would yield HCELL, a potent E-selectin ligand that programs homing to bone marrow, thus driving osteotropism of intravenously infused MSC. We assessed the safety and efficacy of human fucosylated MSC (fMSC) in NOD/SCID mice, comparing 3 groups of 21, 6, and 4 mice receiving 1×10^6 fMSC, 1×10^6 MSC, or saline intravenously. Acute and chronic toxicities were evaluated by clinical and histological examination. Efficacy was assessed by immunohistochemistry using anti-human osteocalcin in sections

of calvarium and femur. There was no deaths or acute toxicity. One mouse in the saline arm had mild chronic toxicity (piloerection, hunched hair, moderate behavior changes and partial alopecia). The histology of heart, liver, kidney, spleen, gonads, brain and bone marrow was normal. There were localized areas of inflammation in the lung of 41%, 17% and 25% of fMSC, MSC, and saline groups respectively ($p=0,44$). Osteocalcin-positive osteoblasts were observed in femur and calvaria of all mice infused with fMSC, in 3 of 6 infused with MSC, and none in the saline group ($p=0,004$). The fMSC were detected inside the bone from the 2nd to the 12th week post-infusion. Our results show that the systemic infusion of human fMSC is safe and effective in guiding the cells to bone with the potential to increase bone deposition. These results form the basis for an ongoing human clinical trial of fMSC in patients with osteoporosis.

Inv091

Engineering of hydrogel-based bioinks for the fabrication of cell-laden 3D constructs

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With advances in tissue engineering, the possibility of regenerating injured tissue or failing organs has become a realistic prospect for the first time in medical history. Tissue engineering – the combination of bioactive materials with cells to generate engineered constructs that functionally replace lost tissue – is a major strategy to achieve this goal. One facet of tissue engineering is biofabrication, where three-dimensional tissue-like structures composed of materials and cells in a single manufacturing procedure are generated. Cell-laden hydrogels are commonly used in biofabrication and are termed “bioinks”. Hydrogels are particularly attractive for biofabrication as they recapitulate several features of the natural extracellular matrix and allow cell encapsulation in a highly hydrated mechanically supportive three-dimensional environment. Additionally, they allow for efficient and homogeneous cell seeding, can provide biologically-relevant chemical and physical signals and can be formed in various shapes and biomechanical characteristics. However, while advances in modifying hydrogels for enhanced bioactivation, cell survival and tissue formation, little attention has so far been paid to optimize hydrogels for the physico-chemical demands of the biofabrication process. The resulting lack of hydrogel bioinks have been identified as one major hurdle for a more rapid progress of the field. The successful development of a “printable” hydrogel that support cell adhesion, migration and differentiation will significantly advance this exciting and promising approach for tissue regeneration.

Inv092

Bone regeneration based on tissue engineering conceptions – A 21st century perspective

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The role of bone tissue engineering in the field of regenerative medicine has been the topic of substantial research over the past two decades. Technological advances have improved orthopaedic

implants and surgical techniques for bone reconstruction. However, improvements in surgical techniques to reconstruct bone have been limited by the paucity of autologous materials available and donor site morbidity. Recent advances in the development of biomaterials have provided attractive alternatives to bone grafting expanding the surgical options for restoring the form and function of injured bone. Specifically, novel bioactive (second generation) biomaterials have been developed that are characterised by controlled action and reaction to the host tissue environment, whilst exhibiting controlled chemical breakdown and resorption with an ultimate replacement by regenerating tissue. Future generations of biomaterials (third generation) are designed to be not only osteoconductive but also osteoinductive, i.e. to stimulate regeneration of host tissues by combining tissue engineering and in situ tissue regeneration methods with a focus on novel applications. These techniques will lead to novel possibilities for tissue regeneration and repair. At present, tissue engineered constructs that may find future use as bone grafts for complex skeletal defects, whether from post-traumatic, degenerative, neoplastic or congenital/developmental “origin” require osseous reconstruction to ensure structural and functional integrity. Engineering functional bone using combinations of cells, scaffolds and bioactive factors is a promising strategy and a particular feature for future development in the area of hybrid materials which are able to exhibit suitable biomimetic and mechanical properties. This review will discuss the state of the art in this field and what we can expect from future generations of bone regeneration concepts.

Inv093

Genome editing with zinc finger nucleases

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The ability to engineer the precise genetic modifications of human primary cells would both accelerate research and extend the range of their potential therapeutic application. This possibility is now being realized via the use of zinc finger nucleases (ZFNs). ZFNs are customizable, sequence-specific endonucleases that can be designed to introduce a discrete cleavage event at any user-chosen location within the genome. By adjusting conditions under which the cleavage event is subsequently repaired, one may efficiently and precisely disrupt or edit the targeted locus, or integrate a larger, gene-sized DNA fragment. This talk will describe our recent work on developing novel cell-based therapies for treating HIV, including preclinical proof-of-concept studies towards the development of autologous, CCR5-disrupted CD34+ hematopoietic stem cells as a treatment for HIV. Data from Phase I studies evaluating infusion of an autologous CCR5-modified T-cell product (SB-728-T) in HIV-infected subjects will also be presented.

Inv094

Targeted Transgene Integration in Human Hematopoietic Stem Cells and Induced Pluripotent Stem Cells from Normal Donors and SCID-X1 Patients

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Gene targeting by homologous recombination holds great promise for gene therapy, as it may overcome the risks of

insertional mutagenesis and uncontrolled transgene expression associated with the use of conventional gene transfer vectors. The development of artificial nucleases, such as Zinc Finger Nucleases (ZFNs), has brought the possibility of targeted integration and gene correction within the reach of gene therapy. We have previously shown that a ZFN-induced DNA double strand break at a predetermined site of the genome can trigger homology-directed repair (HDR), a pathway that can be exploited to insert new sequences with high efficiency and specificity into the ZFN target site. Here we extended these studies to correct mutations in Hematopoietic Stem/Progenitor Cells (HSPC) and induced Pluripotent Stem Cells (iPSC) from normal donors and X-linked Severe Combined Immunodeficiency (SCID-X1) patients, respectively. This disease, which is caused by mutations in the Interleukin-2 Receptor Common Gamma Chain (*IL2RG*) gene, is an ideal candidate for ZFN-driven gene editing, as HSPC-based gene therapy trials performed with randomly integrating vectors showed clinical benefits but also a high rate of leukemia due to insertional mutagenesis and uncontrolled transgene expression. To achieve ZFN-mediated targeted insertion in HSPC, we developed a combined gene delivery protocol based on integrase-defective lentiviral vectors to deliver a donor template DNA for HDR and mRNA nucleofection to drive a short but robust period of ZFN expression. By using this protocol we inserted a corrective *IL2RG* cDNA downstream of its own endogenous promoter with high efficiency and specificity in HSPC. Importantly, gene edited HSPCs sustained long-term, multilineage repopulation in transplanted mice, and gave rise to functional lymphoid cells endowed with selective growth advantage over those carrying disruptive mutations of the *IL2RG* gene. In parallel, we have explored the use of patient-derived iPSC as a potentially unlimited source of gene-corrected HSPC. Using the ZFN technology we inserted the corrective *IL2RG* cDNA in fibroblasts from SCID-X1 patients. Since fibroblasts do not express *IL2RG*, we included downstream of the corrective cDNA a loxP-flanked drug-selection cassette, and efficiently reprogrammed the selected cells to iPSC by using a single-copy, Cre-excisable Lentiviral Vector (LV) expressing the reprogramming factors. Finally, we showed that only the gene-corrected SCID-X1 iPSCs were capable of differentiating into T-lymphocytes *in vitro*. Overall, these studies demonstrate the successful application of ZFN-mediated gene targeting and correction in normal donors and patient-derived HSPC and iPSC, and provide a path to the development of a more precise and safer genetic therapy for SCID-X1 and conceivably, several other diseases.

Ino095

Gene editing

T. Cathomen

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Designer nucleases, such as zinc-finger nucleases (ZFNs) and transcription activator-like effector nucleases (TALENs), are powerful tools to edit the human genome in a targeted fashion. Designer nuclease technology has experienced a remarkable development in the last few years with regard to both platforms to generate these nucleases and ways to apply them *in vivo* and *ex vivo*. As a result, ZFNs and TALENs have been successfully employed to knockout or correct disease-related genes in a variety of primary human cells, including T cells, hematopoietic stem cells, and induced pluripotent stem cells (iPSC). In my talk, I

will provide an overview of ongoing research in my lab, including a side-by-side comparison between ZFN and TALEN technologies as well as our efforts to apply designer nucleases in iPSC to model and treat chronic immune deficiencies.

Ino096

Protein, cell and gene therapies for rare skin disorders

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Currently the vast majority of hereditary skin diseases (genodermatosis) have no cure. However, significant efforts are being made to develop effective therapeutic strategies that include gene, cell and protein therapies. Evaluating the efficacy and safety of these new approaches requires clinically relevant models. Although transgenic mouse models (including knock out/knock in mice) are valuable tools for understanding the molecular mechanisms of the disease, they generally are not faithful substitutes of the human condition in particular in the case of the skin. In fact, histological differences between murine and human skin often leads to lack of significant therapeutic responses in mouse models of skin disease. Thus, humanized models represent a much more reliable system. Using optimized bioengineering and grafting methods our group has been able to model various genodermatoses, including blistering diseases. Currently, we are also focusing on disorders of epidermal differentiation. Based on an strategy of disease deconstruction-reconstruction we have been able to establish models for Pachyonychia Congenita (Garcia M., *J Invest Dermatol.*, 2011, 131 (5): 1053–60), Netherton syndrome (Di *et al.* *Mol Ther.* 2011, 19: 408–16) and Lamellar Ichthyosis (Aufvenne *et al.*, *J Invest Dermatol.* 2012, 132:1918–21). All these human skin regenerated models present reliable hallmarks of the disease. More remarkable, they proved suitable to demonstrate the effectiveness of advanced therapies such as gene therapy in Syndrome Netherton by SPINK5 gene addition to keratinocytes, protein replacement by topical treatment with liposomes containing the transglutaminase in the case of Lamellar Ichthyosis (Aufvenne *et al.*, *Am. J. Hum Gen* in press) and “natural gene therapy” by engraftment of revertant epidermal stem cells for Junctional Epidermolysis Bullosa (JEB) (Gostynski *et al.* *J Invest Dermatol* 2013 in press). The successful results obtained with our models open the way for relevant clinical trials in patients soon.

Ino097

Gene therapy for Recessive Dystrophic Epidermolysis Bullosa: the GENEGRAFT European project and beyond

A. Hovnanian^{1,2,3} and GENEGRAFT partners*

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Recessive Dystrophic Epidermolysis Bullosa (RDEB) is a devastating genetic skin disease caused by loss-of-function mutations within *COL7A1* encoding type VII collagen, a key component of anchoring fibrils which secure attachment of the epidermis to the dermis. Individuals with RDEB suffer from skin and mucosal blistering of variable extend, leading to local and systemic complications, with a drastically increased risk of skin

cancer. Several therapeutic approaches for RDEB have been developed aiming at restoring type VII collagen expression through gene, cell and protein therapies. Cell therapy using local injection of allogenic fibroblasts or bone marrow transplantation have shown a therapeutic benefit in RDEB patients. Protein replacement through local or systemic delivery of recombinant human type VII collagen has also a therapeutic potential. With regards to gene therapy approaches, following the successful treatment of junctional epidermolysis bullosa by transplantation of genetically corrected epithelia by Prof. Michele De Luca's group, we have developed an *ex vivo* gene therapy approach using the transplantation of genetically corrected skin equivalents using a safe SIN retroviral vector expressing COL7A1 cDNA under the EF1alpha promoter. Pre-clinical studies have shown complete phenotype correction with functional anchoring fibrils formation and restoration of dermal-epidermal adherence in an *in vivo* murine model. The current European GENEGRAFT clinical trial aims at treating a limited number of moderate to severe RDEB patients with genetically corrected autologous skin equivalents, in which both keratinocytes and fibroblasts have been genetically corrected. Patients are currently being recruited in the UK and in France for pre-selection and selection. The immune status of pre-selected patients towards type VII collagen and the proliferative capacities of patient keratinocytes will guide patient selection. Skin equivalents will be prepared in GMP facilities certified for skin gene therapy and will be transplanted onto blistered and unblistered skin. In parallel to this approach, alternative therapeutic strategies for RDEB developed in our laboratory include homologous recombination and exon skipping.

*GENEGRAFT partners include: Matthias Titeux and Soëlie Charbonnier (Inserm U781, Paris), Prof John McGrath and Dr Alya Abdul-Wahab (London), Prof. Michele De Luca and Dr Graziella Pellegrini (UNIMORE, Modena), Prof. Christine Bodemer, Dr Sonia Gaucher and Dr Stéphane Guéro (APHP), Klaus Kühlcke and Rainer Löw (EUFETS), Patricia Joseph Matthieu (Inserm-transfert), Domitille D'Alançon (Inserm, promoter), Didier Caizergues and Geraldine Honnet (AFM-Genethon), Clare Robinson (DEBRA UK).

Inv098

***In vivo* mobilization of bone marrow mesenchymal stem cells accelerates cutaneous regeneration in epidermolysis bullosa**

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Recently, bone marrow transplantation (BMT) was shown to ameliorate pathological conditions of recessive dystrophic epidermolysis bullosa (RDEB) of a patient's skin, which genetically lacks functional type VII collagen (Col7) securing the epidermis to the underlying dermis. However, precise mechanism of BMT for improving RDEB skin phenotypes has not been fully elucidated. We previously reported that BMT in fetal circulation provide bone marrow-derived fibroblasts expressing Col7 in the RDEB mouse skin after the delivery. In our succeeding studies for the mechanism of RDEB mouse skin regeneration after BMT, we further clarified that platelet-derived growth factor receptor α (PDGFR α)-positive mesenchymal stem cells (MSCs) in bone marrow sense elevated serum level of high mobility group box 1 (HMGB1) derived from necrotic RDEB epithelia to move out of the marrow into the circulation. The HMGB1-mobilized MSCs in circulation then specifically target the lesional vasculatures expressing SDF-1 α to migrate into the RDEB skin, and differentiate

into both mesenchymal fibroblasts and epithelial keratinocytes expressing functional Col7. With these backgrounds, we are now setting a clinical trial of allogeneic mesenchymal stem cell transplantation for RDEB patients. HMGB1 is ubiquitously expressed in non-histone nuclear protein, which regulate chromatin structure and gene expression. Recent studies, however, showed inflammatory cytokine activity of HMGB1, which is released out from necrotic/inflamed cells, to activate macrophages and neutrophils via TLR-mediated NF κ B activation. Our study may renew a current view of HMGB1 for activating not only innate immunity but also mesenchymal stem/progenitor cells to maintain tissue homeostasis in patients with severe injury, such as RDEB.

Inv099

Towards a Gene Therapy for Neurological and Somatic MPSIIIA

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Mucopolysaccharidosis Type IIIA (MPSIIIA) is an autosomal recessive neurodegenerative metabolic disease caused by the deficiency of sulfamidase, a sulfatase involved in the stepwise degradation of the glycosaminoglycan (GAG) heparan sulfate. MPSIIIA represents an unmet medical need. MPSIIIA shares with many other Lysosomal Storage Disorders (LSD), the characteristic of being a severe neurodegenerative disease accompanied by mild somatic affectation. Thus, the main target organ for the development of new treatments is the Central Nervous System (CNS), but overall clinical efficacy would be greatly incremented by simultaneous correction of the peripheral disease. We have recently developed a novel treatment for MPSIIIA based on delivery to the cerebrospinal fluid of serotype 9 adeno-associated virus (AAV9)-derived vectors. This gene therapy strategy corrected both CNS and somatic pathology in animal models through widespread transduction of CNS, peripheral nervous system (PNS) and liver. Our study sets the grounds for the clinical translation of the approach to treat MPSIIIA in humans. Considerations that further support the applicability of this treatment to MPSIIIA and other LSD with CNS and somatic involvement will also be discussed.

Inv100

AIPGENE: Augmenting PBGD expression in the liver as a novel gene therapy for acute intermittent porphyria

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Acute Intermittent Porphyria (AIP) is a rare genetic disease in which mutations in the porphobilinogen deaminase (PBGD) gene produce insufficient production of a protein necessary for heme synthesis. This leads to an accumulation of toxic intermediates, resulting in a wide variety of problems including acute, severe

abdominal pain, psychiatric and neurological disorders, and muscular weakness. Acute porphyric attacks can be life-threatening and the long-term consequences include irreversible nerve damage, liver cancer and kidney failure. It is estimated that about 1 in 10,000 Europeans or people of European ancestry carries a mutation in one of the genes for acute porphyria. The therapies currently available do not prevent the symptoms or consequences of acute porphyric attacks. The only curative therapy is liver transplantation and thus, new curative options are clearly needed. In 2009, the European Medicines Agency granted Orphan Drug Designation to AAV5-AAT-PBGD for the treatment of AIP. AAV is a replication-incompetent virus that has been modified to deliver genes or genetic material into human tissues or cells. AAV5-AAT-PBGD acts by delivering the PBGD expression cassette directly into hepatocytes, in an animal model for AIP intravenous administration of AAV5-AAT-PBGD. In heterozygous AIP patient that show 50% of the normal activity the additional PBGD activity will be sufficient to prevent the accumulation of toxic metabolites and thus, to prevent porphyric attacks. The aim of this project is the clinical development of the orphan drug AAV-AAT-PBGD for use to treat AIP. The project was performed in two different phases. In the first phase, a GMP-compliant process to produce sufficient AAV5-AAT-PBGD for clinical trials has been developed, and AIP patients have been followed up for a minimum of 6 months before entering the clinical trial. In the second phase, the safety of AAV5-AAT-PBGD is being explored in a dose escalation.

Ino101

Gene therapy of mucopolysaccharidosis VI

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Mucopolysaccharidosis VI (MPS VI) is a rare lysosomal storage disorder caused by deficient arylsulfatase B (ARSB) activity, which results in widespread lysosomal storage of glycosaminoglycans (GAGs). MPS VI is characterized by dysostosis multiplex, organomegaly, corneal clouding and heart valve thickening without central nervous system involvement. The current standard-of-care for MPS VI is enzyme replacement therapy (ERT) that relies on the ability of ARSB to be taken-up by most cells via the mannose-6-phosphate receptor pathway. However, ERT is associated with inconvenient weekly infusions of costly recombinant enzyme and shows limited efficacy on MPS VI cardiac, ocular and skeletal abnormalities features. Alternative strategies with similar or better therapeutic outcome and increased patient compliance to treatment are desirable. Gene transfer to a factory organ like liver may provide a life-time source of secreted ARSB. To this end, adeno-associated viral (AAV) vectors with serotype 8 capsids (AAV2/8) represent a valuable tool for efficient liver gene transfer. We show that intravascular administration of adeno-associated viral vectors AAV2/8 in MPS VI cats results in multi-year ARSB expression and: i) clearance of GAG storage ii) improvement of long bone length iii) reduction of heart valve thickness and iv) improvement in spontaneous mobility. Using a humanized murine model of MPS VI, we additionally show that a single administration of AAV2/8 is, at least as effective, as multiple recombinant enzyme infusions. Our results bode well for further development of AAV2/8-mediated liver gene transfer for MPS VI patients.

Ino102

Effective transplantation of photoreceptors derived from three-dimensional cultures of embryonic stem cells

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Irreversible blindness caused by loss of photoreceptors may be amenable to cell therapy. We have previously demonstrated retinal repair¹ and restoration of vision through transplantation of photoreceptor precursors obtained from postnatal retinas into visually impaired adult mice^{2,3}. Considerable progress has been made in differentiating embryonic stem cells (ESCs) *in vitro* toward photoreceptor lineages⁴⁻⁶. However, the capability of ESC-derived photoreceptors to integrate after transplantation has not been demonstrated unequivocally. In order to isolate photoreceptor precursors fit for transplantation, we have adapted a recently reported three-dimensional (3D) differentiation protocol that generates neuroretina from mouse ESCs⁶. We have now shown for the first time that ESCs can provide a source of photoreceptors for retinal cell transplantation⁷.

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Ino103

Eye diseases

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Adeno-associated virus mediated gene transfer to the retina has been useful for studying retinal structure and function and treating blinding diseases. A major limitation to the broad use of AAV vectors is that gene delivery to photoreceptors and retinal interneurons is inefficient and difficult to target to specific functional subclasses of retinal cells. None of the natural AAV serotypes can transduce the ONL and INL from the vitreous, only a few can infect the inner retina, and these transduce only the fovea of the primate retina when delivered from the vitreous. Generating retinal cell specificity is difficult to achieve by the use of cell specific promoters alone, due to AAV's 4.7 kB packaging limit which limits inclusion of large promoters. One approach to generate specificity is to direct vector tropism at the initial infection step, engineering the virus capsid to recognize rare or

specific cell surface receptors. To overcome shortcomings that limit AAV use, vectors can be engineered at the molecular level. Unfortunately, there is insufficient knowledge of virus structure-function relationships to enable rational design to improve vector function. We have developed approaches to engineer new vectors by a process of “directed evolution”, in which a large, diverse pool of mutants is iteratively generated and screened/selected for improved function, and multiple rounds of this genetic diversification and selection yield variants with progressively improved function. We have evolved AAV variants capable of enhanced gene delivery to cones, Müller glia, on-bipolar cells and subsets of ganglion cells. Because Müller cells both contact the vitreous and span all retinal layers, vitreal transduction of these cells enabled the secretion of molecules throughout the retina. Recently, we have implemented directed evolution to create a novel AAV variant capable of direct gene delivery to photoreceptors after injection into the vitreous. We extended this approach to dog and primate; as there are differences in the viral tropism to retinal cells in other species. Having successfully engineered new AAV variants for gene delivery to the murine retina, we are using the directed evolution strategy to engineer novel AAV vectors with the capacity to mediate enhanced gene delivery to the dog and macaque retina. In preliminary studies, we have generated vectors that expand AAV tropism to numerous cell populations in the retina.

Inv104

Transposon-Based, Targeted *Ex Vivo* Gene Therapy to Treat Age-related Macular Degeneration

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Neovascular age-related macular degeneration (AMD), a major cause of blindness in the elderly, results from over-expression of vascular endothelial growth factor (VEGF) and decreased expression of pigment epithelium-derived factor (PEDF). The current treatment of repeated life-long intravitreal injections of anti-VEGF antibodies may have significant side-effects. A more effective and less costly treatment would be the subretinal transplantation of autologous genetically modified pigment epithelial cells overexpressing PEDF. This gene therapeutic approach, designated TargetAMD, funded within the EU 7th framework program, comprises 13 partners from 3 universities, 2 hospitals, 3 research institutes and 5 industrial partners from 7 countries, who together will develop protocols, reagents and technologies to successfully complete two phase Ib/IIa clinical trials for the treatment of neovascular AMD by collecting and transfecting autologous pigment epithelial cells with the PEDF gene using the SB100X transposon system and transplanting the transfected cells to the subretinal space of patients within one surgical session.

Using the SB100X transposon system we have transfected as few as 10,000 pigment cells, a number that can be obtained from a peripheral retina or iris biopsy, with the PEDF gene. The transfected cells, with efficiencies from 20–55%, have been in culture for longer than one year and expressed and secreted rPEDF continuously. Preliminary results using the safer SB100X mRNA and pFAR4-plasmids (free of antibiotic resistance) have shown similar efficiencies and rPEDF expression. During the first 6

months, the consortium's work has shown that all partners work well together and that deliverables can be accomplished in a timely manner.

Inv105

High-throughput isolation of antigen-specific T cell receptor genes by TCR gene capture

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The transfer of T cell receptor (TCR) genes into patient T cells is a promising approach for the treatment of both viral infections and cancer. While efficient methods exist to identify antibodies for the treatment of these diseases, comparable strategies for TCRs have thus far been lacking. To address this issue we have developed a high-throughput DNA-based TCR gene capture approach for the identification of TCR gene sequences of interest. The approach that we have developed involves the capture and sequencing of genomic DNA fragments encoding the TCR $\alpha\beta$ gene-loci. The quantitative nature of the resulting next-generation-sequencing data reveals relative frequency of all TCR $\alpha\beta$ and β sequences and, because of this, TCR $\alpha\beta$ pairs can be directly determined within oligoclonal T cell populations, through ‘frequency-based matching’. We have exemplified the potential of TCR gene capture in two different applications. First, we demonstrate the possibility to rapidly assemble TCR $\alpha\beta$ gene libraries against antigens of interest, in our case the Cancer/Germline (C/G) antigens that are frequently expressed in human tumors. Second, we demonstrate the ability to analyze TCR repertoires of intratumoral T cell subsets without knowledge of their antigen-specificity, and show that TCR gene capture can be used to identify dominant tumor-reactive TCR $\alpha\beta$ pairs within such populations. Taken together, our data shows that TCR gene capture can be used to describe the TCR repertoire in diverse types of biological samples and to create large collections of TCR genes for genetic engineering of T cell immunity by TCR gene therapy.

Inv106

Immunotherapy of cancer and infectious diseases

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Dendritic cells (DC) as professional antigen presenting cells are pivotal for stimulation of adaptive B and T cell responses and

have vast potential as adjuvant for immunotherapeutic applications in cancer and infectious diseases. The frequency of dendritic cells in peripheral blood mononuclear is less than 1% and several types of protocols have been established for their *ex vivo* production. However, *ex vivo* generated DC administered subcutaneously have very limited viability in trafficking from injection sites to lymph nodes (LN). In addition, complex manufacturing may limit their clinical development. We demonstrate in human and mouse systems, that *ex vivo* gene transfer into DC precursors with integrase-defective lentiviral vectors (ID-LV) for the combined production of cytokines and antigens, induced self-differentiation of DC *in vitro* and *in vivo*. These highly viable "induced DC" (iDC) effectively stimulated antigen-specific cytotoxic T cell responses against melanoma and leukemia. Human monocytes transduced with ID-LV co-expressing GM-CSF/IFN- α resulted in iDC with potent adaptive immune functions in humanized mice. Here, we address the challenges in the field and new creations of genetically programmed iDC as an advanced cell therapy to accelerate immune reconstitution after allogeneic stem cell transplantation to lower the risks of viral infections and leukemia relapse.

Inv107

Adeno-Associated Virus (AAV) vectorology on the move

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Since the first cloning of Adeno-Associated Virus (AAV) more than 30 years ago, recombinant AAV vectors have emerged as one of the most popular gene delivery tools in Gene Therapy. Following local as well as systemic application of AAV vectors, clinical success has been reported for a variety of diseases and an AAV vector encoding for a hyperactive lipoprotein lipase has become the first gene based drug receiving marketing authorization in the European Union. Instrumental for this success was the continuous improvement of the vector system. In particular, genetic engineering of the highly structured, non-enveloped viral capsid has emerged as powerful strategy to improve transduction efficiency, to expanded or re-directed vector tropism and to develop a novel class of AAV-based vaccines. However, anti-AAV immune responses as well as post-entry barriers still pose important challenges. Key to tackle these obstacles is a better understanding of the host-vector interaction. As an example, novel findings concerning intracellular factors influencing AAV-mediated liver-directed gene transfer will be presented.

Inv108

Improved retroviral vector design for gene therapy and regenerative medicine

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Retroviral vectors have demonstrated a promising track record for genetic modifications in experimental systems and more

importantly in clinical studies. However, unfortunately also side effects related to the integrating nature of retroviral vectors were observed. This has been attributed to the integration preference of the underlying retrovirus family member - a feature of the retroviral integrase together with cellular tethering factors - as well as the load of regulatory elements, e.g. promoter/enhancer sequences. Generally, vector design can anticipate some of these issues.

In this talk, we will address three principles describing how the risk-benefit assessment of retroviral vectors can be potentially improved: (I) Towards a more neutral integration: Alpharetroviral SIN vectors, which have a more random integration pattern compared to currently used gammaretroviral and lentiviral vectors. As proof-of-principle, their utility and performance in hematopoietic cells will be shown. (II) "Recycling" of integration sites: We will exemplify this in a clonal setting, which requires multiple genetic interventions, such as the generation and correction of induced pluripotent stem cells (iPSC). Here we re-used the integrated reprogramming vector footprint to insert another expression unit of choice at the same genomic spot. (III) Non-integrating retroviral delivery: Intermediates of the retroviral life cycle can be exploited for transient genetic modifications, e.g. to influence cell fate. This will be demonstrated for the delivery of a key transcription factor during reprogramming.

In summary, rational changes in vector design can help and contribute to improve the risk-benefit assessment of retroviral vectors and to develop useful tools for applications in gene therapy and regenerative medicine.

Inv109

Herpesviral vectors for the gene therapy of Friedreich's Ataxia

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Replication-incompetent helper-dependent vectors derived from herpes simplex virus type 1 (HSV-1) have several advantages for the gene therapy of neurological diseases: (1) minimal toxicity, as they do not encode any virus proteins and elicit low levels of adaptive immune responses; (2) extensive transgene capacity to carry up to 150-kb of foreign DNA, which allows for the delivery of entire genes with regulatory sequences; (3) widespread cellular tropism, though naturally the virus infects mainly neurons and epithelial cells; (4) since the viral genome does not integrate into cellular chromosomes there is a very low probability to induce insertional mutagenesis. Friedreich's Ataxia (FA) is the most common form of hereditary ataxia among the Caucasian population and is caused by recessive mutations in the FRDA (FXN) gene which encodes for a protein referred to as frataxin. FA is a mainly neurodegenerative disease affecting primarily the dorsal root ganglia, spinal cord, brainstem and cerebellum. Our group is using HSV-1 vectors containing the entire 135 Kb FXN genomic locus as gene-delivery vehicles capable of ensuring physiologically-regulated and long-term persistence of FXN gene expression as well as the correct expression of all frataxin isoforms which are generated by alternative splicing. Additionally we are also using HSV-1 vectors encoding for Brain-Derived Neurotrophic Factor which can rescue the degeneration of frataxin-deficient neurons. Major challenges for the clinical translation of these herpesviral vectors are the

optimization of vector delivery (to transduce a significant number of neurons in all the affected regions of the nervous system) and the improvement of vector production.

Inv110

The T-Body/CAR Approach: A long trip to the clinic with challenges yet to be met; the chronicle of an outstanding achievement

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It started 25 years ago, right after the structure and composition of the TCR complex became apparent, that we set forward to study the molecular mechanism underlying the MHC restriction and dependency of TCR mediated T cell activation. Having TCR α and β chain genes cloned from an antigen specific T cell hybridoma, we switched the V-regions of these chains with the V-regions of an anti-TNP mAb, expressed the double chain chimeric antibody-based chains in T cells and found that the double-chain chimeric antibody-based receptor (CAR) modified T cells specifically recognized and underwent stimulation by TNP modified target cells, as well as plate-bound TNP in an MHC un-restricted and independent manner. We nicknamed these CAR modified T cells T-bodies. Next, in order to simplify the transduction we developed the single chain design of CAR by linking a scFv of a specific antibody to an activating molecules such as CD3 β or FcR β and demonstrated that this so-called "1st generation MHC restriction" was functional. While the double chain CAR required the expression of the CD3 complex to function, the scFv-based CAR was functionally independent of it's modular allowing engineering, and into its cytoplasmic region additional domains derived from co-stimulatory receptors (e.g. CD28) (known as 2nd generation CAR), they yielded flexibility in the CAR design and improved its activity. The major function of T-bodies or CAR T cells right from its outset has been to redirect T cells to reject cancers. In this regard, the principle advantage of the CAR over the TCR is that a single CAR with a given specificity could be used against a given tumor associated antigen (or a group of tumors sharing this antigen) independently of their individual HLA. In the last 20 years, following our pioneering studies many CARs were prepared for a variety of human cancers. Coming from a basic research academic discipline, our lab focused on using different configurations of CAR specific to various anti-cancer antigens and tested their antitumor reactivity in different animal models mimicking clinical treatment modalities. Amongst the practical

lessons learnt are: 1. For optimal antitumor activity the affinity of the scFv should be in the range of medium-high. 2. For sustained antitumor activity, persistence of the T-bodies in the recipient is crucial. This can be preset and controlled by selection of the subpopulation of cells to be traduced, expended into the modified cells with certain cytokines, administration of multiple infusions of cells. 3. Careful preconditioning using lymphodepletive protocols before T-body administration. 4. When accessible, primary tumors can be directly infused with T-bodies. 5. Cancer stem cells are the preferred targets for T-body treatment. 6. Allogeneic or haplo-identical donor T-bodies in combination with immunosuppression can be affective against systemic tumors and metastases.

In the last few years, after sporadic preliminary trials, we have seen a few dramatic results in pilot trials in several centers, culminating in complete remissions in an end-stage B cell malignancies and neuroblastoma patients. These cases demonstrate the strength of the T-body approach. These trials should be carefully extended; side effects and failures should be analyzed. The immediate challenge is to make the treatment more accessible and affordable; the largest future challenge is to apply the T-body treatment to solid cancers.

Inv111

Distinct bone marrow niches for distinct hematopoietic stem and progenitor cells

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Hematopoietic stem and progenitor cells interact with heterologous cells in the bone marrow influencing hematopoietic cell differentiation and production. The mesenchymal cells influencing hematopoiesis can be resolved by differential gene expression and appear to have distinctive roles in regulating the parenchymal cells of hematopoiesis by creating pair-wise 'niches' or regulatory units. We have used selective cell depletion to examine these relationships and found that a specific subset of osteolineage mesenchymal cells has unanticipated effects on thymic emigrants, thereby altering T lymphopoiesis. Further, using a method for defining regulatory cells by virtue of anatomic proximity, mesenchymal cells regulating myeloid progenitors can be defined. Therefore, the bone marrow microenvironment provides multiple functionally distinct mesenchymal-parenchymal pairings to accomplish hematopoiesis. These units provide opportunities for modulating cell production to the organism that may be amenable to manipulation.

Selected Oral Presentations

Or001

Collagen VII gene delivery via *Sleeping Beauty* transposon in COL7A1-deficient keratinocytes from epidermolysis bullosa patients

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Autosomal Recessive Epidermolysis Bullosa (RDEB) is a genetic skin adhesion defect caused by mutations in the type VII collagen gene (COL7A1). Although full-length type-VII collagen can be successfully produced in human keratinocytes following retroviral vector transduction, genetic instability due to the large size (9 kb) and highly repetitive nature of the gene sequence remains problematic. The *Sleeping Beauty* (SB) transposon-based integration system can potentially overcome these issues by taking advantage of the hyperactive SB100X transposase in combination with the pT2 transposon. We constructed the pT2 transposon carrying the COL7A1 cDNA driven by the PGK promoter. By co-transfecting the SB100X transposase together with the pT2.Col7 transposon, we observed that there was up to 40% transposition efficiency of the collagen VII expression cassette in immortalized keratinocytes from RDEB patients. Clonal analysis demonstrated that the transposition events occurred in the absence of rearrangements, with the average copy number of 1.5 copies/cell. Despite its enormous potential, the plasmid transfection procedure of the transposon/transposase integrating system in RDEB cells remains an obstacle to its practical application in gene therapy. To overcome this limitation we incorporated the SB100X transposase/T2.Col7 transposon system into first-generation (Ad) and helper-dependent adenoviral vectors (HD), respectively. Furthermore, since the transposition from an adenoviral template required circularization of the vector genome, we incorporated the FRT sites into HD.T2.Col7 vector, providing the Flp recombinase into the integrated defective lentiviral vector. We observed a stable collagen VII expression in RDEB immortalized keratinocytes upon co-infection of transposon, transposase and Flp vectors. Encouraging results lead us to test this approach on primary keratinocytes from RDEB patient.

Or002

An E3-14.7K peptide that promotes microtubules-mediated transportation of plasmid DNA, also increases polyplexes transfection efficiency

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Cationic polymers and lipids are promising chemical vectors for gene therapy. However, the limited cytosolic diffusion of

plasmid DNA (pDNA) impairs its delivery to the nucleus. To improve its intracellular trafficking to the nucleus of the cell, one strategy is to make a pDNA able to interact with cytoskeleton motors, as most viruses do. We have identified a 20 amino-acids peptide (P79-98) of the E3-14.7K early adenoviral protein interacting with the Dynein light chain TCTEL1 *via* FIP-1. Videomicroscopy and Single Particle Tracking clearly demonstrate that a P79-98/pDNA conjugate exhibits a linear transport with large amplitude along microtubules upon 2h polyfection, whereas pDNA conjugated with a control peptide exhibits short non-directional movements in the cytosol. Remarkably, the number of transfected cells is enhanced by a factor 2.5 - up to 76% - upon *in vitro* polyfection with P79-98/peGFP. No improvement was observed with a peptide that interacts directly to dynein. *In vivo* P79-98/pLuc clearly show a 3- to 5-fold transgene expression in skeletal muscles and liver after intramuscular and tail vein hydrodynamic injection in mice, respectively. Comparatively, P79-98/peGFP lipofection do not improve transfection suggesting that the peptide is hidden after the multilamellar assembly of lipoplexes. Our results demonstrate for the first time that *in vitro* and *in vivo* non viral gene transfer can be drastically increased when pDNA is conjugated with a FIP-1 interacting sequence, allowing its migration on microtubules. This is a real breakthrough in the non viral gene delivery field that opens hope to build artificial viruses.

Or003

Non viral gene therapy clinical trial for the pancreatic cancer

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To date, Pancreatic Adenocarcinoma (PDAC) can't be diagnosed early. Consequently, a majority of patient (80%) display an advanced disease that results in a low resection rate leading to a dismal overall median survival of 4 to 6 months. We extensively demonstrated that delivering SSTR2, DCK and UMK genes using non viral vectors strongly inhibit tumor progression and dissemination in relevant experimental models of PDAC. Consequently, we designed a GMP-grade gene therapy product, CYL-02, encoding for the above mentioned therapeutic proteins, delivered by a non-viral vector for the management of patients diagnosed with advanced PDAC. In this phase I clinical trial, gene therapy was administered to 22 patients using endoscopic ultrasound, in dose-escalation in the tumors. Patients received gemcitabine during this protocol. We demonstrate herein that the intratumoral injection of the gene therapy product was safe, feasible and resulted in the presence and the expression of CYL-02 in the tumors. As a consequence, tumor progression was inhibited during the course of the protocol. In patients with locally advanced PDAC at the time of diagnosis, serum levels of CA 19-9 significantly decreased following gene therapy, regardless of the previous lines of

treatment and median progression free survival and median overall survival reached 6.4 and 11.4 months respectively (5 out of 13 patients are still alive). Based on these preliminary, yet very encouraging results, we propose that patients with locally advanced PDAC at the time of diagnosis may clinically benefit from this approach.

Or004

Improved manipulation of hematopoietic stem and progenitor cells (HSPC) for *ex vivo* gene therapy

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The latest lentiviral (LV)-based HSPC gene therapy trials have demonstrated their therapeutic potential in curing genetic diseases. We set out to optimize different steps of *ex vivo* HSPC manipulation. First, we tested the dynamic change of surface markers on CD34 BM and CB HSPC during *in vitro* culture in serum-free maintenance conditions. While positive HSPC markers such as CD34 and CD90 were detectable on a progressively decreasing cell fraction (CD34hi90: 1.9 / -0.6% and 0.5 / -0.09% after 1 and 2 weeks, respectively), negative HSPC markers like CD38 rapidly lost their informative value. Strikingly, after 1 and 2 weeks of culture, >90% of NSG mouse repopulating capacity of BM and CB, respectively, was contained in the CD34hiCD90 fraction, as shown by competitive repopulation assays, and the culture in the presence of an AHR antagonist (SR1) significantly improved the *ex vivo* maintenance of CD34hiCD90 cells. Next, adding dmPGE2 to the culture at the time of thawing, increased CD34 cell recovery by 25–50%, and augmented LV transduction by 30–60%, an effect that was maintained *in vivo*. Several advantages are associated to the use of more highly enriched HSC populations for gene therapy, including the downscaling of culture volumes and vector dose, better maintenance of stem cell properties in pure HSC cultures, and reduced integration load infused into the patient. In respect to the CD34 cell fraction currently used as the standard for HSC gene therapy protocols, we are testing innovative selection strategies to obtain HSPC populations, with a 5–10 fold higher long-term engrafting cell content. In summary, the proposed protocol modifications could substantially improve the efficacy, safety and feasibility of future *ex vivo* gene therapy studies.

Or005

Transient manipulation of haematopoietic stem cells with integrative deficient lentiviral vectors for improved cell expansion, survival and engraftment

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Haematopoietic Stem Cells (HSCs) are successfully used to treat many blood disorders but the inability to expand limited sources is restrictive. HSCs progenitor populations can be expanded *in vitro* with cytokines but this normally leads to partial differentiation, reducing self-renewal potential, homing and engraftment into the bone marrow space. To improve the survival and expansion of progenitors *in vitro* and hence increase the percentage of chimaerism *in vivo* after transplantation, we designed integration-deficient lentiviral vectors (IDLV) to express genes involved in HSC self-renewal, proliferation and maintenance of multipotency. As IDLV-delivered genes do not integrate into target-cell chromosomes, they dilute out the dividing populations. Expression is transient and could produce short-term biological changes, whilst avoiding insertional mutagenesis and undesirable constitutive over-expression of the transgene. IDLV were used to deliver two genes, HOXB4 and ANGPTL3 into Lin-, Sca1, c-Kit (LSK) mouse bone marrow haematopoietic progenitor cells. These genes are involved in HSC growth and homeostasis and the effect of short-term expression in LSK cellular self-renewal, proliferation, survival and engraftment were measured. Expression levels of HOXB4 and ANGPTL3 from IDLV are lower than from integrating vectors but optimisation of the system produces dose-dependent, biologically relevant effects. We demonstrate that transient transduction of these genes in LSK cells enables expansion and maintenance of progenitors in *in vitro* clonogenic assays and improved engraftment during competitive repopulation assays *in vivo*. IDLV are a useful tool to transiently deliver controlled levels of gene expression, suitable for studying biological systems or potentially clinical application.

Or006

Selective regulation of hematopoietic progenitors by estrogens as a basis for anti-leukemic strategies

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Although steroid hormones, such as estrogens, may affect the hematopoietic microenvironment (e.g. through their anabolic effect on bone), their role in the regulation of hematopoietic stem cells (HSCs) remains largely unknown. Here we show differential expression and functions of estrogen receptors (ERs) in primitive hematopoietic cells. Activation of ERs in short-term HSCs and multipotent progenitors directly reduced their numbers by apoptosis, and ER- α was required for complete short-term reconstitution after bone marrow transplantation. In contrast, the selective ER modulator (SERM) tamoxifen did not cause apoptosis but instead, induced cell cycle entry of quiescent long-term HSCs, altered the HSC transcriptome (with repression of self-renewal signatures and activation of the Myc transcriptional program), and compromised hematopoietic reconstitution activity following myeloablation. These effects suggested a novel therapeutic use of SERMs in the treatment of hematological malignancies through a dual mechanism: by compromising self-renewal and

sensitizing quiescent leukemia stem cells to chemotherapy, and by limiting the leukemic burden through a proapoptotic action on mature leukemic progenitors. Indeed, tamoxifen induced apoptosis of MLL-AF9 blasts *in vitro* and enhanced the effect of chemotherapy on MLL-AF9-induced acute myeloid leukemia *in vivo*. In addition, treatment with tamoxifen alone blocked JAK2V617F-induced myeloproliferative neoplasia in mice, abolishing the neutrophilia, thrombocytosis, erythrocytosis and splenomegaly associated with the disease. Tamoxifen inhibited the expansion of JAK2V617F-expressing HSCs and progenitors by counteracting their increased survival signalling and restoring normal levels of apoptosis. These results uncover specific regulation of primitive hematopoietic cells by estrogens, and demonstrate anti-leukemic properties of a SERM already available for clinical use.

Or007

Accurate measurement of NAb status against AAV vector capsids and an approach toward managing its inhibitory effect

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Adeno-Associated Virus (AAV) vectors are suitable in gene therapy approaches targeting various organs. For liver-mediated transduction, IV injection of AAV8 vector seems most practical. On the other hand, inhibitory actions of pre-existing neutralizing the antibody (NAb) against vector capsid, demonstrated even at marginal levels, suggests the necessity of more sensitive assay for NAb detection. To solve this issue, we have improved NAb detection system against AAV capsids with higher sensitivity. As a result, all of the 9 monkeys judged as NAb negative by this method showed transgene expression at the expected levels. The remaining challenge is whether AAV-mediated gene therapy is available to the subjects with positive NAb status. For this purpose, we tested the efficacy of intravascular saline flushing before and after vector injection in order to avoid contact of vectors with neutralizing antibodies. Direct injection of the AAV8 vector carrying the factor IX (FIX) gene into the portal vein of macaques using saline flushing, achieved transgene-derived FIX expression ($4.7 \pm 2.1 \sim 10.1 \pm 5.5\%$ of normal human FIX concentration) in the presence of neutralizing antibodies. This was as efficient in macaques lacking neutralizing antibodies at the same vector dose ($5.4 \pm 2.6 \sim 12.7 \pm 4.8\%$). Next, we tested the efficacy of saline flushing using a balloon catheter-guided injection, which is less invasive and thus more clinically relevant. This approach also resulted in efficient expression of transgene-derived FIX ($2.5 \pm 1.1 \sim 9.0 \pm 2.4\%$) in the presence of neutralizing antibodies ($\times 14 \sim \times 56$ dilutions). Neutralizing antibodies at this range of titers reduced the efficiency of transduction in the macaque liver by 100-fold when the same vector was injected into mesenteric veins without balloon catheters. No apparent toxicity was observed, except the transient increase in serum transaminase levels. Our results suggest that the portal vein-directed vector delivery strategies using saline flushing are efficacious to overcome inhibitory effect of AAV antibodies. This study was performed in collaboration with Tsukuba Primate Research Center, National Institute for Biomedical Innovation, and The Corporation for Production and Research of Laboratory Primates, Japan.

Or008

Uncovering long-term survival and activity *in vivo* in humans of genetically engineered T memory stem cells by retroviral tagging

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The development of efficacious and safe T-cell based therapies is strictly dependent on our understanding of T cell clonal dynamics *in vivo* in humans. Gene therapy of adenosine deaminase (ADA) deficient-SCID patients is based on *ex vivo* retroviral gene transfer into Hematopoietic Stem Cells (HSC-GT) or Mature Lymphocytes (PBL-GT). These unique clinical settings allow studying the molecular and functional profile of engineered T-cell subtypes at clonal level in humans. We found that vector-positive phenotypically naïve T cells (CD45RA /CD62L) survived in PBL-GT patients up to 10 years after last infusion maintaining their plasticity *in vivo*, as testified by the highest percentage of identical insertions shared with other T-cell subtypes (41.2%), without signs of aberrant expansions. A novel T-cell type (T memory stem cell, TSCM) has been recently identified carrying a long-term survival capacity and naïve-like plasticity. By CD95/IL7R α /IL2R β phenotyping we found that 92.5% of CD45RA /CD62L cells from PBL-GT patients were actually TSCM differently from HSC-GT (37.2%) and healthy individuals (10%). IFN γ production and differentiation potential *in vitro* confirmed that TSCM isolated from GT patients were functionally active and distinct from naïve and memory counterparts. Additional *in vitro* data suggest that TSCM were already enriched in PBL-GT patients' lymphocytes prior to infusion, due to PHA/IL2 stimulation and expansion. Overall, our data show that genetically engineered TSCM can survive for several years in patients retaining their activity and plasticity without showing any vector-related genotoxicity. These results have crucial implications for the development of novel T-cell based therapies for hematological diseases and tumors.

Or009

Small peptides blocking SR-A and SREC-I increase HDAd-mediated liver transduction through inhibition of Kupffer and liver sinusoidal endothelial cell uptake

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Kupffer cells (KC) and sinusoidal endothelial cells (LSEC) of the liver are a major barrier to efficient hepatocyte gene delivery by helper-dependent adenoviral (HDAd) vectors. We have previously shown that scavenger receptor type A (SR-A) and scavenger receptor expressed on endothelial cells I (SREC-I) play a crucial role in adenoviral (Ad) vector uptake by these cells and thus, they affect hepatocyte transduction. In the present study, to prevent vector uptake by KC and LSEC, we used small synthetic peptides (PP1 and PP2) designed by phage display to block SR-A and SREC-I

with the goal of increasing hepatocyte transduction efficiency. Pre-infection of J774.1 macrophages with PP1 or PP2 prior to HDAd infection significantly reduced viral vector uptake. Pre-treatment of C57BL/6 wild-type mice with intravenous injections of PP1 and PP2 peptides prior to HDAd injection resulted in a significant 3.7-fold and 2.9-fold increase of hepatocyte transduction efficiency, respectively. Fluorochrome conjugated PP1 was found to co-localize with both CD68 and CD31 thus suggesting that PP1-mediated increase of hepatocyte transduction was dependent upon SR-A blocking on both KC and LSEC. Serum IL-6 was increased in mice injected with PP1 and HDAd achieved higher levels of transduction compared to mice receiving vector alone. In contrast, mice injected with PP2 and HDAd showed no significant increase in serum IL-6, despite the increase in hepatocyte transduction. In conclusion, we show that SR-specific inhibition by small inhibitory peptides resulted in increased hepatic transduction and is a promising strategy to improve the therapeutic index of HDAd vectors.

Or010

Somatotransgenic bioimaging: a novel biosensing platform for *in vivo* bioimaging

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Animal models of disease have been invaluable in elucidating the molecular origins of pathogenesis, progression of disease, and the development of therapeutic interventions. Ultimately, the quantitation of disease biomarkers often requires terminal end-point analysis. The advent of light-emitting transgenics that contain a luciferase reporter gene conditionally activated by an endogenous promoter, or serial transcription factor binding elements controlling a minimal promoter, provide quantitative light emission in response to *in vivo* intracellular signalling. However, transgenesis results in the insertion of the luciferase expression cassette into every cell of the body, which can result in substantial off-target or non-specific activity. Here, we describe a novel technology whereby lentiviral-mediated delivery of a luciferase reporter construct into neonatal mice offers targeted, long-term expression of the transgene in a fraction of the time and cost required for the development of a germ-line transgenic. We have designed, generated and validated *in vitro* a bespoke library of 15 lentiviral vectors that are activated by transcription factors implicated in inflammation, fibrosis and cancer as well as in other diseases. As proof of concept, we show the utility of somatotransgenic bioimaging *in vivo* using NF- κ B signalling as a model of inflammation in several organs, both in rats and mice. Furthermore, we present evidence that this is an effective platform for testing drug efficacy *in vivo* by showing depletion of a mediated LPS-induced NF- κ B response by co-administration of dexamethasone. Other vectors currently being validated include those involved in TGF- β , BMP, WNT, Hedgehog, STAT3 signalling and antioxidant, hypoxic and xenobiotic responses.

Or011

***In vivo* imaging of mesenchymal stem cell recruitment into the tumor stroma of Hepatocellular Carcinoma (HCC) using a HIF-1 α -specific sodium iodide symporter gene system**

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The tumor-homing property of mesenchymal stem cells (MSCs) has led to their use as delivery vehicles for therapeutic genes. The application of the sodium iodide symporter (NIS) as reporter and therapy gene allows non-invasive imaging of MSC biodistribution and functional transgene expression by ¹²³I-scintigraphy or PET-imaging, as well as therapeutic application of ¹³¹I. Hypoxia-inducible factor 1 (HIF-1) is a key mediator of the cellular response to hypoxia and therefore is highly expressed in solid malignancies. Placing NIS under the control of the HIF-1 α promoter should consequently allow tumor-specific NIS expression after MSC-mediated delivery. We stably transfected human MSCs with NIS driven by the HIF-1 α promoter (HIF-1 α -NIS-MSCs) and analyzed functional NIS expression by iodide uptake assay, Western blot and FACS analyses. In a HCC xenograft model, we further investigated distribution and tumor recruitment of HIF1 α -NIS-MSCs by ¹²³I-scintigraphy. After hypoxia was simulated *in vitro* by treatment of HIF1 α -NIS-MSCs with 300 μ M of the hypoxia mimetic agent CoCl₂, a 48-fold increase in perchlorate-sensitive iodide uptake was observed compared to HIF1 α -NIS-MSCs under normal conditions. Western blot and FACS analyses confirmed CoCl₂-induced NIS expression in HIF1 α -NIS-MSCs. After establishment of subcutaneous HCC xenografts in nude mice, HIF1 α -NIS-MSCs were injected intravenously and MSC distribution was analyzed by γ -camera imaging. Injection of 18.5 MBq ¹²³I resulted in a tumor-selective iodide accumulation showing active MSC recruitment and tumor-specific promoter activation. Our results demonstrate selective recruitment of HIF1 α -NIS-MSCs into HCC tumors resulting in tumor-specific iodide accumulation, opening the exciting prospect of NIS-mediated radionuclide therapy of extrathyroidal tumors after MSC-mediated gene delivery.

Or012

Thy1.1, p75^{NTR} or CAR receptor targeting by lentiviral vectors leads to retrograde transport and transduction of spinal motor neurons following peripheral delivery

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To understand how receptors are involved in neuronal trafficking and to be able to utilise them for specific targeting via the peripheral route would be of great benefit. Here we describe the generation of novel HIV-1 targeted lentiviral vectors with tropism to motor neurons (MNs) that were made by co-expressing onto the lentiviral surface of a fusogenic glycoprotein (mutated sindbis G) and an antibody against a cell surface receptor found on the presynaptic neuromuscular junction terminal (Thy1.1 or low-affinity neurotrophin receptor p75^{NTR} or coxsackievirus and adenovirus receptor CAR). These vectors exhibit binding specificity and transduction of receptor positive cell lines and primary motor neurons *in vitro*. Following application of the targeted vectors on the axonal compartment of microfluidic MN cultures, retrograde axonal transport and transduction of cell bodies on

the somatic compartment were demonstrated. *In vivo* delivery of the CAR targeted vectors in leg muscles of mice resulted in predicted patterns of MN labelling in the lumbar spinal cord as demonstrated by bioluminescence whole body *luciferase* imaging and EGFP immunofluorescence. *In vivo* transduction efficiency was in part dependent on transgene promoter strength. Rabies virus glycoprotein pseudotyped HIV-1 vectors tested in parallel were found to be transported in a retrograde direction, yet failed to transduce MNs either *in vitro* or *in vivo*. The potential of these targeted vectors for minimally invasive administration of CNS therapeutics is being currently tested in models of MN diseases.

Or013

ColoAd1 a group B oncolytic adenovirus: pre-clinical characterisation and development of 'armed' variants

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ColoAd1 is a chimeric Ad11p/Ad3 adenovirus, developed by bio-selection from a library of chimeric adenoviruses for the ability to replicate and exit rapidly from tumour cells. The virus is active against a broad range of cancer cell lines demonstrating a shorter time-to-lysis than either wild type Ad11p, Ad3 or Ad5. In normal cells, ColoAd1 is attenuated and shows little or no activity by either cytotoxicity or by quantitative PCR. The mechanism of tumour cell lysis is independent of apoptosis pathways and ColoAd1 readily kills drug resistant cells. *In vivo*, ColoAd1 shows efficacy in a range of subcutaneous and orthotopic metastatic tumour models following intra-tumoral, intravenous and intra-peritoneal injection. When subpopulations of cells are isolated from tumour samples, sphere forming cells (with a self-renewing phenotype) were shown to be disproportionately killed by ColoAd1. The virus capsid is entirely derived from Ad11p for which there are limited circulating levels of neutralising antibodies in the general population. ColoAd1 associates with blood cells reversibly and unlike group C based oncolytic viruses, can efficiently kill tumour cells under clinically relevant conditions in undiluted human blood. Replication and lysis has been demonstrated in primary patient samples and efficacy has been demonstrated in metastatic orthotopic models. A full panel of safety studies has now been completed and a phase I clinical trial is ongoing. ColoAd1 virus variants which can be 'armed' for therapeutic gene delivery in order to enhance ColoAd1 anti-tumour activity, are now under development.

Or014

Results of a trial of compassionate use of Celyvir in children with metastatic and refractory solid tumors

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Celyvir consists of autologous mesenchymal stem cells (MSCs) carrying an oncolytic adenovirus. We published an initial

study on the use of Celyvir in 4 children with metastatic neuroblastoma (Cancer Gene Therapy, 2010, 17: 476) and now present the extended clinical experience of this program of compassionate use of Celyvir in 17 new patients. Diagnoses were neuroblastoma (15), rhabdomyosarcoma (1) and ovarian tumor (1). All patients had failed to at least 3 lines of therapy, and presented a metastatic disease. The children received multidoses of Celyvir in a weekly basis (minimum 4, maximum 70, total 229) with no concomitant treatments. Total cells (min. 70×10^6 , max. 2640×10^6) and viral particles (min. 1.8×10^{12} , max. 5.28×10^{13}) varied among patients. Hematological and biochemical controls were done in blood samples at the time of each infusion. The tolerance was excellent, with very mild and autolimited viral-related toxicities. Peripheral blood lymphocytes raised and the profile of tumor infiltrating lymphocytes (whenever a biopsy was available) changed after Celyvir therapy. Clinical outcomes were progression (13), stable disease (1), partial remission (3) and complete remission (1). The patient with a complete response relapsed after 6 months and received a second round of Celyvir, achieving a partial remission. MSC cultures presented differences in the expression levels of adhesion molecules (CCR9, β_1 integrin, CD44 and VCAM) and immune molecules (HLADR, BH7 family), suggesting interpatient differences in the homing and immune modulation capacities of the therapy administered. In conclusion, Celyvir is a safe therapy that should be explored further in patients with advanced cancers.

Or015

Pancreatic tumor targeting with cell type-specific miRNA-regulated oncolytic adenoviruses reduces local and systemic toxicity

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Oncolytic virotherapy is an emerging treatment that uses replication-competent viruses to eliminate cancer cells. A major concern is raised to confer viral tumor targeting in order to minimize undesirable side effects. In the current work, we explored to engineer replication-competent adenoviruses containing binding sites recognizing robust hallmark microRNAs with pancreatic tumor-selectivity (Ad-miRT). Candidate microRNAs were selected and their expression was validated in a set of primary tumors and non-tumor tissue from patients diagnosed with pancreatic ductal adenocarcinoma. We show that viral E1A-miRT expression and Ad-miRT viral propagation were efficiently controlled *in vitro* and *in vivo* according to miRNAs content. A miRNA-dependent cytotoxic effect on tumoral cells was detected and strong antitumor responses were recorded in Ad-miRT treated mice. We present *in vivo* evidences of Ad-miRT attenuation in liver and pancreas, both in the exocrine fraction and the Langerhans islets. Consequently, Ad-miRT infected mice presented with reduced pancreatic and hepatic damage. Remarkable safety was also observed at higher Ad-miRT injected doses. Fine analysis of the endogenous levels of miRNAs and their targets showed no perturbation derived from the microRNA-binding sites on Ad-miRT infected tissues, highlighting the absence of associated-toxicity to the strategy. Taken together, our study

provides compelling preclinical evidences for the usage of candidate microRNAs binding-sites to confer adenoviral selectivity, improving the safety profile of adenoviral-based therapies while retaining full lytic capacity when administered locally to the pancreas or systemically. Moreover the well-tolerated systemic delivery of elevated viral doses may allow for increasing therapeutic concentrations, leading to improved anticancer efficacy.

Or016

Development of new lentiviral vectors for the gene therapy of LAD-I

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Leukocyte Adhesion Deficiency Type I (LAD-I) is a primary immunodeficiency characterized by recurrent and life-threatening bacterial infections. It is caused by mutations in the *ITGB2* gene, encoding the integrin $\beta 2$ common subunit (CD18). Most of these mutations lead to a defective or absent expression of $\beta 2$ integrins in the leukocytes surface, which implies that leukocytes are unable to firmly adhere to the endothelium and extravasate to infection sites. As other monogenic immunodeficiencies, LAD-I is a good candidate for *ex vivo* gene therapy. Therefore, we have developed four different lentiviral vectors, expressing human CD18 cDNA from ubiquitous (PGK and UCOE) or myeloid-specific (Chim and MIM) promoters. All four CD18-LVs were able to restore the expression of CD18 and CD11a both in a LAD-I patient-derived lymphoblastoid cell line and in cells from a CD18 hypomorphic mice that resemble the moderate form of human LAD-I, including leukocytosis, reduced $\beta 2$ integrin expression and impaired neutrophil migration. To correct the genetic defect of mCD18 defective cells, lineage negative BM cells were transduced with CD18-LVs and transplanted into irradiated CD18-hypomorphic recipients. As a result of this *ex vivo* gene therapy approach, hCD18 could be detected in PB lymphoid and myeloid cells, and increased levels of CD11a expression and normalization of the white blood cell counts were observed. All these results allow us to propose new CD18-LVs that could be good candidates for future clinical applications for LAD-I patients.

Or017

Efficient site-specific integration and *in situ* gene correction of human long-term repopulating hematopoietic stem cells by zinc finger nucleases

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The development of ZFN technology enables *targeted* rather than *random* integration, and gene *correction* rather than *replacement*, as new paradigms for gene therapy. Whereas high levels of

targeted gene modification has been reported in some clinically relevant primary cells, the demonstration of highly efficient targeted integration (TI) in human Hematopoietic Stem/Progenitor Cells (HSPC) remains challenging. Here, we combined Integrase Defective Lentiviral Vectors (IDLV) to deliver a donor template for homology-driven repair and mRNA transfection to drive a spike of ZFN expression in the treated HSPC. Optimization of this protocol facilitated TI of transgene expression cassettes into the *AAVS1* "safe harbor" site or of a corrective cDNA into a mutational hotspot of the *IL2RG* gene of HSPCs with high efficiency and reproducibility (average 6%, n=25 cord-blood donors). Upon xeno-transplantation in NSG mice we observed gene-targeted cells in both myeloid and lymphoid lineages as well as in the early progenitors' compartment of the bone marrow for several months post-transplant across multiple independent mice, indicating successful targeting of long-term repopulating stem cells. Importantly, HSPC targeted with the corrective *IL2RG* cDNA were capable of generating lymphoid cells that express the *IL2RG* protein and are dependent on its signalling. These lymphoid cells expanded in the mice upon challenge with a tumor cell line that expresses human IL7 and IL15, proving that the corrective cDNA was able to functionally restore the gamma-chain coupled receptors. Overall these studies demonstrate that ZFN-mediated TI of a transgene can be obtained in human HSPC with efficiencies compatible with clinical translation.

Or018

Accumulation of proto-oncogene integrations triggering lymphoid as well as myeloid leukemia in WAS gamma-retroviral gene therapy

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The German WAS clinical gene therapy trial showed efficient correction in 9/10 patients and until now more than 120,000 unique integration sites (IS) could be detected. A comparative analysis between the patients revealed a striking high-level clustering of IS in sub-gene regions comprising only 100–300 kb. The most affected gene loci are represented by proto-oncogenes (MDS1-EVI1, PRDM16, LMO2 and CCND2) which have been involved in clonal expansions during other gene therapy trials. 5 patients developed T-ALL, each associated with an LMO2 integration site, triggering the up-regulation of this proto-oncogene and the development of leukemia. Overall, a rapid and abrupt increase of the leukemic clone from less than 0.6% to more than 60% could be observed. 3 patients developed AML

and in contrast to the T-ALL patients, the relative contribution of the leukemic clone increased steadily over time, finally leading to the development of myeloid leukemia. IS analysis revealed that the occurrence of AML was associated with integrations within the myeloid proto-oncogene MDS1. Whole genome sequencing of the leukemias allowed for detection of some large aberrations such as TCR translocations, LOH affecting the tumour-suppressor genes CDKN2a/b as well as large deletions in a Mb range. Further analysis of the data is currently ongoing to comprehensively dissect the lymphoid and myeloid leukemias and to identify possible genetic commonalities between the patients.

Or019

MicroRNA-regulated cassettes aimed at preventing cardiac toxicity of CAPN3 gene transfer

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Limb-Girdle Muscular Dystrophy type 2A (LGMD2A) is due to mutations in the *CAPN3* gene. This disease affects predominantly the skeletal muscles of the proximal limbs. There is no treatment for this disease to date. We showed previously that intramuscular or loco-regional administration of a *CAPN3* transgene, carried by a recombinant Adeno-Associated Virus (rAAV) vector, resulted in restoration calpain 3 expression in a murine model for LGMD2A. The long term expression of calpain 3 in skeletal muscles resulted in the correction of the pathological signs of the disease. However, systemic administration of the same vector led to cardiac toxicity caused by unregulated proteolytic activity of calpain 3 in the heart. To avoid *CAPN3* transgene cardiac expression, we developed a vector regulated by microRNA. We introduced the target sequence of the cardiac specific microRNA-208a downstream of *CAPN3* cDNA and demonstrated *in cellulo* its capacity to down-regulate the expression of the carrier messenger in presence of miR-208a. In order to restrain the expression of *CAPN3* transgene to the skeletal muscle, we also cloned and characterized *in cellulo* the muscle-specific promoters of *CAPN3* and miR-206. The validation of these regulatory elements *in vivo*, using rAAV vectors, resulted in the abolition of *CAPN3* transgene toxicity in the heart.

Or020

Gene transfer of TCIRG1 driven by clinically relevant promoters restores osteoclast function in Infantile Malignant Osteopetrosis

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Infantile Malignant Osteopetrosis (IMO) is a rare, lethal, autosomal recessive disorder characterized by nonfunctional osteoclasts. More than 50% of the patients have mutations in the *TCIRG1* gene, encoding for a subunit of the osteoclast proton pump. Gene therapy could be an alternative treatment to allogeneic stem cell transplantation for IMO patients.

We have previously shown that the phenotype of IMO osteoclasts can be rescued by lentiviral mediated gene transfer of the *TCIRG1* cDNA under the SFFV promoter. The aim of this study is to test two more clinically relevant promoters: Elongation factor 1 α short (EFS) and a chimeric myeloid promoter (ChimP; Grez *et al.*, 2011). CD34 cells from peripheral blood of 3 IMO patients were transduced and cultured with M-CSF, GM-CSF, IL-6, SCF and Flt3L for 2 weeks. Transduction efficiency was evaluated and cells were differentiated to mature osteoclasts on bone slices with M-CSF and RANKL. qPCR analysis and western blot revealed increased mRNA and protein levels of *Tcirg1* compared to controls. Vector corrected IMO osteoclasts generated increased Ca² release and bone degradation products such as CTX-1 into the media, while non-corrected IMO osteoclasts failed to resorb bone. The vector with SFFV promoter gave rise to the highest rescue levels (resorption approximately 80% of controls), followed closely by the EFS promoter (70%) and the ChimP promoter (50%). In conclusion we provide evidence for rescue of the IMO osteoclasts with a human promoter and with a tissue specific promoter, supporting the clinical development of gene therapy of IMO.

Or021

Insight into molecular events associated with partial restriction of Adeno-Associated Vector genome expression in dystrophic muscles

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Improving the efficacy of recombinant Adeno-Associated Viruses (rAAV)-mediated gene transfer is a mandatory step toward successful translation to the clinic. To this end, the molecular mechanisms underlying stability or transcriptional regulation of rAAV episomal genomes must be explored, particularly in pathological tissues often affected by metabolic perturbations. In this study, the molecular behavior of rAAV genomes was evaluated in dystrophin-deficient mice, whose muscle is severely disturbed by oxidative stress compared to healthy tissue. Common to both models, *in situ* chromatin immunoprecipitation analyses showed a robust enrichment in a repressive histone post-translational modification (H3K9me3) despite a histone density comparable to that of endogenous chromatin domains. However, several differences were highlighted between the two tissue contexts: (i) quantitative PCR indicated that rAAV genomes are not only less stable in dystrophic muscles but are also less transcriptionally active; (ii) while DNA methylation along rAAV genomes was hardly detectable in healthy muscles, we noticed a limited and time-dependent enrichment in dystrophic muscles; (iii) several members of the DNA damage response (*Mre11*, *Rad50*, *Nbs1*, *Atm*, *Atr*) were found overexpressed in dystrophic muscles, which in turn may also participate in rAAV genome silencing. A better understanding of these molecular mechanisms is likely to allow the design of dedicated methods to improve the tissue environment and in turn the safety and efficiency of rAAV-mediated gene therapy for muscular dystrophies.

Or022**Development of the manufacturing process for the *ex vivo* gene therapy for ADA-SCID (GSK2696273)**

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GSK2696273 is a novel medicinal product which contains genetically modified cells. It has been developed for the treatment of severe combined immunodeficiency (SCID) due to adenosine deaminase (ADA) deficiency. GSK2696273 contains autologous CD34 cells transduced *ex vivo* with a replication deficient retroviral vector containing the human adenosine deaminase (ADA) cDNA sequence. The product has been administered to 18 paediatric patients, aged approximately 6 months to 6 years, 12 of which were enrolled in the pivotal clinical study. With the exception of the very first patient, all vector, drug substance and drug product has been manufactured at MolMed, Milan, and all patients have been treated at Hospital San Raffaele, Milan. A summary from a systematic review of the data from the manufacturing campaigns of vector produced to date and the 12 batches of drug product produced for the pivotal clinical study, as well as links between the manufacturing process, batch properties and clinical safety and efficacy will be presented. In addition, efforts to further develop the manufacturing process to ensure security of supply and delivery of a safe and efficacious product whilst minimising changes to reduce the comparability risk will be presented. More specifically, changes to the vector process, the key starting material, will be presented. These changes will be implemented to reduce variability between batches and increase scale. For the cell process minimal changes will be implemented, with the main aim being aseptic risk reduction.

Or023**Environmental risk assessment for the placing on the market of the gene therapy product Glybera**

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Glybera (alipogene tiparvovec) is an AAV-1 based gene therapy medicinal product developed by UniQure (Amsterdam Molecular Therapeutics) for long term correction of lipoprotein lipase deficiency (LPLD), a seriously debilitating inherited lipid metabolism disorder. Glybera is the first AAV-based gene therapy medicinal product admitted by the European Commission for Market Authorization in 2012. The environmental risk assessment (ERA) formed an important part of the submission dossier. For the purpose of this ERA, we screened the non-clinical and clinical studies to select data that could be used as a basis for risk assessment considerations. This data was supplemented with use scenarios from clinical trials and literature on the biology of wild-type AAV and modified AAV-based gene therapy vectors, to assess magnitude and likelihood of potential adverse events. The clinical vector shedding from different excreta was the most critical element in the assessment. This data allowed estimating the fraction of the dose excreted after Glybera administration. A thorough description of the characteristics of the recombinant AAV-vector, such as replication deficiency, low infectivity and non-pathogenicity were

pivotal in assessing the hazard of the shed vector in absence of specific environmental data. The process and data will be presented that led us to conclude that the overall assessed risk of Glybera for people and the environment was negligible.

Or024**Peptide-mediated engineering of 12 AAV serotypes**

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AAV vectors are attractive for many reasons, including the option to engineer the viral capsid to expand its tropism to cells that are refractory to natural AAVs. One potent strategy is to insert a randomized peptide library into exposed capsid regions of AAV2, followed by iterative enrichment of candidates mediating improved transduction on target cells. Here, we studied if and to what extent the capsid backbone and its combination with specific peptides rather than the peptide alone determines vector properties. Therefore, we modified the capsid genes of 12 natural AAVs to contain two restriction sites for insertion of peptide-encoding oligonucleotides. Into each capsid, we then cloned 18 different peptides which were pre-selected in AAV2 on various cells. We next produced the resulting >200 AAVs as YFP-expressing vectors and screened them in ~50 human or murine cells, including primary hepatocytes, T-cell lines and stem/iPS cells. Strikingly, many capsid-peptide combinations outperformed the original AAV2 variants, especially those based on AAV1, 7-9 and rh10. Two additional intriguing findings are that our best peptides shared the residues N-R—, and that disruption of one flanking arginine in AAV2 boosted vector potency by two orders of magnitude. Our data illustrate the vast potential of non-2 AAV serotypes as templates for peptide display, and suggest that our set of >400 new AAV capsids will benefit many labs interested in identifying superior variants on their favourite cells. To further aid in this process, we finally also present a novel protocol for AAV spotting and drying in 384-well plates which facilitates the long-term storage and shipping of our vector collection.

Or025**A possible therapeutic strategy for genetic diseases using hematopoietic stem cells generated from induced pluripotent stem cells**

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It has now come to the era when one can expect reality in genetic correction in hematopoietic stem cells (HSCs) as a perfect treatment for inheritable diseases. Hematopoietic stem/

progenitor cells are currently the target cells being used in the clinical gene therapy trials, but careful monitoring of hematopoietic clonality in treated patients is essential to ensure overall safety of the treatment. Utilization of induced pluripotent stem cells (iPSCs) can potentially be a solution because it should allow clonal selection from a bulk population after genetic modification and before transplantation; however, it has proved challenging to generate from pluripotent stem cells genuine HSCs truly transplantable. Here, we demonstrate a unique *in vivo* differentiation system yielding engraftable HSCs from mouse and human iPSCs in teratoma-bearing animals. In mice, iPSC-derived HSCs were shown capable of migrating from teratomas into the BM and their intravenous injection into irradiated recipients led to long-term reconstitution of the multilineage hematopoietic system in serial transfers. Using this *in vivo* generation system, we could demonstrate that X-linked severe combined immunodeficiency mice can be treated by HSCs derived from functionally-corrected clonal iPSCs after therapeutic gene transfer. Of note is that neither leukemia nor tumors were observed in recipients after transplantation of iPSC-derived HSCs. Taken together, our system presented here should provide a useful tool not only for basic research of HSCs biology, but also for possible application of iPSCs in the treatment of genetic diseases where stem cell-gene therapy can be ideal by enabling permanent cure for the patients.

Or026

Retrovirus insertion in iPSCs identifies genes facilitating somatic reprogramming

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Transcription factor-induced reprogramming of induced pluripotent cells (iPSCs) de-stabilizes the differentiated state of somatic cells via intermediate cell populations. The identity and molecular functions of genes that promote reprogramming are highly relevant for understanding the mechanistic roadblocks to this reprogramming process. We here demonstrate that comprehensive retrovirus vector insertion maps in 40 analyzed mouse and human iPSCs show non-random distribution towards genomic loci and genes important for reprogramming mouse embryonic fibroblasts in iPSCs. Our findings are very unique and novel, identifying new genes which in addition to transcription factors, facilitate reprogramming and the gain-of-pluripotency. We found that the systematic selection and ectopic expression of these mechanistically-relevant genes tagged by retroviruses significantly facilitates cellular reprogramming into iPSCs, enhancing the sequential stages of iPSC generation more than 10-fold. Genes that facilitate reprogramming include chromatin modifiers as well as regulators of innate immunity and RNA Polymerase III activity. The capacity of such genes to enhance reprogramming indicates their biological role in overcoming molecular barriers to de-differentiation. Our findings explain the higher frequency of iPSC derivation with integrating vector systems, and predict heteroge-

neity among such iPSC clones. Multi-stage insertional mutagenesis screens with retroviral- or transposon-based vectors enable a further functional dissection of the molecular mechanisms that enhance reprogramming and pluripotency. Functional analysis of these genes indicates that stochastic and deterministic roadblocks accompanying reprogramming can be identified and overcome in the search for more efficient and controlled iPSC derivation.

Or027

Allele-preferred targeted correction of CFTR gene in Cystic Fibrosis induced pluripotent stem cells

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Cellular transplantation of lung stem/progenitor cells represents a potential therapeutic approach for a variety of inherited monogenic diseases. Crucial to the success of such a therapeutic strategy is that the transplanted cells and their progeny are corrected for the disease-causing mutation and that the transplanted cells do not elicit an immune response in the recipient. In order to satisfy these criteria, we are pursuing a patient-specific approach in which, starting with skin or blood cells from patients with inherited lung disorders, autologous induced pluripotent stem cells (iPSCs) are first derived. Utilizing site-specific homology-directed repair, the disease-causing mutation is corrected in the endogenous, chromosomal DNA sequence. Finally, a directed differentiation approach is employed to obtain highly purified populations of the relevant stem/progenitor cells from the corrected iPSCs for purposes of transplantation. We have employed this approach to generate corrected, autologous iPSCs for patients with Cystic Fibrosis (CF). Starting with skin fibroblasts of patients diagnosed with CF, we have derived and characterized iPSCs, confirming their pluripotency. We then utilized zinc finger nucleases (ZFNs), designed to target the endogenous CFTR gene, to mediate correction of the inherited genetic mutation in this locus via homology directed repair in these iPSCs. The corrected CF iPSCs, when induced to differentiate *in vitro*, express the corrected CFTR gene. Importantly, we observed an exquisitely sensitive, homology-dependent preference for targeting one CFTR allele vs. the other. This allele-specific targeting offers the potential for preferential targeting of ZFN-mediated correction to dominant mutant alleles.

Or028

Disease-regulated local interleukin-10 gene therapy diminishes synovitis and articular cartilage damage in experimental arthritis

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Rheumatoid arthritis (RA) is a chronic destructive autoimmune disease with periods of exacerbation and remission. An attractive treatment would provide a disease-regulated therapy that offers flexible drug delivery. Therefore, we expressed the anti-inflammatory interleukin-10 (IL-10) gene under the control of an inflammation-dependent promoter in a mouse model of RA. Proximal promoters of *S100a8*, *Cxcl1*, *Mmp13*, *Saa3*, *IL-1b*, and *TSG6* were selected from endogenous genes differentially regulated in the inflamed synovium of arthritic mice. Mice were injected intra-articularly in kneejoints with lentiviral vectors expressing a luciferase reporter or the therapeutic protein IL-10 under control of selected promoter. After 4 days, arthritis was induced by intra-articular injection of streptococcal cell walls. At different timepoints after arthritis induction, *in-vivo* bioluminescent imaging was performed or knee joints were dissected for histological and RNA analysis. The disease-regulated promoters showed different activation profiles during the course of the disease, subsequently the *Saa3* and *MMP13* promoter were selected for further research, because of differences in activation at day 1. Overexpression of IL-10 resulted in less synovitis and cartilage proteoglycan (PG) depletion and in upregulation of IL-1Ra and *SOCS3* gene expression. IL-1Ra counteracts the detrimental effects of IL-1 on cartilage damage and *SOCS3* inhibits the JAK/STAT pathway and subsequent inflammation; this can explain the diminished synovitis and PG depletion. Probably because IL-10 is expressed at day 1 of arthritis without any treatment, major therapeutic differences between the *MMP13* and *SAA3* promoter were not evident in this study. Yet we can conclude that local inflammation-dependent IL-10 gene therapy suppresses experimental arthritis and is a promising strategy in the development of novel treatments for RA.

Or029

Inflammatory effects of galectin-1-deficient regulatory T cells in a T cell transfer model of inflammatory bowel disease

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Foxp3-expressing regulatory T cells (Treg) are vital for maintaining balance among tolerance, adequate immune responses, and autoimmunity. In recent years, regulatory T cells are being developed as a cellular therapy with the potential to modulated unwanted immune responses. While their suppressive function has been extensively studied *in vitro*, their homeostasis and mechanisms of immunoregulation still remain to be clarified *in vivo*. Previous data from our laboratory have shown that galectin-1, a β -galactoside-binding lectin, is specifically expressed by human and mouse regulatory T cells and contributes to their immunosuppressive function *in vitro*. Using a T cell transfer *in vivo* model of inflammatory bowel disease (IBD), when galectin-1-deficient CD4 CD25 Foxp3 regulatory T cells (Gal-1 KO-Tregs) were co-transferred with CD4 CD25 CD45Rb naïve T cells into Rag-1 mice their survival is compromised. Interestingly, Gal-1 KO-Tregs retained their capacity to modulate intestinal inflammation induced by CD4 CD25 CD45Rb naïve T cells in the surveyor mice compared to

the colitic group. However, this immunomodulation is less efficient than in wild-type Tregs as shown by the increase number of Gal-1 KO-Tregs required for protection against IBD. Strikingly, mice treated with Gal-1 KO-Tregs have clear signs of colon inflammation accompanied by splenomegaly and active mesenteric and caudal lymph nodes suggesting that Gal-1 KO-Tregs can eventually be converted into inflammatory T cells. We conclude that galectin-1 expression on Treg cells is required for the immunomodulatory function of Treg cell-mediated suppression and that adequate expression of galectin-1 should be taken into consideration when cell therapy protocols using regulatory T cells are proposed for clinical use.

Or030

Single intravenous administration of viral vectors carrying IL23R: A promising approach for Multiple Sclerosis therapy

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Multiple sclerosis (MS) is the most common inflammatory disease of the central nervous system (CNS) and the major cause of disability in young adults. Its prevalence in our population is approximately 80–90 cases per 100,000. Although the aetiology of this disease is not clear, it is known that the autoreactive T cells that have lost their tolerance to proteins present in the CNS play a fundamental role in its pathogenesis. Recently, it has been demonstrated the key role of the Th17 pathway in the initiation and development of various autoimmune diseases such as MS. Several molecules belonging to the Th17 pathway have been identified as important in the development of this disease, specially the interleukin (IL)23, which has been described as essential in the maintenance of the Th17 cell population. In this regard, our group has cloned and amplified different constructs of IL23 receptor (IL23R) in both adenoviral and AAV genomes. We have focused specially in a soluble form of IL23R, in order to block the IL23:IL23R interaction and thus to modulate the Th17 pathway by viral vector-mediated gene therapy. We administered a single intravenous dose of different viral vectors carrying IL23R gene or control vectors. Then, EAE was induced in C57BL/6J MOG40-55 peptide. During 30 days, mice were daily assessed for neurological signs using a 6-point scale. Animals were sacrificed and CNS removed in order to performed histo- and immunological studies. In these experiments we observed a significant reduction in clinical progression of the disease in those animals receiving the IL23R compared with those receiving control vectors. Thus, the clinical score for IL23R-treated mice was less severe throughout the experiment compared to the non-treated mice, with final values of 2.7 (± 0.7 SEM) and 4.7 (± 0.3 SEM) respectively. Moreover, a large decrease in cell infiltration, demyelination as well as activated microglia and astrogliosis was observed in the spinal cords of IL23R-treated animals compared to control-treated mice. Therefore, these results confirm the therapeutic potential of a gene therapy strategy for MS based on the expression of soluble IL23R to immunomodulate the Th17 pathway.

Or031

Expression of a small internal fragment of Dyskerin, decreases DNA damage and oxidative stress in Ataxia Telangiectasiacells

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In Ataxia telangiectasia, the ATM gene is mutated and the mechanisms underlying A-T disease are still incompletely understood. A key function of the ATM protein is to sense and regulate cellular redox status, using a complex network of downstream signaling pathways. ATM protein is also a key transducer of DNA damage signals to downstream effectors. ATM deficient cells show increased ROS accumulation, activation of the p38 protein kinase and increased basal levels of DNA damage. GSE24.2 a peptide corresponding to an internal domain of Dyskerin (a protein member of the telomerase complex) has proved to induce telomerase activity by stabilizing hTR (The RNA component of telomerase) and increasing expression of TERT (The catalytic subunit of telomerase). GSE24.2 (Ges-telmir) has been recently approved by EMA for the treatment of Dyskeratosis congenita. Expression of GSE24.2 in human fibroblast is able to protect from DNA damage detected by decreased H2AX foci and ATM and CHK2 phosphorylation. Due to these findings we have explored the use of GSE24.2 in ataxia telangiectasia human cells and studied the consequences in both DNA damage and ROS production. We have used both fibroblast and lymphoblast cell lines obtained from Corriel carrying different mutation in the ATM gene. Infection of A-T human cells with lentiviral vectors expressing GSE24.2 showed a reduction in basal levels of DNA damage, decreased levels of ROS and also lower levels of p38 phosphorylation, than cells infected with control virus.

Or032

Long-term preclinical studies of a FANCA lentiviral vector in a fanconi anemia-A mouse model

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Fanconi anemia (FA) is a rare inherited disease characterized by congenital abnormalities, bone marrow failure and cancer predisposition. To date, hematopoietic transplant is the only curative treatment of the hematopoietic syndrome in FA patients, although it is associated with a significant morbidity and mortality. Gene therapy with optimized lentiviral vectors may constitute a new alternative for the treatment of these patients. Aiming to evaluate the efficacy and the safety of a self-inactivating lentiviral vector developed for FA-A patients,

preclinical studies in *Fanca*^{-/-} mice were conducted. Lineage negative bone marrow cells from these animals were transduced with a PGK-FANCA-wPRE* lentiviral vector (FANCA-LV) designed for an upcoming Phase I-II trial. Robust transduction of *Fanca*^{-/-} HSCs with this vector resulted in long-term healthy hematopoiesis, which upon serial transplantation did not show any symptoms of toxicity or insertion-induced side effects. For a rigorous description of genetically modified cells, the LV insertion sites (LIS) were retrieved by linear-amplification mediated PCR, followed by pyrosequencing. A total number of >225,000 sequences in blood and bone marrow samples from reconstituted mice that mapped in >6,000 different LIS were identified. In contrast to the oligoclonal reconstitution pattern observed in mice transplanted with a genotoxic gammaretroviral vector, FANCA-LV transduced BM cells facilitated a highly polyclonal repopulation of *Fanca*^{-/-} recipients upon serial transplantation and absence of vector-induced malignancies. Our studies provide preclinical data that support the low genotoxicity of the FANCA-LV in upcoming gene therapy trials.

Or033

Towards clinical $\gamma\delta$ TCR gene therapy: the optimal $\gamma\delta$ TCR T cell product

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$\gamma\delta$ T cells are innate immune cells with strong anti-tumor activity and provide anti-tumor receptors that are interesting tools in immune therapy against cancer. The introduction of $\gamma\delta$ TCR genes into $\alpha\beta$ T-cells allows the adoptive transfer of T cells that are not limited by HLA-restriction, in any patient with any cancer. Combinatorial $\gamma\delta$ TCR-exchange (CTE) has been applied to design a $\gamma9\delta2$ TCR with strong and broad anti-tumor reactivity, referred to as TCR $\gamma9$ -G115/ $\delta2$ -cl5. The aim of the study is to design a clinical grade procedure which guarantees an efficient expression of the introduced $\gamma\delta$ TCR in $\alpha\beta$ T-cells, an adequate enrichment of engineered cells, as well as functional efficacy. Firstly, γ and δ chains were introduced into the retroviral vector pMP71, separated by a 2A peptide sequence. We demonstrate that the orientation of the γ and δ chains and the particular 2A peptide sequence influenced TCR expression as well as anti-tumor function as previously reported for $\alpha\beta$ TCR genes. Secondly, the introduction of the optimal $\gamma\delta$ TCR transgene cassette into $\alpha\beta$ T cells was followed by the depletion of non-transduced $\alpha\beta$ TCR positive cells using a clinical grade anti- $\alpha\beta$ TCR antibody. This depletion procedure resulted in a highly pure, but untouched, population of $\gamma\delta$ TCR-engineered T cells with increased $\gamma\delta$ TCR expression. Importantly, introduction of a $\gamma\delta$ TCR into $\alpha\beta$ T cells followed by clinical-grade depletion of non-transduced T-cells abolished residual allo-reactivity. Finally, this clinical grade $\gamma\delta$ TCR-engineered T cell product improved anti-tumor function both *in vitro* and in a humanized mouse model.

All together, we developed a clinical grade GMP vector which allowed a deliberate choice for the most potent clinical $\gamma\delta$ TCR receptor cassette. In addition, clinical grade depletion of non-transduced and transduced T-cells expressing high levels of endogenous $\alpha\beta$ TCRs results in a potent $\gamma\delta$ TCR-engineered T cell product suitable for an autologous but also allogeneic clinical scenario.

Or034

Adoptively transferred TCR gene-transduced lymphocytes persist with anti-tumor reactivity in patients with MAGE-A4+ esophageal cancer

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Engineering the antigen receptor gene in patients' lymphocytes is one promising strategy to create antigen-specific lymphocytes without senescent phenotypes. The strategy provides an opportunity to extend the application of adoptive T cell therapy for cancer patients. However, this concept has not been tested in the epithelial cancer patients. We completed a phase I clinical trial of TCR gene therapy targeting MAGE-A4 to treat esophageal cancer patients without lympho-depleting pre-conditioning. The trial was designed as a cell-dose escalation consisting of three cohorts, 2×10⁸, 1×10⁹ and 5×10⁹ cells/patient. The treatment was tolerable with no adverse events associated with transferred cells. In all ten patients of the 3 cell-doses, the transferred lymphocytes were detected in their peripheral blood in a dose-dependent manner during the first 14 days. In 4 patients, the infused cells have been persisting more than 5 months after the transfer. The T cell clones were established from the transferred lymphocytes that were harvested more than 100 days after the transfer. These clones sustained the reactivity to the antigen-expressing tumor cells. Three patients showed SD or long tumor free status. These results suggest that this approach may extend the availability of adoptive T cell therapy for epithelial cancer patients by providing tumor-reactive and long surviving lymphocytes reducing the risk of intensive pre-treatments.

Or035

T-cell engineering for "off-the-shelf" adoptive immunotherapy

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Adoptive T-cell therapies, where exogenous expression of a chimeric antigen receptor (CAR) confers cancer recognition, have shown significant promise in initial clinical trials. However, present adoptive immunotherapy methods are limited by the need for manipulation of autologous patient T-cells. To permit such an approach in an allogeneic context, Transcription Activator-Like Effector Nucleases (TALENTM) have been used to simultaneously inactivate the endogenous T cell receptor and CD52, a cellular target for a lymphodepleting treatment. This approach reduces the risk of GVHD while permitting proliferation and activity of the introduced T lymphocytes in the presence of the immunosuppressive drug alemtuzumab. Electroporation of primary T cells with mRNA coding for the ap-

propriate TALENTM result in double knock-out (dKO) frequencies of up to 70%. Furthermore, functional characterization demonstrates that the dKO cells are resistant to complement dependent lysis or *in vivo* depletion by alemtuzumab, and show no apparent potential for TCR-mediated activation. Finally, endowing the dKO cells with a CD19 CAR supports their capacity to kill CD19 tumor targets as efficiently as unedited T-cells both *in vitro* and *in vivo*

Or036

A new fully humanized transgenic mouse model for predicting the hematological toxicities of CD44v6-CAR T cells

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Introduction: When developing a new chimeric antigen receptor (CAR) specificity, off-tumor expression of the target antigen in normal tissues, is a matter of concern. We recently developed a CD44v6-specific CAR mediating potent antitumor *in vivo* effects against leukemia and myeloma. Since CD44v6 is expressed at some stages of hematopoietic development, the preclinical evaluation of the hematological toxicities of CD44v6-CAR T becomes mandatory.

Aim: To develop a humanized mouse model for predicting the spectrum of hematological toxicities of CD44v6-CAR T cells.

Results: Differently from NSG mice transplanted with human cord-blood derived CD34 hematopoietic stem cells (HSCs), which mainly reconstituted CD19 B cells, NSG mice transgenic for human IL-3, SCF and GM-CSF (NSG-3GS) showed enhanced myeloid reconstitution, including CD14 monocytes. Similarly to the human situation, reconstituting monocytes expressed CD44v6, while HSCs cells did not, indicating the suitability of this model to profile the hematological toxicities of CD44v6-CAR T cells. The infusion of CD44v6-CAR T cells in reconstituted NSG-3GS mice resulted in the selective elimination of monocytes, but in the preservation of other cell subsets. Importantly, after *in vivo* exhaustion of CD44v6-CAR T cells, NSG-3GS mice reconstituted monocytes *de novo*, indicating preservation of the HSC pool. For a more rapid ablation of CD44v6-CAR T cells *in vivo*, we have co-expressed TK or inducible caspase-9 and validated the suicide gene approach in experimental xenogeneic GVHD surrogating maximal toxicity.

Conclusions: Preclinical safety studies in human hematochimeric NSG-3GS mice predict selective and transient monocytopenia as the sole hematological toxicity of CD44v6-CAR T cells.

Or037

A versatile pre-clinical *in vivo* model to evaluate the efficacy of T cell receptor gene therapy

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T cell receptor (TCR) gene therapy provides a powerful approach for immunotherapy of cancer. The anti-tumor effect of TCR-engineered T cells is largely determined by the transgenic TCR that recognizes tumor cell-derived peptides. Although the function of TCR can be analyzed *in vitro*, current therapeutic approaches are impeded because parameters that predict *in vivo* efficacy are not established. We developed a mouse model to evaluate the efficiency of TCR gene therapy on established cancer. We generated immune-deficient, human MHC-transgenic mice (HHDxRag^{-/-}) and a syngeneic tumor cell line that can be modified to express human tumor antigens of interest. As proof of concept, T cells were engineered with human tyrosinase-specific TCR of different affinities. The anti-tumor effect of the T cells was analyzed *in vitro* and by adoptive transfer into mice bearing tyrosinase-positive tumors. Therapeutic efficacy was assessed by monitoring tumor growth. In parallel, cross-presentation of antigen by tumor stroma was analyzed. Transfer of T cells engineered with the high affinity tyrosinase-specific TCR resulted in complete tumor rejection, whereas low TCR affinity selected for escape variants and relapse. The tumor-derived tyrosinase was cross-presented by stroma cells. Although T cells engineered with both the high or low affinity TCR showed identical *in vitro* tumor cell killing, only T cells expressing the high affinity TCR recognized cross-presented antigen on tumor stroma. This *in vivo* model provides a versatile, pre-clinical test system of TCR gene therapy of cancer and allows the prediction whether or not TCR-engineered T cells eradicate tumors or select escape variants.

Or038

Zinc finger nucleases targeting the beta-globin locus drive efficient correction of the sickle mutation in CD34⁺ cells

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Sickle cell disease (SCD) is an inherited disorder that has lifelong complications and leads to decreased lifespan. An allogeneic hematopoietic stem cell transplant (HSCT) is the only currently available cure, but it faces many limitations such as donor availability and immune complications. We investigated the use of zinc finger nucleases (ZFNs) as a possible way to achieve successful gene therapy for autologous HSCT. ZFNs introduce a site-specific double stranded break upon dimerization, and, with the co-delivery of a donor template, can lead to homologous recombination to correct the sickle mutation. We developed ZFNs targeting the area adjacent to the sickle mutation in β -globin, along with a donor template containing the corrected sickle base and a silent base pair change to introduce a restriction enzyme site for analysis. Initial application of these components led to high levels of gene modification at the β -globin locus in the human erythro leukemia cell line K562 (upwards of 45%). Studies in the more appropriate cell target cord blood-derived CD34 cells resulted in up to 30% allelic disruption when the ZFNs were delivered as mRNA. To mimic a potential therapeutic strategy, when ZFNs were delivered as mRNA and the donor templates as integrase deficient lentiviral

vectors, we could reproducibly achieve 5–10% gene modification. When this delivery strategy was applied to CD34 cells derived from SCD patients, we could achieve up to 7% correction of the sickle mutation using the same delivery methods. These data set the stage for further optimization and studies in humanized mouse models.

Or039

G-CSF + plerixa for results in successful remobilization and single apheresis collections in thalassemic patients after primary mobilization failure

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Thalassemia gene therapy requires high CD34 cell collections because of the lack of a selective advantage at the transduced stem/progenitor cell level and a non-myeloablative conditioning that is, preferably, applied. In two clinical trials, we investigated the safety and efficacy of CD34 cell mobilization by different strategies, based on G-CSF or plerixafor, in splenectomized and non-splenectomized adult patients with thalassemia major as well as the remobilization with G-CSF plerixafor in those patients who failed on plerixafor-alone or G-CSF-alone. G-CSF-induced hyperleukocytosis was a significant dose-limiting factor in the splenectomized, G-CSF-alone-mobilized patients, resulting in poor, or at best, modest CD34 cell yields. Plerixafor rapidly mobilized CD34 cells in all individuals and without inducing hyperleukocytosis in the splenectomized patients; however, 35% of splenectomized and non-splenectomized patients failed to reach the target cell dose of $\geq 6 \times 10^6$ CD34 cells/kg. Four subjects who failed either on plerixafor or G-CSF were remobilized with G-CSF plerixafor. The combination proved highly synergistic; the target cell dose of $\geq 6 \times 10^6$ CD34 cells/kg was readily reached and the per apheresis yield increased by 9 fold (range 3–14x) over initial mobilization, ultimately resulting in single-apheresis collections, despite that a >50% G-CSF-dose reduction was applied in the splenectomized patients to avoid hyperleukocytosis. The total stem and progenitor cells mobilized were higher in G-CSF plerixafor patients as compared to plerixafor-alone patients and G-CSF plerixafor-mobilized cells displayed favorable transplantation features such as a primitive stem cell phenotype and a higher clonogenic capacity over plerixafor-mobilized cells. G-CSF plerixafor represents the optimal strategy when very high yields or/and a single apheresis is required. These data, in addition to providing an optimal mobilization approach for thalassemia, have broader implications for various stem cell gene therapy applications.

Or040

Parallel assessment of a globin lentiviral vector after transduction of ips and somatic hematopoietic stem cells from the same transplanted human β -thalassemia patient

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Patients with β -thalassemia major require lifelong transfusions. The only available curative therapy is an allogeneic hematopoietic transplantation with a high risk of engraftment failure and graft-versus-host disease. Hence, gene therapy (GT) by *ex vivo* transfer of a globin lentivector into the patient's own hematopoietic stem cells (HSCs) is an attractive novel therapeutic modality. The first Phase I/II human clinical trial was initiated by our group and the first treated $\beta E/\beta 0$ -thalassemia patient has become transfusion independent for the past 5 years (Nature, 2010). However, potential oncogenic genotoxicity remains a concern. Induced Pluripotent Stem Cells (iPSCs) are a potential alternative source of HSCs. Comparing HSCs derived from iPSCs with their natural isogenic somatic counterparts had not been performed in the context of therapeutic gene delivery. Here, we derived iPSCs from the thalassemia GT patient. iPSCs were then transduced with the $\beta A(T87Q)$ -globin lentivector used in the clinical trial. If fetal to adult globin class switching does not occur *in vivo* in iPSC-derived erythroid cells, β -globin gene transfer would be unnecessary. In NSG immunodeficient mice, embryonic to fetal and a partial fetal to adult globin class switching were observed, indicating that gene transfer is likely necessary for iPSC-based therapy. We examined the oncogenic risk of the lentiviral vector integration by determining the positions of integration sites (IS) relative to known genes, oncogenes, tumor suppressor genes, miRNA genes or ultraconserved regions. Approximately 15% were in "safe" areas of the genome. Surprisingly, common integration sites (CIS) were identified across iPSCs and cells retrieved from isogenic and non-isogenic gene therapy patients with β -thalassemia and adrenoleukodystrophy, respectively. (CIS) observed in the absence of overt tumorigenesis thus result from non-random lentiviral integration rather than oncogenic *in vivo* selection. These findings bring the use of iPSCs closer to practicality and further clarify both mechanics and interpretation of genome-wide lentivector integration.

Or041

Human cardiac progenitor cells "Tolerogenic/Modulator" immune behavior designates them as low-risk high-benefit allogeneic cardiac repair cells

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Therapeutics using stem/progenitor cells for heart failure are among the most explored in regenerative medicine given the urgent need for a cure. Allogeneic cardiac stem/progenitors

(CPC) are among the most obvious expectative goals. Within the 'Cardio repair European multidisciplinary initiative' (CARE-MI) consortium, we have successfully formulated human CPCs (hCPCs) that have been scaled up for industrial use and to meet regulatory standards. Allogeneic banking methodology has also been incorporated for safe storage. To guide rational design for hCPC clinical use, getting insights into the immunological mechanisms governing their engraftment was mandatory. Through robust and controllable *in vitro* testing we investigated adaptive and innate immune responses, T and natural killer (NK) cells responses, to allogeneic hCPC. Whether under inflammatory conditions or not, hCPC do not trigger conventional allogeneic T cells response but promote a regulatory T cells response and an allogeneic-driven immunomodulation, both dependent on B7 family member programmed death-ligand 1 (PD-L1). Only cytokine-primed NK cells were able to lyse allogeneic hCPC, but an inflammatory context, as after myocardial infarction, provided protection against this NK lysis and reinforced the capacity of hCPC to modulate the NK cytolytic activity towards target cells. Collectively our data revealed that hCPC in allogeneic settings have a "tolerogenic" immune behavior, raised the possibility of using PD-L1 expression as a marker to identify and select low-risk high-benefit allogeneic cardiac repair cells, and paved the way for the "first-in-human" European clinical trial.

Or042

Vascular endothelial and hepatocyte growth factor gene therapy in patients with critical limb ischemia

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Critical limb ischemia (CLI) represents the end stage in the evolution of peripheral arterial occlusive disease (PAOD). PAOD is the term used for patients with chronic ischemic rest pain, ulcers, or gangrene attributed to inadequate blood flow or arterial occlusive disease. The study analyzed the effects of gene therapy in CLI using plasmidial constructs expressing vascular endothelial and hepatocyte growth factors. A total number of 43 patients were included: 29 in the treatment group and 14 allocated to the control group. The main end points were the clinical safety of the method and the rate of major amputations. Secondary we monitored the improvement of pain at rest and walking ability. No significant adverse effects in the treatment group were found. Another important conclusion was the reduction of major amputation rate which was 31.04% in the treatment group and 71.42% in the placebo group ($p=0.029$). Moreover, the rest pain was improved in 65% of patients in the gene therapy group and in 7% in the control group ($p=0.0006$). The gene therapy treatment with vascular endothelial and hepatocyte growth factors is safe, efficiently reduces the rate of major amputations and improves the patient's life quality with critical limb ischemia.

Or043

Combinatorial RNAi- and receptor-based therapy against coxsackievirus B3 exerts additive antiviral effects *in vitro* and *in vivo*

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Coxsackievirus B3 (CVB3) is a major heart pathogen against which no specific therapy exists to date. In the current study the potential of a combination treatment consisting of a decoy receptor, functioning as virus trap, and an RNA interference-based component to prevent CVB3-induced myocarditis was investigated. The decoy receptor sCAR-Fc - a fusion protein composed of the soluble extracellular domain of the coxsackievirus-adenovirus receptor and the Fc domain of human immunoglobulin G1 - was expressed from an adenoviral vector. Additionally, two short hairpin RNAs (shRdRp2.4), directed against the CVB3 genome were delivered by a cardiac-targeted adeno-associated virus vector of serotype 9. Initial cell culture experiments revealed additive antiviral activity of the combined application. In a CVB3-induced mouse myocarditis model, either of the components significantly reduced inflammation and viral load in the heart. The combination of both components exerted an additive antiviral effect. Hemodynamic measurement revealed that infection with CVB3 resulted in impaired systolic and diastolic LV function, as illustrated by a reduced cardiac output and impaired contractility. Both single treatment with sCAR-Fc or shRdRp2.4, significantly improved cardiac output as well as contractility. Strikingly, the combined application again led to a further significant improvement of these parameters. During the time course of our *in vivo* study, no obvious side effects were observed in the animals treated with either of the viral vectors. Therefore the combination of vector-delivered sCAR-Fc and shRdRp2.4 has a significantly higher potency than either of the single gene therapy treatments to inhibit CVB3 myocarditis and prevent cardiac dysfunction.

Or044

AAV vectors produced by a GMP-compliant and scalable production platform mediate a CNS delivery platform using MRI-guided convection enhanced diffusion in NHP

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This study was performed to investigate the delivery of AAV vectors into the putamen, thalamus, or CSF of nonhuman primates. The vectors were delivered to the targeted regions of the brain by MRI guided convection enhanced diffusion. Special attention was given to the analysis of directional axonal transport. Recombinant AAV is an excellent candidate for delivery of therapeutic molecules to the central nervous system to target neurodegenerative diseases. UniQure has succeeded in developing a proprietary platform manufacturing technology that allows safe, effective, cGMP-compliant, economically feasible and commercially scalable manufacturing of AAV. UniQure's novel approach is based on the use of a combination of recombinant baculoviruses and insect cells. Using our production platform, two AAV stocks encoding GDNF or GFP were generated. At eight weeks following infusion into the thalamus, for instance, massive transduction of the thalamus, cortex, striatum and substantia nigra was observed. At the site of injection, transduction was both glial and neuronal, whereas off site transduction was mainly neuronal. Following infusion into the CSF, massive transduction of motoneurons and DRG along the complete length of the spinal cord was observed. Moreover, almost all Purkinje cells in the cerebellum were transduced as well as many cells in the cortex. These data together show that production of AAV using the (scalable) baculovirus-based platform results in an effective vector that is able to mediate expression patterns that can be used to develop and tailor an AAV-mediated therapeutic strategy to treat neurodegenerative diseases.

Or045

A soluble form of wild-type Tau protein is transported in rat brain through a trans-synaptic mechanism : implications for Tau spreading in sporadic Tauopathies

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In Alzheimer's disease and sporadic forms of tauopathies, neurofibrillary degeneration resulting from the aggregation of wild-type (WT) Tau progressively affects brain regions following a specific spatio-temporal pathway. Here, we take advantage of a newly characterized, lentiviral vector-based, model of tauopathy capable of reproducing the sequential progression of tauopathy, to study the cellular mechanisms brought into play in the rat brain following local Tau overexpression in the hippocampus. Through a combination of *in vivo* and *in vitro* experiments, it appears that human WT Tau protein is axonally transported over long distance (>10 mm) and actively transferred to efferent neurons in anatomically-connected brain areas (from ventral Ca1 field to olfactory bulb and frontal areas). No labelling of cell bodies in secondary neurons was observed in rat brains injected with control LVs encoding GFP. This cell-to-cell transfer from primary neurons to secondary ones occurs in an anterograde direction and initiates a Tau pathology as revealed with AT8, MC1 and AT100 labelling. Whether it results from a transfer of hyperphosphorylated Tau or Tau hyperphosphorylation

occurring in secondary neurons remains to be established. Altogether these results demonstrated *in vivo* that a specific trans-synaptic transfer of soluble WT Tau from degenerating neurons may lead to preliminary steps of Tau pathology in secondary neurons through a direct toxicity of human WT Tau and/or a conversion of endogenous Tau into a pathological form in secondary neurons.

Or046

Bone marrow mononuclear cell therapy for amyotrophic lateral sclerosis. Preliminary results of a randomized, double-blind, stratified controlled, parallel group phase I-II clinical trial

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Introduction: Amyotrophic lateral sclerosis (ALS) is an incurable neurodegenerative disease. We have shown that cell therapy has neurotrophic effects in ALS animal models. Therefore we designed a clinical trial to test the feasibility and safety of intraspinal and intrathecal infusion of autologous bone marrow mononuclear cells (aBMNCs). Here we present the interim safety data for those patients that have reached one-year follow-up.

Methods: Inclusion criteria were age between 18–70 years old, diagnosis of definite ALS according to El Escorial criteria, spinal onset and disease duration between 6–36 months, FVC $\geq 50\%$, and 5% of the total time with oxygen saturation level lower than 90%. Eligible patients were stratified by Sniff nasal and randomized between 3 arms: (A) Infusion of aBMNCs into the spinal cord at T3-T4 level. (B) Intrathecal infusion of aBMNCs. (C) Intrathecal infusion of saline, as control group. After the infusion, patients are evaluated every three months for 2 years. The primary endpoint is the absence of severe treatment-related adverse events (AE) that were graded according to the CTCAE.

Results: Forty-four patients have reached one-year follow-up (16 A, 14 B, 14 C). There were sixty-two non-severe treatment-related AE, all grade ≤ 2 . Forty occurred in group A, the majority (58%) during the first two weeks after surgery and only 5% was present at one-year follow-up. The most frequent were surgical wound pain (n=10), intercostal pain (n=6), hypoesthesia (n=6) and constipation (n=5). Twenty-two occurred in groups B and C, mainly headache (n=11) and lumbar pain (n=10) that resolved during the first two weeks after infusion. We did not observe any severe treatment-related AE. However there were seven hospitalizations and five deaths due to ALS progression.

Conclusion: These preliminary results suggest that intraspinal and intrathecal infusions of aBMNCs in ALS patients are feasible and safe. No major complications or significant morbidity was observed.

Or047

Therapeutic efficacy of a systemically delivered oncolytic adenovirus - Biodegradable polymer complex

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Despite great efforts to develop a more effective oncolytic adenovirus (Ad) for eradicating tumors, *in vivo* application via systemic administration is strictly limited to local injection due to host immune responses by Ad surface proteins and liver accumulation by the inherent nature of the Ad. In the last decade, numerous techniques using synthetic polymers have widely emerged to shield the exterior of therapeutic Ad vectors for systemic delivery. We developed a cationic polymer linked with polyethylene glycol for systemically delivering oncolytic Ad. The increased transduction efficiency and oncolytic effect of the Ad vectors physically coated with the polymer were evaluated, showing the optimal size (130 nm) of the Ad/polymer complex for systemic administration and prolonged stability of the Ad/polymer complex. Marked tumor growth suppression of the oncolytic Ad delivered by the polymer through systemic injection was observed in HT1080 and A549 xenograft models. The masking effect of the Ad surface by the polymer elicited evasion of innate adaptive immune responses and the tumor-to-liver ratio of the complex was significantly elevated 1229-fold greater than that of a naked Ad. These results demonstrate that the potential system of oncolytic Ad complexed with the biodegradable polymer may be useful for developing therapeutic vector systems via systemic delivery.

Or048

MicroRNA inhibition by dual-targeting and clustered Tough Decoy inhibitors

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MicroRNAs (miRNAs) are posttranscriptional gene regulators that play a role in almost any cellular process, and disturbed miRNA expression is associated with development of a wide range of diseases. In relation to diverse experimental and therapeutic applications, methods for managing miRNA activity are attracting increasing attention. In contrast to the often used synthetic and chemically modified miRNA inhibitors, vector-encoded miRNA inhibitors expressed from either plasmids or viral vectors allow persistent miRNA inhibition and tissue-specific expression. Currently, the hairpin-shaped "Tough Decoy" (TuD) miRNA inhibitor containing two miRNA binding sites is among the most efficient vector-encoded miRNA inhibitors. Here, we refine the design of the TuD miRNA inhibitor in order to increase the inhibitory potency and to obtain synchronized inhibition of two or more miRNAs. Using RNA polymerase II-transcribed inhibitors carrying clustered TuDs with up to a total of four TuDs in tandem, we demonstrate in luciferase-based assays enhanced miRNA inhibition compared to single TuDs.

Additionally, we show potent co-suppression of pairs of unrelated miRNAs by RNA polymerase III-transcribed dual-targeting TuDs carrying two different miRNA binding sites. Finally, we demonstrate robust inhibition of six miRNAs by a single multi-targeting TuD expression cassette containing a cluster of three dual-targeting TuDs. These refined TuD miRNA inhibitors unveil a new efficient potential for inhibition of miRNA clusters or families and might be adapted to a circularized design, similar to the recently discovered naturally occurring circular RNA sponges, be optimized even further.

Or049

Robust RNAi enhancement via human Argonaute-2 overexpression from plasmids, viral vectors or cell lines

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Argonaute-2 (Ago2) is the only human Ago protein capable of cleaving small RNA-targeted mRNAs, making it a unique and vital player in RNAi applications, including therapeutic gene silencing via small interfering (si) or short hairpin (sh) RNAs. Recently, we showed that cellular Ago2 levels limit the efficiency of si or shRNAs, and that Ago2 saturation by ectopic RNAi triggers can cause cytotoxicity, organ damage and lethality in adult mice, as well as accelerate liver carcinogenesis. Here, we report a new set of versatile tools and widely applicable strategies for transient or stable Ago2 co-expression which overcome these concerns. Specifically, we engineered all-in-one plasmids and viral vectors to co-encode a codon-optimized human Ago2 cDNA along with custom shRNAs. Furthermore, we stably integrated this Ago2 cDNA into a panel of standard human cell lines via plasmid transfection or lentiviral transduction. Notably, we obtained several clones with up to 25-fold higher Ago2 levels that were otherwise identical to their parental cells (growth, morphology, cDNA/miRNA profiles). Using various endo- and exogenous targets, including p53 and hepatitis B virus, we demonstrate that all three avenues increase si/shRNA efficiencies in cell culture by up to ten-fold, and moreover facilitate combinatorial knock-downs. Importantly, these robust improvements were reflected by augmented RNAi phenotypes and accompanied by reduced off-targeting effects. We finally show that Ago2/shRNA-co-encoding vectors can enhance and prolong transgene silencing in livers of adult mice, while concurrently alleviating hepatotoxicity. Our customizable reagents and strategies should broadly advance *in vitro* and *in vivo* RNAi applications in mammalian systems and, hopefully, eventually also in humans.

Or050

Transcriptional and post-transcriptional targeting of FVIII expression to overcome immunological responses to gene therapy for Hemophilia A

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Hemophilia A (HA) is an X-linked bleeding disorder due to mutations in clotting factor VIII (FVIII) gene. To date HA patients are treated with recombinant or plasma-derived FVIII with a high probability of developing inhibitors. Several efforts have been focused on the improvement of lentiviral vectors (LV) to obtain selective targeted expression by transcriptional and post-transcriptional regulation. However, immune responses to FVIII remain the major obstacle. The liver is known to induce tolerance rather than immunity by specialized resident cells, such as sinusoidal endothelial cells (LSEC) and Kupffer cells (KC). To investigate the possible role of LSEC and KC in gene therapy for HA using LV expressing FVIII under the control of cell-specific promoters ± specific microRNA target sequence (miRTs), we prepared LVs containing GFP or FVIII under the control of the ubiquitous PGK promoter, or the CD11b (surface integrin monocyte/macrophage-specific) or VEC (vascular endothelial cadherin) promoters in combination with miRTs alone or in pairs: miR142 (hematopoietic cells), miR126 (endothelial cells), miRT122 (hepatocytes). Co-staining with F4/80 or CD31 and GFP antibodies on liver sections taken at different time points after LV-GFP injection in mice confirmed a widespread pattern of GFP expression. Immunofluorescence showed GFP expression restricted to specific cell types within the liver by addition of one or two miRTs. After injection of LV.VEC.GFP and LV.CD11b.GFP, transgene expression was restricted to LSEC and KC respectively. The addition of the miRT122-142 combination to LV.VEC.GFP restricted GFP-expression to LSEC and miRT126 to LV.CD11b.GFP further increased specific expression in KC, with no off-target expression and sustained GFP expression at all-time points analyzed up to 6 months. We then injected HA mice with LV.PGK.FVIII ± miRT142 and LV.VEC.FVIII ± miRT122-142. In the first group anti-FVIII antibodies were detected, however the presence of miRT142 alone halved the titer of neutralizing antibodies in LV-injected mice. In HA mice injected with LV.VEC.FVIII ± miRT122-miRT142 long term phenotypic correction was shown by functional assay in both groups of the latter injected mice with reduced clotting time and an average of 5% FVIII activity and virtually no inhibitors were detected. Finally, we recently injected LV.CD11b.hFVIII ± miRT126 in HA mice to verify if overexpression of FVIII by KC is associated with tolerance induction and indeed our results with these mice are showing FVIII expression in the therapeutic range. In conclusion our study, using endothelial or monocyte/macrophage-specific promoters in combination of selected miRT showed phenotypic correction in treated HA mice.

Or051

Liver gene therapy by lentiviral vectors provides stable clinical benefit in three hemophilia B dogs and eradicates factor IX inhibitors in mice

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Lentiviral vectors (LVs) are attractive tools for liver gene therapy, by virtue of their ability to stably integrate in the genome of target cells and the lack of pre-existing humoral and

cellular immunity against vector components in most humans. By stringently targeting vector expression to hepatocytes, we previously reported long-term phenotypic correction of hemophilia B and transgene-specific immune tolerance, upon a single intravenous administration of LVs to mice. We have then translated this gene therapy strategy in a large animal model of hemophilia B. We produced large-scale purified LV batches using manufacturing processes and quality assessment previously established for clinical use. Each batch was administered into the portal vein of one adult hemophilia B dog. By incremental improvements in LV potency, we show long-term canine factor IX (FIX) activity up to 1% of normal levels and clinical improvement (substantial prevention of spontaneous bleedings) in the three treated dogs (>5 years cumulative follow up), without long-term adverse effects or anti-transgene immune responses. Moreover, we challenged the tolerogenic properties of our gene therapy approach by treating hemophilia B mice with pre-induced FIX inhibitors and report their eradication and reconstitution of clotting activity. This occurs likely through memory B cell depletion in response to constant exposure to FIX and induction of regulatory T cells. Overall, these studies provide evidence of safe and effective treatment of hemophilia B by systemic administration of LV, even in the presence of pre-existing FIX immunity. In the future, LVs may complement other vector platforms to broaden clinical applications of liver gene therapy.

Or052

Mesenchymal stromal cells reduce graft failure in autologous transplantation models with a high risk of poor engraftment

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Co-transplantation of human mesenchymal stromal cells (MSC) with hematopoietic stem cells (HSC) has been reported to reduce the risk of graft failure in patients subjected to haplo-identical HSC transplants. It is unknown, however, whether the engraftment facilitating role of MSCs is also maintained in an autologous transplantation setting, like the one considered in HSC gene therapy. Using a congenic HSC mouse transplantation model (Ly5.1/Ly5.2) in sublethally irradiated recipients (5 Gy), we have observed that the co-infusion of low numbers of congenic HSCs (1,500 LSK cells/mouse) with 10^6 adipose tissue-derived MSC/recipient (mAd-MSCs) significantly improved engraftment of transplanted recipients with donor cells. With the aim of approaching to a more clinically relevant model, we have conducted similar experiments using Fanconi anemia A (*Fancc*^{-/-}) recipients. While transplants of >1,500 WT LSK donor cells resulted in donor engraftments (considered as $\geq 10\%$ of donor cells in PB) in all WT recipients, these numbers resulted in a graft failure in 25–35% of FA recipients that received the same conditioning regimen of 5 Gy. To guarantee the engraftment of all FA recipients the infusion of at least 5,000 LSK cells per FA recipient was required, reinforcing the idea of a defective supportive hematopoietic stroma as a result of the FA mutation. Significantly, when $6 \cdot 10^5$ mAd-MSCs were co-infused with 1,500 WT LSK cells in FA recipients, all of the transplanted animals showed significant hematopoietic engraftments, reaching 50% and 100% of donor cells in PB at 4 and 8 weeks after HSC transplant, re-

spectively. Taken together, our results demonstrate the hematopoietic facilitating engraftment potential of Ad-MSCs, not only in an allogeneic context, but also in a clinically relevant model of autologous transplantation.

Or053

Combining tissue engineering with metal scaffolds in orthopaedics to improve osseointegration of endo-prostheses

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Electron Beam Melting (EBM) technology allows the fabrication of free-formed metal scaffolds, thus creating the possibility of manufacturing patient-specific endo-prostheses. It also allows the production of highly-porous prostheses with an elastic modulus similar to that of bone. The porous can be filled-in with tissue engineering elements (i.e. osteogenic molecules, biomaterials, cells) in order to promote bone ingrowth inside them, thus improving the prostheses osteointegration. For this purpose, the surface of EBM-sintered titanium should permit cell adhesion, growth and differentiation to ensure a good metal-to-tissue interaction. Our goal was to evaluate the osteoconductivity of EBM-manufactured Ti₆Al₄V porous scaffolds. Porous Ti₆Al₄V discs were manufactured by EBM-sintering, autoclave-sterilized and seeded with human and rat osteoblasts and mesenchymal stem cells (MSC). Cell adhesion, proliferation and differentiation were assessed by vital staining, MTT assay, RT-PCR and immunostaining techniques. Bone organ-explant culture was used to further assess osteoconductivity at tissue level *in vitro*. Both osteoblastic and MSC attached to and grew on the titanium discs, covering up the entire metal surface, and even bridging the pores of the scaffold. Collagen type I, osteopontin, and osteocalcin expression confirmed the osseous differentiation of the cells cultured on the titanium discs. Bone explants placed on EBM-sintered titanium alloy spontaneously released cells that covered up the metal surface. Long-term cultured explants strongly adhered to the titanium. EBM-sintered titanium scaffolds promote cell adhesion and can be populated by osteoblastic and MSC, which can normally differentiate towards the osteogenic lineage upon proper stimulation. These osteoconductive properties should promote the osseointegration of EBM-manufactured endoprotheses for bone replacement.

Or054

Aerosol-based cell therapy as a new strategy for treatment of airway injury

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Persistent obstacles of cell delivery techniques via intraperitoneal (IP) or intravenous (IV) cell injections had resulted in low retention and engraftment of the administered cells in cell-based therapy applications. The effective cell delivery to the lungs is hindered by the complexity of the lung structure. Our current study was aimed to develop an aerosol-based cell delivery as a novel technique to deliver cells into damaged airway epithelium using MicroSprayer[®] Aerosolizer. The effectiveness of aerosol-based cell delivery was validated *in vitro* using skin-derived

fibroblast cells, tracheal-derived epithelial cells, and HeLa cells. The viability and the proliferative capability of the cells were measured from day 1 up to day 21 following aerosolisation. The ability of the aerosolised cells to survive and engraft *in vivo* was studied on the airway of the rabbit following brush-induced injury. The tracheal-derived airway epithelial and fibroblast cells were labelled with BrdU, and later aerosol delivery was performed following 24-hr of brush-induced injury. One day following aerosolization, both fibroblast and tracheal-derived airway epithelial cells showed no sign of vacuolation, attached to the surface of the flask, proliferated rapidly and formed a confluent monolayer of cells at 4–6 days. In contrast, the HeLa cells were unable to proliferate even after 21 days of culture. The *in vivo* study showed that the labelled cells were found to be engrafted in the tracheal epithelium layer following 24 h of cells administration. The fibroblast and tracheal-derived airway epithelial cells were found to be engrafted and localized at the top of the damaged tracheal epithelium layer. Engraftment of cells was also found in the lung area, precisely at the epithelium layer of the bronchiole. The capability of cells to survive and to engraft into the damaged epithelium of the trachea and lungs following force-aerosolisation were found to be a pioneering therapeutic strategy for delivering of cells into the targeted site of the lungs for future treatment of lung-related diseases.

Or055

Gene knock-in using specific TALE nucleases restores erythroid differentiation defect of human iPSC obtained from pyruvate kinase deficient patients

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Pyruvate Kinase Deficiency (PKD) is a rare disease caused by mutations in the PKLR gene that leads to chronic non-spherocytic haemolytic anaemia. For severe cases of PKD the only definitive treatment is allogeneic bone marrow transplantation. Induced pluripotent stem cells (hiPSC) obtained from patients with a diagnosed genetic disease coupled with targeted gene correction via homologous recombination (HR) is a promising alternative for the treatment of hematopoietic inherited disorders. We have generated integration free-iPSCs from peripheral blood mononuclear (PB-MNC) cells of PKD patients (PKDiPSCs) using Sendai viruses and corrected the genetic defect by TALE nuclease (TALENTM) mediated HR in PKLR locus. Different hiPSC clones were obtained from two patients and one healthy donor (PBiPSC). Pluripotency characteristics (RT-PCR gene expression array, immunophenotype, promoter demethylation, teratoma formation) and disappearance of reprogramming vectors were

confirmed. HiPSC had a normal karyotype and neither T nor B cell receptor rearrangements. Interestingly, erythroid differentiation of PKDiPSC was impaired as occurs in PKD patients. To restore the defect, specific TALENTM were used to facilitate Knock-In of a codon optimized RPK cDNA in the PKLR gene. Correct integration was confirmed by PCR and Southern-blot. Surprisingly, allele specific integration was identified in one of the patients (PKD2iPSCs), which was associated to the presence of a SNP in the non-targeted allele, pointing out its potential use in specific allele substitution. Genome integrity was also interrogated by analyzing de novo somatic mutations and Copy Number Variations (CNVs). Few somatic mutations and CNV were found in coPKDiPSC that were not present in original PB-MNC. These modifications are now under validation. More importantly, gene corrected coPKDiPSCs display normal erythroid maturation profile, similar to the one observed in wild-type hiPSCs. All together we show the feasibility of PKD locus specific gene correction inpatient specific iPSCs that could be potentially used for clinical pursues in the future.

Or056

Rescue of T-cell deficiency in PRKDC SCID mice by transplantation of gene-edited haematopoietic stem cells

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The classical Severe Combined Immunodeficiency (SCID) mouse is a model of human DNA-dependent Protein Kinase Catalytic Subunit (DNA-PKCS, PRKDC) deficiency. We have developed an *ex vivo* system to correct PRKDC SCID by genome surgery, using zinc-finger nucleases to induce homologous recombination and lentiviral vectors to deliver nuclease and repair template. Using a Surveyor assay and deep sequencing in fibroblasts and HSCs treated in our system, we have unequivocally observed ZFN-mediated repair of the SCID mutation through the incorporation of the corrected nucleotide and a diagnostic restriction site from the donor template into the targeted locus. In fibroblasts, we have shown rescue of DNA-PKCS activity and increased resistance to DNA damage upon gene correction. Primary transplantation of gene-corrected SCID HSCs into sublethally irradiated SCID mice led to double-positive CD4/CD8 cells in the thymus, and single-positive CD3, CD4 and CD8 cells in peripheral blood. Correction of the SCID mutation and concurrent incorporation of the diagnostic restriction site have been confirmed by deep sequencing of whole blood, whole thymus and purified spleen T-cell DNA. In the purified spleen T-cell population ~50% of the deep sequencing reads corresponded to gene-corrected molecules, confirming their strong selective advantage. Upon secondary transplantation we have observed single-positive CD3, CD4 and CD8 cells in blood, and

proliferative responses in purified spleen T-cells. Our observations suggest that we have been able to correct the T-cell deficiency of PRKDC SCID mice by transplantation of *ex vivo* gene edited SCID HSCs, supporting the potential of gene repair-based rescue for SCID disease.

Or057

***In vivo* gene repair of inherited liver diseases using artificial endonucleases: Application to Crigler Najjar disease**

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Crigler Najjar type 1 (CN1) disease is a liver disease due to UGT1A1 enzyme deficiency. Lifelong cure of the Gunn rat model of CN1 has been obtained with lentiviral and AAV vectors. However, there are still drawbacks, such as risks of insertional mutagenesis. Thus, it is important to develop strategies of targeted gene therapy. Zinc Finger Nucleases (ZFNs) and Transcription Activator-like Effectors Nucleases (TALENs) allow targeted genome editing. They induce a specific DNA double strand break that promotes the insertion of an exogenous custom DNA donor through homologous recombination. *In vitro*, it allows efficient knock-in and gene repair. We constructed liver-specific AAV vectors encoding UGT1A1-ZFNs and carrying a donor to perform gene repair of the Gunn rat mutation. Newborn rats were co-injected with both vectors at 1:1 and 1:5 ratios to evaluate the feasibility, efficacy and safety of gene repair. Sub-therapeutic correction was obtained in the 1:5 cohort. UGT1A1 activity was detected in liver samples as well as bilirubin conjugates in bile. We are now working to evaluate the level of gene correction at a molecular level and to improve gene repair with TALENs, already validated *in vitro*. In conclusion, we showed that the endogenous mutation of UGT1A1 gene in the Gunn rat can be repaired *in vivo* at a level sufficient to obtain a sub-therapeutic effect. Such recent strategies may offer safer therapeutic options to treat inherited monogenic diseases.

Or058

Lentiviral-mediated COL7A1 gene modified autologous cell therapy for Recessive Dystrophic Epidermolysis Bullosa (RDEB)

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Recessive Dystrophic Epidermolysis Bullosa (RDEB) is a severe inherited skin-blistering caused by loss-of-function mutations in *COL7A1* which encodes type VII collagen (C7), a protein

important for anchoring fibril formation at dermal-epidermal junction (DEJ). Presently there are no effective treatments for RDEB. Allogeneic fibroblasts injected directly into the dermis have shown to regulate synthesis and assembly of C7 and anchoring fibrils at DEJ. The main limitations of this approach are that, the cells are rejected within a few weeks. We hypothesize that using gene therapy, *COL7A1* corrected autologous primary fibroblasts and/or keratinocytes injection may restore the full-length C7 and reverse the RDEB skin phenotype. We have produced third generation lentiviral (LV) vectors encoding codon optimized *COL7A1* under the control of a human PGK promoter, which has the ability to express C7 in fibroblasts, keratinocyte stem cells and bone marrow stem cells. LV vectors efficiently transduced *COL7A1* cDNA into RDEB primary fibroblasts and keratinocytes and transduced cells expressed increased levels of recombinant C7. We showed efficient *in vitro* genetic correction of RDEB fibroblasts and keratinocytes with persistent expression of recombinant C7 in ex-vivo culture. *COL7A1* corrected RDEB cells also showed functional restoration in migration assays and gene corrected RDEB cells efficiently deposited C7 at DEJ in an organotypic skin. This preclinical experience provides promising proof of principle data and a route to clinical therapies for RDEB.

Or059

Skin electroporation of a plasmid encoding hCAP-18/LL-37 host defense peptide promotes the healing of non-diabetic, diabetic and ischemic wounds

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Host defense peptides, in particular LL-37, are emerging as potential therapeutics for promoting wound healing and inhibiting bacterial growth. However, effective delivery of the LL-37 peptide remains limited. We hypothesized that skin-targeted electroporation of a plasmid encoding hCAP-18/LL-37 would promote the healing of wounds. The plasmid was efficiently delivered to full-thickness skin wounds by electroporation and it induced expression of LL-37 in the epithelium as demonstrated after immunostaining with anti-LL-37 antibodies. Electroporation of pHCAP-18/LL-37 led to significantly earlier wound closure and wound healing was significantly improved showing in average 77±6% wound closure for LL-37 treatment versus 63±6% for GFP control wounds, on day 12. Electroporation of pHCAP-18/LL-37 also restored delayed diabetic wound healing in db/db mice and wound closure on day 12 was 61±8% versus 43±9% in the control group. Subsequent histology analysis confirmed more efficient wound healing in LL-37 treated diabetic wounds. RT-PCR analysis revealed a significant increase in IL-6 transcription after electroporation of pHCAP-18/LL-37. To shed

light onto the influence of IL-6 we performed *in vitro* wound migration assays and showed that the LL-37-induced cell migration was abolished in the presence of an IL-6 blocking antibody. Finally, in a hindlimb ischemia model, electroporation of the hCAP-18/LL-37 plasmid increased blood perfusion, reduced muscular atrophy, and upregulated the angiogenic chemokines VEGFa and SDF-1a, and their receptors VEGF-R and CXCR-4. These findings demonstrate that a localized gene therapy with LL-37 is promising for the treatment of non-diabetic, diabetic and ischemic wounds.

Or060

Allogenic Bone marrow derived Mesenchymal stem cells for the treatment of Dystrophic Epidermolysis Bullosa

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Background: Dystrophic Epidermolysis Bullosa (DEB) is a devastating skin disease caused by mutations in the type VII collagen gene. Type VII collagen is synthesized by keratinocytes and fibroblasts. Bone marrow mesenchymal stem cells (BM-MSCs) can develop into fibroblasts secreting collagen VII. This is a pilot clinical study to evaluate the safety and clinical efficacy of allogenic BM-MSCs injection in cases of DEB.

Methods: This study included 14 patients with recessive DEB (RDEB), divided into two groups: Group I received cyclosporine while the other did not. 60–80 ml bone marrow were aspirated from donors under aseptic conditions, and MSCs isolated, labelled using iron oxide and injected systemically in a dose of 2 million/Kg. Patients were followed up for six months using severity score, quality of life of EB (QOLEB), and family dermatology life quality index (FDLQI). Skin biopsies from the lesions were evaluated for histology and tracking of injected cells.

Results: Patients ages ranged from 1–11 years with a mean of 4.47 years. They were 8 males and 6 females with 100% showing positive consanguinity (first cousins). After six months, the severity score showed a mean of 35 in Group I and 38 in Group II. The number of new blisters decreased significantly in both groups ($P=0.003$ and 0.004 respectively); with no statistically significant difference between groups ($P=0.46$). The rate of healing of blisters became significantly faster in both groups ($P<0.001$) with no difference between both groups. QOLEB score improved from a mean of 40/68 to a mean of 52 in Group I and 50 in Group II, while FDLQI improved from a mean of 13/40 to a mean of 20 in Group I and 22 in Group II. These differences were statistically significant with no inter-group difference. Skin biopsies revealed iron-staining fibroblasts in the dermoepidermal junction and positive Y chromosome in one female patient who received transplantation from her father. No major side effects were reported with none of the patients showing any signs of rejection.

Conclusion: Our findings highlight the safety and shortterm efficacy of allogenic BM-MSC in the treatment of RDEB.

- number of EB patients: (n=12)
- number of families: 10 (2 families have 2 cases)
- age: 1–11, range=10, mean=4.47, standard deviation=2.76
- Sex: male=8, female=4
- consanguinity: 12 (100% are 1st cousin)
- number of each family (parents and sons): 3–9, range=6, mean=4.92
- severity score (only 3 cases/12): mean 48/100

- Quality of life of EB (QOLEB) [missing 4 cases]: mean=40/68
- family dermatology Life Quality index (FDLQI) [missing 4 cases]: mean=13/30

Or061

Efficacy of combined gene/cell therapy in a murine model of Globoid cell Leukodystrophy

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Globoid cell Leukodystrophy (GLD) is a demyelinating disease caused by genetic deficiency of lysosomal galactosylceramidase (GALC). Our previous studies demonstrated that neonatal intracerebral transplantation of GALC-overexpressing neural stem cells (NSCT) and hematopoietic cell transplantation (HCT) are effective as independent treatments in preventing/delaying pathology in GLD mouse models. NSCT rapidly supplies high and long-lasting GALC activity in the CNS, HC-derived microglia provides functional enzyme in the CNS and PNS. However, the slow kinetics of microglia reconstitution might account for inadequate CNS treatment in GLD mice and patients. Here, we tested the efficacy of a combined treatment (NSCT HCT) performed in neonatal GLD mice in achieving fast and therapeutically relevant GALC activity in all the affected organs. We showed rapid and stable NSC engraftment in the telencephalon and time-dependent caudal-to-rostral increase of HCs in the CNS. GALC activity reached 50% and 100% of the physiological levels in the CNS and PNS, respectively. Importantly, NSCs provided short-term GALC supply in CNS tissues before HC engraftment. Despite no apparent synergy of GALC activity, combination therapy provided benefits when compared to single treatments in terms of: i) reduced storage and astrogliosis in telencephalon, in which NSCs are a local source of GALC; ii) reduced expression of the stress molecule ATF3 and lower number of apoptotic cells in the spinal cord, suggesting the contribution of circulating GALC and local HC-mediated anti-inflammatory activity. Completion of histopathology analyses, survival curves and behavioural studies will disclose whether the proposed combination therapy might represent a suitable approach to effectively treat GLD.

Or062

Gene Therapy using an AAV2/8 vector corrects the biochemical imbalances in a murine model of MNGIE

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Mitochondrial neurogastrointestinal encephalomyopathy (MNGIE) is a fatal disorder caused by mutations in *TYMP*, which encodes the enzyme thymidine phosphorylase (TP). TP

dysfunction causes the systemic accumulation of its substrates, the nucleosides thymidine (dThd) and deoxyuridine (dUrd), which interferes with mitochondrial DNA replication and results in mitochondrial dysfunction in MNGIE patients. All therapeutic strategies have been aimed to reduce the circulating levels of these toxic metabolites. The only effective treatment for MNGIE is allogeneic hematopoietic stem cell transplantation from a healthy donor, but this procedure is associated with high rates of mortality and morbidity. Gene therapy using adeno-associated viral vectors (AAV) constitutes a promising alternative. We have generated an AAV serotype 2/8 vector containing the human TYMP cDNA under the liver-specific promoter of the thyroxine-binding protein. Eight to twelve week-old male *Tymp^{-/-}/Upp1^{-/-}* mice (a murine model of MNGIE) were treated with different intravenous doses of viral particles. Plasma nucleosides were reduced to wild-type levels in 50% of the animals (4 out of 8) treated with the lowest dose (2×10^{11} genome copies/kg) over the entire period of time monitored (8 months). 88% of the animals (7 of 8) treated with 10^{12} gc/kg, and all the animals treated with 2×10^{12} or 10^{13} gc/kg maintained nucleoside levels below the normal range. No signs of hepatotoxicity were detected in the mice, as assessed by monitoring circulating alanine aminotransferase activity. These results suggest that the use of AAV vectors to direct TP expression in liver is a feasible and promising gene therapy strategy for MNGIE.

Or063

Evaluation of intrathecal rAAV vectors in canine mucopolysaccharidosis VII

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Mucopolysaccharidosis (MPS) VII results from lysosomal storage of glycosaminoglycans (GAGs) due to mutations in β -D-glucuronidase (GUSB), which produces a neurological phenotype. Gene therapy could reduce GAGs in the brain, but delivery throughout the brain in a large animal has been problematic. In this study, four MPS VII dogs were injected intravascularly (IV) with 2×10^{13} genome copies (GC)/kg of recombinant adeno-associated virus vector serotype 9 (AAV9) or AAVrh10 at 3 days of age. Two of these dogs were also injected intrathecally (IT) into the cerebrospinal fluid (CSF) via the cisterna magna with the opposite vector at 2 months of age ($\sim 4 \times 10^{12}$ GC/kg). IV injection alone resulted in near normal GUSB activity in CSF and $\sim 5\%$ of normal levels in the brain parenchyma. Both of the IV IT dogs achieved GUSB activity in the CSF ≥ 100 -fold normal, while mean GUSB activity of central nervous system (CNS) parenchyma was ≥ 2.5 -fold normal, correlating to vector copy numbers. Brain GAG levels were reduced by $\sim 66\%$, but did not reach normal. However, immunohistochemical staining revealed clearance of GM3 gangliosides, a secondary storage substrate. Additional histochemical staining revealed widespread GUSB activity throughout the CNS in the IV IT dogs and in the choroid plexus of the IV-only dogs. Two additional MPS VII dogs were injected only IT at 21 days of age with either vector, thus far resulting in CSF GUSB levels ≥ 77 -fold normal seven weeks post-injection. This study demonstrates that IT injection of AAV9 and

AAVrh10 vectors has substantial potential for treating the CNS in lysosomal storage disorders.

Or064

Hyperglycemia and oxygen tension control the expression of the glucose transporter GLUT-1 in models of Diabetic Retinopathy

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Diabetic retinopathy (DR) is a blinding disease characterized by microvascular changes caused by chronic exposure to high glucose levels. It has been hypothesized that an increase of glucose transporters in the retina, such as GLUT-1, is responsible for increased glucose levels in retinal cells, causing cell death. It is also known that GLUT-1 expression is controlled by HIF-1 that is also involved in the development of DR. In this work we evaluated the expression of GLUT-1 *in vitro* and *in vivo*, in models of DR. For the *in vitro* assays, retinal epithelium cells (D407) were subjected to hypoxic and normoxic conditions and were cultured with DMEM supplemented with different concentrations of glucose, corresponding to non-diabetic, pre-diabetic and diabetic conditions for 48 h, GLUT-1 expression was analyzed. For the *in vivo* GLUT-1 evaluation, retinal sections of diabetic *Ins2^{Akita}* mice were used and compared with non-diabetic mice. Our results show that the increase in glucose in the culture medium caused an increase in GLUT-1 expression. These effects were more pronounced in cells under hypoxic conditions, suggesting the involvement of HIF-1 in the regulation of GLUT-1 expression. *In vivo*, GLUT-1 was expressed with higher intensity in retinal sections of the diabetic *Ins2^{Akita}* mice compared with the non-diabetic mice, confirming our *in vitro* results. In this study the expression of GLUT-1 was analyzed in conditions simulating DR. The results indicate that increased GLUT-1 levels in retinal cells during diabetes and oxygen tension, a critical factor for the development of DR, may regulate GLUT-1 via HIF-1.

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Or065

Phase I clinical study of a third-generation simian immunodeficiency virus (SIV)-based lentiviral vector carrying human pigment epithelium-derived factor (PEDF) gene for patients with retinitis pigmentosa

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Retinitis Pigmentosa (RP) is a genetically heterogeneous group of inherited retinal degenerative diseases resulting from photoreceptor

death. Although extensive effort has been made to treat or cure RP, it remains intractable. We recently developed a novel lentiviral vector derived from the non-pathogenic simian immunodeficiency virus (SIV). SIV-mediated gene transfer of human pigment epithelium-derived factor (hPEDF), which is one of the most potent neurotrophic factors found in human, can protect the retinal degeneration and functional defects in two different animal models. Moreover, the safety study using large animals indicated the long-term systemic and local safety of intraocular administration of SIV-hPEDF. These results are encouraging for clinical application of neuroprotective gene therapy for patients with RP. To assess the safety of subretinal administration of SIV-hPEDF, we will enrol 20 RP patients in this open-label, dose-escalation study. The first trial subject was enrolled on March 2013 and here, we report the clinical trial of a SIV-mediated hPEDF gene therapy for patients with RP. This is the first clinical trial for ophthalmologic diseases in Japan. Furthermore, this is the first application of a gene transfer vector based on recombinant SIV all over the world.

Or066

Simple generation of self-organizing neural retina-like structures from human iPS cells

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Human pluripotent stem cells have proven their ability to be differentiated into various retinal lineages, opening the avenue to stem cell-based therapies for retinal degenerative diseases. The increasing number of protocols available to produce retinal cells from either human embryonic stem cells or induced pluripotent stem cells (hiPSCs) confirms their potential but suggests a need for standardization and more robustness. Large-scale production of hiPSC-derived retinal cells for therapeutic approaches or drug screening should comply with certain criteria such as, safety, efficiency, reproducibility, and low production cost. Here, we developed a new, simple and reliable retinal differentiation protocol using confluent hiPSCs derived from adult human dermal fibroblasts by integration-free transgene reprogramming. Under these conditions, retinal pigmented epithelial (RPE) cells and self-organizing neural retina (NR)-like structures were generated in 2 weeks in pro-neural medium without embryoid body formation or addition of exogenous molecules. At Day 14, NR-like structures showed an eye field identity, with a homogenous population of mitotic retinal progenitor cells (RPCs) expressing PAX6, RAX and VSX2. Floating cultures of isolated hiPSC-derived NR-like structures enabled the differentiation of RPCs into all types of retinal cells, which appeared in a sequential manner consistent with *in vivo* vertebrate retinogenesis. Indeed, we observed two waves of differentiation, corresponding to early-born retinal cells identified as early as 21 days in culture (*i.e.* ganglion, amacrine and horizontal cells), and late-born retinal cells appearing after 35 days (*i.e.* photoreceptor, Muller glial and bipolar cells). Interestingly, precocious inhibition of the Notch pathway led to massive differentiation of RPCs into photoreceptor precursors. We thus propose a process that eliminates the need for time- and labor-consuming manual steps, and provides a readily scalable approach to generate large numbers of mitotic RPCs and of both RPE cells and precursors of photoreceptors.

Or067

DAI-armed double deleted oncolytic vaccinia virus displays enhanced anti-tumor activity by eliciting a more robust anti-tumor immune response

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Oncolytic viruses have been engineered to improve their selectivity and their cross-talking to the immune system. The release of tumor associated antigens and damage-associated molecular patterns, consequent to cell lysis promote the cross-priming of naïve T cells. Double deleted vaccinia virus (VVdd) is a very promising tool for oncolytic virotherapy, nevertheless, it is also very immunosuppressive. To overcome this limitation we hypothesized to arm VVdd with DNA-dependent activator of IFN-regulatory factors (DAI), a member of the PRRs family that binds cytosolic dsDNA and induces type I IFNs.

We show in this work that DAI plays a central role in sensing VV's infection. In fact, type I IFNs and pro-inflammatory cytokines secretion was significantly reduced in DAI-silenced Jurkat cells following VV's infection. In addition, DAI expression does not affect the oncolytic activity of VV, since DAI-armed viruses and unarmed control virus feature the same cell-killing activity *in vitro*. Then we assessed the immunostimulatory potential by measuring different markers of immunogenic cell death: ATP, HMGB1 and calreticulin. We found that the levels of these markers were significantly higher in cells infected with DAI-expressing virus. Using B16-OVA murine model we show that the DAI-expressing virus was able to increase by 10 fold the tumor-specific immune response compared to control with no measurable toxicity. Through a micro-array analysis we compared the effects of DAI-VVdd, CD40L-VVdd and unarmed VVdd upon human cancer using a prostate cancer cell line (PC-3MM2). Interestingly we found almost one thousand genes, uniquely induced by DAI-VVdd, with the majority of them belonging to antigen presentation pathways, T cells and DCs maturation, cytokines and chemokines secretion. In conclusion, arming oncolytic vaccinia virus with DAI has proven to be an excellent approach to enhance the immunogenicity VV resulting in better anti-tumor response.

Or068

$\alpha\beta$ -T cell receptor-based gene therapy targeting the common tumor-antigen survivin

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Adoptive transfer of T lymphocytes redirected with abT-cell receptors (abTCRs) has produced complete responses in cancer patients. T-cell redirected against survivin, a broadly expressed tumor-associated antigen, may significantly enhance the applicability of this promising approach. However, targeting survivin by ectopic expression of abTCRs has been previously challenged because survivin-abTCRs isolated from all restricted repertoires produced fratricide effects. Using autologous cultures we isolated by limiting dilution, a new T-cell clone targeting the HLA-A*0201-restricted survivin₉₆₋₁₀₄97M-

epitope that had sufficient avidity to recognize and lyse survivin- HLA-A2 malignancies *in vitro*, but lacked any fratricide effect or toxicity against normal hematopoietic stem/progenitor cells. The TCR α - and β -chains were cloned in an optimized retroviral vector and transduction of CD8 cells resulted in efficient expression of the transgenic abTCR ($89 \pm 4\%$). No fratricide effects by abTCR T cells were detected against HLA-A2 activated T cells and T-cell expansion was comparable regardless of TCR expression by HLA-A2 or HLA-A2⁻ T cells (25 ± 5 vs 26 ± 5 fold, $n=5$ /each). Survivin-abTCR T cells lysed significantly well ($p < 0.01$) the HLA-A2 BV173 ($46 \pm 14\%$) and U266 ($27 \pm 12\%$) targets but not HLA-A2⁻ targets (14 ± 7). Importantly, transgenic cells recapitulated the function of the original clone by inhibiting colony formation (32 – 78% reduction, $n=5$) of primary myeloid leukemias while preserving healthy BM-clonogenic capacity ($n=5$). *In-vivo* in xenografted mice infused intravenously with FFLuc BV173 to mimic a high leukemic burden, survivin-TCR T cells controlled leukemia progression and improved survival ($n=12$ /group, $p=0.01$). In conclusion, our survivin-abTCR provides antitumor activity *in vitro* and *in vivo* without affecting the survival of T cells or normal hematopoietic progenitors.

Or069

Infusion of ZFN CCR5 modified CD4 T-cells (SB-728-T) led to long term reconstitution of CD4 T-cells and reduction of HIV-DNA levels in HIV infected subjects on ART

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Background: Long term improvement in CD4 count was observed in all SB-728-T treated HIV subjects, at levels greater than previous HIV/T-cell studies. Here, we performed phenotypic and transcriptional analyses to elucidate the role of inflammation on CD4 reconstitution and SB-728-T persistence, and evaluated the effect of SB-728-T on the latent reservoir.

Methods: 9 chronic aviremic HIV subjects on ART with CD4 counts btw 200–500 cells/uL received 10-30E9 SB-728-T. Analyses of survival and activation markers were performed on PBMCs collected pre and post infusion. Digital droplet qPCR was performed to measure HIV-DNA level.

Results: Long term CD4 reconstitution is primarily driven by the central memory T-cell subset (T_{CM}). Subjects with greater CD4 improvement showed reduced PD-L1 expression in monocytes, that correlates with lower PD-1 expression in T_{CM} ($r=.77$, $p=.02$) and higher T_{CM} counts ($r=.83$, $p=.01$) at M9. Importantly, the associated improvement in CD4 count at M12 correlates with decreased size of the latent reservoir ($r=-.86$, $p=.02$). Analysis of innate immune activation, as measured by expression of HLA-DR, CD80 and CD40 in monocytes, and IFN type I stimulated gene expression pre infusion showed that suboptimal responders had higher inflammation levels at baseline ($r=-.73$, $p=.03$).

Conclusions: Decreased levels of inflammation may provide a survival advantage of T_{CM} post infusion. This may play a role in the observed decrease in latent reservoir size (0.6 log per 1E6

PBMC). Identification of an inflammatory signature prior to treatment as an important predictor of response will help future study design of SB-728-T.

Or070

Entry targeted gene transfer into functional subpopulations of human T cells

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CD8 cytotoxic T cells and CD4 T helper cells are the two major T cell subpopulations. Specific delivery of genes into each subset separately is an important goal for gene and immunotherapy. We have recently demonstrated specific gene transfer into cytotoxic T cells by a lentiviral vector (LV) based on a previously published method relying on modified measles virus (MV) envelope glycoproteins and a CD8-specific scFv as targeting domain. Here, we describe a CD4-targeted vector (CD4-LV) using a CD4-specific designed ankyrin repeat protein (DARPin) as targeting ligand. Interestingly, titers of CD4-LV were on average more than 10-fold higher than those of CD8-LV reaching 10^8 t.u./ml upon concentration. CD4-LV exclusively transduced CD4 but not CD4⁻ cells. *In vivo* distribution of tail vein injected CD4-LV into immunodeficient mice reconstituted with human peripheral blood mononuclear cells (PBMC) was monitored by imaging luciferase activity. Notably, distinct signals in spleen, thymus and lymph node like structures were observed. Evaluation of GFP expression in organ derived cells by flow cytometry and histology confirmed the specific and exclusive *in vivo* targeting of CD4 human lymphocytes in blood and spleen of these animals. This is, to our knowledge, the first demonstration of exclusive gene transfer into a T cell subset upon systemic vector administration. Together with CD8-LV, both important subsets of T cells can now be genetically modified individually. Thus, these vectors are powerful tools both for gene therapy and basic research.

Or071

Measles virus glycoprotein pseudotyped lentiviral vectors transduce resting long-term repopulation hCD34+ cells at an efficiency without precedent

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Hematopoietic stem cell (HSC) based gene therapy is now moving towards the use of lentiviral vectors (LVs) evidenced by two recent clinical trials. In these trials, VSV-G-LVs were applied at high doses to obtain therapeutically relevant transduction levels. Moreover, high gene transfer was only achieved with very strong cytokine-cocktails compromising the 'HSC' character of the cells. Thus, there is room for improvement. We have shown that measles virus (MV)

glycoprotein displaying LVs (MV-LVs) were able to transduce efficiently into resting human T and B cells. Now, we evaluated these MV-LVs for hCD34 cell transduction under mild cytokine prestimulation in order to better preserve the 'HSC' characteristics. After a single application at a low vector doses, MV-LVs stably transduced 100% of TPO SCF stimulated hCD34 cells, where VSV-G-LVs reached 5–10% transduction at the same vector doses. Even more striking was that these MV-LVs allowed at low vector doses, efficient transduction of up to 50–70% of quiescent hCD34 -cells, an efficacy without precedent. Importantly, reconstitution of NSG mice with MV-LV transduced pre-stimulated or resting hCD34 cells resulted in transduction levels close to 100% or 50%-70%, respectively, of all analyzed myeloid and lymphoid engrafted lineages. This was also confirmed for secondary engrafted mice. Together, these results strongly suggest that the MV-LVs efficiently transduce true HSCs at extremely high levels. This paves the way to HSC-based gene therapy of multiple diseases including diseases characterized by low numbers of HSCs such as Fanconi Anemia, for which high level HSC correction is needed to be successful.

Or072

Bromodomain and extra-terminal (BET) proteins target MLV-based vector integration to transcription start sites

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Stable integration of the viral DNA in the host cell genome is a hallmark of retroviral replication that makes retroviral-derived vector particles attractive tools for gene therapy. Retroviral integration is not random, with lentiviruses preferentially integrating in the body of active transcription units, and gammaretroviruses, like the Moloney Murine Leukemia Virus (MLV), favouring transcription start sites (TSS) and CpG islands. The latter explains the activation of oncogenes and the development of acute leukemia in a subset of patients in several clinical trials using MLV vector-based gene therapy. We identified and characterized the bromodomain and extra-terminal (BET) family proteins (BRD2, BRD3 and BRD4) as specific cellular binding partners of MLV integrase, directing MLV integration near TSS. We show that MLV integration site distribution strongly correlates with the BET protein chromatin binding profile determined by ChIP-seq and that interfering with the chromatin interaction of BET proteins via specific bromodomain inhibitors (JQ-1 and I-BET) blocks MLV virus replication and MLV vector transduction at the integration step. Moreover, we generated an artificial fusion protein that merges the BET integrase binding domain with the chromatin interaction domain of the lentiviral targeting factor, LEDGF/p75. Expression of this chimera in wild-type cells retargets MLV integration away from TSS and into the body of actively transcribed genes, reminiscent of the Human Immunodeficiency Virus integration pattern. Together, these results explain the

molecular mechanism of gamma-retroviral integration targeting and open perspectives to engineer gammaretroviral vectors with a safer integration site profile.

Or073

Integration site analysis in a clinical trial of lentiviral vector based hematopoietic stem cell gene therapy for metachromatic leukodystrophy

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We assessed the safety and efficacy of gene transfer as well as the dynamics of hematopoietic reconstitution in the first 3 patients of an ongoing lentiviral vector (LV)-based hematopoietic stem cell (HSC) gene therapy trial for metachromatic leukodystrophy (MLD). All treated patients showed unprecedented high vector marking levels (50–80%). Analysis of >67,000 integration sites (ISs), obtained from different hematopoietic lineages and time points after transplantation (1, 3, 6, 9, 12 and 18 months), showed the characteristic LV integration profile. Shannon diversity index and abundance analyses indicated that the patients were sustained by a highly polyclonal engraftment without evidence of aberrant clonal behaviour.

To determine the extent of HSC gene marking *in vivo*, we considered for each patient the subset of ISs shared among CD34, mature myeloid and mature lymphoid cells. In patients between 23 and 46% of the CD34 ISs were shared with the myeloid or lymphoid lineage dataset across all time points, providing a stringent evidence of self-renewal and multi-lineage potential of the transduced engrafted HSPCs. By comparing ISs shared between months 6 or 9 and 12, post-transplant by the Petersen/Schnabel estimator method we estimated a population size of about 4,000 marked cells, suggesting a large pool of engrafted transduced self-renewing progenitors. Overall, these data provide evidence that efficient *ex vivo* gene transfer was followed by substantial engraftment and sustained clonogenic activity of the transduced HSCs in the patients, allowing polyclonal reconstitution of hematopoiesis with gene corrected cells to near homogeneity.

Or074

Tracking individual hematopoietic stem cell activity *in vivo* in humans through integration site barcoding

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Understanding human hematopoietic dynamics is crucial for the development of therapeutic approaches based on genetically engineered hematopoietic stem cell (HSC). Our gene therapy (GT) clinical trials, based on retrovirally engineered HSC, constitute a unique setting to study human hematopoiesis through insertion site (IS) barcoding. To this aim, we collected 32,363 IS from purified cell lineages in 3 Wiskott-Aldrich Syndrome (WAS) patients treated with lentiviral GT up to 1.5 years and 4,845 IS in 4 adenosine deaminase (ADA) deficient-SCID patients treated with gammaretroviral GT, up to 6 years after treatment. In WAS patients, we traced self-limiting waves of individual progenitor activity during the first phases stabilizing at 12–18 months after transplant. By mark-recapture models, we estimated that about 1,700–6,300 transduced HSC were active in our patients and we identified and followed HSC clones sharing identical IS in CD34 cells, colony forming units and a wide spectrum of mature cell types. In ADA-SCID patients we showed that identical IS are consistently detected at multiple lineages level even several years after GT. Since gammaretroviral transduction occurs only in actively replicating cells, this finding provides the first evidence that stimulated HSC, “awaken” from dormancy, can still retain *in vivo* long-term activity in humans. Through machine learning network algorithms applied to IS datasets we uncovered the hierarchical relationship among lineages, validating different models of hematopoiesis. Our unique molecular tracking of individual hematopoietic progenitors allowed uncovering HSC dynamics *in vivo* in humans, providing novel information for the treatment of hematological diseases and tumors.

Or075

AAV expression of CD39 and CD73 as a novel therapeutic strategy for the treatment of rheumatoid arthritis

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Adenosine and ATP are known to have important immunomodulatory properties. Extracellular ATP has multiple roles in inflammation and can act as a damage-associated molecular pattern (DAMP) that can activate the immune system. Conversely, adenosine is primarily anti-inflammatory and can inhibit the production of pro-inflammatory molecules by immune cells. The conversion of ATP to adenosine is an important mechanism of immune suppression by Tregs, and this is done by expression of ENTPD1 (CD39) and 5NTE (CD73). CD39 is a membrane bound ATPase that converts ATP and ADP to AMP, whereas CD73 is a membrane bound ecto-nucleotidase that converts AMP to adenosine.

Genes involved in the ATP:adenosine pathway, including CD73, were differentially expressed in high-inflammation synovial tissues, consistent with the hypothesis that there is skewing of the ATP:adenosine balance during inflammation. There was a significant decrease in ATPase activity and increase in adenosine deaminase activity synovial fluid from rheumatoid arthritis (RA) *v.s.* osteoarthritis (OA) patients. RA fibroblast like synoviocyte (FLS) cell lines transduced with CD39-CD73 expressing AAV5 vectors demonstrated high CD39 and CD73 activity. THP-1 (human monocyte cell line) cells stimulated with LPS showed lower levels (>80% reduction $p = <0.05$) of cytokine (e.g. TNF- α , IL-1B) and chemokine (CCL2) secretion when co-cultured with CD39 and CD73 expressing HEK293 cells or FLS cells in the presence of ATP. Together, these data suggest that synovial inflammation in RA is characterized by skewing of the ATP:adenosine balance. This could be reversed *in vitro* by over-expression of CD39 and CD73. Thus, these data show that the ATP:adenosine pathway may be a novel therapeutic target for the treatment of RA.

Poster Presentations

P001

Use of Sleeping Beauty transposase mRNA for safe and efficient gene delivery in pigment epithelial cells

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Previous studies have shown that the non-viral *Sleeping Beauty* transposon system (*SB100X*) ensures stable and consistent transgene expression in pigment epithelial cells. However, the transposase-encoding DNA molecule has the potential to spontaneously integrate into the host cell's genome, possibly leading to transposon re-mobilization and re-integration. To eliminate this risk, transposition using *SB100X*-encoding transient mRNA was evaluated.

ARPE-19 cells were electroporated with the *Venus* reporter gene using *SB100X*-encoding DNA or mRNA at a ratio of 1:16. *Venus* expression was analyzed by fluorescence microscopy and flow cytometry. Expression of both transposon and transposase genes as well as the number of integrated transgene copies into the cell's genome were evaluated by quantitative real-time PCR.

Both *SB100X* DNA and mRNA led to initial transfection efficiencies of 100%. 21 days after transfection, the transposon copy number varied between 3.4 and 5.8. 150 days after transfection, in ARPE-19 cells co-transfected with *SB100X* DNA the amount of transfected cells decreased below 10%, whereas cells co-transfected with *SB100X* mRNA showed transfection efficiencies of 100% for the 180 days that the cells were followed. *SB100X* expression was only detected in *SB100X* DNA-transfected cells within 14 days after electroporation.

SB100X mRNA is a safe tool for efficient transfection of pigment epithelial cells; it led to long-term and stable expression of the inserted transgene without the risk of transposase insertion into the host cell's genome. Transferring the method to primary human pigment epithelial cells resulted in transgene expression for longer than one year.

P002

Efficient and non-toxic siRNA delivery vector for systemic administration

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Small interfering RNAs (siRNA) with a capacity to inhibit expression of pathologic proteins *in vivo* represent a promising approach to the treatment of various diseases. However, even though RNAi is a highly specific technique and has the potential to selectively silence the expression of any gene, the systemic or local delivery of RNAi molecules remains a challenge.

Recently, we developed a clinically acceptable efficient formulation of siRNA. This tri-component delivery system consisted of (i) the cationic lipid 2-[3-[Bis-(3-amino-propyl)-amino]-propylamino]-N-ditetradecyl carbamoyl methyl-acetamide, which we termed DMAPAP, (ii) the neutral lipid 1,2 dioleoyl-sn-glycero-3-phospho-ethanolamine or DOPE and (iii) an anionic polymer that served as an enhancer of lipoplexes efficiency. This siRNA delivery system has proven to be efficient in two rodent disease models.

We assayed a large variety of anionic polymers, in terms of structure, origin or size and showed that various anionic polymers can be used to enhance gene silencing efficiency of this cationic lipid-based siRNA delivery vector.

We then examined how the nature of the added anionic polymer impact the physico-chemical properties of these siRNA particles as well as their gene silencing efficiency, cytotoxicity, *in vivo* localization and toxicity upon i.v. administration to mice. We also deciphered the influence of polymer's nature on the stability of siRNA particles in various biological milieus.

In conclusion, we developed efficient and clinically acceptable siRNA vectors that can be used for systemic injection. These vectors are cationic liposomes-based and contain anionic polymers that are biodegradable and FDA approved for use in humans. These siRNA vectors represent a promising vehicle for siRNA administration in various pharmaceutical and medicinal applications.

P003

Folate-polycationic amphiphilic cyclodextrin-DNA nanocomplexes for gene delivery *in vitro* and *in vivo*

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Folic acid (FA) is an essential vitamin for the biosynthesis of nucleotides and has been widely used as a targeting agent in cancer disease. Non-viral vectors, such as polycationic amphiphilic cyclodextrins (paCDs), are considered as promising gene carriers in the treatment of this disease despite having a low transfection activity. In this work paCD-T2-pDNA CDplexes

have been electrostatically coupled to FA to be used as a novel targeted device to cancer cells in order to increase delivery specificity and activity. The nanoparticles formed with different amounts of FA (up to 2 μg FA/ μg pDNA) presented nanometric sizes and positive surface charge. The transfection activity data showed an increase in the luciferase expression values in HeLa as the FA ratio increased. In HepG2, a FA receptor defective cell line, the behavior was opposite and the transfection activity was reduced for higher amounts of FA. In both experiments, branched polyethyleneimine (bPEI) polyplexes were less effective than CDplexes. Viability studies were performed showing negligible toxicity (viability >80%) for CDplexes whereas bPEI produced acute toxicity. The *in vivo* studies were carried out in BalbC female mice and 24 h after the administration of the CDplexes luciferase activity was mainly detected in the liver followed by the lungs. Luciferase expression levels increased with the amount of FA used in the formulations. Biodistribution studies by bioimaging do also corroborate this fact. Control mice (PBS and pDNA) did not show any activity.

P004

Inhibition of hepatitis B virus replication using guanidinopropyl modified siRNAs

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Chronic hepatitis B virus (HBV) infection remains an important public health concern and currently licensed therapies rarely prevent the life-threatening complications of viral persistence. Silencing HBV gene expression with exogenous activators of RNA interference (RNAi), such as synthetic small interfering RNAs (siRNAs), presents a promising option for development of effective anti-HBV agents. This study aimed to assess the benefits of incorporating novel 2'-O-guanidinopropyl (GP) modifications into siRNAs that target HBV. Single GP residues were incorporated at nucleotide positions from 2 to 21 of the antisense strand of a previously characterised effective antiHBV siRNA. The siRNAs with GP moieties at selected positions improved silencing efficacy *in vitro* and stability of chemically modified siRNAs in 80% serum was moderately improved. The innate interferon response was not induced *in vitro* and the GP-modified siRNAs were not toxic to cultured cells. Unintended microRNA-like silencing caused by incomplete hybridisation of the siRNA guide to its cognate was attenuated by GP moieties, particularly those incorporated within the siRNA seed region (comprising nucleotides 2 to 8 from the 5' end of the guide strand). The cleaved target mRNA product was successfully detected by using 5' RACE. Hydrodynamic co-injection of siRNAs with a replication-competent HBV plasmid resulted in highly effective knockdown of markers of viral replication in mice. Current studies are aimed at assessing siRNA efficacy in HBV transgenic mice, which stringently simulate the human condition of chronic HBV infection. Data from this study suggest that GP modified siRNAs may potentially be utilised for HBV therapy.

P005

Smart[†] bacteriophage nanocomplex: a hybrid multi-component particle for safe and efficient gene transferTeerapong Yata¹, Koon-Yang Lee¹, Charlotte A. Stoneham¹, Tararaj Dharakul², Sirirung Songsivilai², A Bismarck¹, Amin Hajitou¹¹Imperial College London, United Kingdom; ²National Nanotechnology Center, Thailand

Gene delivery can be achieved using either viral or non-viral vectors. In order to obtain the ideal gene delivery vector system, attempts have been made to combine the attributes of two or more different types of vectors. In this study, we combined the advantages of three gene delivery vectors: the filamentous M13 bacteriophage, the genome of a recombinant adeno-associated virus (rAAV) and cationic polymers. In this novel complexed system phage vector, i) the phage particle serves as a vehicle displaying targeting peptides on its capsid for cell internalization, ii) the rAAV cassette has the potential for episomal amplification and long-term transgene expression, and iii) the cationic polymer creates positively charged phage particles resulting in enhanced attachment to the negatively charged surface of eukaryotic cells. Herein, we report a novel hybrid multi-component vector termed the "smart" bacteriophage nanocomplex. We have assessed the efficacy of this vector in two dimensional (2D) as well as three dimensional (3D) cell culture systems. A study of the expression of green fluorescent protein and luciferase reporter genes by the new bacteriophage system confirmed that such hybrid vectors display substantial gene transfer efficacy, that is targeted, compared to uncomplexed parental phage. As a proof of concept, application of the smart bacteriophage vector carrying the cytotoxic suicide gene 'HSVtk' resulted in eradication of brain tumour cells upon ganciclovir treatment.

We conclude that the innovative combination of different types of vectors into a single particle provides significant extension to the development of novel gene delivery platforms which can be used as tool for gene therapy applications.

P006

Targeted lipid-based strategies to silence Machado-Joseph disease through the systemic routeM Conceição^{1,2}, L Mendonça¹, C Pedroso Lima^{1,3}, M Swamy⁴, L Pereira de Almeida^{1,2}¹Center for Neuroscience and Cell Biology, University of Coimbra; ²Faculty of Pharmacy, University of Coimbra; ³ Faculty of Sciences and Technology, University of Coimbra; ⁴Center of Excellence in Infectious Diseases, Texas Tech University Health Sciences Center

Machado-Joseph Disease (MJD) is the most common dominantly-inherited ataxia worldwide, but there is no therapy able to modify disease progression. In this context, small interfering RNAs (siRNAs) represent an extremely promising approach. However, its successful application in the clinic is limited due to the presence of the blood-brain barrier (BBB) and the unfavorable pharmacokinetic and biodistribution properties of siRNAs. Therefore, the aim of this study was to develop an efficient delivery system that would circumvent these barriers, enabling transvascular delivery of siRNAs to the brain.

To cross the BBB a lipid-based formulation encapsulating siRNAs and targeted to the brain associating a short peptide

derived from rabies virus glycoprotein (RVG) or a transferrin receptor binding peptide was developed. Flow cytometry and confocal microscopy studies revealed specific *in vitro* delivery of siRNAs to neuronal cells. These formulations, efficiently knocked down target mRNA resulting in protein level reduction. Importantly, intravenous injection allowed significant delivery of labeled-siRNAs to the mouse brain.

Next, we will test the silencing efficiency of our non-viral system in MJD mouse models. We expect to generate an siRNA delivery system that may be used for MJD therapy and potentially to other neurodegenerative diseases.

P007

Increased Adenovirus density for increased ultrasound-mediated delivery to tumorsR C Carlisle¹, S Mo¹, R Myers¹, S Graham¹, E Mylonopoulou¹, R Cawood¹, L W Seymour¹, C C Coussios¹¹University of Oxford,

Systemic administration of adenovirus (Ad) for the treatment of cancer is limited by poor circulation kinetics and inefficient uptake from the bloodstream into tumors. We have recently reported a method for linkage of highly-PEGylated gold nanoparticles (goldPEG) to Ad by a single reduction cleavable bond. The resulting 'dandelion' structure provides very effective steric shielding with only minimal and reversible modification of the Ad capsid. This reduces the binding of antibodies and enhances circulation kinetics. However, in addition to providing a platform for effective stealthing, the gold nanoparticles also increase the density of Ad. Here we describe how this increase in density imparts a second major advantage on our strategy. Specifically, the response of Ad to ultrasound exposure is dramatically improved upon goldPEG addition.

Electron microscopy confirmed the attachment of 111 goldPEG per Ad and ultra-centrifugation through caesium chloride confirmed a consequent large increase in density compared to Ad or Ad modified with poly(*N*-(2-hydroxypropyl)methacrylamide) (Ad-PHPMA). The movement of Ad, Ad-goldPEG or Ad-PHPMA out of a flow-channel and into a tissue mimicking material was then tested. Notably, application of ultrasound to instigate cavitation and generate shock-waves substantially increased the propulsion of all samples into the tissue mimicking material. However, more Ad-goldPEG (50-fold, $p < 0.001$) was recovered at greater penetration distances (up to 6mm) than Ad or Ad-PHPMA. In murine models, 12% of the intravenously injected Ad-goldPEG dose accumulated in the tumor following exposure of the tumor to ultrasound. This level represents a >20-fold increase compared to alternative strategies and lead to substantial anti-tumor efficacy.

P008

The merging of the antibiotic-free pFAR4miniplasmids with the Sleeping Beauty transposon system mediates higher transgene delivery in human cellsM Pastor¹, S Johnen¹, M Quiviger¹, Z Izsvak¹, D Scherman¹, G Thumann¹, C Marie¹¹CNRS, Université Paris 5, University Hospital RWTH Aachen, Max Delbrück Center for Molecular Medicine Berlin, Hôpitaux Universitaires de Genève

New generations of plasmid vectors lead to elevated and prolonged transgene expression levels, which make non-viral gene therapy an attractive alternative to treat genetic and acquired disorders.

The pFAR4 plasmids are novel antibiotic-free mini-plasmids. Their propagation is based on the suppression of a mutant strain auxotrophy, thus eliminating safety concerns classically attributed to plasmid DNA vectors, such as horizontal antibiotic resistance gene transfer to patient's bacteria. In addition, pFAR4 vectors display high-level and sustained transgene expression in muscle, skin and liver.

To obtain a long-term transgene expression in dividing cells, we combined the pFAR4 technology to the plasmid-based integrating *Sleeping Beauty* (SB) transposon system, composed of a transposase that mediates the excision of a transgene-containing transposon from a donor plasmid and integration into the host cells' genome.

This combination was used in two parallel studies, delivering either the neomycin resistance gene to HeLa cells or the anti-angiogenic pigment epithelium-derived factor (PEDF) gene to primary pigment epithelial cells, in the context of an *ex vivo* gene therapeutic approach with autologous cells for the treatment of neovascular age-related macular degeneration. The combination of the pFAR4 and SB technologies led to an increased number of neomycin resistant colonies, as compared to conventional gene plasmid vectors, and to an increased level of secreted PEDF for more than 5 months.

Thus, pFAR4 is an efficient SB transposase expression vector and donor substrate for SB-mediated transposition. Studies are ongoing to determine whether this increased efficiency results from a higher transfection level and/or an increased transposition rate.

P009

Using *Sleeping Beauty* transposons as a non-viral approach for the correction of Fanconi Anemia cells

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Sleeping Beauty (SB) transposon system has been developed as an integrative tool with a wide range of gene therapy applications and virtually random profile of integration, diminished immunogenicity and reduced handling costs as advantages versus viral vectors. Our goal is to develop *Sleeping Beauty* as a technology to deliver Fanconi A and D1 genes, belonging to the Fanconi Anemia (FA) pathway implicated in the resolution of DNA interstrand cross-links (ICL). FA is a rare inherited disorder in which paediatric patients suffer from progressive bone marrow failure and an increased susceptibility to malignancies among other symptoms. Human cDNA corresponding to *FANCA* and *FANCD1* (*BRCA2*) genes were cloned together with a GFP marker in a "sandwich" SB transposon, conformation developed to improve large-size transgene transposition¹. Nucleofection was used to deliver FA transposons plus SB100x transposase in trans to patient fibroblasts (deficient *FANCA* gene) or a *FANCD1* deficient Chinese Hamster cell line (V-C8). Stable and long expression of GFP was achieved and the presence of both FA genes could be proved by an immunoblot analy-

sis. Functional analysis of *FANCA* and *FANCD1* proteins were performed: the recovery of *FANCD2* (in case of *FANCA* deficiency) or Rad51 (*FANCD1* deficiency) nuclear foci detected by immunofluorescence and the survival improvement after treatment with a DNA crosslinking agent, like mitomycin C -as deficient cells are hypersensitive to the deleterious effects of this agent- indicated the recovery of the normal phenotype in comparison to the uncorrected cells. SB transposons mediate robust genetic modification of deficient cells *in vitro*, thereby offering a non-viral alternative for FA gene therapy.

1. Zayed *et al.* Mol.Ther. 2004 9:292-304.

P010

Enhancing plasmid nuclear import and delivery system: room for improvement

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The use of plasmid DNA vectors for gene transfer represents an important platform for clinical applications, notably with DNA vaccination wherein large-scale vaccine production is not easily manageable with other forms of vaccine including recombinant protein, whole tumor cells, or viral vectors. A variety of clinical trials against cancer have provided evidence that DNA vaccines are well tolerated and have an excellent safety profile. Nevertheless there is still a gap between the experimental data and their application in a clinical setting due to low level of antigen expression.

DNA vaccines allow incorporating multiple components to activate and direct selected immune effector pathways and are prone to be delivered in many different manner. To achieve more efficient gene expression from plasmid vectors CMV promoter driven, DNA nuclear targeting sequences (DTSs) are introduced to increase the efficiency of nuclear plasmid uptake from cytoplasm. Electrotherapy is promising for the treatment of muscle disorders, as well for the systemic secretion of therapeutic proteins, DNA vaccination, immunotherapy and cancer therapy. The inclusion in a DNA vector backbone of a DTS localized in a non-coding region of the SV40 virus sequence is established to increase *in vivo* expression up to 20-fold using electroporation delivery in muscle tissue.

We have used this delivery technique in combination with plasmids containing a tandem repeat of two 72-bp DNA elements from the SV40 enhancer in preclinical immunization protocols.

To evaluate the increase in performance we analyzed over time the IL-2 expression of anti-idiotypic DNA vaccines, developed against an aggressive murine B-cell lymphoma model and co-expressing IL-2 as immunomodulating agent. The anti-idiotypic humoral response was assayed as well.

Our *in vivo* results show that the combination of electroporation and a plasmid vector carrying DTSs elements results in efficacious DNA vaccines.

P011

The Initiation of Replication from the human beta-globin locus enhances plasmid establishment and transgene expression in an S/MAR based episomal vector

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The Initiation of Replication region, (IR), from β -globin gene locus is capable to promote DNA replication ectopically and can rescue DNA replication within the context of Scaffold/Matrix Attachment Region (S/MAR) based episomal vector. Based on episomal vector pEPI-eGFP (6.7Kb) we constructed vectors pEP-IR and pEPI-SFFV carrying the Spleen Focus forming Virus (SFFV) promoter in place of CMV promoter to drive eGFP expression. Additionally, vector pEP-IR carries the IR element at the 5' site of SFFV promoter. All three vectors showed excellent episomatic properties upon transfer into K562 cells and can transfect human Mesenchymal stem cells too. All three vectors were transferred into CD43 cells from peripheral blood, from adult, mobilised, healthy donors, with transfection efficiency 24%–30%. FACS sorted eGFP cells were placed in semisolid cultures to generate differentiated cell-colonies within 14 days. Fluorescent microscopy revealed that control vector pEPI-eGFP does not support the presence of fluorescent colonies (0%), pEPI-SFFV supports 56% fluorescent colonies and pEP-IR –most efficiently- 100% fluorescent colonies. Non-fluorescent colonies deriving from transfections with pEPI-eGFP or pEPI-SFFV were shown by PCR to be devoid of plasmids. Plasmid copy number pEPI-SFFV and pEP-IR per fluorescent cell was estimated by Real time PCR to be 1.12 and 1.78 copies/ differentiated fluorescent cell respectively. Similarly, estimation of eGFP mRNA revealed that pEP-IR supported 3 times higher eGFP expression per plasmid copy, compared to pEPI-SFFV. These data show that IR enhances plasmid establishment, increases plasmid copy number per cell and transgene expression per plasmid copy in transfected CD43 cells progeny.

P012

Engineered dynein light chain Rp3: a strategy to exploit cell's machinery for an enhanced intracellular trafficking of transgenes

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Despite all the advances on the development of more efficient non viral gene delivery vectors, intracellular trafficking of transgenes remains an important limiting step. We designed a recombinant dynein light chain (Rp3) fused to a C-terminal TAT peptide, a N-terminal DNA-binding domain, and a histidine tail. Named T-Rp3, the protein was able to interact and condense plasmid DNA, resulting in positively charged (28,6 mV) and small (~100nm) particles. The vector was highly efficient during transfection of HeLa cells, also presenting low cytotoxicity. Unlike many non viral vectors, the kinetic of transgene expression medi-

ated by T-Rp3 was fast, similarly to Lipofectamine 2000TM. The intracellular trafficking of pDNA:T-Rp3 complexes were found to be highly dependent on microtubules, since reporter gene expression decreased 92% when microtubules were depolymerised using Nocodazole. Confocal microscopy studies together with time lapse imaging of live cells revealed a high rate of pDNA internalization and centrosomal accumulation. Perinuclear accumulation of pDNA was detected in just a few hours post-transfection, even in the presence of chloroquine, a lysosomotropic drug known to disrupt endosomes. These are promising results and may contribute to the development of more efficient non viral gene delivery vectors.

P013

Study of modular peptide-based vehicles for targeted gene delivery

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Development of peptide-based gene delivery systems constitutes an essential challenge in gene therapy. Carrier's modification with nuclear localization signal (NLS) and ligand for certain receptor can promote effective targeted gene delivery. SDF-1/CXCR4 is an important ligand/receptor pair involved in cancer metastases and stem cells trafficking. SV40 NLS has been renowned to enhance nuclear uptake of DNA. This work aimed to study cross-linking peptides modified with CXCR4 ligand and SV40 NLS as vehicles for gene delivery to CXCR4-expressing cells.

We studied eight variants of DNA/peptide complexes modified with SV40 NLS and N-terminal fragment of SDF-1. Study of physicochemical properties included DNA-binding and protection, dynamics of complex formation, resistance to polyanions and size measurement. Transfection efficacy was studied in three cell lines: HeLa, A172, CHO and in human mesenchymal stem cells (hMSC). DNA-complexes' toxicity was investigated with Alamar blue test. CXCR4 expression in hMSC was induced by valproic acid (VPA).

We showed that all peptides can protect DNA from endonuclease degradation after completing DNA condensation. Carrier's modification with CXCR4-ligand or NLS increases transfection activity of DNA/peptide complexes and contribute hMSC transfection. VPA treatment resulted in 4-15-fold increase in transfection efficacy of the complexes in hMSCs. Toxicity of the complexes in the most cases was lower, than in control PEI. Thus CXCR4-targeted peptides should be considered as useful tools for non-viral gene delivery into cancer cells and hMSC.

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Keywords: gene delivery, CXCR4, SDF-1, SV40 NLS

P014

Development of synthetic DNA reference material for accurate analysis of gene therapy vectors

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Accurate analysis of gene therapy vectors is required to establish the administered dose and in vector biodistribution

studies. Such analyses are usually performed by PCR using internal laboratory standards based on a plasmid containing the same PCR target sequences as the vector. Internal laboratory standards are often poorly characterised and, in case of inadvertent contamination of negative samples with such controls, false positive results will be obtained.

To improve vector analysis, we developed a synthetic DNA reference material (RM), using as a model, a plasmid vector incorporating a human erythropoietin transgene. The RM was designed to contain several sequences for various PCR assays that target multiple transgenic sequences within the vector. The RM is amplified by these assays with the same specificity and sensitivity as the vector itself, allowing accurate vector quantification using a standard curve based method. Importantly, the target sequences within the RM are modified to generate amplicons different in size and sequence to those from the vector, permitting discrimination of false positive results. The developed RM was characterised for purity, quantity, homogeneity and stability in accordance with ISO Guide 34 using latest technologies including digital PCR and next generation sequencing. In addition, we used the RM to *in vitro* validate accuracy and reproducibility of analysis of a nonviral vector containing erythropoietin transgene in blood samples.

The developed RM can serve as a prototype for preparing other RM for accurate analysis of vectors carrying any transgene and can be used to validate internal laboratory standards. Moreover, our RM design can be applied to generate a RM that contains transgenic sequences from several vectors used in a laboratory; this will allow only one RM for analysis of multiple vectors.

P015

Inefficient RNA translation limits the efficacy of hydrodynamic gene transfer to pig liver "in vivo"

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Introduction: The inefficient expression of eGFP after hydrodynamic gene transfer, in surgically watertight pig liver, was studied.

Material and Methods: Pig liver was isolated by vascular clamping (cava, portal veins and hepatic artery); then, the eGFP gene transfer was performed under different conditions by implantation of a 12F catheter in anesthetized pigs. Animals were injected with 200 mL of saline solution bearing p3c-eGFP plasmid (20 µg/mL), containing the eGFP gene via cava or portal veins at different rates. The gene transfer and expression efficiency of anterograde vs retrograde venous injection and the effect of flow rate, 10 and 20 mL/s, were studied.

Results: The results showed: (i) the highest gene delivery (0,1-1 eGFP DNA copies/diploid genome) occurred in anterograde injection; (ii) anterograde gene transfer mediated a higher rate of transcription (10-100 eGFP RNA copies/cell) despite a lower plasmid amount; (iii) the fastest flow rate, 20 mL/s, reached better transcription efficiency than 10 mL/s (1-10 vs 10-100 eGFP RNA copies/cell) in anterograde injection; (iv) unexpectedly, higher eGFP expression (>1ng/mg prot) was detected with

milder perfusion conditions (10ml/s) and lower RNA presence what could be due to cell stress-induced translation blockade.

Conclusion: Data suggest that, probably, high volumes and pressures are not only unnecessary but counterproductive for gene expression efficiency and mild conditions are the optimal to achieve a safe efficient model of hepatic gene transfer with potential application. Partially supported by AP-151-11 and SAF2011-27002.

P016

Extracellular DNA as a potential cause of preeclampsia

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Background: Extracellular DNA induces an inflammatory response involved in the pathogenesis of several diseases. Preeclampsia is associated with higher concentrations of fetal DNA circulating in maternal plasma. It is currently unknown, whether this is the consequence of placental damage due to preeclampsia or fetal DNA is itself involved in the pathogenesis of preeclampsia.

Materials & Methods: Preeclampsia was induced by LNAME in drinking water given to pregnant Wistar rats. In another experiments pregnant Wistar rats received daily i.p. injections of saline (negative control), lipopolysaccharide (positive control) or rat fetal DNA for 5 days during the first, second or third week of gestation. Blood pressure, proteinuria and placental as well as fetal weight were measured at the end of gestation one day before delivery. Fetal DNA was assessed in maternal plasma using SRY-targeted real time PCR.

Results: Blood pressure did not differ between groups in any experiment. Proteinuria and pup weight were affected by LNAME, lipopolysaccharide and partially also by fetal DNA administration during the third week of gestation. In the LNAME-induced animal model of preeclampsia fetal DNA was not increased compared to the control group.

Conclusions: Fetal DNA circulating in maternal plasma is not increased in this model of preeclampsia. Injections of exogenous fetal DNA into pregnant rats induced preeclampsia at least partially suggesting that fetal DNA is one of the causes rather than a consequence of preeclampsia. These results might be of importance for the treatment of pregnant women with non-viral vectors.

P017

Catheter mediated suprahepatic HAAT gene transfer: Effect of portal outflow blockade

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Introduction: The efficiency of catheter mediated pig liver gene transfer and the benefits of pressuring the organ by blocking the portal outflow were evaluated.

Material and Methods: After jugular dissection, a catheter with balloon was introduced and placed into the hepatic vein from the inferior cava vein targeting an area of the liver. In other

experiments, two balloon catheters were introduced simultaneously through jugular and femoral veins and were placed in cava vein close to liver entry restricting the perfusion area, the whole liver was targeted; portal outflow was blocked by intrahepatically placing another balloon catheter in main portal branch. Then, pTG7101 plasmid bearing the hAAT gene in 200 ml saline solution (20 µg/ml) was retrovenously injected (20 ml/s). The gene transfer efficiency and protein expression of both models were compared.

Results: The results showed: (i) the highest gene delivery (10–100 hAAT DNA copies/diploid genome) occurred with free outflow; (ii) outflow blockade mediated a slightly higher rate of transcription (~10 vs ~1 hAAT RNA copies/cell) despite a lower plasmid amount; (iii) conjugating these results, the intrinsic efficacy of outflow blocking model resulted higher (10²-10³ vs 1–10 RNA/DNA copy per cell); (iv) outflow blockade model achieved efficient protein expression (~10⁸ copies/cell).

Conclusion: Data suggest that retrovenous catheter mediated liver gene transfer is an efficient model of hepatic gene transfer with potential clinical applications and the portal outflow blocking could improve the gene expression. Partially supported by AP-151-11 and SAF2011-27002.

P018

Epidermal growth factor targeted novel cationic lipoplexes Enhance transgene expression in HepG2 cell line *In vitro*

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For augmented efficiency, cationic liposomes can be modified as cell-specific gene therapy systems. Of the different ligands used for modification and exploitation of receptor mediated endocytosis, the epidermal growth factor (EGF) was chosen for this investigation. The aim of this study was to evaluate the hepatotropism of targeted liposomes for enhanced transgene expression in the HepG2 (Human hepatocellular carcinoma) cell line, known to over-express EGF.

Four liposomes, two consisting of cytofectins 3β[N-(N',N'-dimethylaminopropane)-carbamoyl] (Chol-T) and N,N-dimethylaminopropylamidodistearoylphosphatidylethanolamine-polyethyleneglycol 2000 (DSPE-PEG 2000), were formulated with the neutral lipid DOPE through thin film re-hydration. Preformed cationic liposomes were conjugated to the (EGF) polypeptide through a simple adsorption method prior to characterization and cell culture studies. All liposome formulations were investigated for their ability to bind, condense and protect plasmid DNA (pCMV-Luc), using the agarose gel retardation, ethidium bromide dye displacement and nuclease protection assays. All liposomes and lipoplexes were subjected to electron microscopy and zeta-sizing to determine lamellarity and size distribution. *In vitro* cytotoxicity was determined using the MTT assay, and gene expression using the luciferase reporter gene assay and fluorescence microscopy in the HepG2 cell line.

Overall targeted liposomes showed good binding and protection of plasmid DNA. These novel lipoplex systems were able to successfully transfect the HepG2 cell line with minimal cytotoxicity and greater transgene expression than the receptor negative control cell line. Initial results show that with further optimization clinically viable gene or drug delivery vehicles can be formulated.

P019

Viral vs. non-viral gene therapy to improve tendon healing: Electrotransfer leads to rapid gene expression compared to AAV-based delivery.

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Injury and repair of the flexor tendons of the human hand are often complicated by fibrotic adhesions limiting the hand function. In order to restore the tendon gliding function new treatment options are needed. Gene therapy using AAV has shown ability to improve tendon healing in model systems. In this study we sought to develop a non-viral method, in order to avoid potential inflammatory response induced by the viral vector. Indeed, inflammation is one of the factors thought to increase adhesion formation. We used electrotransfer of plasmid DNA to muscle as delivery strategy.

Using an established murine model of flexor tendon injury and healing, we transected and repaired the flexor digitorum longus tendon in the mouse foot. Following surgery we injected 10 µl of either pDNA (15 µg) or rAAV (2×10⁹ particles) encoding a firefly luciferase reporter gene into an adjacent muscle (flexor digitorum brevis). Luciferase activity were recorded by live bioimaging at 2, 4, 8, 12, 24 hours post treatment and followed for up to 28 days. We detected luciferase activity as early as two hours after gene delivery by electrotransfer. In contrast the onset of gene expression in the viral vector treated group did not exceed our detection limit until day 3.

Electrotransfer and the rapid onset of gene expression opens new possibilities for flexor tendon gene therapy. Currently we are investigating a number of possible anti-adhesive genes of their potential to improve the tendon healing and gliding function in our murine model.

P020

Muscle-targeted incretin gene therapy for type 2 diabetes

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Type 2 diabetes affects 350 million people worldwide. Current treatments are somewhat effective but usually do not halt disease progression relying heavily on patient adherence. Our objective was to evaluate plasmid-mediated GLP1 gene therapy in normal and diabetic mice. Constitutively active pVR1012-GLP1; pVR1012-Ex4 (long-acting GLP1 homologue), and pVR1012-eGFP plasmids were injected into both anterior tibialis and gastrocnemius muscles in CD1 or db/db mice, with adjuvant hyaluronidase pre-treatment and electroporation. Sustained eGFP reporter gene expression was confirmed by IVIS spectrum imaging throughout study duration (CD1: Day 8–30 (n=5); db/db Day 3–41 (n=6)). Circulating Exendin-4 levels following pVR1012-Ex4 injection in normal mice (n=5) peaked at Day 15 (7095±9405 pmol/l) and were maintained throughout study duration (Day 30: 1250±634 pmol/l) with comparable end-point levels in db/db mice (n=6) (pre-plasmid: 95.5±22.3 pmol/l; Day 14: 234±35 pmol/l; Day 42: 1684±1310 pmol/l). Circulating GLP1 levels in db/db mice following pVR1012-GLP1 injection were low (Day 42: 3.9±3.0 pmol/l (n=6)). Glucose tolerance in db/db mice improved in the group receiving pVR1012-Ex4 (IPGTT AUC: Day 42 5022 pmol.min vs Day 0 3536 pmol.min

($p < 0.05$) and was significantly better post-plasmid compared to pVR1012-eGFP / pVR1012-GLP1 groups ($p < 0.05$). Db/db mice in all groups became increasingly obese during the study with no differences in weight between groups. Glucose-stimulated circulating insulin levels were equally elevated at study end-point in all groups (pVR1012-Ex4: 531 pmol/l; pVR1012-GLP1: 897 pmol/l; pVR1012-eGFP: 870 pmol/l) and were >40-fold higher than in normal mouse serum (13 pmol/l). Sustained plasmid expression has been attained in normal and diabetic mice leading to therapeutically active concentrations of Exendin 4 but not of unmodified GLP1.

P021

Phenotypic correction of dwarfism in immunodeficient dwarf mice following intramuscular hGH plasmid administration analyzed via different growth parameters

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Growth hormone deficiency (GHD) is a major deficiency of the pituitary gland and our group investigates an alternative strategy for its treatment based on the *in vivo* administration of a plasmid containing the human growth hormone (hGH) gene. Highly significant weight increase of ~23% in immunodeficient dwarf (lit/scid) mice, up to 60 days after a single hGH-DNA administration has been reported in previous work. Aiming at the maximum phenotypic correction, a 6-month bioassay was performed by injecting 50µg hGH-DNA or saline on the quadriceps muscle of lit/scid mice, followed by electrotransfer.

After 104 days, having reached a 35% weight increase, against 11% for the control group, the body weight of treated mice had reached a plateau. A second administration was thus carried out obtaining a further 10% increase, while a third one at day 160 provided only 5% increase, the final weight gain stabilizing at ~46% after 188 days: catch-up growth towards normalization was 22.6%.

Analyzing growth parameters more directly related to linear growth, a 16.4% increase (catch-up=26.6%) for nose-to-tail, a 16.4% increase (catch-up=24.5%) for tail and a 24.3% increase (catch-up=55.5%) for femur length were obtained. Mouse IGF-I serum levels of up to 162 ng/ml, i.e. 6-fold higher than the basal value (27 ng/ml) for untreated mice, but still ~4-fold lower than non-dwarf scid mice (683 ng/ml), were also determined.

These results are very promising and pave the way to more effective preclinical assays for the treatment of GHD, via plasmid DNA muscular administration.

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P022

PDMAEMA is an efficient retinal gene therapy vector

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Ocular pathologies are among the most debilitating medical conditions affecting all segments of the population. Gene therapy

is an alternative approach to the traditional treatment of several diseases.

Methacrylate are versatile polymers that due to their cationic nature, can form polyplexes with DNA and deliver it to cells, thus constituting a therapeutic alternatives. In recent years, several studies have demonstrated the potential of poly(2-(N,N'-dimethylamino) ethylmethacrylate) (PDMAEMA) as gene therapy vehicles. We synthesized PDMAEMA by RAFT (Reversible Addition-Fragmentation chain Transfer) and prepared with DNA at 5 and 7.5 nitrogen/phosphorous (N/P) ratio. The nanosize and positive charge of the polyplexes was measured. Cytotoxicity was evaluated using a retinal pigment epithelium (RPE) cell line and was found to be concentration and time dependent for the polymer, as expected, and inexistant for the PDMAEMA-DNA polyplexes. Transfection efficiency was evaluated by fluorescence microscopy and flow cytometry, showing that the retinal cells were in efficiently transfected by polyplexes.

These results suggest PDMAEMA to be a delivery system for non-viral gene therapy to the retina.

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P023

PhiC31-integrase and chitosanmediated gene delivery potentiates transgene expression

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A successful gene therapy approach relies on efficient gene transfer and stable transgene expression. One of the strategies recently devised to promote safe, site-specific integration and long-term gene expression, exploits the use of phage phiC31 integrase. We aim to develop a novel system for sustained gene transfer by coupling polymeric vectors with phiC31-integrase to promote transgene integration and therefore sustained transgene expression.

Chitosan-pDNA nanoparticles were produced using pGFPattB with or without pCMVINT at a molecular ratio of 2:1. These nanoparticles were characterized for size, surface charge and morphology, pDNA encapsulation and transfection studies on cultured HEK293 cells. Co-transfection with pCMVINT was done by delivering the two plasmids complexed in the same particle or separately. GFP expression was evaluated by fluorescence microscopy, flow cytometry and western blot analysis.

Characterization of the particles revealed that their size and charge varied with the size of the polymer used. DNA encapsulation efficiency, particle size and morphology were not affected by the use of different plasmids, even when they were complexed simultaneously. Transfection studies indicate that transfection efficiency and transgene expression is affected both by polymer size and mode of integrase delivery with the polyplexes. GFP expression was visible 16 weeks after transfection and selection of transfected cells with Geneticin®.

We here show that long-term GFP expression can be achieved with a non-viral approach using a combined strategy of polymers and integrase, proven more efficient than non-integrative strategies. Long-term transgene expression is currently being further evaluated *in vivo* in mice.

P024

Novel oligopeptide-terminated poly(β -amino ester)s for highly efficient gene delivery and controlled intracellular localization

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The majority of protocols in gene therapy employ viral vectors. Although they are highly efficient, viral vectors have certain disadvantages, including safety risks and high costs of scale production. Non-viral vectors offer potential advantages, including infinite packing capacity, ease of production and low immunogenicity, but are less efficient than viral vectors. Poly(β -amino ester)s (pBAEs) are promising gene delivery vectors capable of condensing nucleic acids into discrete nanoparticles. In a previous work, we have successfully generated induced pluripotent stem cells from human fibroblasts using poly(β -amino ester)s at higher efficiencies, when compared to commercial transfection reagents.

Here we present a new family of pBAEs that incorporate terminal oligopeptide groups capable of condensing both DNA and siRNA into discrete nanoparticles. This new family of polymers showed improved features in terms of particle formation, transfection efficiency and biocompatibility, when compared with basic pBAEs or commercially available transfection agents. Interestingly, DNA particles obtained from oligopeptide-modified pBAEs showed different intracellular distribution – i.e. perinuclear or cytoplasm – depending on their oligopeptide composition. In addition, formulations of mixtures of different poly(β -amino ester)s showed differential gene expression levels depending on the cell line, which revealed that the chemical composition of the oligopeptides had a deep effect on transfection.

We have developed a new family of bioinspired polymers based on oligopeptide-modified poly(β -amino ester)s that has demonstrated efficient delivery of nucleic acids. Our approach of adding oligopeptides to the termini of poly(β -amino ester)s is of great interest for the design of tailored complexes having specific features, such as preferential intracellular localization or cell-specific transfection.

P025

Minicircle DNA - how small is "mini"?

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Minicircle DNA is a safe and efficient vector system for gene therapy and genetic vaccination approaches. Their size is significantly reduced in comparison to regular plasmid DNA and miniplasmid DNA vectors. The minicircle technology is removing needless sequences like marker genes and the bacterial origin of replication that is only used for the stable maintenance and am-

plification of plasmids in bacteria. The resulting minicircle consists almost only of the gene of interest, leading to a significant size reduction and improved application possibilities in comparison to plasmids. In addition to their safety profile, minicircles have shown their potential to increase the efficiency of transgene expression. The first challenging step in minicircle production is to construct the parental plasmid containing the gene cassette of interest framed by recombination sequences needed for the *in vivo* recombination. The reduction of size for the gene of interest shifts the recombination sites into a quite close position. We show that such a short distance still allows the proper recombination of the parental plasmid into minicircle and miniplasmid. We identified no lower size limit so far by generating a 500bp ccc minicircle encoding a shRNA against GFP. Throughout the DNA manufacturing process certain analytical samples are taken to ensure the quality. Additionally to gel electrophoresis and capillary gel electrophoresis we show results obtained by atomic force microscopy (AFM) which is a versatile tool that depicts and measures surface structures with unprecedented resolution and accuracy at the nm-scale.

P026

Bacteria mediated gene silencing in mouse model of inflammatory bowel disease

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Delivery of therapeutic genes into colonic mucosa has been previously demonstrated in mice. In the current study we tested nonpathogenic *Escherichia coli* as a vector for delivery of short hairpin RNA (shRNA) against tumor necrosis factor alpha (TNF α) in cell cultures and in a mouse model of inflammatory bowel disease. The bacterial strain contained the plasmid with genes encoding invasin (*inv*), listeriolysin (*hlyA*) and an expression cassette encoding shRNA against TNF α or luciferase (LUC). Bacterial strains were used for invasion into mouse macrophages cell lines J774 and Raw264.7 and several multiplicities of infection (MOIs) were tested. After 2 hours of bacterial invasion the expression of TNF α (both mRNA and protein) was significantly reduced in TNF- compared with LUC-treated cells in most of the MOIs tested. We also administered the engineered strain to mice with dextran sulfate sodium (DSS)-induced IBD and checked for the expression of TNF α in the colon. Mice receiving the therapeutic TNF strain showed significantly reduced expression of TNF α compared with the control LUC strain in both proximal and distal colon on day 5 of DSS intake. This finding, however, diminished later in the course of colitis. Colon length as a measure of intestinal inflammation was significantly improved in the therapeutic TNF group compared with LUC group on days 5 and 8 of DSS intake. Bacteria mediated silencing of target genes provides a potential new therapeutic technology for treating IBD.

P027

Goldnanoparticles for the enhancement of adenovirus stealthing

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Oncolytic adenoviruses (Ads) have demonstrated high anti-tumor efficacy upon direct administration. Unfortunately Ads show rapid clearance and poor tumor uptake following intravenous delivery which limits their utility in treating disseminated disease. Polyethylene glycol (PEG) and poly(*N*-(2-hydroxypropyl)methacrylamide) (PHPMA) have previously been used in an attempt to 'stealth' Ad and thereby extend its circulation by reducing bloodstream interactions. Here we report a novel strategy whereby many highly PEGylated gold nanoparticles (goldPEG) were attached to Ad via single disulphide bonds. This 'dandelion' structure was designed to provide a dense steric shield with minimal and reversible modification of the Ad capsid. Dynamic light scattering and zeta potential demonstrated changes to the size and surface charge of Ad, commensurate with the addition of a layer of goldPEG nanoparticles. Electron microscopy was used to image and calculate an average of 111 goldPEG per Ad capsid. Upon exposure of Ad-goldPEG to reducing conditions that mimicked those evident within tumors, the goldPEG was removed proving the utility of the disulphide linkage. The protection provided by goldPEG was tested using ELISAs for anti-adenovirus antibody binding and human blood for erythrocyte and leukocyte binding and complement activation. Ad-goldPEG showed 4-fold, 8-fold and >8-fold lower antibody binding than Ad-PHPMA, Ad-PEG or Ad respectively. Binding to human blood cells was reduced >10-fold with Ad-goldPEG ($p < 0.001$) vs. Ad. In murine models circulation kinetics were improved providing a >10-fold ($p < 0.001$) enhancement in tumor uptake. This approach could potentially enhance the stealth and circulation kinetics of a range of biological therapies extending their clinical utility.

P028

Bis-MPA dendritic derivatives as gene therapy vectors

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Gene therapy is the subject of great interest because of its potential to prevent or treat numerous diseases by delivering therapeutic genes into cells. Viral vectors have high delivery efficiencies, but they are expensive to produce and may cause adverse immune responses. Non-viral delivery systems offer a non-immunogenic and safe method of gene delivery, but they have low transfection efficiency. High transfection efficiency is often combined with high cytotoxicity, as it is the case of one of the most widely used cationic polymers, polyethylenimine (PEI), and two of the most commonly used dendrimers, PAMAM and PPI.

2,2-bis(methylol) propionic acid (bis-MPA) based dendrimers are biodegradable polyesters, biocompatible *in vitro* and *in vivo* that do not display cytotoxicity to various cell lines. Two types of derivatives of bis-MPA were synthesized: one set of modified hyperbranched Boltorn, which are easier and cheaper to synthesize than dendrimers, and other set of self-assembly Janus dendrimers, to facilitate at the same time micelle formation and DNA binding. Also, the formation of different nano-systems can im-

prove the therapeutic potential of the treatment towards cancerous cells.

Promising results in terms of DNA complexation, encapsulation and release and biocompatibility were obtained with these derivatives compared to similar bis-MPA-based dendrimers, thereby showing a promising future as delivery agents for gene and cancer therapy.

P029

Influence of PAMAM-based dendrimers on mesenchymal stem cells growth

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Mesenchymal stem cells present tumor-homing properties and potential for delivering anticancer agents, but the effects of propagating them *ex vivo* greatly alter their phenotype and expression of surface markers. Also, the strict cell culture conditions make the scale-up production extremely complicated and expensive. An efficient method for expanding MSCs could help to retain efficient proliferation rates and to preserve their multipotency.

Dendrimers are three-dimensional, highly branched, synthetic polymers with a well-defined chemical structure. In particular, PAMAM (poly(amidoamine)) dendrimers have been extensively investigated for their biological applications and have clinically-relevant carrier properties that are facilitated by controlling charge, functionality and size.

A PAMAM dendrimer completely covered with mPEG was developed to study the growth changes on the MSCs. We evaluated proliferation rate, morphology, surface markers and pluripotency status of the cells. The data obtained suggests that our PAMAM-modified dendrimer is highly biocompatible and low concentrations of it promote proliferation of mMSCs without affecting the phenotypic characteristics or the differentiation potential.

P030

MSCs and Hollow Gold Nanoparticles as tumor treatment

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Stromal components provide factors and structural support for malignant cells. The formation of tumour stroma closely resembles wound healing and scar formation that increase

proliferation of stem cells. Some groups have hypothesized that exogenously administered stem cells would preferentially engraft at the tumor site and contribute to the population of the tumour stroma. These observations, have led us to postulate that stem cells could deliver different therapies (such as nanoparticles) to tumour sites in a cell therapy approach.

Amongst the different nanoparticles to be employed, Hollow Gold Nanoparticles (HGNs) have the capacity to resonate in the near infrared (808 nm) when they are irradiated by a laser. Along that process, HGNs are capable to rise the temperature and theoretically they could kill cells and so eliminate tumors. Moreover, at this particular wavelength, biological tissues do not absorb radiation, so the tissues without HGNs should not be affected by the laser effects. Some other advantages about this nanoparticles type are their biocompatibility and their easy synthesis.

At first we demonstrated the low HGNs toxicity when Alamar Blue studies at different time points were performed. Moreover, we proved the HGNs internalization in MSCs and U251MG cells by confocal microscopy and transmission electron microscopy.

Finally, we studied the *in vivo* treatment effects, where the treated group had HGNs and laser carried together with MSCs. At the end of the *in vivo* experiment, we were able to stop or even reduce completely the tumor size in the treated group.

P031

Use of gold nanoparticles to enhance the MSCs adenoviral infection for celltherapies

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Mesenchymal stem cells (MSCs) are adult pluripotent cells with plasticity to be converted to different cell types. Their self renewal capacity, relative ease of isolation, expansion and inherent migration to tumours and damaged areas, make them perfect candidates for cell therapies. MSCs resist significantly infection by adenovirus, mainly because only a small fraction of these cells express the coxackie adenovirus receptor (CAR), restricting the use of recombinant adenovirus that might carry therapeutic genes or markers.

In the last years, nanoparticles have attracted a great deal of attention as potential candidates for gene delivery vehicles. Their size and optical features make them suitable for bio-tagging or labelling. The possibility of using nanoparticles to coat adenovirus for MSCs infection (or other CAR- cells) opens a new perspective on gene therapy of cancer.

This study shows a new approach on which gold nanoparticles are able to increase the efficiency of adenovirus infection and expression of the carried genes. Not only it was clearly shown that these formulations increased gene transfer without altering cell viability but also that they exerted an important transduction of exogenous genes without altering cell phenotype. Finally, our data showed that the use of RGD-functionalized 13 nm gold nanoparticles represent a new approach for the use of adenoviral vectors to transfer genes towards MSCs or non-infectable cells by using a simple, reliable and efficient method.

P032

Gene delivery potential of bio functional carbonate apatite nanoparticles for lung gene transfer

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Although carbonate apatite (CO₃Ap) has been recognized as an effective plasmid DNA (pDNA) delivery vector to many mammalian cells, no study has been performed to lungs as yet. The aim of this study was to assess the pDNA delivery potential of CO₃Ap to Human non-small cell lung carcinoma (NSCLC) cell line and in mouse lung. The characteristics of the CO₃Ap/pDNA complex formulations based on its appearance, pDNA protection against DNases and particles surface charge distribution were also investigated. NSCLC cells were transfected with various formulations of CO₃Ap/pDNA complexes in serum and serum free media. Significantly high level of reporter gene expression was detected in the NSCLC cells transfected with CO₃Ap/pDNA complex, particularly when 8 µl of 1M CaCl₂ was used to prepare the complex [CO₃Ap(8µl)/pDNA]. Cytotoxicity study revealed that the percentage of the viable cells treated with CO₃Ap/pDNA was similar to the untreated cells. Characterization analysis showed that the CO₃Ap/pDNA possessed aggregated spherical structures and were in nanometer range. Particle surface charge densities of the complexes tended to be more negative when high amount of CaCl₂ was used in the CO₃Ap formulations. CO₃Ap was found to offer considerable protection of pDNA against nuclease degradation. In the lung of BALB/c mice, highest level of reporter gene expression was observed when CO₃Ap(8µl) was complexed with 40 µg of pDNA at day 1 post administration. These findings suggest that CO₃Ap exhibits attractive gene delivery properties well suited for gene delivery into the lung.

P033

Extracellular vesicles of mesenchymal cells in culture

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Microvesicles play important roles in cell communication between close or distant target cells. Their physiological role includes immunomodulation and intercellular signalling. They are also being tried as delivery vehicles for therapeutic molecules. Here we present an initial characterization of the release exosomes and shedding vesicles by human adipose tissue-derived stromal cells (ADSCs) in culture. These mesenchymal cells are capable of releasing large amounts of vesicles which could be used for immune and gene therapies. Atomic force microscopy (AFM) was used to characterize microvesicle-related structures on ADSC surfaces. In fixed preparations, circular, crater-like depressions with raised rims, were interpreted as multivesicular bodies fusing to cell membranes. Buddings appeared as abrupt

profiles, occasionally raising to 1.5 micrometer height. In living cell preparations at 37° C, similar depressions and buddings were observed, showing a fast turnover (shorter than 1 minute), suggestive of high rates of vesicle production. Using NanoSight, we could estimate that more than 30.000 microvesicles were released to the culture medium by each ADSC in 24 hours, which supported AFM observations. AFM analysis was also used to estimate the number and size distribution of microvesicles deposited by gravity onto polyornithine-coated coverslips. Various treatments stimulated microvesicle production by ADSCs; as an example of physiological signals, progesterone increased several parameters, like microvesicle number and size as well as the size of depressions on ADSCs surface. The present studies confirm the capability of ADSCs to produce large numbers of extracellular vesicles and support the use of AFM for analysing such process.

P034

How to make siRNA lipoplexes efficient In Vivo? Formulate with proper anionic polymer

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RNAi represents a promising strategy for the treatment of various disorders. Important tasks however still remain before in vivo applications, such as stability of siRNA after injection. Indeed, the half-life of unmodified siRNA in vivo is short due to rapid degradation by endogenous nucleases and renal elimination.

We report an efficient formulation to deliver siRNA both in vitro in the presence of serum, and in vivo. With siRNA targeting various cytokines, the formulation was able to restore immunological balance in a mouse arthritis model following intravenous injection and has shown efficiency in a tumor osteosarcoma model.

This efficient formulation comprised the final addition of a cationic lipid, with various lipids displaying similar activity. The unique and basic originality of this formulation is that it is based on the pre-association of siRNA with an anionic polymer, which could be either a double stranded DNA, or a polyglutamate polymer or polyalginate. The respective potency and toxicity of the three formulations will be described.

Our results suggest that the addition of DNA cargo to siRNA complexes is an easy procedure that leads to more efficient complexes to transfer siRNA at low concentration and in the presence of serum. This increased efficiency could result from a modified stability of the complexes towards cellular components.

P035

Peptide-mediated anti-VEGF siRNA delivery targeting angiogenesis in glioblastoma and endothelial cells

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Angiogenesis plays an important role in the growth of most solid tumors. Of particular importance is vascular endothelial

growth factor (VEGF), which stimulates endothelial cell migration and proliferation. Delivery of anti-VEGF siRNA is a well-known approach to anti-tumor therapy. For this purpose targeted delivery system has to be developed. Previously, we studied peptide carriers modified with ligand to CXCR4, a receptor overexpressed in some metastasizing tumors.

We studied vehicles composed of cross-linking peptides conjugated with CXCR4 ligand and ligand-free peptides at different molar ratios. Physicochemical and protective properties of the siRNA/peptide complexes were tested. Ability of carriers to deliver nucleic acids (NA) to endothelial hybridoma E.A.Hy926 cells and to A172 glioblastoma cells was evaluated *in vitro*. For downregulation of VEGF expression the cells were transfected with siRNA against VEGF. We also evaluated the ability of siRNA/peptide complexes to inhibit migration of E.A.Hy926 cells *in vitro*.

It was shown that carriers efficiently bind siRNA and protected it from enzymatic degradation. Modification with CXCR4 ligand significantly increased their transfection efficacy. siRNA delivery by means of ligand-conjugated carriers resulted in 65–70% decrease of VEGF expression in A172 cells and in 40–50% decrease in E.A.Hy926 cells. Significant inhibition of cell migration was observed.

Recent study shows that utilization of peptide carriers modified with CXCR4 ligand is a promising approach to development of targeted NA delivery system to human endothelial and glioblastoma cells.

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P036

'Enhancer Therapy': emerging strategy targeting cell-specific enhancer RNAs

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Coordinated transcriptional response to extra- and intracellular signals requires the combined activity of promoters and enhancers, the latter are largely responsible for cell-specific regulation of gene expression. Recent studies have led to the unexpected finding that many enhancers direct the expression of RNA transcripts (eRNAs), in a manner that is correlated with the expression of nearby genes. In this work, we studied the expression of eRNAs in response to Toll-like receptor 4 signalling and regulation of Rev-Erb-nuclear receptors using global run-on sequencing. Knock-down of eRNAs using siRNAs or ASOs reduced the expression of eRNAs and led to corresponding reduction in mRNAs in vitro and in vivo. As the expression of many widely expressed genes appears to be controlled by cell-restricted enhancers, the ability to target eRNA could potentially provide a new strategy called 'enhancer therapy' for altering gene expression in a cell-specific manner.

P043

Lentiviral episomes provide long-term transgene expression in dividing cellsS Chakkaramakkil Verghese^{1,2}, N Goloviznina^{1,2}, P Kurre^{1,2}¹*Papé Family Pediatric Research Institute, Oregon Health & Science University, 97239, Oregon, USA;* ²*Department of Pediatrics, Oregon Health & Science University, 97239, Oregon, USA*

Genomic integration by retrovirus vectors can induce a number of adverse transcriptional events. Non-integrating vectors provide a potentially safer approach, but their genome is successively lost with cell division. Here we incorporated human β -interferon derived Scaffold/Matrix Associated Regions (S/MARs) sequence in a 3rd generation lentivector to 'anchor' the expression cassette to the host cell genome (termed 'aLV'). Our approach exploits the preferential circularization of vector DNA in the absence of proviral integration that generates LTR circles when using an integrase-defective gag packaging plasmid. When we transduced 293T cells with VSV-G pseudotyped aLV (GFP cassette) we saw an initial drop off in the proportion of transduced cells, but GFP expressing S/MAR episomes were successfully established in ~10% of aLV transduced cells. Next, a subset of clonal aLV- and iLV- 293T cell isolates were propagated and characterized in detail over time. GFP fluorescence intensity from aLV-293T clones, was consistently lower than iLV, but stable through more than 100 cell divisions to date. Long-term persistence of episomes was also suggested by interphase and metaphase FISH studies of select clones. We further detected LTR-circle episomes by PCR in aLV transduced cells, confirmed by sequencing of episomal junctions. Similarly, Southern analysis of a subset of aLV clones detected band of the size predicted for episomes. We next tested the performance of aLV in lineage-depleted murine hematopoietic progenitor cells (HPCs) and observed GFP expression in clonogenic CFU-C after aLV exposure. Transplantation of aLV transduced HPCs into irradiated hosts revealed low-level GFP expression (0.2–3%) in peripheral blood leukocytes for up to 11 weeks. In aggregate, our studies provide proof of principle for lentivector delivery of S/MAR episomes, their persistence and transgene expression in dividing target cells. Our studies suggest that S/MAR elements anchor non-integrating lentiviral episomes through successive rounds of cell division and progenitor differentiation.

P044

Towards a direct comparison of HC-AdV vectors utilizing different genetic elements for stable transgene expressionP Boehme¹, R Voigtlander², M Mück-Hausl³, W Zhang^{1,2}, E Schulz¹, A Ehrhardt¹¹*Institute of Virology and Microbiology, Center for Biomedical Education and Research, Department of Human Medicine, Faculty of Health, University of Witten/Herdecke, 58453 Witten, Germany;* ²*Virology, Max von Pettenkofer-Institute, Ludwig-Maximilians-University Munich, 80336 Munich, Germany;* ³*Institute for Virology, Technical University Munich, 81675 München, Germany*

High-capacity adenoviral vectors (HCAdV) efficiently deliver the genetic information into the nucleus but due to the absence of efficient nuclear retention and replication mechanisms, recombinant adenoviral DNA molecules remain episomal and transgene expression levels decline due to loss of vector molecules in dividing cells. Herein, we aimed at establishing and comparing

two optimized HCAdV hybrid-vector systems capable of maintaining the administered extrinsic DNA by different genetic elements.

Our first hybrid-vector system synergizes HCAdV for efficient delivery and the S/MAR based pEPito plasmid replicon for episomal persistence. In our previous study we demonstrated that this plasmid replicon can be excised from adenovirus genomes and colony forming assays in U-87 glioblastoma cells revealed an up to 7-fold increased number of colony forming units (Voigtlander *et al.*, Molecular Therapy Nucleic Acids 2013).

To directly compare this S/MAR-based vector system with Sleeping Beauty (SB) transposase based hybrid-vectors, we generated a HCAdV/SB100X hybrid-vector system delivering the identical transgene expression cassette along with the hyperactive SB transposase SB100X for somatic integration. Our initial experiments for the HCAdV/SB100X hybrid-vector system were plasmid based and we could show that SB100X results in significantly enhanced integration frequencies (up to 100-fold) in U-87 cells. Next, we generated an HCAdV encoding SB100X. We could show that compared to the control group which received an inactive version of SB transposase, this viral vector results in enhanced integration efficiencies (up to 10-fold) in the presence of SB transposon donor DNA.

In a further step, the two hybrid-vector systems will be directly compared with respect to stability of the transgene and its safety profile. If the S/MAR-based vector system results in similar transgene stabilities as shown for the transposase system, this episomal S/MAR based system may be superior to hybrid-vectors which utilize somatic integration for stable transgene expression, because potential genotoxic side effects can be avoided.

P045

Tightly regulated Doxycycline (Dox)-inducible lentiviral vectors for human myeloprotective gene therapy: *in vitro* and CD34⁺ - xenotransplant studiesN Lachmann^{1,2}, R Hillje^{1,2}, A Kuhn^{1,2}, S Brenning^{1,2}, J Dahlmann³, N Heinz², I Gruh³, B Schiedlmeier^{2,3}, C Baum², T Moritz^{1,2}¹*Reprogramming and Gene Therapy Group, REBIRTH Cluster of Excellence, Hannover Medical School, Hannover, Germany;* ²*Institute of Experimental Hematology, Hannover Medical School, Hannover, Germany;* ³*Leibniz Research Laboratories for Biotechnology and Artificial Organs (LEBAO), Hannover Medical School, Hannover, Germany*

Myeloprotective gene therapy using drug resistance (CTX-R) genes such as cytidine deaminase (CDD) or O6-methylguanine-methyltransferase (MGMT) has proven an effective way to protect lymphohematopoiesis from anti-cancer chemotherapy. As administration of anticancer cytotoxic agents usually is only transient we here describe use of a Dox inducible "all-in-one" lentiviral vector construct. In our construct hCDD-cDNA is expressed from a pTET-T11 minimal-promoter, while the reverse transactivator protein is provided by a PGK-promoter on the same construct. CDD-mediated drug resistance was evaluated in K562 and primary human-CD34 cells. Both, transduced K562 and CD34 cells showed robust transgene induction within 24–48 h of Dox-application at doses of >0.2 μ g/ml. In K562 cells this resulted in protection from apoptosis and cell-cycle arrest for Ara-C doses of 600 - 2000 nM, while control cells were affected at 10-fold lower doses. Primary human CD34 cells were protected from Ara-C doses of up to 600 nM versus 25 nM for control cells.

Furthermore, NSG-xenotransplantation studies not only showed efficient engraftment with 5–20% human-CD45 cells in the peripheral blood 8 weeks after transplantation but also revealed robust transgene expression within 4 to 7 days of Dox administration. Of note, 5–30% transduced cells were observed in peripheral blood human B- (CD19) and myeloid- (CD11b) cells by flow-cytometry and qRT-PCR. No significant background transgene expression was noted in non-Dox-treated animals similar to the K562 or CD34 *in vitro* models. Our data demonstrate Dox-inducible transgene expression as an elegant tool for robust and rapidly controlled transgene expression in human hematopoietic cells which effectively avoids background expression.

P046

The role of furin in recombinant adeno-associated viral vectors infection and transduction

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Recombinant adeno-associated viral vectors (rAAVs) have been widely used for gene transfers in animal models and are currently evaluated for human gene therapy clinical trial. However, little is known about the mechanism involved in rAAV transduction - endocytosis, capsid processing, *intracellular trafficking*, nuclear import and genome release - and these processes limit rAAV gene delivery and transduction efficiencies. In the present study, we investigated the role of furin protease on rAAV transduction. We have found that LoVo cells, human colon adenocarcinoma derived cells, are refractory to AAV transduction. AAV serotype-1,2,5,6,8 were tested and all of them failed, or are very inefficient, in transducing LoVo cells. The virus enters into the cell but the viral genome fail to enter into the nucleus. However, LoVo cells expressing furin showed a very high transduction efficiency. Furin is an endoprotease that participates in the maturation of many important proteins in the cell. One of these enzymes is the uncovering enzyme (UCE). UCE is a Golgi enzyme that participates in the synthesis of the mannose 6-phosphate (Man-6-P) lysosomal targeting signal on acid hydrolases. Interestingly, LoVo cells expressing a furin-independent active form of the UCE protein, are efficiently transduced by AAV. Indicating that, UCE is important for AAV transduction. These results suggest that an intracellular process dependent on UCE activity is essential for AAV transduction. Furthermore, experiments performed in parallel demonstrated that the transduction efficiency of LoVo cells by AAV can also be recover by coinfection with nuclear replicating virus like Adenovirus or HSV.

P047

DNA transposition by protein transduction of the piggyBac transposase from lentiviral Gag precursors

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DNA transposon-based vectors have emerged as gene vehicles with a wide biomedical potential. So far, genomic insertion of such vectors has relied on co-delivery of genetic material encoding the gene-inserting transposase protein, raising concerns related to persistent expression, insertional mutagenesis, and cytotoxicity. Here we investigate direct protein delivery of the hyperactive variants of *piggyBac* transposase (hyPBBase) and *Sleeping Beauty* transposase (SB100X). We adapt integrase-deficient lentiviral particles (LPs) as protein carriers by fusing the transposase to the N-terminus of the Gag polypeptide. We demonstrate robust incorporation of hyPBBase and SB100X into LPs and show that both are efficiently liberated from the viral proteins by the viral protease upon particle maturation. However, LP-directed delivery of SB100X protein does not lead to transposition most likely due to the inactivating effect of five amino acid residues left from HIV-1 protease cleavage. In contrast, delivery of hyPBBase facilitates rates of DNA transposition that are comparable to state-of-the-art plasmid-based strategies. We show lentiviral co-delivery of the hyPBBase protein and vector RNA carrying the transposon sequence, allowing robust DNA transposition in several cell types treated with VSV-G-pseudotyped vectors. Importantly, this novel delivery method facilitates a balanced cellular uptake of hyPBBase, as evidenced by confocal microscopy, and allows high-efficiency production of clones harboring a single transposon insertion. Our findings show new means of combining DNA transposition with lentiviral gene delivery and establish engineered LPs as a new tool for transposase delivery. We believe that protein transduction methods will increase applicability and safety of DNA transposon-based vector technologies.

P048

Serum proteins impact efficiency of rAAV vectors in a species-specific manner

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Clinical relevance of gene therapy using the recombinant adeno-associated vectors (rAAV) often requires widespread distribution of the vector and in this case systemic delivery is the optimal route of administration. The success of future clinical trials depends much on the adequacy of the results obtained in animal models. We previously demonstrated that blood proteins, galectin 3 binding protein (G3BP) and C-reactive protein (CRP) can interact with different AAV serotypes in a species specific manner (Denard *et al.*, J Virol. 2012: 6620–31). While human and dog G3BP were able to interact with rAAV-6 and diminished its transduction efficiency, the mouse CRP protein increased more than 10 times the transduction efficiency of rAAV-1 and rAAV-6 under systemic delivery. Taking into account the importance of the blood proteins for the efficiency of rAAV vectors, we undertook systematic studies of proteins interacting with different AAV serotypes in sera from mice, dog, cow, macaque and human. We demonstrate for the first time that the blood of each tested species contain specific set of proteins to interact with a given serotype. The role of these particular proteins in the transduction efficiency of rAAV vectors and for preclinical studies and clinical trials will be discussed.

P049

Alpharetroviral SIN vectors for T-cell based therapyJ D Suerth¹, V Thies¹, T Maetzig¹, J Meyer¹, C Baum¹, A Schambach^{1,2}¹*Institute of Experimental Hematology, Hannover Medical School, Hannover, Germany;* ²*Division of Hematology / Oncology, Boston Children's Hospital, Harvard Medical School, Boston, USA*

Retroviral vectors are highly efficient in integrating therapeutic transgenes into cellular genomes making them valuable tools for human gene therapy. However, retroviral integration can lead to insertional mutagenesis, which has been involved in adverse events in hematopoietic stem cell-based clinical trials and which has been shown to potentially contribute to the immortalization of mature T-cells.

Especially the integration spectrum of the vector and the presence of promoter and enhancer elements influence its genotoxicity. Because of the comparatively neutral integration spectrum of alpharetroviral vectors we have developed an alpharetroviral self-inactivating (SIN) vector system with an advanced split-packaging design, not only removing viral enhancer, promoter, and splice elements from the vector, but also removing potentially immunogenic viral coding sequences. Using this safety-optimized alpharetroviral SIN vector system we were able to efficiently transduce primary human T-cells with different clinical relevant pseudotypes. Side-by-side analyses revealed RD114/TR-pseudotyped vectors to outperform amphotropic and VSVg-pseudotyped particles, allowing for high transduction rates in T-cells at low multiplicities of infection. To demonstrate proof-of-concept, we transferred suicide genes and demonstrated their functionality *in vitro*. As T-cell based therapies usually require large amounts of vector particles for clinical applications, an economic, large-scale vector production is of utmost importance. Therefore, we generated stable packaging cell lines for RD114/TR-pseudotyped alpharetroviral particles and could show sustained productivity over time.

Our proof-of-principle studies along with the favorable safety profile and the production perspective highlight the potency of the alpharetroviral SIN vector platform for future T-cell based therapies.

P050

The efficacy and safety of DVC1-0101 for intermittent claudication secondary to peripheral artery disease: study protocol of a randomized phase IIb trialY Fujino¹, M Tanaka¹, Y Yonemitsu¹¹*R&D Laboratory for Innovative Biotherapeutics, Graduate School of Pharmaceutical Sciences*

Background: We have developed a new gene transfer vector based on nontransmissible recombinant Sendai virus expressing the human fibroblast growth factor-2 gene (DVC1-0101) to treat peripheral arterial disease (PAD). A phase I/IIa open-label four dose-escalation clinical trial for critical limb ischemia was completed and concluded safe and well tolerated, and resulted significant improvements of limb function.

Methods: The phase IIb clinical trial is a randomized, placebo-controlled, parallel design, single-dose blinded, and single center clinical trial in Japan. The study will enroll 60 patients diagnosed PAD with intermittent claudication. Subjects who meet eligibility criteria will be randomized to receive a single dose of either pla-

cebo, 5×10^9 ciu/limb of 1-0101, or 1×10^9 ciu/limb of DVC1-0101 administered by direct intramuscular injection. The total length of participating in this trial for individual subject will be approximately 12 months with 9 visits. The primary endpoint is to evaluate the efficacy of DVC1-0101 versus placebo on peak walking time and to evaluate the safety and tolerability of two dose levels of DVC1-0101. The secondary endpoints are 1) to evaluate the effect of DVC1-0101 on treadmill claudication onset time and Quality of Life, as measured using the Walking Impairment Questionnaire, 2) to determine the effect of DVC1-0101 on qualifying limb hemodynamics, and 3) to explore the pharmacodynamics of DVC1-0101 by evaluating exploratory biomarkers.

Discussion: The results of this trial will give a insight in the potential of DVC1-0101 for improve walking activities, and will inform the design of a possible phase III study.

P051

Vpx improves nuclear import and uncovers a restriction to lentiviral integration in quiescent human hematopoietic stem and progenitor cellsC Petrillo^{1,4}, F Piras^{1,2,4}, S Bartolaccini¹, N R Landau³, L Naldini^{1,2}, A Kajaste-Rudnitski¹¹*San Raffaele Telethon Institute for Gene Therapy (HSR-TIGET), San Raffaele Scientific Institute, Milan, Italy;* ²*Vita Salute San Raffaele University, Milan, Italy;* ³*New York University School of Medicine, New York, USA;* ⁴*Equal contributors*

The HIV-2/SIV accessory protein Vpx counteracts SAMHD1-mediated restriction to reverse transcription in myeloid cells. In an effort to dissect the restriction bottlenecks that hamper LVs in human hematopoietic stem and progenitor cells (HSPCs), we investigated the capacity of Vpx to improve HSPC transduction. Human monocyte-derived macrophages (MDM) and cord-blood (CB) or bone marrow (BM)-derived CD34 HSPC were challenged with Vpx-packaging GFP-expressing LVs. To track the LV genome fate, retroviral DNA replication intermediates were quantified at early times post-transduction. In MDM, the Vpx LV generated 8.4-fold more late RT products, 27.9-fold more 2LTR circles and 6.8-fold more integrated proviruses as compared to the Vpx⁻ vector ($n=5$, $p<0.004$), leading to a 3-fold overall increase in the number of GFP cells. In quiescent CD34 cells, there was no difference in the number of late RT products but the Vpx LV lead to a 3-fold increase in the number of 2LTR circles in both CB and BM-derived HSPCs ($n=4$, $p<0.007$). This increase did not generate a larger number of integrated proviruses nor GFP cells and was no longer detected in cytokine-stimulated HSPC, in which all replication intermediates increased by 10-fold compared to unstimulated cells, independently of Vpx ($n=8$, $p<0.001$). Overall, our results suggest that LV nuclear import is limiting in quiescent human HSPC and can be overcome by Vpx, likely in a SAMHD1-independent manner. The lack of increased transduction by Vpx LV underscores the existence of a restriction bottleneck after nuclear import in human HSPCs.

P052

Inducible (shRNA-mediated) deoxycytidine-kinase (dCK) knock-down confers myeloprotective properties to human hematopoietic *in vitro* modelsK Czarnecki^{1,2}, N Lachmann^{1,2}, M Hetzel^{1,2}, S Brenig^{1,2}, N Heinz², B Schiedmeier², T Moritz^{1,2}

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Drug resistance (CTX-R) gene transfer has been advocated to protect the lymphohematopoietic system from the side effects of anti-cancer chemotherapy. However, resistance genes for a variety of clinically relevant nucleotide analogs with anti-leukemic properties such as fludarabine, clofarabine or cladribine have been missing, so far. As reduced expression of deoxycytidine kinase (dCK) has been linked to nucleotide analog resistance, we here introduce a novel Dox-regulated lentiviral vector harbouring a minimal pTET-T11-Pol(II) promoter and an exchangeable miRNA-30 based shRNA to mediate specific dCK knock-down in hematopoietic cells. Robust and Dox dependent knock-down of 80% of dCK expression within 2–3 days of Dox administration was demonstrated in human pro-myelocytic HL-60 cells by western blot and quantitative real-time-PCR analysis. In addition, dCK knock-down by Dox concentrations of >0,2µg/ml conferred more than 10-fold increased Ara-C resistance compared to control cells by LD50 values of 700 nM to 60 nM, respectively. Furthermore, dCK knock-down allowed for highly efficient selection of transduced cells by 200 nM Ara-C (from 10 to >95% within 7 days). Most importantly, dCK knock-down also protected HL-60 cells effectively from clofarabine and fludarabine as evident by 6–10-fold increased LD50 concentrations as well as cladribine (5-fold increase). We here describe a novel Dox-inducible, lentiviral “knock-down” vector expressing a dCK-specific shRNA in a miRNA-30 backbone from a minimal pTET-T11-Pol(II) promoter. This construct not only induces resistance to Ara-C in hematopoietic cell lines and efficient *in vitro* selection of transduced cells, but also provides profound resistance to highly clinically relevant cytostatic-drugs such as clofarabine, fludarabine, and cladribine.

P053

TetR-regulated all-in-one Lentiviral vectors for conditional expression in human stem cells

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Transient and inducible expression of transgenes in human stem cells is instrumental in studying the role of specific proteins in stem cell biology and in achieving safer gene therapy strategy. However genetic manipulation of human pluripotent and multipotent stem cells to generate conditional gene expression has been very difficult to achieve due to the low efficiency of existing delivery methods, the strong silencing of the transgenes (Stewart R, 2008) and the toxicity of the regulators. In this work we describe several lentiviral vectors (LVs)-TetR-based systems that can generate conditional stem cell lines (MSCs and hESCs) in less than a week. The new regulated LVs derived from the previously published CEST All-In-One LV. The CEST LV efficiently generated primary

cells responsive to doxycycline but required an average of 3 vectors per cell and performed very poorly in MSCs and hESCs. We improved the CEST all-In-One LV by 1- Modification of the TetR protein to include two different nuclear localization signals (*TetRnls1* and *TeRnls2*), 2- Using the EF1alpha promoter to express TetR and 3- Using insulators to favour stability of expression of the different components. Our final constructs, named CEEtNls2 and CEEtNls2IS2 were able to obtain conditional expression in MSCs and hESCs after one single transduction. This conditional expression was maintained in differentiated MSCs as well as in hESC-derived cells such as hemogenic progenitors (CD31 CD45-) and hematopoietic cells (CD34 CD45 and CD34-CD45). This is to our knowledge the first All-In-One Dox-regulated vector system that allows the generation of Dox-regulated hESCs lines in the absence of selection.

P054

Elimination of packaging sequences from lentiviral vector DNA delivered to target cells

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Lentiviral vector (LV) packaging sequences (Ψ , Rev Response Element) up to 2 kb long are necessary for viral RNA genome packaging into virions in producer cells. In standard LVs, packaging sequences are reverse transcribed into DNA and persist in target cells after transduction. This persistence creates several known and potential problems for LV gene therapy applications. Splice sites within packaging sequences have been shown to splice with nearby host genes, creating aberrant fusion transcripts. The CpG island within the packaging sequence undergoes DNA methylation in some target cells, potentially contributing to transgene silencing. Packaging sequences enable remobilisation of LV genomes in cells expressing viral proteins, which could be problematic in HIV-positive patients. Large packaging sequences within the reverse transcript may reduce the size of the transgene cassette which can be accommodated.

In standard LVs, packaging sequences are located between the two viral long terminal repeats (LTRs), within the region of the vector that is reverse transcribed. We have developed a novel transfer vector in which the packaging sequences are located downstream of the 3' LTR so are present in the RNA genome during virion packaging but are outside of the region of the genome that is reverse transcribed into DNA in the target cell. We can produce these vectors to high titre (3×10^8 TU/ml by eGFP flow cytometry of 293Ts, pCCL parallel preparation 1×10^9 TU/ml) and the proportion of eGFP cells is stable between 3 and 14 days post-transduction. We suggest that the use of this configuration to eliminate most of the remaining viral DNA from target cell proviruses could be a feature of the next generation of gene therapy vectors based on HIV-1 and other retroviruses.

P055

A Chimeric HS4-SAR insulator (IS2) that prevents silencing and enhances expression in pluripotent stem cells

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Chromatin insulators are DNA sequences that separate active transcriptional domains in the genome making possible its differential regulation and avoiding the spreading of heterochromatin to these active areas. Scaffold attachment regions (SARs), also named Matrix attachment regions (MARs), have also been described to play important roles in defining boundaries between different chromatin regions. Both elements have been incorporated separately or in combination into retroviral vectors in order to improve their efficiency (expression pattern and stability) and safety (reduction of genotoxicity) with different outcomes. Different publications have demonstrated that the effects of the different insulators and SAR/MARs elements are highly dependent on the retroviral backbone and on the cell under study. We have developed a chimeric insulator, IS2, based on the chicken β -globin locus control region hypersensitive site 4 (HS4) and a synthetic SAR/MAR element containing 4 MAR/SARs recognition signatures (MRS). The IS2 element was able to enhance expression, avoid silencing and to reduce variegation (variability of expression depending on the integration site) of constitutive lentiviral vectors on human embryonic stem cells. Neither the HS4 nor the SARs elements on their own had these effects. The IS2 element is therefore a novel insulator that confers expression stability and that enhance expression of lentiviral vectors.

P056

Novel ubiquitous chromatin opening elements

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Background: An approach developed by Antoniou and colleagues involves the use of a novel enhancer-less ubiquitous chromatin opening element (UCOE), derived from the human *HNRPA2B1-CBX3* locus (A2UCOE), to control transgene expression within a SIN-LV (Self inactivating lentiviral vector) context. This element has been shown to increase the number of cells that express transgenes at highly reproducible and stable levels in a variety of different cell lines and especially *in vivo* following *ex vivo* gene transfer to mouse bone marrow HSCs.

Aims: The overall aim of this project is the identification and testing of novel dual divergent housekeeping gene promoters in the genome and tests them for UCOE activity and to build artificial UCOEs by linking single housekeeping gene promoters back to back in a divergent configuration.

Methodology/Results: A number of vectors harboring candidate novel UCOEs linked upstream of an SFFV-eGFP cassette were constructed within lentiviral vectors. These vectors were used to transduce murine embryonal carcinoma P19 and F9 cells. The stability, potency, and reproducibility of expression of the eGFP transgene was then analysed in differentiated and undifferentiated P19 and F9 cells.

Conclusion: P19 and F9 cells were successfully transduced with novel candidate UCOE-based vectors plus controls. The results show that the novel candidate SETD3-CCNK and RPS11-HNRPA2B1 UCOEs were functional in both orientations when linked to an SFFV promoter suggesting greater potency than the prototypical HNRPA2B1-CBX3 A2UCOE element.

P057

Improved lentiviral transgene expression in murine and human pluripotent stem cells and their differentiated progeny utilizing ubiquitous chromatin opening elements (UCOEs)

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Epigenetic silencing of retroviral transgene expression in pluripotent stem cells (PSCs) and their differentiated progeny represents a substantial problem for iPSC-based gene therapy. Recently, we demonstrated that a defined 1.5 kb ubiquitous chromatin opening element (A2UCOE) sustains transgene expression driven from the truncated elongation factor-1 α (EFS)-promoter during differentiation of murine (m)iPSC into derivatives of all three germ layers. Now we extended these data to viral promoter/enhancer-elements and demonstrated, that the A2UCOE also effectively stabilized transgene expression driven by the spleen focus forming virus (SFFV) promoter in undifferentiated miPSCs (~80% vs. ~3% dTomato -cells on d14) as well as thereof differentiated CD41 hematopoietic progenitor cells (~80% vs. 1-3% dTomato -cells). Bisulfite-sequencing revealed protection of the SFFV-promoter from CpG-methylation to be associated with this effect.

In addition, we investigated the properties of the A2UCOE in human PSCs. Whereas profound silencing of EFS-driven transgene-expression was observed in undifferentiated hESCs/hiPSCs, this was prevented by the A2UCOE (~20% vs. 80% dTomato cells in passage 6). Again, the effect was associated with reduced EFS-promoter methylation (~33% vs. 90% methylated CpGs). Even more important, analysis of hESC during cardiac differentiation revealed sustained, A2UCOE-mediated transgene expression in ~70% of differentiated cells (EFS-driven controls ~10%). Moreover, following hematopoietic differentiation of hiPSCs, transgene expression could only be detected in monocytes/macrophages derived from A2UCOE.EFS-transduced hiPSCs. Also SFFV-driven transgene expression was effectively stabilized by the A2UCOE in non-differentiated hESCs.

Thus our data furthermore support the concept of UCOEs as a generalized strategy to stabilize transgene expression during the generation of PSC-derived transgenic cell therapy products.

P058

Lentiviral vector for p47phox gene therapy

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Recent clinical gene therapy trials for Chronic Granulomatous Disease (CGD) have demonstrated the feasibility of this approach for the treatment of patients for which no other therapeutic alternative is available. However the therapeutic effect was mitigated in these patients due to *EVII*-driven clonal expansion and methylation-dependent transgene silencing.

Oncogene transactivation in stem and progenitor cells may be prevented by the use of the highly myelospesific miR223 promoter. Therefore we developed a lentiviral GT vector for gene therapy of CGD patients with mutations in p47phox, one of the subunits of the phagocytic NADPH oxidase. miR223 driven p47phox transgene expression was restricted to phagocytes and sufficiently high to reconstitute reactive oxygen species (ROS) production in p47-KO mice transplanted with gene modified cells and in primary p47phox^{-/-} CGD phagocytic cells derived from transduced progenitors. An *E.coli* killing assay conducted in granulocytes derived from transduced primary p47phox^{-/-} CD34 bone marrow cells confirmed the restoration ROS production and *E.coli* killing by miR223 driven p47phox transgene expression. We then used the same construct to transduce iPSCs derived from p47phox-deficient CGD patients. DNA methylation analysis in p47phox^{-/-} hiPSCs and phagocytes derived thereof revealed that the vector encoded miR223 promoter was methylated in stem cells. Upon differentiation of hiPSCs to monocytes the miR223 promoter was actively demethylated and activated resulting in p47phox expression and reconstitution of ROS production in hiPSCderived monocytes and macrophages. From these observations we expect our new p47phox CGD GT vector to facilitate stable long term p47phox transgene expression and correction of the CGD phenotype.

P059

Construction and efficient transduction of lentiviral vector with p22^{phox} gene in peripheral granulocytes

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Purpose: To demonstrate newly developed lentiviral vector with target gene (p22^{phox} gene) efficiently transduces in peripheral granulocytes.

Materials and Methods: The lentiviral vector pLL3.7-EF1 α -p22^{phox} was constructed by replacing the U6 promoter in pLL3.7 with the EF1 α promoter. pLL3.7-EF1 α -p22^{phox} lentiviral particles were generated by transient cotransfection with two packaging plasmids (pMDG2 and pCMV- Δ 8) into HeLa and HEK 293T cells. Viral supernatant was harvested at 48 and 72 hours after transfection, and viral titers were quantified by flow cytometric analysis. The peripheral granulocytes from chronic granulomatous disease (CGD) patients were infected with recombinant retroviruses carrying human telomerase reverse transcriptase (hTERT) and B lymphoma Mo-MLV insertion region 1 (Bmi-1) genes in order to prolong the life span and then, transformed granulocytes were transduced by pLL3.7-EF1 α -p22^{phox}. After transduction, the efficiency was compared and the NADPH oxidase activity was quantified by dihydrorhodamine (DHR) 123 flow cytometry.

Results: The transduction efficiency of 10-fold dilution of viral supernatant in HeLa cells was highly efficient, with 70–96% of cells displaying eGFP fluorescence. The transduction efficiency of high-level granulocyte^{hTERT Bmi-1(-)} cells was 40% higher than that

of granulocyte^{hTERT Bmi-1(-)}. The eGFP expression was detectable at 3 days after transduction, and its expression persisted for up to 3 weeks. The production of superoxide was significantly increased in granulocyte^{hTERT Bmi-1(-)} cells after PMA stimulation, and the number of DHR-positive cells increased by as much as 2-fold.

Conclusion: This study described the lentiviral-mediated transduction of CGD-derived peripheral granulocytes in an experimental setting to increase the efficiency of gene transfer and its compatibility with clinical constraints.

P060

Enhancing myeloid specific gene expression by improving transcription factor binding to the miR-223 promoter

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Integrating vectors pose the risk of insertional activation that can be reduced by targeted expression using cell type restricted promoters. However, weak expression levels might require multiple integrations for efficient therapeutic reconstitution counteracting the reduced transformation potential expected from cell specific expression. Therefore, our intention was to enhance myeloid specific expression by improving myeloid transcription factor binding to the miR-223 promoter.

In silico predicted and experimentally validated transcription factor binding sites (TFBS) within the proximal human miR-223 promoter (CEBP, c-MYB, SP1, PU.1) were converted into their respective consensus motive in various combinations. The resulting promoter mutants were cloned into retroviral vectors and expression levels of reporter genes (eGFP, gp91phox) were analyzed after transduction of myelomonocytic PLB-985 X-CGD cells and subsequent myeloid differentiation. Three promoter mutants revealed enhanced expression (up to 1.5 fold) and the regulatory potential was maintained as determined by fold induction upon differentiation. Following transduction of human CD34 the low expression levels of the original promoter were recapitulated in the immature subpopulation (CD133) whereas a more than 2-fold increase in GFP expression was achieved in CD11b cells after *in vitro* differentiation by our novel promoter mutants.

We propose here a simple method to significantly enhance promoter activity by combining *in silico* prediction with optimizing TFBS according to consensus motives. Future *in vivo* studies addressing effects on specificity as well as binding studies are warranted to support our hypothesis, however, regulated expression as observed in our *in vitro* studies serves as a first indicator of increase specificity.

P061

Optimization of lentiviral gene therapy for ADA-SCID

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Adenosine Deaminase (ADA) deficient-SCID is a severe autosomal recessive disease characterized by impaired lymphocyte

development and function with subsequent recurrent infections. Currently available therapeutic options include bone marrow transplantation (BMT), enzyme replacement therapy with bovine ADA (PEG-ADA) or hematopoietic stem cell gene therapy (HSC-GT) using a retroviral vector carrying ADA gene. Nevertheless, correction of systemic metabolic toxicity including non-immunological alterations may require increased ADA expression levels.

Aim of this study is to improve the efficacy of ADA gene transfer in HSC and increase expression level using a lentiviral vector (LV) carrying a codon-optimized human ADA gene. We compared this novel LV with a LV carrying the non-optimized transgene (Mortellaro, Blood, 2006). In both vectors transgene expression was driven by a PGK promoter. We transduced BM progenitors from ADA^{-/-} mice using different vector concentrations. Integrated vector copies (VCN/genome) and ADA enzymatic activity were measured two weeks after *in vitro* transduction.

The optimized LV induced ADA overexpression from 10 to 30 fold depending on the VCN/genome (2.5-10) of transduced cells. At similar VCN/genome codon-optimized LV produced 6 fold higher ADA enzymatic activity.

Ongoing studies in human CD34 cells and *in vivo* in ADA^{-/-} mice are aimed at evaluating gene transfer, immune reconstitution, metabolic detoxification and correction of systemic abnormalities after gene therapy with the codon-optimized LV.

P062

Novel Dual-Insulated Lentiviral Vectors for Gene Therapy of Sickle Cell Diseases

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Autologous stem cell gene therapy is an alternative treatment for Sickle Cell Disease (SCD) without some limitations of allogeneic hematopoietic stem cell (HSC) transplant. Lentiviral vectors (LVs) developed for the treatment of hemoglobinopathies, require inclusion of regulatory and strong enhancer elements to achieve sufficient expression of the β -globin gene; the resulting LVs have complex cassettes with a deleterious effect on titers. Moreover, the efficacy of the LVs may be limited by transgene silencing by DNA methylation and heterochromatinization due to the genomic environment around the integration site. Barrier insulators can be used to resist silencing and give more consistent expression with lower vector copies. LV carrying an anti-sickling human β -globin gene (β AS3) was engineered to contain the FB (Ramezani *et al.*, 2008) and Ankyrin (Ank) (Gallagher *et al.*, 2010) small insulators in different orientations. In single vector copy MEL clones, a single copy of the Ank insulator in reverse orientation (AnkR) demonstrated barrier activity that most closely resembled that observed with the *chf4* positive control. However, erythrocytes derived from human SCD CD34 cells transduced with the AnkR- β AS3-FB LV did not exhibit enhanced β AS3 expression or increased phenotypic correction of sickling compared to cells transduced with the analogous vector without an insulator. Since short-term *in vitro* culture of primary cells did not demonstrate the benefits of the AnkR- β AS3-FB LV,

chromatin analysis of the provirus, and *in vivo* studies using serial transplants of murine HSCs are in progress to determine the extent of the barrier activity in the AnkR- β AS3-FB LV.

P063

Replication-Inducible HSV-1 Vectors: Construction and Application in Gene Therapy

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Replication-inducible viruses hold importance in terms of making helper-free packaging cell lines and recombinant vectors for gene therapy. We generated a haploid HSV-1 mutant in which we have deleted one set of ICP0 and ICP4 genes. In additional mutations, we modified the promoter of the remaining copy of ICP0 by insertion of a Tetracycline responsive element in combination with deletion of regions of the native promoter. We are testing if these mutant HSV-1 viruses can infect cell lines where they can be artificially forced to enter a latent-like state by tetracycline-dependent repression, and/or exit this state by tetracycline-dependent activation. This strategy will provide a transfection-independent system for vector/amplicon packaging. In parallel these same mutants are being tested to generate recombinant genomic vectors which can only grow under induced conditions.

We found a large fold decrease in cellular toxicity with haploid virus compared to wild type diploid virus. Replication-inducible recombinant vectors based on this haploid backbone will provoke less cellular toxicity and possible inducible control of transgene expression.

The neurotropic and neuroinvasive properties of HSV-1 hold great potential for gene therapy vectors. An understanding of inducible replication control will improve the production of HSV-1 vectors for therapeutics.

P064

Gene Therapy of Friedreich's Ataxia: Generation of Recombinant Herpesvirus

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Friedreich's ataxia (FRDA) is an autosomal recessive and progressive neurodegenerative disorder. Currently there is no available therapy. In the majority of the patients it is due to homozygous expansion of guanine-adenine trinucleotide repeats within intron 1 of the FXN gene resulting in diminished expression of the mitochondrial protein frataxin and consequently in neuronal atrophy. Among the first sites of neurodegeneration in FRDA are the dorsal root ganglia, followed by the corticospinal and spinocerebellar tracts of the spinal cord and at late stages, atrophy of the brainstem and cerebellum is observed.

Since FRDA is caused by a deficiency of frataxin protein, one solution would be to increase the expression of this protein. This could be achieved by delivering a fully functional normal FXN allele to the main site of pathology.

Recently, in cancer pain therapy, a recombinant replication-defective HSV-1 vector has reached phase 2 clinical trials for gene delivery to the dorsal root ganglia of the spinal cord, and intradermal injection of this vector (termed NP2), even at high doses, demonstrated that it is non-cytopathic and safe for use in the human nervous system. However, the NP2 vector cannot

accommodate the large FXN genomic transgene, which we previously used in preclinical experiments to achieve persistent physiological expression in the adult nervous system.

Thus, on one hand, we are currently working on reduction of the FXN transgene, without losing long-term regulated expression, and on the other hand, we are constructing a vector similar to NP2 with further deletions to increase transgene capacity.

P065

The role of adenovirus replication cycle on the production of helper-dependent vectors

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Adenoviral helper-dependent (HD) vectors, devoid of any viral coding regions and eliminating the problems associated with residual viral gene expression, are promising tools for gene delivery applications, achieving high transduction efficacies, long-term gene expression, and a cloning capacity of >30 kb. However, their production yields are ~10-fold lower than E1-deleted (Δ E1) vectors.

Our goal was to identify the steps of canine adenovirus type 2 (CAV-2) propagation that were altered in HD vectors production versus Δ E1 vectors. CAV-2 vectors were selected as case study due to their attractive features to address fundamental neurobiological questions and to develop potential treatment of neurodegenerative disorders.

We found an equivalent accumulation of intracellular viral genomes up to 6 hpi in HD- and Δ E1- infected cells. However, in HD-producing cells viral genome replication occurred faster originating higher levels of late phase proteins. Such high viral protein content might be responsible for the increasing apoptotic cells and lower viability observed during HD CAV-2 production. Moreover, despite similar cell specific productivities of HD and Δ E1 virions, infectious to physical particles ratio obtained in HD CAV-2 was 4-times lower. These results indicated that the deregulated CAV-2 cycle progression was impairing producer cell homeostasis and the resulting virus outcome. Vector genome replication during HD CAV-2 production should be therefore balanced to pursue the typical adenoviral productivities.

This work identifies the bottleneck in HD CAV-2 production from a viral replication perspective, highlighting the importance of vector genome design on virus-cell interaction and production process yields.

P066

Soluble coxsackievirus and adenovirus receptor (sCAR-Fc) has prophylactic and therapeutic potential in adenovirus infections

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Background: Adenoviruses (Ad) are pathological agents inducing under certain circumstances severe infections of the liver and heart, sometimes leading to death. Currently, no curative anti-adenoviral therapy is available. Here we investigated the ability to treat Ad infection with soluble coxsackievirus and adenovirus receptor (sCAR-Fc).

Methods: The endothelial cell line EA.hy926 and the liver cell line HuH7 were infected with wild-type Ad2 and Ad5 and treated with sCAR-Fc containing cell culture medium, which was produced by transduction of HeLa cells with a sCAR-Fc expressing viral vector. Cell killing-assay, Plaque-assay and qPCR were used to determine the sCAR-Fc-mediated inhibition of Ad infection.

Results: *In vitro* prophylactic employment of sCAR-Fc resulted in an up to 20-fold reduction of cytopathic effect and up to 2log₁₀ reduction of virus replication compared to untreated controls. For therapeutic purpose sCAR-Fc was added at different time points after Ad infection. Early therapeutical sCAR-Fc application resulted in strong inhibition of Ad-induced cytopathogenicity. The protective effect reduced gradually as later as sCAR-Fc was applied after Ad infection but remained still visible if it was applied 72 h after virus infection. For *in vivo* usage we constructed an AAV9 vector expressing a codon optimized sCAR-Fc variant. After intravenous application to mice sCAR-Fc expression (1,5 µg/ml) was already detectable in mouse serum one day later and increased to 40 µg/ml at day 28 days p.i. No side effects were detected.

Conclusions: Our data suggest sCAR-Fc as a new promising biological antiviral therapeutic for treatment of Ad infections. Studies are currently underway proving its antiviral efficacy *in vivo*.

P067

Very efficient production of adenovirus vector lacking genes of virus-associated RNAs that disturb cellular RNAi machinery: safer alternative to current vector

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Although first-generation adenovirus vectors (FG AdVs) are frequently used, virus-associated (VA) RNAs that act as small-interference RNAs are, in fact, always expressed from the vector genome. Because they are expressed by RNA polymerase III, their expression is independent of E1A transactivation of polymerase II. It has been reported that VA RNAs trigger innate immune responses. Moreover, VA RNAs are processed to functional viral miRNAs and disturb the expressions of numerous cellular genes. Therefore, VA-deleted AdVs lacking VA RNA genes are advantageous not only for basic studies but also for gene therapy as a safer vector. We established a very efficient method of producing VA-deleted AdVs (Maekawa *et al.*, *Scientific Report*, 2013). First, a VA RNA-substituted “pre-vector” lacking original VA RNA genes but alternatively possessing an intact VA RNA region flanked by a pair of FRTs was constructed. The pre-vector grew as an FG AdV and gave a high titer. Then, the pre-vector was converted to VA-deleted AdVs by removing the VA genes through infection to 293hde12 cells, which highly express FLP. The conversion efficiency was about 97–99% and VA-deleted AdV showed the titer near to pre-vector, i.e. only one-order lower than FG AdV. The titer is sufficient for practical use *in vitro* and *in vivo*, and perhaps for clinical trial. However,

the vector tends to rearrange, and therefore we here report its improved version. The improved vector substituted for current FG AdV in our lab and may become widely used.

P068

Dual-safe adenovirus vector lacking virus-associated RNA genes enhanced shRNA activity

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The first-generation adenovirus vector (AdV) has been considered an attractive tool for gene therapy because of high titer and high transduction efficiency. However, since AdVs cause high immune responses, application of AdVs were limited. We have reported low-inflammatory AdVs using EF1a promoter in the leftward orientation, which does not induce aberrant expression of viral pIX protein (Nakai *et al.*, *Hum. Gene Ther.*, 2007). Another important problem is that two different virus-associated RNAs (VA RNAs) transcribed by Pol III are always expressed from the AdV genome. We here report development of novel AdVs lacking genes of VA RNAs. Although VA RNAs are not essential for adenovirus replication, VA-deleted AdVs have been very difficult to construct or showed extremely low titer. We succeeded in establishing an efficient production method of VA-deleted AdVs using a site-specific recombinase. Notably, the titer was almost comparable with current AdVs. Actually, we have already constructed more than twenty VA-deleted AdVs of high titer. Moreover, we combined the VA-deleted vector with low-inflammatory vector (Dual-safe AdVs). As one example, we used this vector system for aiming gene therapy of chronic hepatitis caused by hepatitis C virus (HCV). Because VA RNAs interfere maturation of miRNA and/or compete with short-hairpin RNA (shRNA), VA-deleted AdVs were, in fact, able to enhance shRNA suppression of HCV replication. So this Dual-safe AdV carrying both interferon gene and anti-HCV shRNA cassette may be an efficient and safe tool for gene therapy of chronic hepatitis C.

P069

First-generation adenovirus vector expresses viral-associated (VA) RNAs that disturb cellular gene expressions

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Adenovirus vector (AdV) has widely been used not only for basic studies but also for gene therapy. We have been reported that viral pIX protein, one of the causes of severe immune responses, is often expressed but that this problem can be avoided by modifying this vector (low-inflammatory AdV; Nakai *et al.*, *Hum. Gen. Ther.*, 2007). However, this vector has another problem that, in fact, it always expresses not only the transgene but also viral-associated (VA) RNAs. VA RNAs, VAI and VAIL, are non-coding small RNAs and disturb cellular RNA-interference machinery. We recently developed AdVs lacking the expression of these miRNAs (VA-deleted AdVs) that solve this problem (Maekawa *et al.*, *Sci. Rep.*, 2013). Although the current AdVs used worldwide abundantly express VA RNAs, their influence on the cellular gene expression are still not clear. In this study using this vector we identified its possible target gene that was

downregulated in response to VA RNAs by comparing current AdVs with VA-deleted AdVs by microarray analysis. This gene has been reported to correlate with cell growth and sometimes its expression level was accelerated in tumours. This result demonstrated that VA RNAs actually influence on cell functions and suggested that VA-deleted AdVs may be safer vector for gene therapy than current AdVs.

P070

An advanced toolbox for molecular AAV evolution through DNA family shuffling

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AAV vectors are amongst the most promising vehicles for therapeutic gene transfer owing to their unique combination of apathogenicity, efficiency and amenability to genetic engineering. The latter includes a variety of powerful methods to modify vector tropisms, most notably, DNA family shuffling (DFS) whereby capsid genes from several AAV isolates are fragmented and then reassembled into chimeras ideally merging the best properties of the parental viruses. Here, we present three developments that fundamentally advance AAV-DFS technology and should foster its application in other labs. The first is a set of new plasmids carrying the capsid genes from 12 commonly used AAV isolates that we engineered to be fully compatible with a robust, simple and straight-forward DFS protocol which we have implemented in parallel. Second, we have adapted the DNA sequence of AAV-4 and -5, two of the most heterologous AAV isolates, to that of the AAV-2 prototype in order to improve their recombination frequency during AAV-DFS. Indeed, comparisons of libraries comprised of AAV serotypes 245, 24589, or 1-9 showed up to 25-fold more cross-overs of AAV-4 and -5 with the other serotypes in clones generated with the optimized capsid sequences. Third, we report a comprehensive and freely available software package termed Salanto that greatly facilitates the bioinformatical and statistical analysis of individual AAV chimeras or entire libraries. As a whole, the advanced toolbox reported here paves the way for a much wider use of AAV-DFS methodology within the gene therapy community and will thereby hopefully further accelerate its clinical translation.

P071

Liver inflammation affects the formation of AAV transcriptionally active forms but not preexisting AAV infections

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Background and aims: Clinical trials showed that steroid treatment controls immune responses against adeno associated viruses (AAV), however few studies have focused on the effect of

inflammation over AAV-mediated transgene expression. The aim of this study is to analyze how liver inflammation affects to AAV-mediated gene expression.

Methods: AAVs hepatospecifically and constitutively expressing interleukin-12 (AAVIL-12) and luciferase were co-injected into C57BL/6, IFN- γ R^{-/-}, Rag^{-/-}, Rag^{-/-} γ c^{-/-} and NK-depleted mice. Luciferase expression was analyzed by *in vivo* bioluminescence imaging at different time points and, 14 days post-injection, animals were sacrificed for *ex vivo* luciferase measurement and viral genome and mRNA quantification.

Results: Mice injected with both vectors showed an early 90% decrease on luciferase expression when compared to controls. This inhibitory effect occurred at a transcriptional level, as only mRNA was affected, and it was mediated by IFN- γ , mainly produced by T and NK cells.

In vivo studies revealed that methylation, but not acetylation, was involved as only 5'-azacytidine treatment avoided the reduction of transgene expression. Administration of dexamethasone also prevented this inhibitory effect. Together, these results suggest that IFN- γ exerts some epigenetic regulation over AAV-mediated expression.

However, this epigenetic regulation might not affect to a pre-established AAV infection, as AAV8-IL12 did not affect to luciferase expression in a liver with a previous AAV5-luciferase infection.

Conclusions: Our results show that IFN- γ -mediated liver inflammation downregulates AAV-mediated transgene expression at a transcriptional level by a mechanism in which methylation is involved. This inhibitory effect does not affect pre-established AAV infections and can be counteracted by anti-inflammatory treatments.

P072

Delivery of anti-HBV primary-microRNA mimics using gutless adenoviral vectors and adeno-associated viral vectors

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As a consequence of an ineffective current therapy against hepatitis B virus (HBV) infection, ~350 million people are estimated to be chronically infected with the virus globally. Application of RNA interference (RNAi) gene silencing technology against HBV has yielded promising outcomes and effective anti-HBV RNAi activator sequences have been designed. However, an efficient way of delivering these sequences still remains to be established. Viral vectors are the most promising carriers of genetic material. This study investigates the application of both gutless adenoviral vectors (HdAds) and adeno-associated viral vectors (AAVs) for the delivery of anti-HBV primary microRNA (pri-miR) mimics as RNAi activators. We have recently observed efficient HBV replication silencing in mice injected with HDAd expressing pri-miR mimics. However, the HBV silencing effect was short-lived (2 weeks). This could be attributed to the cytomegalovirus (CMV) promoter used, being switched off and/or the immunostimulatory effect of the β -galactosidase reporter encoded by these vectors. Several groups have illustrated long-term transgene expression from AAVs. In this study, the CMV promoter was substituted with liver specific phosphoenolpyruvate carboxykinase promoter or murine transthyretin receptor promoter. These expression cassettes were incorporated in to the *lac-Z* lacking HDAd plasmid or the AAV plasmid. Currently the HDAd and AAV vectors are being propagated *in vitro*.

Following propagation, pri-miR expression, pri-miR processing and anti-HBV effect from the vectors will be tested in transgenic mice. These may allow long-term anti-HBV effect, making these vectors more suitable for application in RNAi effector delivery directed to HBV chronic infection.

P073

Rep-mediated persistence of AAV

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Through its endogenous Rep protein, wildtype adeno-associated virus (wtAAV) integrates its genome site-specifically into the AAVS1 region on Chromosome 19, which is considered one of the most promising "safe harbors" in the human genome. In recombinant AAV (rAAV), Rep is deleted and the ability to integrate site-specifically is lost. The knowledge about wtAAV integration remains limited. Two reports of wtAAV integration site (IS) distribution exist that showed IS clustering near Rep binding site (RBS)-motifs. These analyses however present few IS numbers or map IS with a low nucleotide resolution and have exclusively been performed in HeLa cells. To avoid the bias that might arise from HeLa cell aneuploidy, we analyzed efficiency and specificity of Rep-mediated wtAAV integration in HeLa cells and diploid human dermal fibroblasts (HDF). We obtained a total of 3659 unique IS (HeLa: 2403 IS; HDF: 1256 IS) and identified 276 common IS (CIS) besides AAVS1. Notably, only few CIS were shared between HeLa cells and HDFs (Shared CIS: 23; HeLa CIS: 162; HDF CIS: 91). 16.5% of HeLa IS and 9.1% of HDF IS were located in the AAVS1 region. wtAAV IS were almost exclusively distributed at one side of the RBS-motifs, while the viral genome is mostly integrated in one orientation. Within CIS we identified sub-clusters, whose positions may help to elucidate the mechanism of Rep-mediated AAV integration. Comprehensive high resolution mapping and analysis of wtAAV integration sites in diploid cells reveals new insights in the potential of targeted, Rep-mediated rAAV gene therapy.

P074

Use of the Monobac for the production of rAAV8 vectors against the γ -sarcoglycanopathy

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We have developed a single baculovirus, named "Monobac", for the production of recombinant AAV8 vectors using the insect cell/baculovirus system. In a bacmid devoid of the *chitinase* and *cathepsin* genes, the AAV *rep2* and *cap8* genes have been inserted in the *egt* locus, while the recombinant AAV was cloned in the Tn7 site. This production system was used for the production rAAV2/8 encoding the human γ -sarcoglycan gene, of clinical interest for the treatment of LGMD2C (γ -sarcoglycanopathy). A gain by a factor 3 in rAAV8 productivity was observed in the cell culture and was maintained after purification, compared to the recently developed production system based on the use of 2 baculoviruses. The produced rAAV8 capsids displayed a reduced degradation profile of

the capsid proteins VP1/VP2 due to the elimination of the baculovirus *cathepsin* protease gene. This optimized system allows the production of an improved quantity of rAAV vectors with improved vector quality, resulting in enhanced infectivity of the rAAV. The genetic stability of this baculovirus vector displaying both the replicase gene and the recombinant genome on the same DNA backbone is studied. This presentation gives an overview on recent results including *in vivo* studies.

P075

AAV9 mediated gene delivery in peripheral tissues is greatly reduced in old mice

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Although aging is the main risk factor for the prevalent diseases of developed countries preclinical studies of gene delivery in old mice are very scarce. Systemic-gene delivery holds great promise for many gene therapy applications. Among vectors, AAV9 stands out as a particularly attractive vehicle because of its superior performance in different tissues. We therefore studied the peripheral tropism of AAV9 in aging mice. Young (6 weeks, n=8) and old (22 months, n=8) male C57BL6/J mice received a single i.v. injection of 4×10^{11} vg AAV9-GFP. Six weeks later expression of GFP in skeletal muscle, heart and liver was analyzed using immunohistochemistry, Western-blot and RT-PCR. The results showed a large decrease in GFP expression in all tissues examined of aged mice compared to young. To explore the possible mechanisms involved in the pronounced reduction of GFP, titers of IgG anti-AAV9 were analyzed in the mice-sera. There were no significant differences between young and old mice. Since it has been recently reported that cell-surface N-linked glycans with terminal galactosyl residues serve as the primary receptor for AAV9, we also assessed sialylated and desialylated cell-surface glycans in these organs by fluorescence staining using the biotinylated lectins, MAL-I and ECL. The results indicate that the substantial decrease in AAV9 tropism in the peripheral organs of aged mice is due to a very low infectivity of AAV9 in the vascular endothelium. This correlates with the significant reduction in desialylated galactose residues of cell-surface glycans that occurs in aging.

P076

Protein transfer vectors as targeted and effective antigen carriers

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To induce and trigger innate and adaptive immune responses, antigen presenting cells (APCs) have to take up and process an antigen. Retroviral particles are capable of transferring not only the vectors' genome, but also foreign cargo proteins into transduced cells when the cargo is genetically fused to structural proteins of the vector (Voelkel *et al.* 2010). Here, we demonstrate the advantage of lentiviral protein transfer vectors (PTV) for targeted antigen transfer directly into APCs.

Targeting of lentiviral PTVs is conducted analogous to lentiviral gene transfer vectors by pseudotyping the vectors with truncated wild-type measles virus (MV) glycoproteins (GPs), which use SLAM as main entry receptor. SLAM is expressed on stimulated lymphocytes and APCs, e.g. dendritic cells, and thus represents a potential target structure on APCs. As cargo proteins, the reporter protein eGFP or the model antigen Ova were analyzed.

Analysis of lentiviral eGFP-PTVs on receptor-transgenic CHO cells revealed that, in contrast to VSV-G pseudotyped vectors, vectors pseudotyped with MV-GPs mediated specific delivery only into SLAM-positive cells. Simultaneously, transfer of viral RNA encoding red-fluorescing Katushka protein into the same cells as the protein cargo was demonstrated. Accordingly, specific protein transfer of Ova into SLAM-positive CHO cells was evident. Moreover, *ex vivo* treatment of primary APCs with Ova-PTVs resulted in stimulation of Ova-specific T-cells (IL-2 and IFN- γ secretion) upon co-cultivation with transduced APCs.

SLAM-targeted PTVs have demonstrated specificity and efficacy of antigen transfer into primary APCs to trigger antigen-specific immune activation. Assessment of *in vivo* targeting and immunogenicity will complete this picture.

P079

TALENs mediate genome editing with superior specificity and lower toxicity than matched ZFNs

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Zinc-finger nucleases (ZFNs) and TALE nucleases (TALENs) have been employed successfully to modify genomes of various complexities. To perform a comprehensive side-by-side comparison of these two designer nuclease platforms, we have engineered several TALEN pairs targeting three human loci (CCR5, AAVS1, IL2RG) for which benchmark ZFNs exist. The activity of these TALENs was comparable to the ZFNs, with allelic gene disruption frequencies between 15–30% in HEK293T cells and primary human fibroblasts. However, nuclease associated cytotoxicity, as measured by cell survival, cell cycle progression and cell viability, was significantly lower for the TALENs than the similarly active ZFNs. Specificity of the CCR5- and AAVS1-specific designer nucleases was profiled at off-target sites predicted by bioinformatics. We confirmed considerable off-target activity of the CCR5-specific ZFN at

CCR2 and five previously reported loci. Furthermore, we identified five new off-target sites for the AAVS1-specific ZFNs. In contrast, out of 27 and 22 predicted off-target sites for CCR5- and AAVS1-specific TALENs, respectively, we detected marginal off-target cleavage activity at three genomic sites for two CCR5-specific TALENs and none for an AAVS1-specific TALEN. In accordance, the best performing CCR5-specific TALEN pair did not induce chromosomal rearrangements between CCR5 and CCR2, as was observed for the ZFN pair. For the first time, these results link nuclease-associated toxicity directly to off-target cleavage activity and corroborate TALENs as a highly specific platform for human genome editing.

P080

Development of ZFN-based pre-clinical in vitro cell model in human embryonic stem cells for Wiskott-Aldrich syndrome

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The Wiskott-Aldrich syndrome (WAS) is an X-linked primary immunodeficiency characterized by recurrent infections, eczema, severe bleeding and increased risk to develop autoimmune diseases and malignancies. WAS is caused by mutations in the WAS gene that is expressed specifically in the hematopoietic lineage. Absence of the WAS protein (WASp) produces important defects in the function of almost all hematopoietic lineages resulting in thrombocytopenia with small platelets and abnormal lymphoid and myeloid function. Although the role of WASp in terminally differentiated lymphocytes and myeloid cells are well characterized, its role on early hematopoietic differentiation and on platelet biology is poorly understood. We have developed the first human model for WAS disease, hESCWASKO, based on human pluripotent stem cells. We knocked out the WAS gene in the AND-1 human embryonic stem cell line using a zinc-finger nuclease (ZFN) pair targeting the WAS intron 1 and a homologous donor DNA that disrupted WASP expression. We have generated two clones homozygous for the desired WAS mutation that didn't show alterations neither in the karyotype nor pluripotency. Interestingly both hESCWASKO cell lines showed significant alterations during early hematopoietic development generating higher number of CD34 CD45 hematopoietic progenitors that had altered responses to stem cell factor (SCF) but similar colony-forming-unit potential. Surprisingly, hESCWASKO cells differentiated toward the megakaryocytic lineage produced higher number of CD34 CD41 progenitors, megakaryocytes and platelets than wild-type hESCs. However, megakaryocytes and platelets derived from hESCWASKO cells have altered responses to ADP and thrombin and have increased F-actin content in basal conditions. These data corroborate and validate the hESCWASKO cell lines as a human cellular model for WAS and point to a new role for WASp on early hematopoietic differentiation as well as on megakaryocytic development.

P081

Generation of disease free hematopoietic progenitors from FA patient cells using a combined gene targeting and cell reprogramming strategy

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Fanconi anemia (FA) is a DNA repair syndrome characterized by the progressive depletion of the hematopoietic stem cells (HSCs). In this study, we aimed the generation of disease-free HSCs from non hematopoietic tissues through the specific insertion of FA genes in safe harbor locus, followed by the reprogramming of these cells to generate iPSCs. Initially, we demonstrated the efficient phenotypic correction of FA-A fibroblasts by gene editing in the AAVS1 safe harbor locus, using ZFNs and an IDLV vector containing a promoterless EGFP gene and the PGK-FANCA sequence flanked by AAVS1 homology arms. This approach allowed us to target between 0.2–4.5% cells. Notably, gene-edited FA fibroblasts showed an increased proliferation capacity with respect to uncorrected cells, facilitating that up to 40% of cultured cells became EGFP+. Gene edited FA-iPSCs (geFA-iPSCs) were generated from these samples using an excisable reprogramming vector (STEMCCA). PCR and Southern blot analyses confirmed the specific integration of the therapeutic gene in the AAVS1 locus. The pluripotency and correction of the FA phenotype of geFA-iPSCs was also demonstrated. In subsequent experiments, the reprogramming cassette was excised from geFA-iPSCs and in vitro hematopoietic differentiation allowed the generation of hematopoietic cells which contained up to 6% of CD34+ /CD45+ progenitor cells. Finally, hematopoietic colonies resistant to the interstrand cross-linking agent, mitomycin C, were obtained from these progenitors. Studies focused in the repopulation capacity of disease free hematopoietic progenitors are currently on going. Our results confirm the efficacy of gene targeting and cell reprogramming approaches to generate disease-free hematopoietic progenitors cells.

P082

Mitochondrial Gene Targeting in Mammalian Systems using Novel 'Mitochondriotropic' Liposomes

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Mitochondrial research has made a giant leap since the 1980/90s when mitochondrial DNA mutations were first identified as a primary cause for human diseases and the organelle's role in apoptosis was elucidated. This makes the mitochondrion a prime candidate for organelle-specific delivery of exogenous materials such as DNA and drugs, for therapy of diseases caused by mitochondrial dysfunction. Hence vector design and development is of paramount importance. The success of liposomes viz cationic liposomes, in chromosomal gene therapy make them potential

vectors for mitochondrial gene targeting. In this investigation novel 'mitochondriotropic' liposomes were synthesised to evaluate their mitochondrial localisation activities *in vitro* and *ex vivo*. Cationic cholesterol derivative, 3 β [N-(N',N'-dimethylaminopropyl)-carbamoyl] cholesterol (CHOL-T) was formulated with dioleoylphosphatidylethanolamine (DOPE) to produce cationic liposomes, to which a mitochondrial signalling peptide was attached via two different cross-linking agents. Size and lamellarity of liposomes and lipoplexes were assessed by zeta sizing and electron microscopy. Their ability to bind, condense and protect plasmid DNA (pBR322), was determined using the band shift, dye displacement and nuclease protection assays. *In vitro* cytotoxicity was determined using the MTT assay in the hepatocyte-derived human cell line (HepG2). Radiolabeled DNA was used with rat liver mitochondria to determine ability of liposomes to cross the mitochondrial membrane. Confocal and fluorescence microscopy was used to determine nucleic acid localisation. These mitochondriotropic liposomes protect plasmid DNA, are well tolerated by HepG2 cells in culture and were seen to successfully traverse the mitochondrial membranes. Furthermore these liposomes showed positive mitochondrial localisation activities *in vitro*.

P083

ZFN Mediated Targeting of Albumin: a Platform for Expression of Multiple Therapeutic Genes *in vivo*

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Albumin represents an attractive target for ZFN-based, liver-derived protein replacement as it is highly expressed in the liver and the most abundant protein in plasma. Here, we investigated whether ZFN-mediated gene insertion into the endogenous mouse albumin locus could represent a general strategy to express a wide variety of proteins *in vivo*.

An AAV vector encoding ZFNs targeting mouse albumin intron 1 (AAV8-ZFN) was constructed. Adult mice were treated with AAV8-ZFN and AAV8-hF9-Donor, containing a cDNA cassette with exons 2–8 of the human F9 gene. ZFN Donor-treated mice exhibited stable circulating F.IX levels >3000 ng/mL (60% of normal) while F.IX expression in Mock Donor mice (no ZFN) was below the assay limit of detection. Importantly, the 3 amino acid substitutions to the F.IX pro-peptide caused by splicing with endogenous albumin exon 1 were functionally tolerated, supporting correction of the hemophilic phenotype, as treatment reduced the clotting time from 70 seconds pre-AAV administration to wild-type levels of 42 seconds (measured by the activated partial thromboplastin time assay) 2 weeks post administration. Dose-response studies showed that hF.IX expression was proportional to AAV8-ZFN dose.

An ideal safe harbor locus should be able to express various transgenes for the treatment of different indications. We show evidence of successful expression of human factor VIII in hemophilia A mice as well as protein expression of four enzymes deficient in patients with lysosomal storage diseases.

These data provide the first demonstration of ZFN-mediated *in vivo* genome editing of an endogenous safe harbor locus for therapeutic protein production.

P084

Baboon retrovirus envelope pseudotyped LVs allow efficient transduction of progenitor T cells, thymocytes and adult T and B cells

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Efficient gene transfer into quiescent T and B lymphocytes for gene therapy or immunotherapy purposes may allow the treatment of several genetic dysfunctions of the hematopoietic system, such as immunodeficiencies, and the development of novel therapeutic strategies for cancers and acquired diseases. Previously, we have shown that measles virus gps LVs (MV-LVs) were able to transduce efficiently stimulated or resting T and B cells. We now showed that the newly engineered Baboon retrovirus envelope pseudotyped LVs (BAEVgp-LVs) allowed in addition to hematopoietic stem cell transduction, high level transduction of activated T and B cells and resting B cells, where VSVG-LVs fail. Moreover, both naive and memory cells were efficiently transduced and their phenotypes conserved. Since the thymus plays a key role in the induction of self-tolerance and by intra-thymus injection of an LV *in situ* correction of a genetic immunodeficiency might be envisaged, it is important to develop vectors for efficient thymocyte transduction. BAEVgp-LVs allowed high level transduction of the thymocytes in the early stages of development with preference for early thymic progenitor T cells while MVgp-LVs, showed an equivalent transduction level of all thymic subpopulations. BAEVgp-LVs are especially superior over VSVG-LVs for the transduction of the very immature T cell progenitor subpopulation, which is long-lived *in vivo* and might confer a permanent therapeutic effect. Currently, the vectors are tested for intra-thymic transduction in human immune system mice model.

P085

Meganuclease-based therapy for recessive dystrophic epidermolysis bullosa

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Dystrophic Epidermolysis Bullosa (DEB) is a rare and severe genetic skin disease, inherited in a dominant (DDEB) or recessive (RDEB) manner, responsible for blistering of the skin and mucosa. DEB is caused by a wide variety of mutations in *COL7A1* encoding type VII collagen, the major component of anchoring fibrils, which are key attachment structures for dermal-epidermal adhesion. Genetic correction of primary cells by engineered site specific nucleases provides powerful tools for gene therapy. Our goal is to demonstrate the feasibility of correcting *COL7A1* mutations in RDEB patient's keratinocytes and/or fibroblasts through tailor-made Meganucleases (MN) mediated Homologous Recombination (HR) in a safe and efficient manner. For this proof-of-concept study, we have successfully developed tools indispensable for *COL7A1* targeting. Four MNs variants specific for *COL7A1* intron 2 were designed. One of them showed efficient expression, activity and low toxicity. This MN and corresponding Repair Template were cloned into an HIV-1 derived lentiviral vector and delivered as integration deficient lentivirus

(IDLVs). A keratinocyte cell line and primary keratinocytes and fibroblasts from two RDEB patients homozygous for *COL7A1* null mutations in exons 2 and 3 were transduced by IDLVs encoding the MN and Repair template. Gene correction was demonstrated by allele specific PCR in bulk transduced cells and type VII collagen protein re-expression by immunocytochemistry. These results establish that MN can achieve *COL7A1* genetic correction, and lead to evaluate the therapeutic potential of this approach in future studies.

P086

Adenoviral targeting using genetically incorporated camelid single domain antibodies

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Adenovirus (Ad) vectors possess unique attributes which have led to their employment for a wide range of gene therapy applications. Recent studies have highlighted the additional utilities that may accrue strategies to modify Ad tropism to allow cell specific targeting. Strategies to retarget Ad have sought to capitalize on the available repertoire of antibodies species. Specifically it has been proposed that genetic incorporation of widely available single chain antibody (scFv) molecules could realize a single unit retargeted Ad compatible with transitional applications. Limiting such efforts is the relative instability of scFv in the redox environment of the natural Ad assembly site within the cytosol. We have previously shown that the use of genetically stabilized scFv could circumvent this restriction. However the limited availability of such stabilized scFv led us to explore an alternate antibody species which potentially addresses this key biological issue. In this regard, single domain antibodies (sdAb) derived from the camelid family have been exploited for molecular targeting purposes. Of note, their molecular structure confers exceptional stability properties. Herein, we have thus endeavored initial studies to evaluate the utility of camelid sdAb to be incorporated within the Ad capsid to allow altered tropism for targeting applications. Specifically we have explored the genetic incorporation of a novel anti-human carcinoembryonic antigen (hCEA) sdAb and its specific cell targeting.

P087

Targeted genome editing by lentiviral delivery of zinc-finger nuclease proteins

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Engineered nucleases, like zinc-finger nucleases (ZFNs), have emerged as versatile tools for precise genomic editing. Future clinical use of this technology relies on safe and effective means of nuclease delivery. Here we demonstrate the feasibility of delivering ZFN protein in the context of engineered lentiviral particles (LPs). By fusing ZFNs to the N-terminus of Gag, we incorporate ZFN protein in VSV-G-pseudotyped LPs and demonstrate the release of ZFN protein from Gag upon

particle maturation. Gene disruption frequencies up to 24% are achieved in cells (HEK293, normal human dermal fibroblasts, and primary human keratinocytes) exposed to LPs containing pairs of ZFNs targeting the *egfp*, *CCR5*, and *AAVS1* loci, respectively. To establish targeted genomic editing by lentiviral ZFN protein delivery, we create 'all-in-one' lentiviral vectors carrying both (i) a pair of locus-directed ZFNs and (ii) vector RNA that allows reverse-transcribed DNA to serve as a donor for gene editing by homologous recombination. In HEK293 cells harboring a mutated *egfp* gene, integrase-defective lentiviral vectors carrying *egfp*-directed ZFN proteins and an *egfp* donor sequence induce eGFP expression in a VSV-G- and vector dose-dependent manner, leading to editing in 8% of the cells. We demonstrate the feasibility of this approach also for targeted editing within the *CCR5* and *AAVS1* loci and additionally provide evidence of site-directed transgene insertion into the *CCR5* locus. Our findings establish lentiviral transduction of ZFN proteins for targeted modification, correction, and insertion of genes and generate an entirely new platform for safe and customized application of ZFNs for genome engineering.

P088

Targeted integration of a large transgene cassette by TALEN and ZFN-mediated homologous recombination

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Targeted transgene integration by homologous recombination (HR) represents a promising strategy for gene therapy as it may overcome the issue of insertional mutagenesis associated with retroviral vectors. We recently published the feasibility of using adenoviral vectors (Ad) to package and deliver functional TALEN genes into human cells, demonstrating that Ad-TALEN-mediated transduction results in efficient site-specific DSB formation at the chromosomal safe harbor site *AAVS1*. Moreover, we demonstrated efficient targeting at *AAVS1* in human repopulating epidermal stem cells upon Ad-ZFN cleavage. Here we investigate the feasibility of TALEN- and ZFN-mediated targeted integration of a large expression cassette (10 kb) by HR into *AAVS1* locus. We evaluated the targeting efficiency in human immortalized keratinocytes co-infected with *AAVS1*-specific Ad-TALENs or Ad-ZFNs and integration-defective lentiviral or retroviral vectors (IDLV or IDR) carrying a GFP donor cassette. To exploit the possibility of targeted gene addition of a large therapeutic gene (9 kb) with a repetitive structure (*COL7A1* gene) into a safe harbor, we generated a large IDR carrying a donor cassette bearing the GFP reporter gene and a non-coding stuffer DNA sequence (9.5 kb). Preliminary experiments showed that IDRs are competent to deliver short (1.5 kb) and large donor GFP cassettes (9.5 kb) suitable for HR, although with lower efficiency when compared to that of their cognate IDLVs. *AAVS1*-specific PCR analyses on genomic DNA extracted from bulk populations co-infected with both donor vectors and Ad vectors to express the engineered nucleases confirmed on-target integration of the delivered GFP cassettes.

P089

PPRHs as a tool to allow specific oligonucleotide DNA targetingA Solé¹, V Noé¹, C J. Ciudad¹¹School of Pharmacy, University of Barcelona

Polypurine Reverse-Hoogsteen Hairpins (PPRHs) are a new gene therapy tool, formed by two antiparallel homopurine domains, linked by a five-thymidine loop (Coma *et al.*, 2005), with the ability to bind dsDNA displacing the fourth strand away from the newly formed triplex. The main goal of this research was to explore if this property of opening dsDNA could be used as a technology to attempt gene repair of single point mutations using oligonucleotides carrying the corrected base. Given that one of the homopurine strands of the PPRH binds with antiparallel orientation (by Watson-Crick) to the polypyrimidine target sequence forming a triplex, we used as a model two polypyrimidine target sequences within the sixth exon of the *dihydrofolate reductase (dhfr)* gene, flanking a nonsense mutation of this gene. Three different approaches using PPRHs were tested in binding experiments, varying the length of the polypyrimidine target, the length of the repair oligonucleotide and the number of PPRHs used. In all cases the polypurine strand of the dsDNA was displaced by the hairpins. In addition, the repair oligonucleotide was able to bind the displaced strand containing the point mutation, regardless of its length. As the *in vitro* experiments were performed maintaining several important parameters, such as temperature, pH and salt concentration, close to physiologic conditions, our research suggests that antiparallel purine-hairpins are a promising tool to allow sequence-specific targeting within the cell. This could be especially useful in gene therapy, to replace point mutations occurring in monogenic diseases.

P090

Gene silencing polypurine reverse hoogsteen hairpins: *in vivo* effect, stability and immunogenicityL Rodríguez, X Villalobos¹, C Oleaga¹, J Prévot¹, N Mencia¹, C J. Ciudad¹, V Noé¹¹School of Pharmacy University of Barcelona

Gene silencing is a promising approach in cancer therapy. We recently developed a new gene silencing tool called Polypurine Reverse Hoogsteen Hairpins (PPRHs), which are non-modified DNA molecules formed by two antiparallel polypurine stretches linked by a five-thymidine loop (1). There are two types of PPRHs capable of decreasing gene expression: Template-PPRHs, which bind to the template strand of the dsDNA (2), and Coding-PPRHs (3), which bind to the coding strand of the dsDNA and the mRNA.

We evaluated the *in vitro* effect of PPRHs in different cancer cell lines (PC3, MiaPaCa2 and HCT116) using survivin as a target because of its role in the evasion of apoptosis. An *in vitro* screening of several PPRHs led us to choose a Coding-PPRH against the promoter of survivin for *in vivo* studies. We performed two types of administration, either intratumorally or intravenously, using a subcutaneous xenograft model of prostate cancer (PC3). The chosen PPRH induced a significant decrease in tumour volume.

We explored important properties of PPRHs, such as their stability and immunogenicity. Stability experiments performed in different conditions revealed that PPRHs half-life is much longer than that of siRNAs. The innate immune response was evaluated analyzing the levels of the transcription factors IRF3

and NF- κ B, the cleavage of Caspase-1, and the expression levels of several pro-inflammatory cytokines: type-I interferons, TNF α , IL-6, IL-1 β and IL-18. These results indicated that PPRHs do not activate the immune response, unlike siRNAs.

These findings represent the preclinical proof of principle of PPRHs for *in vivo* approaches.

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P091

Generation of a human reporter cell line HCT116-GFPtrunc to test conditions that improve the frequency of gene targetingM A Bernardi^{1,2}, R Torres^{1,2}, L Pérez^{1,3}, M Simarro², M A de la Fuente^{1,2}¹Instituto de Biología y Genética Molecular (IBGM), Valladolid, Spain; ²Universidad de Valladolid, Valladolid, Spain; ³Consejo Superior de Investigaciones Científicas (CSIC), Valladolid, Spain

Introduction: Gene targeting is a tool that uses homologous recombination (HR) to modify an endogenous gene. HR is an inefficient process in human cells. Thus, understanding the mechanisms that increase the efficiency of HR is essential to advance strategies for gene therapy.

Objectives: To generate a system consisting in a reporter cell line and a donor adeno-associated virus (AAV2) which will allow studying changes in HR frequency through monitoring GFP expression.

Materials and methods: GFPtrunc HCT-116 reporter cell line was generated by co-nucleofection of a zinc finger nuclease targeting AAVS1 site in chromosome 19 together with a pMCS-GFPtrunc plasmid containing a N-terminus fragment of the GFP gene unable to express fluorescent protein.

Donor virions were produced by co-transfection of the recombinant plasmid, which contains the C-terminus fragment of the sequence of the GFP required to express fluorescent protein, together with pRC and pHelper in AAV293. Donor virions will be then used to infect the reporter cell line.

Results and Conclusions: A reporter cell line and a donor virus were obtained to visualize changes in GFP expression as a result of the HR between the DNA inserted in the reporter cell line and the sequences of the donor AAV. A 2×10^{-3} gene targeting frequency was estimated by counting the number of green events under UV microscopy. We are currently using this system to find novel proteins and chemicals able to improve the HR frequency.

P092

Genome editing of a mouse model of the Crigler-Najjar SyndromeL Bočkor¹, A De Caneva², F Porro³, A F Muro⁴¹International Centre for Genetic Engineering and Biotechnology (ICGEB)

Crigler-Najjar Syndrome (CNSI) is a rare genetic recessive disorder characterised by extreme unconjugated hyperbilirubinemia,

caused by UDP-glucuronosyltransferase 1A1 (UGT1A1) deficiency. High blood levels of unconjugated bilirubin can lead to severe brain damage known as bilirubin encephalopathy or, in extreme cases, death by kernicterus. Presently, the only known treatments are intensive phototherapy, the efficacy of which decreases with time, and liver transplantation, a procedure that is not devoid of severe complications. In order to search for new therapies we are using a mouse model of the human CNSI that has a one-base deletion in the exon 4 of the mouse *Ugt1* (*mUgt1*) gene, resulting in a stop codon immediately downstream of the mutation. Mutant mice have high bilirubin levels and die a few days after birth if left untreated. Recent advances in the field of site-specific nucleases have made genome editing possible, reaching a previously unpredictably high frequency. As only 5–10% of normal physiological levels of UGT1A1 are sufficient to reduce bilirubin levels to life-compatible levels, we devised *in vivo* and *ex vivo* strategies to repair the mutated gene. To this end we designed Transcription Activator-Like Effector Nucleases (TALENs) that target the mutated exon 4. We first verified their efficacy by targeting the exon 4 of *mUgt1a1* in NMuLi mouse hepatoma cell line. To increase the efficiency of exon 4 gene editing, we are using TALENs and oligonucleotides to repair the mutation by homology directed repair, both *in vivo* and *ex vivo*.

P093

Targeted Integration of Transgenes in a Safe Harbor Locus using Engineered Meganuclease and TALENTM-based Homologous Recombination Strategies

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Gene therapy is one of the most promising treatments for monogenic diseases, though one of its main limitations is the potential risk of insertional mutagenesis. Recent approaches aimed at the specific insertion of therapeutic genes in "Safe Harbor" (SH) loci, thus limiting the impact on surrounding genes. Here we aimed to insert an EGFP transgene in a specific human locus (so called SH6) using engineered meganucleases (MN) and transcription activator-like effector nucleases (TALENTM). This locus is far from coding regions, regulatory sequences or microRNA transcription sites. In order to select the best nuclease for its application in gene targeting strategies, we have compared both the gene targeting efficacy at a defined SH locus (SH6) and the toxicity of the SH6-MN and the SH6-TALENTM in immortalized HEK-293H cells. Homologous recombination at the SH6 locus was achieved both by the SH6-MN and the SH6-TALENTM, although at different efficiencies depending on type or concentration of the respective nuclease. Additionally, under optimized conditions, very limited toxicity was associated to the transfection of these DNA constructs on HEK-293H cells. The efficacy of MN and TALENTM was also compared in primary cord blood CD34 cells. Preliminary results showed EGFP hematopoietic progenitors with HR events determined by fluorescence and PCR analysis. Our results open the possibility of using new safe harbor loci and specific nucleases to develop improved gene therapy approaches based on gene editing.

P094

Towards TALEN mediated targeting of a safe harbour locus in mouse models of Fanconi anemia

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Fanconi anemia (FA) is a rare inherited disease associated with aplastic anemia and cancer predisposition. Mutations in any of the 16 FA genes so far discovered can disrupt a common DNA repair pathway, known as the FA pathway. Within the FA patients, mutations in *FANCA* are the most frequent (>60%), justifying that most FA gene therapy studies have been developed with cells of this complementation group. Based on previous studies showing the advantages of the *AAVS1* locus for conducting homologous gene targeting strategies in humans, we aimed to specifically insert the therapeutic *FANCA* gene into the murine *Mbs85* locus, homolog of the human *AAVS1* locus, in murine *Fanca*^{-/-} cells. To this end, specific TALE nucleases targeting this locus were designed with the final goal to test whether FA-A gene-edited cells would allow for the reversion of the FA hematopoietic phenotype in this FA mouse model. We have generated two different TALEN pairs with variations in the RVDs towards the murine *AAVS1* locus, and donor constructs harbouring the *FANCA* gene flanked by EGFP or tdTomato selection markers. Assessment of TALEN activity by T7 endonuclease1 assay demonstrated that both TALEN pairs were active in mouse cell lines. In parallel to these experiments, and because of the mild hematopoietic phenotype of the FA-A mouse model compared to FA-A patients, we have worked towards the development of *in vitro* and *in vivo* selection strategies to facilitate the repopulation of gene targeted cells in *Fanca*^{-/-} recipients. Data showing our current achievements in this new targeting model will be presented.

P095

Non-integrative lentiviruses stably and specifically target safe humangene harbour by efficient recombinase-mediated cassette exchange

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Optimized gene transfer into human cells are still challenging the promise of human stem and induced pluripotent stem cells as resources for disease models, diagnostic screens, and personalized cell therapy. These potential applications require precise control of the spatio-temporal action of gene switches and the coordinate regulation of modulators, effectors and differentiation factors during pluripotency, differentiation and homeostasis. Most studies require identical transgene environments for comparable analysis; however, this cannot be achieved by standard methods for transgenesis in human cells due to unintended epigenetic modifications, genetic instability, dose-dependent effects, and disruption or activation of host genes. Although gene targeting can circumvent these problems, human cells have proved difficult to target, and there is

therefore a need to develop tools for targeted transgenesis at efficiencies similar to those achieved in mice. We present a simple strategy, KASTRINA 2.0, for reliable transgenesis in human cells, based on targeted recombinase-mediated cassette exchange and the safe episomal status conferred by integrase-deficient lentivirus (IDLV). By driving limited cre recombinase expression, the IDLV yields single site-specific recombination of a selectable donor cassette (TRINA) at the 'safe harbour' *AAVS1* locus previously edited by zinc-finger nuclease to contain an acceptor site (KAS 2.0).

P096

Gene editing of *CFTR* and *CTNS* mutations using ZFNs and CRISPR/Cas-9 guide RNAs

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Cystic fibrosis is characterised by defective secretory epithelial affecting multiple organs and is caused by mutations in *CFTR*. Cystinosis is a lysosomal storage disorder characterised by end stage renal disease and is caused by mutation in *CTNS*. Attempts to treat both diseases by cDNA addition have not been successful; gene editing could potentially be used for both these disorders.

We have described repair of disease-causing mutations in *CFTR* (Lee *et al.*, 2012) and targeting of *CTNS* (Kaschig *et al.*, 2011) using ZFNs and TALENs. Here, our goal is to establish cDNA based mini-gene recombination to repair a wide range of mutations and/or tag genes with GFP using existing nucleases and newly created CRISPR/Cas9 gRNAs.

To repair *CFTR* mutations, a mini-gene was constructed comprising splice acceptor, exon 10–24 *CFTR* cDNA, T2A and GFP with flanking homology arms and transfected with our existing *CFTR*-specific ZFNs or CRISPR/gRNAs in a human tracheal epithelial cell-line homozygous for *CFTR* ΔF508. GFP expression was only observed when donors were co-transfected with ZFNs or gRNAs/Cas9 indicating successful recombination.

To monitor the trafficking of the *CTNS* gene product expressed from its endogenous locus, we have designed CRISPR gRNAs to cleave exon 12 to enable the in-frame incorporation of the GFP cDNA. Analysis of PCR products generated from modified genomic DNA will be analysed by next gen sequencing to confirm the frequency of recombination.

Successful *CFTR* and *CTNS* correction would result in normal spatiotemporal expression of these genes, and should last the lifetime of the cell. This methodology is compatible with existing clinically approved delivery methods, and could serve as an alternative strategy to cDNA addition for gene therapy.

Lee CM *et al.*, 2012. *BioResearch Open Access* 1:99–108.

Kaschig K *et al.*, 2011. *Human Gene Therapy* 22: A1-A138.

P097

Modification of lentiviral vector surfaces using molecular painting

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Manipulation of lentiviral vector surfaces is useful for three main reasons: (i) easier preparation and/or detection of the vector particles, (ii) modulation of the immune response of the target organism and (iii) targeted attachment to and subsequent infection of cells. We have developed a method, termed Molecular Painting, which transfers functional protein onto retro- or lenti-viral particles post-exit, using proteins carrying post-translationally added glycosylphosphatidylinositol (GPI) anchors. We could demonstrate that viral particles acquired novel protein species responsible for facilitating the fields of application mentioned above: both green and red fluorescent markers could be attached, as well as factors stimulating or inhibiting immune functions (Interleukin 2, CD55, CD59). Additionally, the ability of GPI-anchored ligands, such as epidermal growth factor (EGF), to induce changes in attachment behavior to facilitate targeted infection of specific cell types is being tested. Experiments for functional proof-of-principle of these modifications are currently ongoing and we believe that this quick and flexible method holds great promise as a platform technology for the fine-tuning of lentiviral vectors, both in biomedical and research settings.

P098

CD19 and CD20 targeted vectors induce minimal activation of resting B lymphocytes

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B lymphocytes are an important cell population of the immune system. However, until recently it was not possible to transduce resting B lymphocytes with retro- or lentiviral vectors, making them unsusceptible for genetic manipulations by these vectors. Lately, we demonstrated that lentiviral vectors pseudotyped with modified measles virus (MV) glycoproteins hemagglutinin, responsible for receptor recognition, and fusion protein were able to overcome this transduction block. They use either the natural MV receptors, CD46 and signaling lymphocyte activation molecule (SLAM), for cell entry (MV-LV) or the vector particles were further modified to selectively enter via the CD20 molecule, which is exclusively expressed on B lymphocytes (CD20-LV). Here, we generated a vector specific for another B lymphocyte marker, CD19, and demonstrated that this vector (CD19ds-LV) was able to transduce unstimulated B lymphocytes, albeit with a reduced efficiency compared to CD20-LV. Since CD20 as well as CD19 are closely linked to the B lymphocyte activation pathway, we investigated if engagement of CD20 or CD19 molecules by the vector particles induces activating stimuli in resting B lymphocytes. Although, activation of B lymphocytes often involves calcium influx, we did not detect elevated calcium levels compared to background. However, the activation marker CD71 was up-regulated upon CD20-LV transduction. Importantly, B lymphocytes transduced with CD20-LV or CD19ds-LV entered the G1 phase of cell cycle, whereas untransduced or MV-LV transduced B lymphocytes remained in G0. Hence, CD20 and CD19 targeting vectors induce activating stimuli in resting B lymphocytes, which most likely renders them susceptible for lentiviral vector transduction.

P099

Targeting the kidney with *in vivo* phage displayP Celec¹, E Lengyelová¹, J Bábíčková¹, L Tóthová¹¹Comenius University

Increased prevalence of renal diseases and therapeutic deficit makes molecular approaches to the therapy highly needed. Gene therapy of renal diseases is complicated by the difficult accessibility of the kidney. Targeting the renal vasculature using homing peptides would be a way to restrict gene therapy towards the kidney. *In vivo* phage display can identify ligands for organ-specific receptors of the endothelium. The aim of this study was to identify specific binding peptide sequences in renal cortical blood vessels using *in vivo* phage display. Three rounds of *in vivo* phage display panning were performed in C57BL/6 mice. Heptapeptide phage library was administered intravenously into the tail vein. Phages were recovered from the renal cortex and used for subsequent rounds of panning. After the third round the phage with the peptide HAQLPMP was most abundant. The binding ability of the selected phage to cortex was confirmed by comparison with the unselected phage library. In further experiments the identified peptide sequence will be used for targeting transgenes to the renal cortex.

P100

Increasing the repertoire: framework engineering of a CD30-specific scFv enables targeted gene delivery to iPS cellsT Friedel¹, S Klawitter², L J Dürner³, H Abken⁴, A Sebe², Z Ivics², G G Schumann², I C Schneider¹, C J Buchholz¹

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Restricting gene transfer to the relevant cell type by cell entry targeting of retroviral vectors is a key issue to achieve efficient *in vivo* gene transfer. We developed a flexible and highly specific targeting method for lentiviral vectors (LVs) relying on the recognition of cell surface antigens by single-chain antibodies (scFv). Proper folding of the scFv being fused to the C-terminus of the measles virus (MV) hemagglutinin (H) is essential for its transport to the cell surface and the efficient incorporation into vector particles.

From about 30 different scFv tested so far about half were non-functional as targeting domain, usually due to inefficient cell surface expression as H-scFv fusion protein. To convert a CD30-specific scFv into a targeting-compatible scFv we identified amino acid residues in its framework regions potentially critical for stability and folding. A series of scFv variants was then generated and characterized. While the framework modifications did not impair the affinity of the optimized scFv for CD30 they increased the melting temperatures. The optimized H-scFv variants showed an improved surface expression and, compared to the original scFv, an up to 100-fold increase in titer. CD30-LV was absolutely specific for CD30-positive cells and mediated stable gene transfer. Interestingly, when added to iPS cell cultures exclusively Oct-4 positive iPS cells were transduced.

Framework engineering of scFv can increase the repertoire of targeting ligands for LVs and may lead to an optimized universal scFv framework. CD30-LV may become a novel tool for optimized gene transfer into iPS cells.

P101

Development of a Myeloid cell-binding Adenovirus for the potential gene therapy of alpha-1 anti-trypsin deficiencyM Buggio¹, C Towe², D T. Curiel¹

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Alpha-1 anti-trypsin (A1AT) deficiency lung disease represents an ideal candidate for definitive gene therapy treatment. As the liver is the major locale for A1AT synthesis, the majority of gene therapy approaches have been directed towards induction of functional wild type A1AT within hepatocytes. Despite the diverse range of gene therapy strategies applied, the levels of serum A1AT achieved have not been sufficient to correct the elastase/anti-elastase balance within the lung. To this end, we hypothesized that *in vivo* gene delivery to pulmonary endothelium could provide A1AT augmentation of Epithelial Lining Fluid (ELF) more effectively than liver-based transduction methods.

To re-target the expression of transgene in the lung, the knob domain of Ad5 has been successfully modified genetically in our lab. We have identified a novel peptide sequence called myeloid binding peptide (MBP) which increases the lung targeting. Based on this we created a recombinant adenovirus expressing A1AT with MBP in the fiber protein (AdMBPA1AT). When we injected this Ad systemically in mice we saw a high expression of the transgene in the serum in comparison to the control Ad expressing A1AT without MBP in the fiber protein. In order to confirm that the AdMBPA1AT localizes to the lung, we analyzed viral copy number using total DNA extracted from the organs of mice by qPCR and the expression of the protein in serum and ELF. Our strategy will test the hypothesis that A1AT deriving from the pulmonary endothelium can augment more effectively ELF A1AT to provide a corrective anti-elastase screen.

P102

Reduction of liver transduction by fiber-modified Ad following their strong uptake by Kupffer cellsN Raddi¹, F Vigant¹, C Bressy¹, J Paulo Portela Catani¹, O Bawa², P Opolon² & K Benihoud¹

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Adenovirus 5 (Ad5) use in gene therapy is hampered by its strong liver tropism associated with hepatotoxicity. Ad hexon protein binding to blood factor X was shown to be responsible for hepatocyte transduction via heparan sulphate proteoglycans receptors. Unexpectedly, Ad5 pseudotyping with the fiber from serotype 3 results also in a significant reduction of liver transduction.

To understand how fiber modification affects in vivo Ad5 tropism, we characterized in vivo behaviour of fiber-modified Ads using two pseudotyped viruses bearing whole (AdF3) or only the shaft (AdS3K5) of Ad3 fiber. Two days after their systemic delivery, both fiber-modified Ads led to a reduction of transgene expression and viral DNA content in liver and spleen. For both viruses, liver viral DNA content was comparable to Ad5 at 1 h p.i. demonstrating an unmodified liver uptake. In addition, hepatocyte transduction was dramatically decreased, after depletion of factor X, thereby indicating that fiber-modified Ads are still able to use FX for liver transduction. The ability of fiber-modified Ads to bind FX was confirmed by slot blot and ELISA and both viruses were able to use FX to increase CHO cell transduction in vitro. Remarkably, Kupffer cell depletion either by predosing or liposomes-clodronate treatment restored transgene expression level of AdF3 and AdS3K5, thus demonstrating their stronger uptake by Kupffer cells compared to Ad5. Interestingly, both fiber-modified Ads transduce tumours xenografted on nude mice as efficiently as Ad5.

Altogether, our findings contribute to a better understanding of the role of the fiber protein in controlling Ad biodistribution in the liver.

P105

A role for NIK (NfκB Inducing Kinase) in the response of hematopoietic stem cells to stress

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HSCs are characterized by self-renewal and multipotential capacity. We studied the role of the alternative NFκB pathway to maintain hematopoiesis in steady state and after stress conditions. We used *aly/aly* mice, which present deficit at the alternative NFκB pathway because of a spontaneous mutation in the NFκB Inducing Kinase, *NIK*. We analyzed the blood cell counts, bone marrow cellularity, presence of different lineages in peripheral blood (PB) and myeloid progenitors content. Except for the already reported defects in B-cell maturation, the steady state hematopoiesis in *aly/aly* mice was essentially normal. The deep analysis of the HSC compartments [LSK phenotype (Lin-Sca1 cKit); LT-HSC (LSK CD34-CD135-) and ST-HSC (LSK CD34 CD135-) and SLAM population (LSK CD34-CD135-CD150 CD48-)], showed lower contents of LT-HSC in NIK-deficient mice versus controls. We observed an impaired competitive repopulation capacity of *aly/aly* HSC in primary transplants, and confirmed this result upon secondary transfer. Thus, NIK-deficient HSCs have an impaired capacity to cope with stressful situations. Lin-HSCs from *aly/aly* mice showed a defect in their proliferation capacity in *in vitro* cultures, compared to that of wild type (wt) HSCs. This defect was not due to higher levels of apoptosis among NIK-deficient HSCs. However, NIK-deficient HSCs needed longer time than wt HSCs to complete rounds of cell division, as found in CFSE-dilution studies. Since NIK can transduce

signals through p100/p52 or through c-Rel, we investigated the pathway downstream NIK in HSCs. All the defects described for the NIK-deficient HSCs were recapitulated using HSCs from a NFκB2-deficient mouse, but not with a cRel-deficient one. Our results suggest that the alternative NF-κB pathway has an important role in the response of HSC to stress.

P106

SCL/TAL1 enhances megakaryocytic specification from human embryonic stem cells by activating a complex megakaryocytic transcriptional network

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Human embryonic stem cells (hESCs) are an exceptional in vitro model to study human developmental biology. Moreover, hESCs have become a potential source for cell replacement strategies. Lately, the production of human platelets from hESCs is acquiring relevance as a promising strategy for therapeutic use. The molecular mechanisms that control megakaryocytic differentiation from cord blood hematopoietic stem cells are well understood. However, the regulators of the in vitro megakaryocytic specification from hESCs are still undefined. We have recently shown that SCL overexpression accelerates the emergence of hemato-endothelial progenitors from hESCs and promotes subsequent differentiation into blood cells with higher clonogenic potential. We thus hypothesized that SCL may potentiate megakaryocytic commitment from hESCs. In this study, we showed that ectopic SCL expression enhances the appearance of mature megakaryocytes and platelets in vitro. SCL-overexpressing cells and derived platelets respond to different activating stimuli (ADP, fibrinogen coating or thrombin) similarly to their control counterparts. Importantly, analysis of early megakaryocytic precursors demonstrated that SCL overexpression accelerated their appearance and increased their commitment during the embryonic body differentiation stage. Gene expression profile analysis of these precursors showed that SCL-overexpressing cells contained a megakaryopoietic molecular signature. Bioinformatics analysis using the Connectivity Map tool showed that SCL-overexpression mimicked the effects induced by thricostatin A (TSA), a histone deacetylase inhibitor (HDI). Finally, we confirmed that TSA treatment promoted megakaryocytic precursors emergence. We propose the manipulation of megakaryocytic transcriptional regulators, such as SCL, and the use of HDIs as a tool to improve human platelets' generation from hESCs.

P107

Transcriptional repression of Bmp2 by cell cycle inhibitor p21 links quiescence to neural stem cell maintenance in the subependymal niche

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Relative quiescence and self-renewal are defining features of adult stem cells, but their potential coordination remains unclear. Subependymal neural stem cells (NSC) lacking the cyclin-dependent kinase (CDK) inhibitor (CKI) p21^{WAF1/Cip1} exhibit rapid expansion that is followed by their permanent loss later in life. Here we demonstrate that bone morphogenetic protein 2 (*Bmp2*) transcription in NSCs is under the direct negative control of p21, through actions that are cell cycle-independent. Loss of p21 in NSCs results in increased levels of secreted BMP2 which induce premature terminal differentiation of multipotent NSCs into mature non-neurogenic astrocytes in an autocrine/paracrine manner. We also show that the cell non-autonomous p21-null phenotype is modulated by the Noggin-rich environment of the subependymal niche. The dual function described here provides a physiological example of combined cell autonomous and non-autonomous functions of p21 with implications in self-renewal, linking relative quiescence of adult stem cells to their longevity and potentiality.

P108

The role of RUNX1c in hematopoietic specification from human embryonic stem cells

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The elucidation of the molecular determinants that orchestrate hematopoietic differentiation of human embryonic stem cells (hESCs) has immense implications not only in future potential cell therapies but also in developmental hematopoiesis and disease modeling. The transcription factor RUNX1 has multiple transcripts originated from two different promoters and by alternative splicing. The three main isoforms are denominated RUNX1a, b and c. Runx1 deficient mice die during early embryogenesis (E11.5- E12.5) due to inability to establish definitive hematopoiesis. We thus hypothesized that RUNX1 may be a master regulator during human embryonic hematopoietic development. In this study, we analyzed the expression of the three main RUNX1 isoforms throughout hematopoietic differentiation of hESCs. As previously described, all three isoforms are observed in hematopoietic derivatives (CD45). Interestingly, RUNX1c is exclusively expressed in hemato-endothelial progenitors (CD31 CD34 CD45-), suggesting an essential role in the emergence of this specific early pre-hematopoietic population. Consequently, constitutive expression of RUNX1c in hESCs enhances the appearance of hemato-endothelial progenitors and

promotes subsequent differentiation into primitive (CD34 CD45) and total (CD45) blood cells. Furthermore, these hematopoietic derivatives exhibit higher clonogenic (CFU) potential. RUNX1c specific deletion using TALEN technology did not affect hematopoietic potential of hESCs. Importantly, shRNA-based silencing of all endogenous RUNX1 isoforms impairs hematopoietic differentiation of hESCs, demonstrating the early hematopoiesis-promoting role of RUNX1 during human development. Unfortunately, RUNX1c over-expression does not confer *in vivo* engraftment to hESC-derived hematopoietic cells, suggesting that additional genes participate in the process leading to the generation of definitive functional hematopoiesis from hESCs *in vivo*.

P109

Novel *in vivo* method to test the differentiation potential of mesenchymal progenitor cell cultures

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Clinical interest on human Mesenchymal Progenitor Cells (hMPC) relies on their potential applicability in cell-based therapies. An *in vitro* characterization is usually performed in order to define MPC potency. However, *in vitro* predictions not always correlate with *in vivo* results and thus there is no consensus in how to really assess to define cell-potency. Our goal was to provide an *in vivo* testing method to define cell behavior before therapeutic usage. Focused on bone tissue engineering applications, we wondered whether hMPCs would proceed in a osteogenic microenvironment.

Based on previous ectopic ossicle formation approaches, we developed a fibrin/ceramic/BMP-2/hMPCs compound and we implanted it subcutaneously in NOD-SCID mice during only 2 weeks. Then we assessed by fluorescent cell-labeling and by human-specific immunohistochemistry the cell differentiation to bone, bone marrow, cartilage and fibrous tissue.

Seven bone marrow-derived hMPCs primary cultures were tested. Some of them undergo differentiation in osteoblast, calcified cartilage and adipocytes, while others were not able to differentiate to all of mentioned tissues. These data indicate differences in cell-potential between tested hMPCs. Additionally we implanted other primary hMPCs derived from adipose tissue and mobilized peripheral blood. Our data indicates that presented method is reliable, rapid and reproducible to define hMPCs potency. Primarily it would be useful for testing those cells destined to bone tissue engineering purposes. However it seems also useful for testing any potentially implantable mesenchymal cells and also for other purposes, as could be bone marrow niche and also bone tumor studies.

P110

Expansion of hematopoietic stem/progenitor cells from bone marrow, peripheral blood and umbilical cord blood under serum-free conditions by co-culture with plastic adherent or CD271+ mesenchymal stromal cells

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Introduction: Hematopoietic reconstitution after hematopoietic stem cell (HSC) transplantation may be delayed in patients for whom limited numbers of HSCs are available. *Ex vivo* HSC expansion could be used in these cases. Here, we compare the effects of Stem Cell Factor/Thrombopoietin/Insulin growth factor-binding protein-2/Flt3Ligand/Angiopoietin-like 5 (STI-FA5) with or without Interleukin (IL)-3/6 or Hepatocyte growth factor (HGF), in presence or absence of plastic adherent (PA) or CD271 Mesenchymal Stromal Cells (MSCs) for the optimization of HSC expansion.

Materials and Methods: Bone marrow (BM) PA-MSCs and magnetically isolated CD271 MSCs were expanded up to passage 3 (P3) in DMEM-LG/MCDB201 supplemented with 10% batch-selected heat inactivated FBS, 1% penicillin/streptomycin and 2 mM L-Glutamine. CD34 cells were magnetically isolated from BM, peripheral blood (PB) stem cell collections or umbilical cord blood (UCB). Cells were analysed for immunophenotype and colony assays at day 0, 7 and 10.

Results: Culture of BM CD34 HSC in serum-free medium supplemented with STI-FA5 resulted in maintenance of highest percentages of early CD34 /CD38- cells, whereas culture in presence of PA-MSCs or CD271 MSCs resulted in a shift towards more mature CD34 /CD38 cells. However, in terms of absolute numbers, expansion of CD34 /CD38- and CD34 /CD38 cells, as well as total colony numbers (CFU-GM, BFU-E, CFU-GEMM), was the highest after co-culture with CD271 MSCs, followed by co-culture with PA-MSCs and least in HSCs cultures without MSCs at day 7 and 10. Addition of IL-3/6 to PB CD34 cultures resulted in less expansion, whereas addition of HGF to UCB CD34 cultures resulted in increased expansion of CD34 /CD38-cells, but only in cultures in absence of PA-MSC or CD271 MSC feederlayers.

P111

TNF α signaling in neural stem cells

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The subependymal zone (SEZ) of the adult murine brain is a very active neurogenic niche in which a relatively quiescent population of radial glia/astrocyte-like GFAP neural stem cells (NSC) continually produce new neurons and oligodendrocytes. Although some intrinsic determinants are known to regulate stem cell division, the observation that stem cells can respond to excessive cellular demand in pathological situations or after traumatic injury suggests that signals present in their microenvironment or niche contribute to the regulation.

Tumor necrosis factor (TNF) has been characterized as a pro-inflammatory cytokine prototypically involved in the innate immune response, but it is a multifunctional protein with a broad range of activities in different systems. Although TNF α is char-

acteristically produced by immune cells, it is also expressed in many other cell types. In the adult brain, TNF α is produced by microglia and infiltrating macrophages, but can also be produced by astrocytes and neurons upon injury. Increasing evidence indicates that immune cells and immunological mediators modulate neurogenesis. In this context, effects of pro-inflammatory cytokines that are produced under non-physiological conditions, such as irradiation, inflammation, status epilepticus or stroke, on neurogenesis have been described. However, their effects appeared sometimes contradictory, suggesting potentially distinct effects depending on the cell or receptor type involved. TNF α interaction with the TNF-R1 generally triggers apoptosis and cytotoxicity whereas engagement of the TNF-R2 is usually associated with cell protection and proliferation.

TNF α receptors knock-outs exhibit alterations in neurogenesis in the dentate gyrus under basal conditions, indicating that endogenous TNF α might regulate neurogenesis in this niche. In the present work, we have evaluated the involvement of TNF α signaling in the SEZ by analyzing the role of the two TNF α receptors, both *in vivo* and *in vitro* neurosphere cultures. We find that each receptor mediates a different biological response under physiological conditions and upon inflammation.

P112

A unified model for the neural competence of adult human connective tissue stem/precursor cells

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Resident neural precursor cells (NPCs) have been reported for a number of adult tissues. Understanding their physiological function or, alternatively, their activation after tissue damage or in vitro manipulation remains an unsolved issue. Here we investigated the source of human dermal NPCs in the adult. By following an unbiased, comprehensive approach of cell surface marker screening, cell separation, transcriptomic characterization and *in vivo* fate analyses, we found that p75 Sox2 dermal stem/precursor cells of human trunk skin can be traced back to the Schwann cell (CD56) and pericyte lineages (CD56-), which seem to be developmentally interrelated. Moreover, neural differentiation of dermal stem/precursor cells was restricted to the Schwann-like cells. These cells were similarly obtained from human heart stromal tissue. We postulate a model by which neural competence of connective tissue stem/precursor cells is dependent on Sox gene expression levels. We further hypothesize that Sox2 resident precursors arise by dedifferentiation of Schwann cells at peripheral nerve endings of multiple organs.

P113

Differential genomic imprinting regulates paracrine and autocrine roles of IGF2 in adult neurogenesis

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The imprinted *Insulin-like growth factor 2 (Igf2)* gene is not imprinted in the choroid plexus and leptomeninges of the brain. Using an *Igf2*-reporter mutant mouse, we address the relationship between *Igf2* imprinting and adult neurogenesis in the subventricular zone (SVZ) and hippocampus *in vivo*. Paracrine IGF2 functioning as a CSF-derived neurogenic factor requires biallelic expression with reduced activation of the stem cell pool in the SVZ and impaired olfactory bulb (OB) neurogenesis in mutants. In contrast, in the hippocampus *Igf2* is imprinted, and is an autocrine factor that is paternally expressed in the NSCs themselves. Reduction in the stem cell population is observed in the dentate gyrus (DG) but only when the *Igf2* mutation is paternally transmitted. Our data also show that biallelic *Igf2* expression may control the dosage of paracrine IGF2 which is taken up by the NSCs in the SVZ *via* activation of insulin receptor (IR) and/or insulin growth factor 1 receptor (IGF1R). In the DG the action of imprinted autocrine IGF2-signalling likely involves a different mechanism. Our findings emphasize the importance of selective imprinting in the control of adult neurogenesis, and indicate that a regulatory decision to imprint or not may be an important functional mechanism of dosage control.

P114

Rabbit amniotic membrane is a high throughput source for multipotent mesenchymal stem cells with adipogenic and osteogenic differentiation potential

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Introduction: Amniotic membrane (AM) is actually being considered as an excellent alternative source for obtaining multipotent mesenchymal stem cells (MSCs). AM-MSCs possess a high proliferative potential and immuno modulatory properties, as well as low immunogenicity and tumorigenicity *in vivo*. These cells have been previously used in animal models underlying their potential application in cell therapy. Our aim was to study the rabbit AM-MSC immunophenotype, growth and multipotent differentiation in order to its further application in cellular therapy of multiple diseased tissues.

Methods: To confirm the immunophenotype of the isolated AM-MSCs, these cells were analyzed to determine the expression of CD90, CD44, CD45, CD34 and HLA-DR by flow cytometry. To analyze the proliferation of AM-MSC, cells were seeded at different concentrations and allowed to grow for 7 days at 37°C. Proliferation rate was determined by BrdU incorporation. AM-MSCs were analyzed with respect to their ability to differentiate

into adipocytes and osteoblasts. Cells were put in culture with cell-specific differentiation medium and the presence of differentiated cells was demonstrated by Oil Red O or Alizarin Red staining.

Results: AM-MSCs analyzed were mostly positive for CD90 (98.3%) and CD44 (99.8%) and negative for CD45 (1.2%), CD34 (0.8%) and HLA-DR (4%). Regarding its growth, cells reached the confluence after 3–5 days. AM-MSC differentiated into adipocytes and osteoblast as demonstrated by the presence of Oil Red O and Alizarin Red staining.

Conclusions: Rabbit amniotic membrane is a promising source for obtaining MSCs for further applications in animal models of disease.

P115

Multiple neural markers are spontaneously expressed by human adipose tissue-derived stromal cells (ADSCs) in culture

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Aiming to find a source of nerve cells for transplantation, various reports have described induction of “neural like” characteristics from mesenchymal cells in culture by using diverse cocktails of molecules. Most of those studies were based on the expression of typical neural markers, like β -tubulin III for neurons, GFAP for astrocytes, galactocerebroside for oligodendrocytes or nestin for neural stem cells. In our attempts to reproduce such results, we found that not only those markers, but a whole repertoire of specialized neural molecules were already spontaneously expressed by ADSCs in growing culture conditions (α MEM 20% FBS). Besides the above mentioned molecules, ADSCs were positively immunostained for neuronal markers (NeuN, NSE, PGP9.5), neuronal cytoskeleton proteins (MAP1b, MAP2a b, Tau, α -inter-nexin, peripherin, GAP-43) synaptic vesicles (synaptophysin, chromogranin A), transmitters (CGRP, VIP, somatostatin, neuropeptide Y), neural development markers (doublecortin, neuroD, SLUG) and oligodendrocyte markers (MBP, olig2, MOG, CNPase).

Western blot analysis confirmed the immunocytochemical findings. However, in some instances, the cellular location of these antigens did not correspond to their usual distribution in neural cells, which suggest that they accomplish a different function. The tested “neural induction” strategies (including combinations of retinoic acid, dibutyryl cAMP, PDGF, FGF2, NT4 and/or neurobasal medium B27 supplement) had an effect on the morphology of ADSCs in culture but did not differentiate them into functional neurons nor into myelinating glia. We interpret the expression of neural molecules in ADSCs not as the substrate for their neural potential but as the capacity of these mesenchymal cells to present a great repertoire of antigens meant for immunomodulatory purposes.

P116

Isolation and characterization of a spontaneously immortalized multipotent mesenchymal cell line derived from mouse subcutaneous adipose tissue

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The emerging field of tissue engineering and regenerative medicine is a multidisciplinary science, based on the combination of a reliable source of stem cells, biomaterial scaffolds and cytokine growth factors. Adult mesenchymal stem cells are considered to be important cells for applications in this field and adipose tissue has revealed to be an excellent source of them. Indeed, Adipose derived Stem Cells (ASCs) can be easily isolated from the stromal vascular fraction (SVF) of adipose tissue. During the isolation and propagation of murine ASCs we observed the appearance of a spontaneously immortalized cell clone, named m17.ASC. This clone has been propagated for more than 180 passages and stably expresses a variety of stemness markers, such as Sca-1, c-kit/CD117, CD44, CD106, islet-1, nestin and nucleostemin. Furthermore these cells can be induced to differentiate towards osteogenic, chondrogenic, adipogenic and cardiogenic phenotypes. m17.ASC clone displays a normal karyotype and stable telomeres, does not proliferate when plated in soft agar, nor give rise to tumors when injected subcutaneously in NOD/SCID- g^{null} mice. The analysis of gene expression highlighted transcriptional traits of SVF cells. m17.ASCs were genetically modified by lentiviral vectors carrying GFP as a marker transgene and efficiently engrafted in the liver, when injected in the spleen of NOD/SCID- g^{null} monocrotaline-treated mice.

These results suggest that this non-tumorigenic spontaneously immortalized ASC line may represent a useful tool (cell model) to study the differentiation mechanisms involved in tissue repair as well as a model for pharmacological/toxicological studies.

P117

CD105⁺ murine MSCs defines a new multipotent sub-population with distinct differentiation and immunomodulatory capacities

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Administration of ex vivo expanded mesenchymal stromal cells (MSCs) represents a promising therapy for regenerative medicine and autoimmunity. Both mouse and human MSCs ameliorate autoimmune disease in syn-, allo- and xenogeneic settings. However, MSCs preparations are heterogeneous which impairs their therapeutic efficacy and endorse variability between experiments. This heterogeneity has also been a main hurdle in translating experimental MSCs data from mouse models to human patients. The objective of the present manuscript is to further characterize murine MSCs (mMSCs) with the aim of designing more efficient and specific MSC-based therapies. We have found that mMSCs are heterogeneous for endoglin (CD105) expression and that this heterogeneity is not due to different stages of MSC differentiation. CD105 is induced on a subpopulation of mMSCs early upon in vitro culture giving rise to CD105⁺ and CD105⁻ MSC. CD105⁺ and CD105⁻ cells represent independent subpopulations that keep their properties upon several passages. CD105 expression on CD105⁺ mMSCs was affected by passage number and confluence while CD105⁻ mMSCs remained negative. The CD105⁺ and CD105⁻ mMSCs sub-populations had similar growth potential and maintain almost identical mMSCs markers (CD29 CD44 Sca1 MHC-I and CD45⁺CD11b⁻CD31⁻) but varied in their differentiation and immune-regulatory properties. Interestingly, CD105⁺-mMSCs were

better at T cell suppression and more prone to differentiate into adipocytes and osteocytes than CD105⁻ mMSCs. Based on these studies we propose to redefine the phenotype of the mMSCs based on CD105 expression.

P118

Isolation and Characterization of Prospectively Isolated CD271⁺ Human Bone Marrow-derived Mesenchymal Stromal Cells

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Introduction: Bone marrow (BM) multipotent mesenchymal stromal cells (MSCs) have the capacity to differentiate into adipogenic, osteogenic and chondrogenic lineage. MSCs are readily isolated from BM mononuclear cell fractions based on their capacity to adhere to culture plastics. However, plastic adherent (PA) MSCs generally consist of a heterogeneous cell population. Prospective isolation of MSCs is hampered by the absence of MSC-specific antibodies. Recently the CD271 antigen (Low affinity Nerve Growth Factor Receptor) has been identified on MSCs with colony forming (CFU-F) capacity. Here, we characterized prospectively isolated CD271 BM-MSCs and compare these with PA-MSCs.

Materials and Methods: CD271 positive cells were isolated from BM mononuclear cells using magnetic beads (Miltenyi Biotec) and plated in DMEM-LG/MCDB201 medium supplemented with 10% FBS-HI, 1% pen/strep and 2 mM L-Glutamin. Cells were cultured up to passage 3 and then used for analysis of immunophenotype, differentiation capacity, and support as feeder layer for CD34 hematopoietic stem cells.

Results: BM-MNC fractions contained on average $2,3 \pm 1,2\%$ CD271 cells. Recovery of CD271 cells after isolation ranged from 0,0 - 1,85% of total cells and resulted in an average level of $61,5 \pm 23,8\%$ CD271 cells, of which about 10% qualified as non-hematopoietic CD271 /CD45⁻ cells. CFU-F capacity of PA-MSCs was $23 \pm 20/10^5$ plated cells versus $884 \pm 750/10^5$ plated CD271 /CD45⁻ cells for CD271 cultures ($p < 0,05$). Upon culture, the CD271 antigen was rapidly downregulated to 1,3% (0,2 - 4,2%). Similar to PA-MSC, CD271 MSCs abundantly expressed CD29 (97,3%), CD44 (78,6%), CD73 (98,5%), CD90 (96,6%), CD105 (93,5%) and CD166 (77,9%), and were negative for hematopoietic (CD14, CD45, CD34) and endothelial cell markers (CD31). CD271 cells showed adipogenic and osteogenic differentiation potential similar to PA-MSCs and similar or better capacity to support hematopoiesis *in vitro*. In conclusion, CD271 isolation is an effective method to enrich for multipotent MSCs from BM.

P119

Human placenta-derived multipotent mesenchymal stem cells exhibit unique features that make them ideal candidates for cancer and wound healing therapies

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Placenta tissue is studied as an important reservoir of stem cells with great potential in regenerative medicine and cell therapy because of its unique features: phenotypic plasticity, immunomodulatory properties and ease of isolation procedure. This application involves: removal of cells from a donor, cell expansion in culture, administration to the recipient and migratory capacity of cells toward injury *in vivo*. *In vitro* cell culture is a tricky step since cells must overcome a "traumatic" situation preserving all its characteristics and properties. This work has focused in determining whether human placenta mesenchymal stem cells (phMSCs) maintain their specific phenotypic and genotypic profile and multipotent properties when expanded in culture. Moreover, we studied phMSCs migratory potential *in vitro* and *in vivo*. Our group characterized phMSCs further culture amplification from a genotypic, phenotypic and functional standpoint. We monitored the proliferative capacity of phMSCs at different passages and in two different cell culture conditions, low and high density. An analysis of the genes related to pluripotency Sox-2, Oct-4 and Nanog was performed by qRT-PCR. The expression pattern of mesenchymal markers such as CD73, CD105 and CD44 was examined by flow cytometry analysis. In relation to the immunomodulatory capability of these cells, intracellular and extracellular TGF- β 1 expression was determined by flow cytometry. We also analyzed: the cellular potential to differentiate into different lineages, together with their migratory capacity *in vitro* and *in vivo*: wound healing and trans-well 8 μ m pore size assays, *in vitro*, and tumor in animal models, *in vivo*, were conducted. Our data revealed that phMSCs show a remarkable stability at phenotypic, genotypic and functional level when expanded in culture and a migratory ability *in vivo*. These results together with their demonstrated migratory ability *in vivo*, point to them as interesting candidates in raising clinical applications.

P120

Isolation and culture of mouse amniotic membrane-derived stem cells

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Introduction: Recent studies have shown that amniotic membrane tissue is a rich source of stem cells (SCs) in human. Because the mouse is the model of choice for many areas of scientific investigation, isolating epithelial and mesenchymal stromal cells from murine amniotic membrane (mAECs and mAMSCs) is an important issue to study the therapeutic potential of AM-based therapies. The present research aimed to amplify *in vitro* and characterize murine amniotic membrane-derived SCs (mAMSCs).

Methods: An amniotic membrane distinct from the yolk sac was carefully collected and followed by collagenase digestion. We characterized the immunotype of mAMSCs by flow cytometry and immunofluorescence. To determine the differentiation potential, the cells were cultivated under osteogenic and adipogenic conditions, and followed by specific staining.

Results: We established a method to isolate murine amniotic cells that are equivalent to human amniotic epithelial and mesenchymal cells. The cell yield was approximately $1.5-2 \times 10^6$ cells per pregnant female, (2×10^5 cells per fetus), roughly in proportion to the age of fetus used and isolated cells were attached to the dish under culture conditions. Characterization by immunofluorescence showed morphological and functional differences between the two amniotic-derived cell types. mAM-ESCs cultures displayed homogeneous cells with a small size cuboid shape, central nuclei. Instead mAM-MSCs cultures were characterized by their spindle-shaped morphology and are able to form colonies. Only mAM-MSCs showed proliferative potential in culture. *In vitro* differentiation assays showed plasticity by differentiating into osteocytes and adipocytes. mAM-MSCs showed high expression of CD44, moderate expression of nestin, CD90 and ki67, low expression of CD73, and were negative for CD45, CD34 and CD19.

Conclusions: Thus, murine AMSCs may represent sources of characterized pluripotent SCs that can be collected and amplified *in vitro*. Furthermore, these murine SCs may be useful as a cell therapy application for preclinical studies to develop future human therapies.

P123

Large scale LV production and purification: process optimization for *ex-vivo* gene therapy

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MolMed is a biotechnology company focused on research, development and clinical validation of innovative therapies. Cell and gene therapy technologies have been developed for new approaches including large-scale manufacturing and downstream purification, concentration and sterilisation of retroviral vectors (RV) and lentiviral vectors (LV).

A GMP process for LV production and purification was developed in collaboration with TIGET and Généthon and is currently used for LV manufacturing for *ex-vivo* gene therapy clinical trials sponsored by Telethon and applicable to 3rd generation LV carrying different transgenes.

Besides the manufacturing process, a quality control strategy has been defined and appropriate analytical methods have been developed and validated; this approach is largely applicable to different LV vectors.

The original manufacturing process was standardized, robustness of upstream phase was improved and further optimization was achieved in downstream phase both in terms of yield and in reduction of contaminants such as plasmid and host-cell DNA.

DNA removal was improved by the addition of a second endonuclease step, while process yield was increased by the optimization of the sterilizing filtration step. The overall process yield increased from 25 (± 5)% to 52 (± 5)%, alongside a reduction of residual host-cell and plasmid DNA was achieved.

The improved production and purification process is applicable to vectors carrying different transgenes and gives high quality purified vectors, suitable for clinical applications.

Improved yields allow the transduction of a larger number of patients' cells with the same vector lot. GMP transduction

process guarantees the quality of the product in terms of identity/potency, purity and safety.

P124

Single step cloning-titration method: accelerating the development and engineering of high-titer retro and lentiviral vector producer cell lines

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Stable cell line development for viral vector production is a tedious and time consuming work as viral components are split in several cassettes – for engineering and safety purposes – requiring the transfection, clonal selection and screening for each. Herein, we describe a novel method for fast screening of high-titer virus producing clones by merging cloning and titration in a single step.

The method makes use of split-GFP, a green fluorescent protein separated into 2 fragments – S10 and S11 – which fluoresce only upon transcomplementation. A cell population producing infectious virus with a S11 transgene is cloned and co-cultured with a target cell line harboring the S10 fragment. S11 viruses produced by the clone infect the target cells and reconstitute the GFP signal. Only the clones yielding high signal are isolated, avoiding growth/titration studies for the low titer clones.

The method was validated by establishing a retrovirus producer from a nude cell line allowing for the screening of 200 clones in two weeks; clones producing up to 1×10^8 infectious particles *per* mL were isolated. It was additionally used to evaluate the stable production performance of different cell substrates – HEK293 *vs.* HEK293T – showing that the later sustain increased titers. Finally, it was used in cell metabolic engineering; clones producing up to 12× more than non-manipulated cells, after the over-expression of glutathione metabolism genes, were obtained.

The method herein described, demonstrated its potential in important aspects of viral vector production, from high-titer clone selection, engineering cell metabolism, and screening for high-producing cell substrates. It should be adaptable to stable cell lines producing other types of infectious virus and, with the proper modifications, extendable to transient systems. Ongoing is the modification for lentiviral vector producer cells. This method will contribute to the progress of gene therapy by accelerating cell line development and engineering.

P125

RD3-MolPack packaging cells for stable production of Tat-independent self-inactivating lentiviral vectors

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We have previously reported on the generation of the RD2-MolPack-Chim3 clone for stable production of LTR-driven lentiviral vector (LV) expressing the anti-HIV therapeutic gene Chim3, a transdominant HIV Vif. RD-MolPack system grounds on the HEK-293T derivative PK-7 clone, which contains an in-

tegration cassette expressing the HIV-1 *gag*, *pol*, *rev* and the *hygro*-resistance genes flanked by AAV inverted terminal repeat (ITR) sequences. From PK-7, we separately derived not only the Tat-dependent RD2-MolPack packaging clone, suitable for generating LTR-driven and self-inactivating Tat-dependent LV, but also the RD3-MolPack packaging clone, which expresses the RD114-TR envelope glycoprotein, but lacks the Tat trans-activator and it is therefore fitting only for Tat-independent LV production. To obtain a prototype RD3-MolPack producer clone, we integrated a SIN-GFP LV by transient transfection of the linearized plasmid. The selected RD3-MolPack-GFP clone 24 grows in continuous culture in disposable two-compartment bioreactor for longer than 3 months producing, on average, not concentrated supernatant with around $1-5 \times 10^6$ TU/ml titer when calculated on either CEM A3.01 or primary CD34 cells. We also confirmed the preferential transduction ability of the RD114-TR pseudotyped SIN-GFP of RD3-MolPack-GFP respect to equivalent VSV-G pseudotyped LV produced by transient transfection on human CD34 cells as previously described for the LTR-driven Chim3-LV. The major features, including the safety ones, and the potential of the RD-MolPack system to be a valid alternative for clinical manufacturing to the current LV technologies will be discussed.

P126

HEK293-platform for the generation of clinical-grade gamma-retroviral vectors

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Stable producer cells are the source of choice for the production of clinical-grade-retroviral self-inactivating (SIN) vectors since the recombinase mediated cassette exchange technology (RMCE) has been exploited for their generation (Verhoeven *et al.*, 2001, Schucht *et al.*, 2006). The major advantages of this technology were the reproducibility and predictability of SIN-vector production allowing the reduction of screening work. However, initial SIN-vector supernatants generated by such producer cells were contaminated with vectors bearing full LTR activity as a result of recombination of the therapeutic vectors with the vector used for integration site selection in the packaging cell genome. This particular problem was solved by inverting the direction of expression of the therapeutic vector within the targeting construct (Loew *et al.*, 2010) but unfortunately was accompanied by a significant loss of vector titer. Because of its advantages, a further optimization of the RMCE technology for the introduction of retroviral SIN-vectors into packaging cells appears to be important.

The data we present show that HEK293-based packaging cells (Ghani *et al.*, 2007 and 2009) could be successfully modified to contain a targetable locus. The newly designed vector employed to introduce the tag into the host cell genome, combines FLP- and Cre-recombinase recognition sites. This combinatorial approach allowed RMCE and, once the producer clone is established, the removal of residual tag-vector sequences eliminating the possibility for accidental recombination. The resulting producer cells were highly productive and titers achieved with the new producer cells reached about 4×10^7 infectious particles (IP)/ml for

AMPHO- and GALV- pseudotyped experimental SIN-vectors and up to and 1×10^8 IP/ml for RD114-pseudotypes. The potential to generate therapeutic vectors was demonstrated for the amphotropic packaging cell by targeted integration of a therapeutic SIN-vector transferring the large (9 kb) collagen 7A1 cDNA. With this producer cell we achieved titers around 5×10^6 IP/ml. Into GALV- and RD114-pseudotyping cells a LTR-driven therapeutic vector transferring the TCR150m T-cell receptor was integrated via targeted exchange. Functional titers were determined by tetramer-stain revealed around 6×10^6 and 2×10^7 IP/ml. In all cases, precise targeting and clean-up reactions were confirmed by locus-specific PCR and sequencing, thus the fc-technology has matured into an applicable process.

P127

Cholesterol as an enhancer of lentiviral vector production in serum-free transient transfection

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Current serum-free lentiviral production methods for gene therapy generally generate disappointing titres. As a consequence, multiple production enhancers have been tested in an attempt to improve these yields, including a number of media supplements. Of these, cholesterol in particular has been shown to significantly improve viral titres in established cell lines.

In this study, media supplementation with cGMP compliant cholesterol is shown to also be effective in the HIV transient transfection of suspension cultured 293T cells using branched PEI. Importantly, in this context, the time point at which cholesterol is added is crucial. It was found that supplementation with cholesterol pre-transfection drastically impairs lentiviral production. However, when added post-transfection the resulting titres more than doubled. This effect was observed when using a number of permutations of lentiviral vector constructs (different transgenes or surface proteins). However, issues of biomaterial compatibility need to be evaluated if such measures are to be used in large-scale lentivirus production. Methyl-beta-cyclodextrin, a component of cholesterol supplements, can interact with both cholesterol and the plastic surface of disposable bioreactors, leading to cell death. Post-transfection addition of cholesterol is therefore an effective supplementation in a transient transfection to enhance lentiviral vector production, but care must be taken in the selection of the growth parameters.

P128

Setting up a transfection-based rAAV production process in the Integrity iCellis™ single use fixed-bed bioreactor

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In July 2013 the Journal of Gene Medicine counted a total of 1902 gene therapy clinical trials. 5.1% of these are using AAV as vectors to deliver the therapeutic gene. In fact AAV technologies show a strong upward trend in view of current preclinical work. Despite

the relatively small genome (4.7kb) that limits its packaging capacity AAV is getting increasingly popular for the advantages it has to offer: lack of pathogenicity, low immunogenicity, ability to infect quiescent cells, long-term transgene expression without risk of insertional mutagenesis, etc. We are currently establishing a technology platform to manufacture high-quality vectors based on rAAV that will allow for fast and easy switch of capsid and vector appropriate for our customer's needs.

Since many virus-based biologics (viral vectors, virus based vaccines and oncolytic viruses) are still produced on anchorage-dependent cells we as a CDMO are preparing for production processes of up to 1000 m² for cell culture technologies such as roller bottles and multitray systems to meet the future demands of the growing market. However, these conventional technologies have some serious disadvantages: they are bulky, require a lot of handling operations and offer only limited process control. Hence, we are also evaluating novel cell cultivation technologies such as ATMI's Integrity iCellis™ single use fixed-bed bioreactors. This technology is available in different formats which offer surface areas of 0.5–500 m².

In this case study we present data for transfection-based production of rAAV in the iCellis™ laboratory scale bioreactor. Cell growth, metabolic data and virus titers are compared to the benchmark values of conventional multitray production.

P129

Stressed, accelerated and real-time stability testing on retroviral and lentiviral platform: parallelism among batches and among vectors

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MolMed is a biotechnology company focused on research, development and clinical validation of innovative gene therapy technologies and particularly on large-scale manufacturing, downstream purification, concentration and sterilisation of both retroviral (RV) and lentiviral (LV) vectors. Both LV and RV vectors have undergone stability and shelf-life studies to track and examine how different environmental conditions affect a product over a specified period of time. Different temperature conditions: stressed, accelerated and real time (storage condition) have been investigated. Stability data have been evaluated with a statistical *ad hoc* macro program and with the covariance analysis (ANCOVA) that allows to compare one variable in 2 or more groups taking into account (or to correct for) the covariates, in order to verify the parallelism among different batches. Stability studies for LV vectors has been conducted in a matrix approach on two vectors carrying two different transgenes considering that they are produced with the same procedure, except for the transgene plasmid. LV data have been analyzed also with the variance analysis (ANOVA) to look for significant differences between different vectors in terms of physical and infectious titer. The data analysis obtained for LV vectors indicates not only a shelf life of 36 months, but that the two vectors present a parallel stability trend. The stability is not conditioned by the transgene plasmid used for manufacturing. Stability study on RV vectors indicates a shelf life up to 60 months with no statistical significant differences among different harvests and among different batches inside the same manufacturing procedure.

P130

Xeno-free isolation and expansion of GMP fibroblasts for cell therapy

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Many studies describe the isolation of human fibroblasts from skin biopsies by either collagenase-based techniques or mechanical dissociation-based approaches (“explant technique”); however, it is not clear which option is preferable when developing cell-based human therapies under good manufacturing practice (GMP) conditions. Likewise, it is also uncertain which is the best anatomical area of the body to obtain fibroblast and which is the best culture media for fibroblasts expansion for a clinical use. The purpose of this pilot study was to compare the efficacy of fibroblasts isolation from different areas of the body using both mechanical and enzymatic protocols. Two skin biopsies from different donor areas were processed by either the collagenase technique or “explants technique”, comparing their efficiency in isolating fibroblasts in terms of time and number of fibroblasts obtained. The quality of the isolated fibroblasts was also assessed by measuring fibroblasts proliferation capability, viability, identity, morphology and genetic stability. The use of different antibiotics and serum bathes was also optimized in terms of growth capability. Our results indicate that 1) The “explant technique” is a more efficient approach to obtain fibroblasts under our experimental conditions; 2) once the primary fibroblast culture is established there are no significant differences between the techniques although fibroblasts isolated by the collagenase approach acquire an earlier senescent-like morphology; 3) The back area, seems to be an appropriate area to isolate dermal fibroblasts; 4) AB human serum and gentamicin are preferable in a GMP setting however they decrease fibroblast proliferation rate when compared to the combination of FBS and P/S.

P131

Cytotoxicity of HIV-1 protease in 293T and 293-EBNA cells is independent of procaspase-8: implications for lentiviral vector production

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Lentiviral vectors (LVs) allows stable expression of a transgene for long-time period in both dividing and non-dividing cells unlike simpler genera of retroviruses. This feature contributed for the growing interest of LVs as research tools and as vector for

gene therapy. However, large scale production of LVs is still an issue. To improve productivities and safety, a packaging cell line is desirable but its development is hampered by the toxicity of some viral proteins like protease [1]. The cleavage of procaspase-8 by HIV-1 protease has been implicated in activation of apoptotic pathways in T cells [2]. Furthermore this cytotoxicity decreases in cells with a mutated procaspase-8 resistant to protease cleavage [3] and in cells with lower expression of procaspase-8 [4].

The aim of this work is to elucidate the relation between protease cytotoxicity and cleavage of procaspase-8 in the most used cell lines for LV production. The expression of procaspase-8 was compared in both 293T and 293-EBNA cells before and after expression of HIV-1 gag-pro-pol gene with a wild-type protease and a mutated one less active. Transcriptomic and proteomic results show that expression of procaspase-8 in both cells is significantly low before and after gag-pro-pol expression, indicating that cytotoxicity should be mainly mediated by other cellular HIV-1 protease targets. These results are the first evidence that cytotoxicity mediated by HIV-1 protease in 293T and 293-EBNA cells could not be related with cleavage of procaspase-8. This work will contribute for the development of robust LV packaging cell lines through the understanding of viral vector cytotoxicity.

P132

Comparison of different strategies for the establishment of retroviral cell lines: random vs. targeted integration

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Retroviral vectors constitute a powerful tool as gene delivery vehicles, being largely used in cancer immuno-gene therapy. However, development of retroviral producer cell lines is time consuming, requiring extensive screening and its integration process is random, leading to variable expression levels. Herein, a stable retroviral producer cell line, 293OuaS, which transgene contains an Ouabain resistance gene (OuaSelect), was established. OuaSelect is a safe, rapid and cost-effective selection marker based on Na K -ATPase. Mutations in this ATPase, usually inhibited by Ouabain, confer an exceptional resistance to this drug, allowing elimination of non-modified cells in 48 h^{1,2}. Previously, we established an optimized retroviral packaging cell line by targeted integration³ and reported that viral components balanced stoichiometry is of high importance⁴. In this work, a novel packaging cell line was established by random integration of all viral components, using 293Flex stoichiometry level for decision making and to rationally select the best clones, thereby reducing the amount of clones screened. The importance of the stoichiometric optimization was also revised but instead of stably expressing the transgene in the first step, this component was the last to be introduced. A ten-fold higher producer clone was obtained, producing 6×10^7 IP.mL⁻¹ and presenting higher gag-pol and GaLV expression levels than 293Flex. Results show that gag-pol is a pivotal component and that GaLV expression seems to dramatically influence viral infectivity. In order to further compare both cell lines the

transgene was introduced in 293Flex by Flp/FRT recombination system⁵. Viral productivity, gene expression and transgene copy number are under evaluation.

P133

Development of a validated QPCR Assay for ADA SCID and an IHC assay for human CD45+ cells in NSG mice to support ADA-SCID pre-clinical biodistribution studies

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In October 2010 GSK initiated a research and development alliance with the Telethon Institute of Gene Therapy and the San Raffaele Foundation. The alliance launched a GSK funded gene therapy development program using first generation retroviral vector for the treatment of Adenosine Deaminase Severe Combined Immunodeficiency (ADA-SCID).

Here we describe the experimental approach taken for the validation of an assay system to support biodistribution studies in NSG mice transplanted with human hematopoietic stem cells (HSCs), transduced with a retroviral GIADAI vector containing the human adenosine deaminase (hADA) gene sequence. The assay used Quantitative Polymerase Chain Reaction (QPCR) to detect DNA sequences specific to the 3000 bp region of the retroviral GIADAI vector containing the Human Adenosine Deaminase (hADA) gene sequence and also to detect the presence of sequences specific to Human GAPDH and Murine Beta Actin housekeeping genes. The assay was validated in accordance with the ICH harmonised tripartite guidelines. The following parameters were addressed in the validation; Linearity, Range, Precision, Quantification Limit, Limit of Detection with 95% confidence, Specificity, Accuracy and Robustness. This assay was supported by the complementary development of an immunohistochemical assay to assess the biodistribution of human CD45 cells in GIADAI transduced UCB CD34 transplanted NSG mice. This assay used rabbit polyclonal antibody for human CD45 to specifically test for the biodistribution of human CD45 cells in transduced NSG mice.

P134

Manufacturing of HSV-TK engineered donor lymphocytes: a comparability study to improve the production process

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TK is a cell therapy that allows to practice hematopoietic-stem-cell-transplantation from haploidentical donors for treatment of high risk leukemia. Donor T lymphocytes are transduced by the SFCMM-3 Mut2#48 retroviral vector encoding HSV-TK and the cell surface marker ΔLNGFR, making them susceptible to gangciclovir. Infusion of the medicinal product (*i.e.* TK-transduced

lymphocytes), promotes early immune reconstitution of patients and allows to exploit the anti-leukemia effects of lymphocytes, while maintaining under control GvHD.

The selection step of the manufacturing process was modified to improve the procedure and to comply new regulatory requirements. The current reagent consist of anti-LNGFR mAb conjugated with SAM-dynabeads (Invitrogen). MolMed proposes the use of CliniMACS CD271-microbeads (Miltenyi). Prior to adopt the CD271-microbeads for production of clinical batches, a comparability exercise was performed to support the change and to warrant quality, safety, efficacy of the post-change medicinal product and to ensure consistency of production throughout the on-going phase III trial. The successful results of the comparability study and the approval by the regulatory agencies are prerequisites for the introduction of the change in the manufacturing process. The comparability exercise was articulated in three phases: a preliminary small/medium scale study; comparability experiments at full scale and a retrospective analysis with data from the phase III study. We evaluated the following process parameters: cell purity and viability, immunophenotype and cytokine secretion profile, potency and *in vivo* functionality in immunodeficient mice. Overall, no statistically significant differences are observed for each parameters analysed, thus demonstrating that pre- and post-change medicinal products are comparable.

P135

Novel tools could give stable producer cell lines their break for commercial manufacturing of recombinant AAV vectors

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Recombinant Adeno-associated virus (rAAV) based vectors increasingly emerge as promising candidates for viral gene therapy due to a large toolbox available and favourable properties such as non-pathogenicity of AAV, low B-/T-cell immunogenicity against transgenes and long-term transgene expression from a non-integrating vector. A boost of the gene therapy field among others driven by approval of Glybera® in the European Union, raises the demand for robust and cost-effective manufacturing of gene transfer vectors for market supply. Producer cell lines enabling stable rAAV production represent an auspicious perspective to circumvent existing issues implied with standard transient productions like high costs, regulatory effort and product quality. The aim of the underlying study is to provide all tools necessary to generate a versatile and high-titre rAAV manufacturing platform based on a stable mammalian producer cell line. First, the general feasibility to establish a producer cell line with stably integrated adenoviral helper functions was investigated applying classical protocols and investigating rAAV productivity of HeLa cells using proprietary plasmids. Overall rAAV yields could be increased by optimizing and modifying plasmids of the used split-packaging system. Different temperatures were examined for optimal production conditions. Furthermore, we investigated differential gene expression in response to temperature downshifts. The final goal is to use identified temperature-inducible switches to control expression of the key inducer gene of rAAV production cascade.

Making use of a temperature downshift as primary switch for rAAV production, we would combine the inevitable induction event due to the formation of toxic proteins with conditions presumably enhancing rAAV production.

P136

Vector Production Unit (UPV, CBATEG-UAB): a technological platform for the generation, production and characterization of viral vectors

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Viral vectors are widely used tools for gene transfer and gene expression. Their use is an attractive choice given their high transduction efficiency, and the ease and flexibility to genetically express or inhibit one gene or a combination of genes in specific areas and periods of time, while avoiding compensation phenomena or other drawbacks associated with animal models.

Despite the availability of standardized procedures for their application in both *in vitro* and *in vivo*, and their low risk level when used in a controlled setting, the production of viral vectors requires the application of specialized techniques, access to expensive equipment and biological safety laboratories. The Vector Production Unit (UPV) is a non-profit technological platform of the CBATEG at the Universitat Autònoma de Barcelona. It has Biological Safety Level 2 and 3 facilities and it is staffed by experienced and highly qualified personnel. Since its opening in 2003 it has been dedicated to the design, development, production and purification of more than 400 viral vectors for basic research and gene therapy pre-clinical studies for both public and private research laboratories.

The UPV currently produces marker and custom adenoviral vectors (of human (Ad5) or canine (CAV-2) origin) as well as several pseudotypes of adenoassociated vectors. Standard productions (20 × 15 cm plates, scalable upon request) yield averages of 4×10^{12} PP/ 3×10^{11} IU for Ad5 vectors, and 1×10^{13} gc for AAV vectors. Average titers are $1,5 \times 10^{12}$ PP/ml, 1×10^{11} IU/ml for Ad5 and 7×10^{12} gc/ml for AAV. Production times (without cloning) are of 2,5 months for Ad5 and 1-1,5 months for AAVs.

P137

Adeno-Associated Virus production using a disposable fixed-bed bioreactor from bench-scale to industrial scale

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Background and novelty Recombinant viruses (e.g. lentivirus and adeno-associated-virus) can be used as human gene therapy vectors. They are mainly produced in adherent cell cultures (e.g. HEK293T, A549, VERO) in Roller Bottles (RB) or multiple-tray-stacks (CF) using either transient transfection (e.g. PEI, PO4 pre-

cipitation) or infection (e.g. recombinant viruses) strategies. Therefore, iCELLis® bioreactors offer a new production alternative with stronger process controls and ease of scale-up. Adherent cells grow on microfiber carriers packed in a fixed-bed providing up to 500 m² of growth surface area in a small reactor volume. Environmental conditions, combined with the large growth surface area in the iCELLis yields high cell productivity. Experimental approach First, a mirrored approach of the previous production in CF was set-up in the small scale iCELLis bioreactor. Transfection efficiency through the fixed-bed of the iCELLis system is evaluated by Flow Cytometry (measuring the expression of protein marker) and viral productivity by transduction assay. Cell disruption is carried out directly in the bioreactor by physico-chemical methods to harvest the intra-cellular viruses. Results and discussion Results of transient transfection processes by PEI showed similar transfection efficiency in iCELLis than in CF. Results in iCELLis demonstrated that the system allows high biomass growth, regulation, and virus productivity with identical infectious units for viral genomes than in control vessels. The technology can be considered as an efficient tool for the production of viral vectors with a minimum space requirement.

P138

Production of customized minicircle DNA

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PlasmidFactory offers the service to construct customized minicircle DNA consisting almost only of the functional gene (cassette) of interest (GOI). The patented minicircle production technology is applied to deliver circular and supercoiled (ccc) DNA without sequences of antibiotic resistance or other selection markers and free of other undesired vector sequences (e.g. bacterial origin of replication – ori) and hence a significantly reduced number of CpG motifs. In order to construct and produce the minicircle, the following working steps are applied: 1) Cloning of a parental plasmid (PP) 2) Transformation of a characterized E. coli strain with PP 3) Transformation of a production cell bank for reproducible DNA quality 4) Small-scale evaluation of minicircle productivity 5) Cultivation of cells 6) Production by proprietary minicircle DNA production technology 7) Comprehensive QC 8) Fill and finish of minicircle DNA. With this technology PlasmidFactory managed to produce the smallest functional circular DNA molecule available so far. Although any minicircle production, independent from the recombinase used, is covered by our patents, certain significant differences influence the success in clinical application: Especially those made by ϕ Chi show multimers, whereas we fulfil regulatory requirements by producing a monomeric and supercoiled ccc minicircle DNA. Besides fulfilling requirements concerning product safety, minicircles show a significantly higher efficiency with respect to gene expression levels and duration *in vitro* and *in vivo*, making them useful tools for future therapeutic applications.

P139

GMP manufacturing process for allogeneic cell therapy treatment of acute myocardial infarction

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Ischemic cardiac disease is a crucial challenge for health systems due to high prevalence of the disease and the absence of effective treatments that can prevent cardiac remodelling and development of chronic heart disease after an acute myocardial infarction. In addition to the mortality and the lack of life quality of patients, health systems expend huge amount of money for palliating the effects of this disease. The use of stem cells has been proposed as an effective way to regenerate cardiac damaged tissues.

Coretherapix is developing a cardioregenerative cell therapy based on the use of allogeneic progenitor-stem cells obtained from cardiac biopsies.

To efficiently translate cell therapies to the health system, it is essential to make the manufacturing product affordable in terms of costs, to guarantee the bioequivalence between different cell batches and availability for administration at the most effective time using techniques widely available in hospitals.

Coretherapix has developed a cell manufacturing process compatible with scalable procedure and GMP compliance. We have characterized these cells using different techniques showing the bioequivalence between cell products and their therapeutic capabilities. In addition, we also developed storage, delivery and clinical administration protocol that allows cell based medicinal product administration from the earliest stage of disease. *In vivo* analysis using small and big animals models showed the final product is safe and promotes cardioregeneration. We expect to initiate a first in human clinical trial to check for safety and efficacy of these cellular products, during the first quarter of 2014.

P140

Analysis of stem-cell derived extracellular matrices in chemically modified plastic surfaces usable under GMP-conditions

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There are increasing numbers of studies investigating the application of therapeutic cells. For optimal culturing of these cells under GMP regulations new devices like chemically modified plastic bags must be developed. Plastic surfaces were chemically modified by atmospheric plasma. Thin films of plasma-polymerized 3-Aminopropyl-trimethoxysilane (pp-APTMS) were achieved by dielectric barrier discharge. This surface modification supports growth of adherent cells on the plastic surface. As model cell lines murine MC3T3 and human M7 cells, which can differentiate into fat cells, cartilage cells and bone cells *ex vivo*, were used. These cells produce during culturing their own extracellular matrix (ECM).

Modern LC-MS/MS methods allow the analysis of the total protein content. Here we describe a method to analyze the ECM during the process of cellular multiplication, maturation and differentiation. ECM-proteins were cleaved *in situ* with porcine trypsin to get characteristic peptides. Raw MS data were converted to a data format compatible with the Mascot search engine

(Matrix Science). Mascot Parameters were: peptide mass tolerance -10 ppm, fragment ion tolerance - 0.3 Da. The assigned spectra from all peptide fractions were merged and loaded into the Scaffold 3 viewer for further interpretation.

Preliminary results indicate that secreted ECM matrices showed the typical composition of well-known proteins, but serum proteins bound to a different degree dependent on the state of differentiation of the stem cells. Our newly developed method is suitable analyzing the newly synthesized ECM-proteins derived from the cultured cells. The ECM influenced the attachment of the cells.

P141

In-process characterization of multipotent and pluripotent stem cells

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Introduction: Regenerative medicine using stem cells is facing of several technical challenges to encounter the regulatory points associated to the pharmaceutical guidelines. Cell therapy products emerge from a long process of cell culture, differentiation and purification steps where appropriate cell identity and purity monitoring are mandatory. The validation of relevant quantitative phenotypic markers is required to monitor in-process expansion of mesenchymal stem cells (MSC), induced pluripotent stem cells (iPS) and their derived products.

Material and Methods: Multicolor stainings were designed to deeply characterized MSC, iPS and differentiated cells (in-process impurities or cells of interest) using flow cytometry. To detect very few cell populations, absolute counting mode was assessed. The assays were qualified for accuracy, precision, specificity, limit of detection and quantification.

Results and conclusions: Flow cytometry experiments have shown that MSC are, as expected, positive for CD105, CD90, CD73 and CD44 markers while these cells are negative for CD34, CD45, CD11b, CD19 and HLA-DR. iPS cells are positive for TRA1-60, TRA1-81, alkaline phosphatase and Oct3/4 while they are negative for CD13 and CD15. Specifications for in-process controls were defined. Ongoing works consist in designing complementary multicolor panels to quantify differentiated cells using markers such as osteopontin, adiponectin or aggrecan. In conclusion, panels of surrogate markers that we developed allow characterization and precise follow-up of MSC and iPS during the course of cell expansion. Additional panels on differentiated cells will allow monitoring of multipotent or pluripotent markers associated to markers of spontaneous differentiation or targeted phenotyping shifting.

P142

The effects of integrated biometrics and automated orchestration on the failure modes associated with autologous therapy manufacture

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Unlike traditional pharmaceutical products which have linear supply chains, autologous therapies have circular supply chains with patients receiving therapies generated from their own cellular starting material. Following errors in autologous therapies supply chains, patients receiving therapies generated from other sources may suffer from graft versus host disease and other unwanted responses. Supply chain complexity is exacerbated when

considering the time sensitive nature of these products, for some therapies, cellular starting material must be subjected to cell processing within six hours of removal from the patient. Furthermore, all movements of cellular therapies must observe stringent temperature control.

We examined the failure modes associated with manufacture and treatment of a patient with an autologous therapy, including; harvesting cellular starting material, the movement of cells between treatment facility and manufacturing facility, processing cells to produce the final therapy, the movement of the therapy to the treatment site and engraftment. Each step in the autologous supply chain was assigned a risk score based upon potential harm to the patient, the probability of the activity occurring and the ability to detect an error at that point in the supply chain.

We postulate that using TrakCel, an automated system for tracking, tracing and scheduling treatments, the probability associated with each failure mode occurring will be significantly reduced while the detection rate of errors will be significantly increased, thus improving autologous supply chain efficiency and patient safety. Orchestration technologies would be of great benefit when scaling up/scaling out autologous production.

P143

Pathology of donor, harvest site and culture medium could determinate mesenchymal stem cell properties

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Objective: To compare different harvest site, and culture media for expansion of Mesenchymal Stem Cells from adipose tissue (AMSC), obtained from different donors.

Methods: The fat was obtained from 6 patients by liposuction. Four patients had morbid obesity; in two of them we obtained the fat from abdominal flap during reconstructive surgical procedure post-bariatric surgery. And in the other two patients the fat was harvested from hips and thighs. The two others are healthy donors submitted to cosmetic lipectomy, and the fat was aspirated from hips and thighs too.

Results: The AMSC culture harvested from abdominal flap has failed. These AMSC have limited their ability to duplication and differentiation. However the AMSC obtained from thighs and hip have the same stem cell properties in patients like in healthy donors.

In addition we have cultured AMSC with two different culture media. There are no-differences among AMSC from healthy donors *vs* patients, but there is difference in AMSC duplication and differentiation ability according to the culture media used.

Conclusion: Although it had demonstrated that the abdominal fat contains significant higher frequencies of AMSC compared with fat from the hip/thigh region, we have seen that the abdominal flap is not useful like AMSC source, and in these patients is better use hip and thigh like AMSC source. These sites

seem that are not influenced by the age and the body mass index of the donors. On the other hand the culture medium used to expand AMSC could modified their properties.

P144

Cell therapy for peripheral artery disease: product characterization and stability

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The Cardiocentro Ticino Cell Therapy Unit is authorized for the production of experimental advanced therapy medicinal products (ATMP).

The ATMP for the upcoming CIRCULATE study ("Bone Marrow Derived Cell Therapy in Peripheral Arterial Disease"), currently under evaluation by Swiss regulatory authorities, is named VASASTIM and consists of fresh mononuclear cells isolated from autologous bone marrow through density gradient centrifugation on low density Ficoll-PaqueTM; cells are formulated in saline solution containing 5% human serum albumin. The process has been successfully validated according to Good Manufacturing Practices, for safety (sterility, endotoxin), identity (CD45/CD34/CD133), purity (contaminant granulocytes and platelets) and potency aspects (viability, CFC, CFU-F and invasion assays).

The aim of the present work was to further characterize the product and evaluate its stability, according to specific requests formulated by regulatory authorities.

Fourteen pre-clinical VASASTIM batches were manufactured and extensively tested. All of them comply with viability and purity specifications (viability $\geq 70\%$, granulocytes $\leq 55\%$, platelets/WBC ≤ 10) and contain CD34 ($2.67 \pm 1.86\%$) and CD133 ($0.33 \pm 0.64\%$) cells; a consistent fraction of the cells express CXCR4 ($32 \pm 13\%$), a marker known to correlate with functional activity of bone marrow-derived mononuclear cells. The product contains hematopoietic precursors (CFC assay: 6698 ± 4363 colonies/1E6 cells), mesenchymal precursors (CFU-F assay: 56 ± 40 colonies/1E6 cells) as well as cells with invasion capacity (Fluorimetric invasion assay: Invasion Index $47 \pm 18\%$) and angiogenic potential (CFU-EC assay: 31 ± 19 colonies/1E6 cells).

Nine batches entered a stability program: they were stored at 10°C, previously defined as the optimal temperature for this product, and tested for several parameters at 0-6-20-24 hours. Overall, the results indicate that VASASTIM is stable for at least 20 hours at 10°C.

P145

Fucosylation to control cell fate. Protocol validation under GMP conditions for clinical use

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Introduction: The anti-inflammatory, immunomodulatory and regenerative properties of bone marrow mesenchymal cells (MSCs) configures them as a promising tool for cell therapy in multiple pathologies. However, when intravenously infused, they fail to effectively reach their destination. Adding a fucose residue in the α 1-3 position of the CD44 surface molecule in MSC increases its affinity for E-selectin, which is over-expressed in the endothelial cells of bone marrow, skin, and sites of inflammation. This modification confers the MSCs tropism for these tissues, increasing their recruitment at the needed place. We describe a new fucosylation protocol of human bone marrow MSCs to facilitate their GMP production.

Methods: Mononuclear cells from bone marrow were isolated by Ficoll gradient and seeded in factories with Alpha-MEM supplemented with platelet lysate. MSCs were harvested after the third pass and they were incubated for 1 hour at 37 °C with fucosyltransferase VI enzyme and its substrate. Fucosylation of CD44 and its affinity for the E-selectin ligand were studied by flow cytometry. The MSC were also studied to assess its morphology, phenotype, and differentiation potential.

Results: We obtained a fucosylation efficiency >85% in 10 different samples, with a cell viability after processing of 93%. All samples maintained their phenotype and their osteogenic and adipogenic differentiation potential. Conventional cytogenetics were performed before and after fucosylation in the samples and were normal in all cases.

Conclusion: Our fucosylation protocol is able to effectively modify the MSC CD44 molecule, increasing its E-selectin affinity, without affecting the cell properties. The protocol is easily scalable for clinical production under GMP conditions.

P146

Second generation EPODURE Biopump markedly extends duration of EPO delivery in mice, could prolong therapeutic effect in patients

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Autologous EPODURE Biopumps are processed from dermis needle biopsies, typically measuring 30 mm by 2 mm diameter, harvested from the patient's skin under local anesthesia, transduced ex-vivo with Helper Dependent Adenoviral EPO vector (HDAd-EPO) to continuously secrete erythropoietin. We have reported months of sustained hemoglobin elevation from a single subcutaneous implantation of a few first generation EPODURE Biopumps (BP-1) in anemic patients with renal failure, without EPO injections. However, protein expression from implanted Biopumps could be limited post-implantation by various factors, including local inflammation, causing reduced metabolic function, loss of gene copies, or expression cassette instability. To minimize these factors and further improve duration of clinical effect, anti-inflammatory agents were tested for use during implantation and second generation EPODURE Biopumps (BP-2) were developed incorporating HDAd gene expression cassettes designed to increase stability of expression: i) CpG free HDAd-MAR-EF1 α -optEPO, and ii) HDAd-MAR-CAG-optEPO-WPRE. BP-1 and BP-2 producing comparable daily EPO amounts *in-vitro* were each implanted subcutaneously into SCID mice,

with or without administration of Depo-Medrol (DM) at the implantation site. Without DM, serum EPO levels remained 7–20 folds higher using BP-2 compared to BP-1 for over 100 days post implantation. With DM administered, serum levels remained 6–7 folds higher with BP-1 and 40–50 folds higher with BP-2 compared to BP-1 without DM. Since clinical studies to date have used BP-1 without DM, these results suggest that BP-2 with Depo-Medrol may potentially increase sustained therapeutic levels of target proteins and could prolong therapeutic effect in patients.

P147

Validation of a nucleic acid amplification technique for detection of mycoplasma in cell-based medicinal products (CBMPs)

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Introduction: The contamination of cell cultures by mycoplasma is a widespread and serious problem in biopharmaceutical production. The European Pharmacopoeia (7.0, 2.6.7) prescribes empiric test methods for determination of mycoplasma biosafety: the broth and agar cultivation procedure and a DNA staining technique using indicator cell cultures. Both methods are time-consuming (28 days). This long period of time is not relevant for CBMPs. The Regulator accepts the use of nucleic acid amplification techniques as an alternative method after suitable validation study. Material and

Methods: Amplification of 16S rRNA gene by quantitative PCR was performed on 8 mycoplasma species (*M. arginini*, *M. hyorhinitis*, *M. fermentans*, *M. synoviae*, *M. orale*, *M. pneumoniae*, *S. citri* and *A. laidlawii*). Each mycoplasma reference strain was spiked in a CBMP at 10 GC/reaction and specificity was tested at 105 GC/reaction. The assay was qualified for limit of detection, specificity and robustness.

Results and conclusions: All spiked samples were positive for the presence of mycoplasma DNA at 10 GC/reaction. Specificity study showed that the assay did not detect DNA from human, yeast or gram-positive bacteria with close phylogenetic relation to mycoplasmas, such as *Clostridium acetobutylicum*, *Lactobacillus acidophilus* and *Streptococcus pneumoniae*. Robustness was assessed using different thermocyclers and several dilution buffers. Ongoing study is performed on culture spiked with CFU-quantified mycoplasma mid-log phase culture preparations in order to validate this method at 10 CFU/ml as required by European Pharmacopoeia (comparability study). In conclusion, data from PCR-based Mycoplasma test demonstrate that this assay exceeds European Pharmacopoeia requirements and constitutes an alternative of choice for rapid testing of CBMPs.

P148

How to meet the challenges associated with the development of cell-based medicinal products

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During the past decades, advances in cell biology, developmental biology and stem cell research have fostered the

development of new potential therapies for diseases for which no therapeutic option had been available. Marketing authorization of cell-based medicinal products requires, as for all medicinal products, the demonstration that the product is consistently manufactured to a predefined quality, and that it is safe and efficacious in patients. Since cell-based products are often complex mixes of cell types, clinical performance depends on rigorous control of the manufacturing process and specifications which is in turn limited by the difficulty to design analytical methods to characterize cell mixtures. Investigational medicinal products intended for clinical use should be manufactured in compliance with current Good Manufacturing Practice regulations (cGMP). This is a challenge for small companies or academic laboratories aiming at obtaining proof-of-concept in man. An additional hurdle in manufacturing is the need to upscale production while conserving the product characteristics and specifications when moving away from the bench to clinical studies. Advices will be provided on how to face main challenges associated with the development of cell-based medicinal products with the aim to deliver high quality therapeutic cells fit for clinical phases.

P149

Transplantation of bone marrow mononuclear cells for the treatment of ischemic syndromes

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Bone marrow-derived mononuclear cells (BM-MNCs) are a heterogeneous population exerting haematological, immunological and other physiological functions. There is evidence that the endothelial progenitors in BM-MNCs have a fundamental physiological role in the processes of post-natal neovascularization. Ischemic or damaged tissues physiologically recruit BM-MNCs which stimulate the mechanisms of neovascularisation and tissue repair. This process is cumulative with continuous recruitment of cells over a varying period of time. Therefore, when these cells are used therapeutically for vascular restoration in ischemic tissues, they are carrying out nothing other than their physiological mission. Nevertheless, the European Medicines Agency classifies autologous BM-MNCs as Advanced Therapy Medicinal Product (ATMP) when used for clinical purposes different from haematological restoration because they consider this as their unique essential function. Surprisingly, when the same identical heterogeneous group of progenitor cells, comprising endothelial progenitors, is infused for haematopoiesis restoration it is not considered ATMP. Hence we long for the same rule being applied to BM-MNCs when therapeutically used to treat ischemic syndromes. If these products are considered ATMP, it will impact negatively on European Public Health Services and hospitals authorized for bone marrow transplantation (BMT) will suffer a contradictory situation being able to process the bone marrow – for BMT in immunocompromised patients receiving an allogeneic product – but being obliged to send it to a company for processing (using the same procedure and technology), with the attendant costs, for immunocompetent patients receiving their own cells. Regulating these products as cell transplant streamlines the entire process and offers affordability and safety.

P150

Off-label use of GMO medicines: Blessing or Burden?

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The market authorization for a medicinal product contains a precise description of the therapeutic uses for which the medicinal product is intended. In practice, however, medicinal products are regularly prescribed for treatments outside the scope of their registered therapeutic uses, a practice referred to as 'off-label use'. It is estimated that in Europe for example around half of the drugs prescribed to children is off-label.

Recently, the first human medicine based on a genetically modified organism (GMO medicine) was authorized for use in the European market. This GMO-medicine, called Glybera is based on an AAV vector and is developed to treat lipoprotein lipase deficiency. Besides efficacy and human safety, a key issue in the evaluation of GMO medicines for market authorization is the environmental safety. Despite the experience with off-label prescription of 'regular' medicines, the off-label use of GMO medicine is not accounted for in EU legislation and is not included in the environmental risk assessment that is performed for a market authorization. Consequently, the off-label prescription of GMO medicines fall under each member state's national environmental legislation and might imply that the off-label use of a registered GMO medicine requires a license for deliberate release into the environment. This might put the physician in a dilemma with the interest of the patient on one hand and the interest of the population and the environment on the other. The authors will discuss the consequences of this undesirable situation and will suggest possible solutions to circumvent the issue.

P151

Cell expansion system for scale up of cellular immunotherapies

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The widespread adoption of autologous cellular immunotherapies requires the development of robust, scalable manufacturing workflows to generate sufficient, high quality cells for infusion into patients. The Cell Expansion System, formally known as the WAVE Bioreactor™ system, is often the equipment of choice for the final expansion phase of the immune cell therapy manufacturing process before patient infusion. The 5 W and 25 W systems are closed, automated, single use systems based on the premise that simple rocking motion generates sufficient mixing and aeration to enhance cell growth and, when combined with perfusion, enables high density culture thereby reducing manufacturing footprint. The 5 W system which combines basic rocking motion, temperature control and perfusion allows for initial scale up during product development. The newly launched 25 W allows for complete control over the cell culture environment with the addition of data logging and remote monitoring and control. This makes it an ideal instrument for process development and ultimately clinical manufacturing. We will present data showing that densities of 2×10^7 cells/mL in 1 L volume can be routinely achieved. Furthermore, the population of cells can be shown to be phenotypically and functionally healthy.

P155

iPSC-based strategy to correct the bleeding phenotype in haemophilia AM Talmon¹, G Ranaldo¹, C Olgasi¹, A Lombardo², S Merlin¹, E S Cannizzo¹, A Raya³, L Naldini², P Schinco⁴, A Follenzi¹¹University of Piemonte Orientale, Novara, Italy; ²Tiget, H San Raffaele, Milan, Italy; ³Inst. for Bioengineering of Catalonia, Barcelona, Spain; ⁴Az. Osp/Univ S.Giovanni Battista-Molinette, Torino, Italy

Hemophilia A (HA) is a X-linked bleeding disorder caused by mutations in the coagulation factor VIII (FVIII) gene. Existing therapy includes recombinant or plasma-derived FVIII infusion. Reprogramming of genetically corrected somatic cells can be used to generate high amount of autologous, disease-free induced Pluripotent Stem Cells (iPSCs), which can be differentiated into progenitor cells relevant for gene and cell therapy applications. In hemophilic patients, to harvest fibroblasts from skin biopsies is risky; thus, we utilized patient-derived peripheral blood cells as an easy-to-access source of cells to reprogram.

Development of a novel strategy for HA treatment, generating human B-domain-deleted FVIII (hBDD-FVIII)-corrected patient-specific iPSCs and differentiating them into functional endothelial cells (ECs), secreting FVIII in hemophilic mice after transplantation.

Mononuclear cells (MNC) were isolated from healthy and hemophilic donors by ficoll gradient. Cells were corrected by LV-hBDDFVIII transduction and reprogrammed with a cre-excisable polycistronic LV carrying OCT4, SOX2 and KLF4. MNC-derived iPSCs were characterized by Alkaline Phosphatase (AP), immunofluorescence stainings and RT-PCR of specific stem cell markers, telomers length and demethylation of Oct4 promoter. The iPSCs in vitro differentiation potential was analyzed by RT-PCR of three germ layers markers in embryoid bodies (EBs) and by adipogenic, osteogenic and chondrogenic differentiation. iPSCs were differentiated in ECs using EB medium containing 50 ng/ml of hVEGF. Cells were evaluated for morphology, expression of endothelial markers and GFP-expression when transduced with LV carrying GFP under the control of endothelial-specific promoters: Flk-1 and Tie2. Two millions Flk1-GFP cells were transplanted by portal vein injection in MCT-treated NOD-SCID HA mice. Mice were killed 96 hours later and cell engraftment analyzed by GFP-staining of liver sections.

Reprogrammed MNC gave rise to iPSCs in about 45 days. iPSCs displayed embryonic stem cells (ESC)-like morphology: colonies were compact, uniform and with defined borders when grown on feeders. iPSCs were positive for AP staining and expressed specific stem cell markers at RNA and protein level, showing activation of the endogenous reprogramming factors. Moreover, iPSCs differentiated in osteogenic, chondrogenic and adipose tissues. EB expressed markers of the three germ layers: alpha-fetoprotein, brachyury and nestin. Nevertheless, iPSCs showed demethylation at CpG islands at the core of Oct4 promoter, epigenetic marker of complete reprogramming to a pluripotent state. Additionally, telomeres length of original and reprogrammed cells did not show changes indicating telomerase reactivation. Importantly, iPSCs differentiated into ECs, acquiring a typical endothelial-like morphology with an increased expression of ECs markers, such as CD31, KDR, vWF and FVIII. Then, iPSC-derived ECs were transduced with LV-expressing GFP under the control of Flk-1 and Tie2. Flk-1-GFP cells were transplanted in NOD-SCID HA mice. GFP cells were detected in liver sections up to 1 week post transplantation.

Overall, these data will be instrumental to assess the engraftment, the proliferation and the levels of FVIII expression from differentiated, gene corrected and reprogramming factor free iPSCs to confirm the suitability of this approach for HA gene-cell-therapy.

P156

Generation of induced pluripotent stem cells (iPSCs) using non-viral vectorsB Fernández¹, C Prieto¹, C Marín¹, A Revilla¹, A Iriando¹, C González¹, F González², I Liste¹¹Unidad de Regeneración Neural; Unidad Funcional de Investigación de Enfermedades Crónicas. Instituto de Salud Carlos III (ISCIII), Madrid; ²Memorial Sloan Kettering Cancer Center, New York

Somatic cells can be reprogrammed to induced pluripotent stem (iPS) cells by ectopic expression of specific sets of transcription factors. The delivery of these transcription factors has mostly entailed the use of integrating viral vectors (retroviruses or lentiviruses), carrying the risk of both insertional mutagenesis and oncogenesis due to misexpression of these exogenous factors; thereby limiting the therapeutical application of the generated cells.

The main goal of this work was the generation of iPS cells from different sources of somatic cells using non viral vectors, and the characterization of the generated iPS cells. For that we have used polycistronic vectors expressing three or four transcription factors (Oct4, Sox2, Klf4 with/without c-myc). The vectors were introduced by nucleofection. In order to increase reprogramming efficiency, several reprogramming enhancer factors have also been tested.

We have successfully generated and established iPS cells from mouse embryonic fibroblasts (MEFs) and human fibroblasts. These cells have been characterized morphologically, by the expression of endogenous pluripotency transcription factors, and the potential to differentiate into specialized cells from the three germ layers, including dopaminergic neurons. Systematic comparison with Embryonic Stem Cells (ES cells) and already established viral iPS cells it has been also performed.

Our results demonstrate that although the reprogramming efficiency is extremely low, it is possible to generate "bona fide" iPS cells using non-viral and non-integrative vectors. This opens up a whole range of biomedical applications such as cell therapy, drug development and disease modeling.

P157

Development of a liver-specific marker transgenic mouse for the study of alternative pathway of hepatic regeneration from bone marrow cellsM García-Bravo¹, A Fernández², R Chinchón¹, M Ángel Martín¹, N Rubio³, J Blanco³, L Montoliú², J C. Segovia¹¹Cell Differentiation and Cytometry Unit. Hematopoietic Innovative Therapies Division, Centro de Investigaciones Energéticas, Medioambientales y Tecnológicas (CIEMAT) and Centro de Investigación Biomédica en Red de Enfermedades Raras (CIBER-ER), Madrid, Spain; ²Departamento de Biología Molecular y Celular. Centro Nacional de Biotecnología (CNB-CSIC), Madrid, Spain; ³Cellular Therapy, Department of Chemical and Biomolecular Nanotechnology, Instituto de Química avanzada de Cataluña

The development of new clinical approaches for treating chronic liver diseases is urgently needed. Several approaches are being explored, including the potential use of non-hepatic adult stem cells based on their ability to promote hepatic regeneration in mouse models of liver damage. The *Fech^{m1Pas/m1Pas}* mouse model show, besides the erythropoietic symptoms, hepatic damage from birth and an atypical ductular reaction recapitulating severe cases of erythropoietic protoporphyria in humans. In this model, we demonstrated non-fusion mechanism of generation of bone marrow-derived hepatic cells, including myofibroblasts, hepatic progenitor cells (HPCs) and hepatocytes, which contributed to restore and regenerate the hepatic tissue, improving significantly the liver condition (Garcia-Bravo *et al.*, 2009). A more detailed analysis revealed a spatial gradient of bone marrow-derived hepatocytes, suggesting that the hepatic regeneration could be in charge of bone marrow-derived HPCs. With the objective of obtaining a broader portrayal of these processes, we are developing a mouse system that will facilitate the identification of hepatocytes originated from bone marrow-derived cells through a HPC stage. For that, we have developed a *luciferase:EGFP* specific hepatic marker transgenic mouse that will allow us to trace the transplanted cells in their way to the liver, their engraftment and their contribution to the regenerative response. We show here the characterization of this specific transgenic mouse as a validated tool for monitoring hepatic regeneration. This transgenic mouse will contribute to the multiple transgenic mouse system that will help to outline the liable mechanisms for alternative hepatic regeneration in chronic liver disease that could open a window for therapeutic modulation.

P158

Efficient somatic cell reprogramming using low doses of self-complementary AAV-DJ vectors

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The field of induced pluripotent stem cells (iPSC) is in continuous search for new methods that allow efficient reprogramming of somatic cells without integration of the reprogramming factors into the iPSC genome. AAV vectors should represent an ideal tool for this purpose as they efficiently transduce a wide variety of cells and rarely integrate. We therefore generated self-complementary AAVs expressing the four Yamanaka factors (Oct-4/3, Klf4, Sox2, c-Myc) and pseudotyped them with the chimeric DJ capsid (a hybrid of serotypes 2-8-9). Remarkably, this unique combination of potent genome and capsid allowed us to efficiently reprogram mouse embryonic fibroblasts using marginal doses of 1e3 vectors per cell. In fact, use of higher doses resulted in drops of colony numbers and delays in their appearance, suggesting a threshold of the four factors for optimal reprogramming. In addition, we obtained evidence for cytotoxicity from overexpression especially of Sox2. Notably, the AAV-derived miPSC express a panel of typical stem cell markers as confirmed by immunofluorescence and RT-PCR. Reprogrammed colonies also spontaneously differentiate into beating cardiomyocytes (mesodermal lineage), further confirming their pluripotency. Curiously, we detected traces of AAV vector sequences by PCR in miPSC genomic DNA. We are thus currently conducting LAM-PCR to dissect whether these are episomal or integrated, and, in the case of

integration, whether this occurred randomly or with a bias towards hot spots that favor reprogramming. Based on our vectors' great performance in murine fibroblasts, we are now assessing them for reprogramming of various types of clinically relevant human somatic cells.

P159

Efficient mRNA-based reprogramming by use of the Vaccinia virus proteins E3, K3 and B18R

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The use of conventionally derived induced pluripotent stem (iPS) cells for clinical applications is limited by the risk of possible genomic alterations. RNA-based reprogramming is able to overcome this major obstacle. Nevertheless methods published so far are relatively time-consuming and expensive. Here we present a method to enhance efficiency of RNA-based reprogramming by use of synthetic mRNA coding for the *Vaccinia virus* proteins E3, K3 and B18R (EKB).

Recognition of foreign RNA by cells initiates defense mechanisms including RNA-degradation and a halt in protein translation which obviously interfere with gene transfer based on synthetic mRNA. This is especially the case, when the transfer has to be repetitive as it is necessary for mRNA-based reprogramming of somatic cells.

Here we show that the expression of mRNA-coded proteins is greatly enhanced by co-transfer of synthetic mRNA coding for EKB. Reduction of the interferon response to synthetic mRNA allows thereby to enhance the survival of target cells. Shown for the first time, EKB mRNA enables us to transfer unmodified synthetic mRNA repetitively. With further addition of microRNAs 302a-d, 367 it permits RNA-based reprogramming by transfer of reprogramming transcription factors. The reprogramming efficiency is thereby considerable higher than accomplished with the use of modified synthetic RNAs.

Established Ribo-iPS cells display high quality confirmed by stem cell morphology and high expression of embryonic stem cell-markers like OCT4, REX1, TRA-1-60 and alkaline phosphatase.

We conclude that the use of EKB is a powerful tool to enhance RNA-based reprogramming and in general RNA-based transfer of genetic information.

P160

Endothelial differentiation from iPSC and ESC

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Conventional treatments of coronary artery disease, such as angioplasty and bypass surgery, are not always sufficient or suitable for all patients. Additionally, re-endothelialization of vessel walls may be impaired after coronary artery stenting. Therefore, it is essential to develop additional regenerative therapies to promote therapeutic angiogenesis in ischemic myocardium and endothelialization of stented vessels.

ESC and iPSC can divide indefinitely and they have the capacity to differentiate into any cell type. This ability gives great potential to regenerative medicine and personalized cell therapy. However, the pathway of EC differentiation from ESCs is not fully understood. Consequently, *in vitro* derivation of vascular cells from pluripotent cells and their characterization have proved to be difficult.

We have developed an efficient 2D EC differentiation method using human iPS and ES cells. The protocol employs specific EC-favoring cell culture media and appropriate growth factors, such as VEGF-A, BMP-4 and TGF- β inhibitor with precise dosage and timing. Characterization and functional assays of these EC includes cobble-stone morphology, EC-specific marker expression analyses by FACS, tube forming ability, uptake of acetylated LDL, NO production and Matrigel plug assay in immunodeficient mice. Laminar shear stress, hypoxia and FACS-sorting might also be important contributors in stable EC differentiation and maturing.

P161

Impaired generation of DNA-PK deficient iPS discloses a role of NHEJ in pluripotency induction

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High expectations in regenerative medicine awoken by the discovery of induced pluripotent cells (iPSC) demand for a thorough characterization of pathways affecting the efficiency and safety of its generation. Non-Homologous End Joining (NHEJ) is the preferred pathway for DSB repair during interphase and thus its possible interaction in the reprogramming process is of utmost interest. We used *Prkd^{scid/scid}* murine embryonic fibroblasts (MEFs), deficient in DNA-PKcs, to investigate whether defective NHEJ had any influence in somatic cell reprogramming. We found that, independently of the reprogramming platform used, the generation of iPS is hampered in DNA-PKcs deficient cells, resulting in four to sevenfold decrease of AP-positive colonies and, in most cases, followed by either premature differentiation or loss of the clones generated after the delivery of the reprogramming factors. Several intrinsic factors were tested in the Scid MEFs including growth ratio, transduction susceptibility and telomere length among others, but the only factor that could account for their impeded cell reprogramming was a premature senescence evidenced after SA- β -Gal staining coherent with an overexpression of p16^{INK/4a}. The implementation of Sleeping Beauty reprogramming transposons (Tn) resulted in increased yield of AP-positive colonies and allowed us to isolate Scid Tn-iPS clones with demonstrated pluripotency and confirmed parental radiosensitive phenotype. Our results show the link between NHEJ and cell reprogramming. Besides, this new disease-specific iPSC model will help to understand the physiological consequences of DNA-PKcs mutation during development, hence facilitating future improvements on cell and gene therapy strategies for the disease.

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Genetic correction of CSF2RA deficiency in patient derived iPSCs allows generation of functional macrophages and serves as a therapeutic model for congenital pulmonary alveolar proteinosis

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Pulmonary Alveolar Proteinosis (PAP) due to a deficient GM-CSF/IL-3/IL-5 receptor on monocytes/macrophages (M/M) constitutes a severe lung disease caused by the functional insufficiency of alveolar macrophages, which require GM-CSF signalling for terminal maturation. While treatment options for PAP are rare, we here evaluated the suitability of iPSC-derived M/M for functional disease modelling and (after gene correction) as a donor source for intra-tracheal transplants. PAP-iPSC were generated from CD34 bone marrow cells of an GM-CSF α -chain (*CSF2RA*) deficient PAP patient by OCT4/SOX2/KLF4/c-Myc-based reprogramming, demonstrating SSEA4/Tra1-60 expression, reactivation of endogenous OCT4, SOX2, and NANOG, OCT4-promoter de-methylation, differentiation into cells of all three germ layers, as well as lack of chromosomal abnormalities. Hematopoietic differentiation yielded M/Ms of typical morphology and phenotype (CD14, CD11b, CD45) for all clones. Upon functional analysis GM-CSF independent characteristics of M/Ms (cytokine secretion, basal phagocytosis) were maintained, whereas GM-CSF dependent functions (CD11b activation, GM-CSF uptake, and pSTAT-5 downstream signalling) were profoundly impaired, thus establishing M/M differentiation of PAP-iPSC as a functionally relevant disease model. When a PAP-iPSC clone was transduced with a 3rd gen. SIN-lentiviral vector expressing a codon-optimized *CSF2RA*-cDNA from a combined ubiquitous-chromatin-opening-element (UCOE)/ EFS1a-promoter sequence, stable *CSF2RA*-expression was observed with no detectable effects on pluripotency behaviour. Furthermore, upon differentiation to M/Ms *CSF2RA*-expression was maintained and complete reconstitution of GM-CSF dependent functions was achieved. In summary, we have established iPSC-lines from a *CSF2RA*-deficient PAP-patient. M/Ms differentiated from these cells represent a functional disease model in which complete reconstitution of GM-CSF dependent functions is observed upon *CSF2RA* gene transfer.

P163

Genomic engineering of the cystic fibrosis gene in patient-derived iPSC cells

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Cystic Fibrosis is a hereditary disease produced by the absence or malfunctioning of the Cystic Fibrosis Transmembrane Conductance Regulator (CFTR) gene. To date over 1,200 alterations in the DNA composition of the CFTR gene have been detected, although the deletion of the phenylalanine in position 508 ($\Delta F508$) is responsible for more than 70% of the cases described in the European Population. CF is a degenerative disease, which can be considered as the main genetic cause of death in Caucasian children. Its first manifestations occur in early childhood, generally affecting the respiratory tract, and later extending to other organs.

The identification and isolation of the gene responsible for the disease raised great expectations of finding a treatment. However, such hopes have yet to be realized. Different attempts to develop effective gene therapy protocols have not provided satisfactory results. We have opted instead for a genomic engineering approach, in which homologous recombination aided by gene editing nucleases has been used to eliminate the genetic defect of the gene.

The method was applied simultaneously in human and mouse cells, and includes the following steps:

- (I) Production of iPSC cells from keratinocytes obtained from $\Delta F508$ CF patients and $\Delta F508$ mutant mice;
- (II) Correction of the mutation in the iPSC cells with a combination of gene-editing nucleases and PiggyBac technology;
- (III) in vitro differentiation of repaired iPSC cells up to a state in which the derived cell expresses CFTR;
- (IV) Functional analysis of the repaired CFTR protein.

Steps I through III have been completed and results will be provided at the meeting. Step IV is now under investigation

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Congenital Pulmonary Alveolar Proteinosis iPSC-derived hematopoietic progenitor cells (HPCs) reveal functional defects upon GM-CSF administration

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Congenital Pulmonary Alveolar Proteinosis (PAP), caused by a mutation in the GM-CSF receptor β -chain (*CSF2RB*), is an extremely rare lung disease resulting from the inability of alveolar macrophages to clear the alveolar spaces from surfactant phospholipids. Since current treatment options are extremely limited, we here investigate the suitability of a gene therapy approach based on hematopoietic cells derived from induced pluripotent stem cells (iPSC). Studies were performed in a murine model for *CSF2RB*-deficiency (*CSFRB*^{-/-}). Therefore iPSCs were generated from lin⁻ bone marrow cells of *CSFRB*^{-/-} mice utilizing lentiviral overexpression of the standard Yamanaka-factors OSKM. Generated β c-PAP-iPSCs displayed all major pluripotency criteria such as SSEA-1 expression, alkaline phosphatase activity, endogenous *Sox2*, *Oct4*, *Klf4*, *Nanog* re-

activation, as well as three germ layers differentiation capacity assessed by teratoma formation. Hematopoietic differentiation of β c-PAP-iPSC lines yielded CD41 HPCs on day 8, which further directed differentiation generated granulocyte-, monocyte-, and erythrocyte-containing colonies comparable to control iPSCs. However, upon differentiation with GM-CSF, β c-PAP-iPSCs - in contrast to control iPSCs - were unable to form GM-type colonies, recapitulating the defect found in primary lin⁻ bone marrow cells of *CSFRB*^{-/-} mice. Furthermore, in the presence of M-CSF β c-PAP-iPSC-derived day 8 CD41 HPCs differentiated into CD45, CD11b, F4/80 monocyte/macrophage-like cells.

In summary, we generated murine *CSF2RB*-deficient iPSC lines, which upon hematopoietic differentiation recapitulated GM-CSF dependent functional defects characteristic of PAP. These cells - following genetic correction - appear as a promising source for future cell and gene therapy strategies.

P165

GDNF prevents dopaminergic neurodegeneration in iPSC cell-based models of familial and sporadic Parkinson's disease

AUTHORS PENDING

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Parkinson's disease (PD) is among the most frequent human neurodegenerative diseases characterized by a gradual degeneration of nigrostriatal dopamine-containing neurons (DAN). None of the currently approved drugs significantly prevents PD progression. Thus, it is urgent to establish new therapies that can be combined with existing treatments to further improve the quality of life of patients and possibly slow down or halt disease progression.

We have recently generated a series of induced pluripotent stem cell (iPSC) lines representing both idiopathic (ID) and familial PD patients associated to LRRK2 mutations, and identified distinct PD-related neurodegeneration phenotypes arising upon long-term culture in PD patient-specific iPSC-derived DAN. To search for possible modulators of this increased susceptibility to undergo neurodegeneration, we exposed ID and LRRK2 iPSC-derived neural cells to GDNF for 6 weeks. GDNF-treated samples showed increased neurite numbers and length, as well as increased cell size of DAN in both ID-PD and LRRK2-PD cell lines, compared with non-treated samples. Exposure to GDNF promoted neuronal survival, as judged by the decrease in the number of TH/CASPASE3 cells in GDNF-treated versus untreated samples, consistent with a GFR1 α /RET activation of Akt and its downstream target mTORC1.

Our data show that iPSC-derived neuronal cells are valuable models for measuring responses to neuroprotective strategies and they may help to identify potential new drugs and future treatments for PD.

P166

iPSCs generation from genetically-corrected *Brca2* deficient cells (*Brca2*^{Δ27/Δ27})

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Recent studies have demonstrated that cell reprogramming is particularly inefficient in cells defective in DNA repair pathways. Mutations in the *Brca2/Fancc1* gene cause defects in DNA damage repair systems that lead to genomic instability, due to its essential role in homology directed repair (HDR) through the interaction with *Brca1* and *Rad51*. Based on these observations we studied the involvement of HDR in cell reprogramming and evaluated the molecular processes involved in the generation of iPSCs using Fanconi anemia cells with a hypomorphic mutation in *Brca2* (*Brca2*^{Δ27/Δ27}).

Our results showed that iPSC from *Brca2*^{Δ27/Δ27} MEFs were only efficiently generated when these cells were gene-complemented with a *BRCA2*-lentiviral vector (LV). Both the efficiency of cell reprogramming and the generation of nuclear *Rad51* foci during the reprogramming process were very limited in *Brca2* mutant cells, in contrast to observations made in either WT or *Fancc1*^{-/-} cells, demonstrating that implication of HDR during cell reprogramming. The generation of disease-free *Brca2*^{Δ27/Δ27} iPSC indicates that the reprogramming defects observed in *Brca2*^{Δ27/Δ27} cells can be efficiently overcome by gene therapy strategies performed prior to the reprogramming process.

P167

Horizontal transfer of RNAs mediated by induced pluripotent stem cell-derived microvesicles

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Background: Microvesicles (MVs) are circular membrane fragments shed into the extracellular environment by direct budding from the cell plasma membrane or are derived from the endosomal compartment. MVs originated from a given cell type may act as mediators of cell-to-cell communication by transferring surface determinants and genetic material in the form of mRNA and microRNA.

Objective: In this study, we have analyzed biological content of microvesicles derived from human induced pluripotent stem cells (hiPS) and show that they can transfer endogenous as well as exogenous RNAs to recipient cells.

Results: Human iPSCs were generated from umbilical cord-derived mesenchymal stem cells (UC-MSC) with Sendai virus in serum-free and feeder-free conditions. Cells were characterized by expression of surface antigens, genotyping, immunohistochemistry and teratoma formation assay, showing their pluripotential nature. hiPS-MVs were harvested by ultracentrifugation. The

transcript levels in MVs and hiPSCs were compared by real-time RT-PCR method demonstrating that hiPS-MVs are particularly rich in miRNAs and contain several mRNAs. Moreover, we showed that hiPS-MVs can transfer their biological content to target cells, human heart-derived MSCs. Lastly, we engineered hiPSCs to express a marker gene, green fluorescent protein (GFP) and we showed that exogenous material can also be transferred to hiPS-MVs.

Conclusions: this study suggests that hiPS-MVs may be important mediators of signaling between various stem cell types and may be useful therapeutic tools for horizontal transfer of RNAs to target cells.

P170

Systemic administration of adeno-associated viral (AAV8) vectors expressing human ADA as in vivo enzyme replacement therapy in the ADA-deficient mouse

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Adenosine deaminase-deficient Severe Combined Immune Deficiency (ADA-SCID) combines a severe primary pan-lymphoid immune deficiency and a systemic purine metabolic disorder affecting multiple organs. Without treatment, ADA^{-/-} mice perish at 3 weeks of age; however, they were rescued by intravenous injection of a lentiviral vector expressing human ADA (5×10¹⁰ TU/kg) which resulted in high ADA expression in liver and lung. Given the high lentiviral vector dose required for adequate ADA expression, we evaluated serotype 8 adeno-associated virus (AAV8) vectors expressing human ADA. ADA^{-/-} and ADA^{-/-} neonates received 1×10⁹, 1×10¹⁰ or 1×10¹¹ genome copies (gc)/kg of AAV8/EFS-ADA or AAV8/MND-ADA. Survival of ADA^{-/-} mice required ≥10¹⁰ gc/kg of AAV8/MND-ADA or ≥1×10¹¹ gc/kg of AAV8/EFS-ADA. Surviving ADA^{-/-} mice (6 months) that received 10¹¹ vg/kg of AAV8/MND-ADA or AAV8/EFS-ADA had immune reconstitution with very low VC (0.001–0.00001) in bone marrow, thymus and spleen. In cardiac muscle, the mean VC was 2.1/–0.9 with 10¹¹vg/kg AAV8/EFS-ADA and 2.8/–1.7 with 10¹¹ vg/kg AAV8/MND-ADA. In liver, lung, kidney and brain there were 0.01–0.1 vector copies (VC) with both vectors. In the treated ADA^{-/-} littermates, liver VC and ADA activity were dose-dependent from 10⁹–10¹¹ vg/kg. In surviving ADA^{-/-} mice, VC was dose-dependent in heart, kidney and brain, but not in liver, such that only mice with VC in the liver above a threshold level survived within cohorts that had lower ADA expression (lower vector dose or weaker EFS promoter). In conclusion, AAV-mediated gene transfer for in vivo ADA enzyme replacement represents an additional modality for the treatment of ADA SCID.

P171

Persistent multilineage engraftment and WASP expression after lentiviral mediated CD34⁺ cell gene therapy for the treatment of Wiskott-Aldrich Syndrome

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Wiskott-Aldrich Syndrome (WAS) is an X-linked immunodeficiency characterized by thrombocytopenia, infections, autoimmunity and lymphomas. Gene therapy (GT) with ex vivo transduced hematopoietic stem cells (HSC) could represent a valid therapeutic option for patients lacking an HLA-identical donor. Our phase I/II clinical trial is based on infusion of autologous CD34 cells, transduced with lentiviral vector encoding for WAS under the control of the homologous 1.6 kb WAS, combined to reduced intensity conditioning. We recently described the safety and efficacy results in the first 3 patients. (Aiuti *et al.*, Science, epub 2013 Jul 11) and three additional patients have been treated. Transduction of clonogenic progenitors was highly efficient ($94.4 \pm 4.2\%$), with a mean VCN/genome in bulk CD34 cells of 2.7 ± 0.98 . Robust and stable engraftment was observed in all hematopoietic lineages from PB and BM, persisting at stable levels over time. At the latest follow up, the proportion of vector transduced BM clonogenic progenitors ranged from 22 to 60%. We estimated that each transduced progenitor cell contained on average 1 to 1.6 copies of the vector. Accordingly, WASP expression, measured by flow cytometry, was observed in PB platelets, monocytes and, as expected due to the selective advantage, in a higher proportion of lymphoid cells (WASP cells: $70.9 \pm 4.8\%$). Among lymphoid cells, CD8 cells and memory T cells showed the highest proportion of WASp expressing cells (80–90%). Moreover, immunological functions, including responses to anti-CD3 proliferation, improved after gene therapy. All six patients are currently clinically well, independent from platelet transfusions, with the current follow up ranging from 4 to 36 months. In conclusion, LV-WAS gene therapy results in stable engraftment of multilineage transduced HSC even when combined to reduced intensity conditioning.

P172

New Detection method for p22-phox-deficient Chronic Granulomatous Disease Heterozygote Carriers in Jeju

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Chronic granulomatous disease (CGD) is a genetically heterogeneous disease caused by defects in the genes encoding any of the NADPH oxidase components. The estimated prevalence of CGD is between 1 in 200,000 and 1 in 250,000 individuals, with variable rates in different countries. According to a compilation by the Korean College of Pediatric Clinical Immunology, the prevalence of CGD in Korea was 0.9 in 1,000,000 individuals. Surprisingly, the prevalence of CGD on Jeju Island is 20.7 in 1,000,000 individuals. We found that 12 patients from 10 unrelated families on Jeju Island had an identical homozygous single-

base substitution of C to T in exon 1 (c.7C>T) of the CYBA gene. We hypothesized that the high prevalence of CGD on Jeju Island is associated with an identical mutation inherited from a common ancestor or proband. The aim of this study was to develop an assay to detect heterozygote carriers of the genotype specific to Jeju Island. We developed three specific primers, and nested PCR was employed using whole blood samples as a source of genomic DNA. Using the new detection method, 704 individuals were tested. Nine individuals were detected as carriers and the expected number of carriers is 1.3 in 100 individuals.

P173

Biosafety studies of a clinically-applicable lentiviral vector for gene therapy of RS-SCID

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Radio-sensitive severe combined immunodeficiency (RS-SCID) patients lack the Artemis protein, fail to produce T and B cells and have increased sensitivity to ionizing radiations. In the perspective of a phase I/II gene therapy trial, we conducted a controlled preclinical biosafety study in a murine model of the disease. The vector, a SIN rHIV pseudotyped with VSVg expressing the human Artemis cDNA under control of the short intronless EF1a promoter, was purified by chromatography according to a standard method and was quality-controlled. The vector achieves non toxic and physiological levels of transgene expression in cell lines. The biosafety protocol evaluated histological and biological parameters in treated mice throughout the long-term follow-up of primary (64 mice, 6 months) and secondary recipients (92 mice, 6 months). Transduced hematopoietic progenitor cells engrafted into irradiated Artemis-deficient female mice and provided long-term immune reconstitution of T and B cell lineages with the restructuring of lymphoid tissues. In 17% and 21% mice from primary and secondary grafts respectively, histopathology analysis showed lymphomas that developed irrespective of Artemis vector treatment and were essentially of host origin (75%-80% of the tumors). In a few tumour samples (5), transduced donor cells were found but associated with multiple genomic insertion sites from the Artemis or GFP vector. While further exploration is ongoing, the study provides little evidence for insertional mutagenesis caused by the Artemis vector and shows that the gene therapy protocol is well-tolerated in this murine model. Such data support further development towards gene therapy of RS-SCID.

P174

High dose of transduced HPSCs provides safe long term correction of beta-thalassemia

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Gene therapy for beta thalassemia is based on the use of lentiviral vectors (LV) expressing the beta globin gene from a regulated cellular promoter. Clinical trials have been started in Europe and USA and the results from the first patient have shown both the benefit and the limit of this approach. To support the use of hematopoietic stem/progenitor cells (HPSCs) transduced by the GLOBE LV for the treatment of thalassemia major and to assess the risk/benefit ratio, we treated by gene therapy thalassemic th3 mice (n=30), conditioned by busulfan, using cell dose and vector copy number (VCN)/cell superior to that expected in human cells (6.3 to 9.7). Control groups include mice transplanted with mock-transduced HPSCs and untreated age-matched animals. Mice have been monitored for 12 months post treatment and since the donor and recipient strains are co-isogenic for the allelic form of CD45 antigen, chimerism and cell origin can be monitored using specific antibodies. At termination mice are sacrificed and full necropsy and histopathology examination are performed along with clinical chemistry, hematological evaluation and integration site analysis. Results from interim analysis show: 1) no transplant related mortality; 2) efficient engraftment of transduced cells in blood; 3) correction of hematological parameters and transgene expression. Currently this study is ending and final results will be available.

P176

Transduction susceptibility and engraftment potential of genetically-modified human thalassemic CD34+ cells collected by different mobilization approaches

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The optimal autologous graft for thalassemia gene therapy will be one that is easily accessible, rich in CD34 cells, susceptible to transduction and highly engraftable after transplantation. In two clinical trials, we investigated CD34 cell mobilization using G-CSF- or plerixafor-based strategies, in adult β -thalassemia major patients; mobilization with G-CSF plerixafor yielded the highest CD34 cell doses obtained by single apheresis. We here explored whether human thal CD34 cells mobilized by G-CSF(n=6), Hydroxyurea G-CSF(n=6), plerixafor(n=13) or G-CSF plerixafor(n=4) and transduced with the TNS9.3.55 β -globin lenti-viral vector, display differences in clonogenic capacity, susceptibility to transduction or engraftment potential after xenotransplantation. No differences in clonogenic capacity of transduced CD34 cells mobilized by all different modes were observed. Plerixafor-alone-mobilized cells exhibited a trend for higher CFU transduction rates, over G-CSF, Hydroxyurea G-CSF, G-CSF plerixafor-mobilized cells (% positive CFUs, VCN > 0.45: 55.5±18.8 vs 44.7±10.08 vs 47.4±13.05 vs 39.3±12.9, respectively, $p=0.1$) whereas G-CSF plerixafor-mobilized cells displayed a significantly lower average colony VCN over G-CSF-, Hydroxyurea G-CSF-, plerixafor-mobilized cells (1.35±0.31 vs 1.84±0.43 vs 1.87±0.25, vs 2.01±0.71, respectively, $p<0.0001$). The higher transduction rates of plerixafor-mobilized cells could be at least partially attributed to the higher content in cells at the G1 phase of cell cycle. Ongoing HPLC analysis in erythroid differentiation cultures, shows that the normalized TNS9.3.55-

derived β -globin expression in all groups is close to the normal hemizygous output. The engraftment potential was assayed in IL2Rgamma^{null} mice after partial myeloablation and infusion of 1×10^6 CD34 cells/recipient. TNS9.3.55-transduced G-CSF plerixafor cells displayed superior multilineage short-term ($p<0.004$) and a trend towards superior long-term ($p=0.1$) engraftment over cells mobilized by all other modes. The higher CD34 cell yields obtained by G-CSF plerixafor in conjunction with their engraftment benefit in a non-myeloablative setting, may outweigh their lower susceptibility to transduction. The optimal autologous graft for thalassemia however, will be ultimately determined by the amount and persistence of transduced cells in vivo in addition to the level of β -globin gene expression.

P177

Long-term safety of clinical grade LentiGlobin vectors in β -thalassemic and normal mice

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* These authors participated equally to this work. The long term non-clinical efficacy and safety of an improved LentiGlobin lentiviral vector (BB305) were evaluated and compared to those of the HPV569 lentiviral vector, currently used in a clinical trial for the treatment of β -hemoglobinopathies. The therapeutic efficacy and potential toxicity (histopathology, hematology, and insertion sites) were analyzed in 58 β -thalassemia mice (Hbbth1/th1) transplanted with BB305-, HPV569- or Mock-transduced cells. A significant improvement of the phenotype, polyclonal reconstitution and no toxic effect were observed in all mice transplanted with vector-transduced cells. Four months after primary transplantation, bone marrow samples from 54 primary β -thalassemic mice were injected into 108 secondary C57BL/6 recipients to study the long-term toxicity. Mice were followed for engraftment, fitness and necropsied six months later. Extensive investigations were performed in order to assess hematopoietic cell reconstitution, to evaluate potential toxic effects (biochemical parameters, bone marrow cytology, organ damages and tumors) and to analyze insertion sites. Malignant cells were identified in a number of secondary transplant mice; however, the tumor frequency was not significantly different between the three treatment groups. Additional immunohistochemical analyses for the CD45.1/CD45.2 markers and vector sequence specific qPCR carried out on bone marrow and thymic cell DNA demonstrated that the malignant cells were derived from non-transduced primary recipient cells or from cells derived from secondary recipients indicating that the emergence of these cells were most likely related to lethal irradiation and due to the spontaneous high rates of malignancies in the treated animals.

P178

Mouse models to assay osteoclasts and genetic correction in osteopetrosis

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IMO can only be treated with hematopoietic stem cell transplantation (HSCT) it can be performed if the patient has suitable stem cell donors. Therefore IMO is a candidate disease for development of gene therapy.

It has been demonstrated in our laboratory that the osteopetrotic phenotype in oc/oc mice can be reversed by transplantation of fetal liver HSCs cells retrovirally transduced to express non-mutated TCIRG1.

In order to demonstrate that CD34 IMO patient cells are able to repopulate stem/progenitors cells we created a new mouse model. We backcrossed the oc/oc mouse strain onto NSG strain and we selected the oc/oc mutation and NSG mutations through seven generations. Recent results show that NSG/oc/oc offspring developed osteopetrosis and is immunodeficient because the lack of T, B and NK cells. In the further experiments NSG/oc/oc mice will be transplanted neonatally with cord blood and IMO CD34 cells transduced with a suitable therapeutic vector to investigate the disease-correcting ability of vectors and human cell population in vivo.

P179

Lentiviral-based gene therapy for Pyruvate Kinase deficiency

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Pyruvate Kinase Deficiency (PKD) is an autosomal recessive disease caused by mutations in the PKLR gene. It produces chronic nonspherocytic hemolytic anemia (CNSHA), being fatal during early childhood in some severely affected patients. Hematopoietic stem cell (HSC) gene therapy emerges as a therapeutic option for patients with a severe disease lacking an HLA-compatible donor. Our preclinical results in a PKD mouse model aimed to investigate the efficacy of two different ubiquitous promoters, CMV and hPGK, driving the expression of the human PKLR cDNA. While CMV was associated with transgene silencing events, hPGK promoter was potent enough to express clinically relevant levels of RPK transgene in both, primary and secondary deficient transplanted mice. With LV dosages within the clinical standards, ectopic RPK expression was able to normalize the erythroid compartment, correcting the hematological phenotype and reverting organ pathology. Metabolomic studies based on targeted and untargeted mass spectrometry performed on blood cells demonstrate the functionality correction of the glycolytic pathway in genetically corrected RBCs, with no metabolic disturbances observed in leukocytes. Current studies aiming the analysis of the LV insertion sites in the genome of hematopoietic cells from primary and secondary transplanted animals are in progress. These results underscore the therapeutic potential of the hPGK-RPK lentiviral vector, providing for the first time encouraging expectations towards the gene therapy of PKD.

P182

A pilot study of quality of life for patients with chronic critical limb ischemia after gene therapy

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Background: The effects on quality of life (QOL) after the first-in-man Phase I/IIa clinical trial using non-transmissible recombinant Sendai virus vector carrying fibroblast growth factor-2 gene (SeV/dF-hFGF2) were investigated for patients with chronic critical limb ischemia (CLI).

Method: The QOL was carried out using the Short Form 36 health survey (SF-36) for 12 patients, who participated in the gene therapy, on pre-administration, and 28 days, 3, 6, and 12 months after administration. The score of SF-36 was analyzed by one-way ANOVA. The correlation of the rate of change in absolute claudication distance (ACD) and QOL was also analyzed.

Results: The scores excluding 2 amputated subjects significantly improved the bodily pain for the 12 months ($p < 0.043$) and the role-physical for 3 months ($p < 0.047$) and 12 months ($p < 0.039$) after administration. There was no significant correlation with the rate of change in ACD and QOL.

Conclusions: CLI affects patient' QOL and functional status. Our findings indicate that SeV/dF-hFGF2 gene therapy improved or preserved the bodily pain and role-physical activities for CLI patients in a long-term period.

P183

Molecular changes and gene therapy of the infarcted myocardium

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Molecular mechanisms responsible for the pathology of acute myocardial infarction (AMI) as well as for the healing of infarcted myocardium are not completely understood. Moreover, the treatment strategies are still limited.

In the present study, C57Bl6 mice were subjected to left anterior descending (LAD) coronary artery ligation. The assessment of left ventricle function with VEVO2100 ultrasound at day 7 revealed significant impairment of contractility and decreased ejection fraction when compared to healthy individuals. Gene expression analysis revealed significantly increased mRNA levels of atrial natriuretic peptide (ANF), brain natriuretic peptide (BNP) and tenascin C (TnC) in the regions of the heart affected by LAD ligation. Additionally, we have observed significant differences in the levels of miRNAs expressed by heart muscle - down-regulation of miR-126, miR-208, miR-378, miR-499 and up-regulation of miR-199. Modification of changes caused by the infarction can be potentially achieved by gene therapy, assumed to be more feasible when applied systemically. To this end the mice were subjected to systemic application of 5×10^{10} – 5×10^{11} viral genomes of adeno-associated viral vector serotype 9 (AAV9), claimed to specifically target heart. Transduction was assessed intravitaly with IVIS Lumina detecting luciferase gene expression. Surprisingly, except efficient cardiac gene transfer, we observed strong expression of the transgene also in other organs such as liver, skeletal muscle, and fat tissue.

Our results suggest an important role of the post-transcriptional regulators of gene expression in the infarcted heart. Moreover, cardiotropic properties of AAV9 appear to be undermined.

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Comparison of intrapericardial versus intracoronary delivery of Mesenchymal Stem Cells in a swine model of myocardial infarct

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The increase in mortality and morbidity associated to cardiovascular disease determines the development of new therapies to help reduce their impact on society. Despite the increase in research focused on Cell Therapy for myocardial regeneration since 2000, as at today there are still many unanswered questions. The aim of this study is to compare two inoculation routes intracoronary and intrapericardial through a thoracoscopic approach.

Methods: 14 domestic female swine were randomly allocated to each group. The infarction model was performed occluding the mid-left anterior descending artery using a balloon catheter during 90 minutes. 30×10^6 MSC were injected two days after model creation. Imaging studies were serially performed to assess the evolution of cardiac morphology and function. The animals were sacrificed after 3 months of follow-up.

Result: The myocardial infarction model was successfully created in both groups. Changes in the ECG with ST segment elevation were observed in all animals during occlusion of the artery. There was a significant difference in end systolic and end diastolic volumes during the study. The percentage affected by myocardial ischemia was not significant different between groups or between the different study times. We believe that both approaches provide partial benefits, on the one hand, the thoracoscopic approach allows a more accurate placement of the cells in the area affected by ischemia, preventing migration while the intracoronary approach requires a lighter anesthetic level.

P185

IRES-based vectors for a combined gene therapy of heart ischemia

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Despite of considerable advances in the treatment of cardiovascular diseases, left ventricle dysfunction and heart failure remain an important problem of public health. Our laboratory develops an original approach of combined gene therapy, based on translational activators, IRESs (internal ribosome entry sites), to generate gene transfer vectors co-expressing several therapeutic molecules. We have previously validated the benefit of IRES-based vector co-expressing FGF2 and Cyr61 for therapeutic

angiogenesis of hindlimb ischemia (1). This association creates a synergistical effect of the two molecules that are more efficient at low doses.

Here we have developed gene transfer vectors applicable to gene therapy of myocardial ischemia. The aim is to express combinations of molecules to stimulate both angiogenesis and myocardium contractile function. Bicistronic IRES-containing lentivectors expressing two luciferase genes have been successfully assessed in transduction of mouse cardiomyocytes and cardiac fibroblasts *ex vivo*, as well as in infarcted myocardium *in vivo*. In addition we show that the lentivector is more efficiently expressed in the ischemic heart than in the control, probably due to IRES activation by hypoxia. We have thus validated intracardiac injection of lentivectors in a mouse model of heart ischemia. Lentivectors expressing combinations of two or three therapeutic genes including apelin, FGF2 and Cyr61 have been designed and produced. First experiments with a lentivector expressing apelin alone show encouraging data on decrease of heart hypertrophy and fibrosis. Bicistronic and tricistronic lentivectors are presently assessed in order to obtain a synergistic effect of the therapeutic molecules, and an optimal therapeutic benefit on heart perfusion and contractile function.

1) Rayssac *et al*, Mol Ther 2009.

P186

Non-inflammatory angiogenesis is impaired, while the inflammation-driven revascularization of ischemic tissue is accelerated by the lack of Nrf2

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Antioxidant genes, like heme oxygenase-1 (HO-1; *Hmox-1*) are involved in blood vessels formation. Importantly, lack of HO-1 impairs response of endothelial cells (ECs) and proangiogenic progenitor cells (PACs) to VEGF or SDF-1 stimulation. Accordingly, wound healing and revascularisation in ischemic limbs of *Hmox-1* knockout (KO) mice is impaired.

Here we investigated angiogenic role of Nrf2 (*Nfe2l2*), a transcription factor upstream of *Hmox-1*. Stimulation of ECs with VEGF, IL-8 or SDF-1 stabilized Nrf2 and led to HO-1 induction. Silencing of Nrf2 by RNAi attenuated angiogenic response of ECs, similarly as Nrf2 deficiency in bone marrow-derived PACs *in vitro*. Moreover, PACs from *Nfe2l2* KO mice demonstrated decreased production of proangiogenic mediators in hypoxia, such as VEGF. *In vivo*, role of Nrf2 was demonstrated when in GFP transgenic mice subcutaneously injected Matrigel containing Nrf2-deficient PACs recruited less GFP-positive ECs than wild type PACs. Moreover, mobilization of PACs to peripheral blood after hind limb ischemia tended to diminish in *Nfe2l2* KO mice. Surprisingly, regeneration of blood flow was accelerated in ischemic limbs of such animals, what was accompanied by increased inflammatory response in the ischemic muscles. Treatment of mice with etodolac (10 mg/kg b.w./day), a cyclooxygenases inhibitor, diminished blood flow in Nrf2-deficient mice to the level observed in wild type counterparts, and decreased revascularisation in ischemic limbs of the latter.

In sum, lack of Nrf2 impairs non-inflammatory angiogenesis, while the inflammation-driven revascularisation is accelerated in

the absence of Nrf2. Additionally, the role of PACs in inflammation-dependent reparative angiogenesis is undermined.

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Correction of murine ADAMTS13 deficiency using the 'Sleeping Beauty' transposon system

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Introduction: Congenital deficiency of the metalloprotease ADAMTS13 causes the life-threatening disease thrombotic thrombocytopenic purpura (TTP). Current treatment of choice is plasma infusion, which, although effective, might expose patients to allergies, infections, etc.

Aim: Investigating whether the non-viral 'Sleeping Beauty' (SB) transposon system could be an alternative treatment strategy for TTP.

Methods: The SB transposon plasmids were delivered by hydrodynamic tail-vein injection in 5 ADAMTS13^{-/-} mice. Transgene expression of muADAMTS13 and presence of anti-muADAMTS13 antibodies were measured using immunoassays. The proteolytic activity of muADAMTS13 was analyzed. VWF multimer patterns were detected using SDS-agarose gel electrophoresis.

Results: The co-injection of the transposon plasmid with the transposase-expressing plasmid resulted in high levels of transgene, active muADAMTS13 expression (880 ± 909%, 475 ± 230%, and 199 ± 179% at day 3, 7 and 14 after injection respectively). The expression of active muADAMTS13 also resulted in the disappearance of ultra-large VWF multimers in plasma. However, 2 weeks after injection a decrease in muADAMTS13 expression was observed which was due to the formation of antibodies against the transgene protein.

Conclusion: Using the SB transposon system we can correct the deficiency of the ADAMTS13 gene. The development of antibodies, however, hampered the long-term expression. Ongoing studies will focus on suppressing this antibody response.

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A comparative study of the reparative and angiogenic properties of mesenchymal stem cells derived from the bone marrow of BALB/c and C57/BL6 mice in a model of limb ischemia

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Introduction: BALB/c and C57/BL6 mice have different abilities to recover from ischemia. C57/BL6 mice display increased vessel collateralization and VEGF expression with a consequent rapid recovery from ischemia compared to BALB/c mice. Mesenchymal stem cells (MSCs) are one of the main cells that contribute to the recovery from ischemia because, among their biological activities, they produce several pro-angiogenic paracrine factors and differentiate into endothelial cells. The objective of this study was to evaluate whether the MSCs of these two mouse strains have different inductive capacities for recovering ischemic limbs.

Methods: MSCs from these two strains were obtained from the bone marrow, purified and characterized before being used for *in vivo* experiments. Limb ischemia was surgically induced in BALB/c mice, and MSCs were injected on the fifth day. The evolution of limb necrosis was evaluated over the subsequent month. Muscle strength was assessed on the thirtieth day after the injection, and then the animals were sacrificed to determine the muscle mass and perform histological analyses to detect cellular infiltration, capillary and microvessel densities, fibrosis, necrosis and tissue regeneration.

Results: The MSCs from both strains promoted good recovery from ischemia, with all recovery indicators showing similar results.

Conclusions: The similar therapeutic results obtained with MSCs from BALB/c and C57/BL6 mice, which have a clear genetic difference in term of angiogenesis, when treating ischemic tissue demonstrate the important role of MSCs in the recovery of ischemic tissues, regardless of the genetic background.

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Intrapericardial delivery of bone marrow mesenchymal stem cells in a porcine model: *in vivo* cell tracking by MRI and histological analysis

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Purpose: The mesenchymal stem cells (MSCs) have been widely used in clinical and preclinical trials. They are particularly attractive for their availability, multipotentiality, self-renewal ability and low immunogenicity. The appropriate route for MSCs administration is a fundamental step for the success of stem cell-based therapies and could determine the degree of therapeutic effect. The intrapericardial delivery is the least studied delivery approach for cardiac cell therapy.

Methods: Allogeneic porcine bone marrow MSCs (pBM-MSCs) were isolated from male Large White pigs. For viability and proliferation assays, pBM-MSCs were cultured in the presence of different concentrations of pericardial fluid. In order to detect pBM-MSCs by magnetic resonance imaging (MRI), the cells were magnetically labeled with superparamagnetic iron oxide (Endorem®). The labeling efficiency was determined by Prussian-Blue. A total of 100 × 10⁶ pBM-MSCs were injected into the pericardial cavity via thoracotomy. Samples were obtained 7 days post-injection, fixed for histology and frozen for Y chromosome PCR detection.

Results: *In vitro* cell culture with pericardial fluid demonstrated that phenotype, viability and proliferation rate was comparable to standard culture conditions. The *in vivo* cell tracking by MRI (7 days follow-up) demonstrated a preferential distribution of magnetically labeled pBM-MSCs in the left ventricle. Finally, the histology and Y chromosome PCR showed coincident results and MSCs were detected in the pericardium, ventricles and atrium.

Conclusions: Intrapericardial administration allows an optimal cell retention and implantation of MSCs. The presence of MSCs adhered to myocardium or pericardium may have a beneficial effect through the release of paracrine factors. We hypothesize that intrapericardial administration would enhance the therapeutic potential of MSCs in heart failure.

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Human placental derived stem cells grown on a synthetic-fibrin scaffold for cardiac tissue engineering

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Tissue-engineering strategies combining scaffolds and stem cells to enhance cardiac repair is considered a very promising approach for the treatment of damaged myocardium. For this goal a suitable cell delivery system to improve tissue regeneration, without affecting cardiac contractile function is required. For their multi-lineage differentiation potential and low immunogenic properties Mesenchymal Stem Cells (MSCs) isolated from term Amniotic Membrane (AM) are considered a very attractive source for cardiac repair. In this study an elastic scaffold, obtained combining a fibrin layer with a poly(ether)urethane-polydimethylsiloxane (PEtU-PDMS) layer, was used as a substrate for "in vitro" growth and differentiation of human amniotic mesenchymal stromal cell (hAMSCs).

Cell proliferation and metabolic activity of these cells, grown on Combined Synthetic-Fibrin scaffold, were evaluated by bromodeoxyuridine and WST-1 assays for up to 14 days of culture. Their cardiomyogenic commitment was induced, for the first time in this cell population, by a nitric oxide (NO) treatment. The NO treated hAMSCs, showed morphological changes, an increase of the messenger cardiac differentiation markers [troponin I (TnI) and NK2 transcription factor related locus 5 (Nkx2.5)] and a modulation of the endothelial markers [vascular endothelial growth factor (VEGF) and kinase insert domain receptor (KDR)] by RT-PCR analysis.

The results of this study propose a combined synthetic fibrin scaffold, able to support growth and cardiomyogenic commitment of hAMSCs, as a new strategy that could be used in cardiac regenerative medicine.

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Secretome-based approach for therapeutic angiogenesis outclasses proangiogenic bone marrow-derived cell transplantation

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Rationale: The experimental studies have definitely evidenced the role of proangiogenic myeloid cells (previously referred to as EPCs) in postnatal angiogenesis in ischemic conditions. Contribution of such cells to tissue repair is claimed to be achieved both by incorporation of the cells into developing capillaries and their paracrine effect. We compared which approach, cell transplantation or conditioned media injection, might be more effective for therapeutic angiogenesis.

Principal findings: Proangiogenic bone marrow-derived cells (BMDCs) were injected intradermally around the cutaneous wounds in mice. We observed no statistical difference in time course of wound closure between cell treated and control animals.

About 60% of BMDCs transplanted intradermally to the mice were eluted from the tissue within 6 h after injection. Next, 70% did not survive for subsequent 3 days. Thus, of 200 000 cells injected, only about 5000 cells were still present in the skin 7 days after injection, and one week later their number was less than 50. In another model, mice underwent femoral artery ligation and were subsequently intravenously injected with PBS or BMDCs. Unexpectedly, the adoptive transfer of BMDCs impaired tissue reperfusion in comparison to PBS treated animals. In contrary, blood flow recovery could be rescued by local injections of conditioned media harvested from BMDCs. Mass spectrometry identified HSPA8 and osteopontin as the main potential factors facilitating revascularization.

Conclusions: Cell-free strategy facilitated revascularization of the ischemic tissue in our experimental settings, whereas cell therapy was not effective. Therefore, secretome-based approach seems to be more feasible.

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Safety of allogeneic pCSCs intracoronary delivery: a pathological and MR study assessment in the porcine model

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Purpose: Intracoronary delivery of cardiac derived cells is a promising strategy that has produced positive results in recent studies. Clinical trials have generally administered up to 25×10^6 CDCs. Higher doses are considered to place the subject at infarction risk. We undertook this study to assess the effects of intracoronary administration of 35×10^6 pCSCs in healthy swine using clinically available tools (cMR and Troponine I assay) and to confirm the findings with pathology, in order to evaluate the possibility that the cells in themselves exert toxicity or cause infarction.

Methods: Six healthy domestic swine were subjected to delayed enhancement cardiac magnetic resonance (DE-CMR) to confirm that the myocardium was healthy. 35×10^6 pCSCs were then injected via a microcatheter placed at the mid-LAD. DE-CMR was repeated 24 h and 7 days after injection. Cardiac TpnI levels were analyzed prior to injection and at 4 and 24 hours. Animals were euthanized at 3 weeks for pathological study of the target heart area.

Results: No ST elevations or cardiac events were seen during the procedure. cTpnI increased after intervention but it remained within clinically acceptable values (baseline $0.012 \pm 0.003 \mu\text{g/L}$; 4 h $0.028 \pm 0.021 \mu\text{g/L}$ and 24 h $0.41 \pm 0.69 \mu\text{g/L}$). Neither DE-CMR nor pathological examination showed evidence of cardiac lesion in any animal.

Conclusion: The intracoronary delivery of 35×10^6 pCSCs in healthy swine appears to be safe. Our results suggest that human trials with these cells can be initiated. The increase in cTpnI seen after injection could be attributed to the cardiac catheterization, as suggested by the lack of myocardial injury on the MR and pathological examinations.

P193

Characterisation of porcine cardiosphere-derived stem cells line for preclinical studies in a large animal model leading to expand the current purposes of CDCs therapy

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Cardiosphere-derived cells (CDCs) have recently emerged as an effective cell type for cardiac cell therapy. Swine is the most adequate animal model to study cardiovascular pathologies and therapies. The aim of this work was to develop and characterize a porcine CDCs line appropriate for preclinical studies.

CDCs were expanded from porcine cardiac explants and subjected to different selection processes and culture conditions to test if these differences affect CDCs phenotype and differentiation ability. Stemness, early cardiac differentiation and mature cardiomyocytes markers expression, as well as growth factors and receptors were analyzed by RT-PCR and flow cytometry. The differentiation potential was *in-vitro* assayed.

Our expansion protocol of porcine CDCs allows the procurement of 10 to 100 million cells in 90–120 days, a suitable quantity for preclinical studies. The characterization assays showed that these CDCs can differentiate into cardiomyocytes, although they preserve the ability to differentiate into other cell lineages. Also they showed a high expression of growth factors such as IGF-1 (and its receptor), HGF and TGF- β 1. Finally, their phenotype and genetic expression are similar to human CDCs. These porcine CDCs are currently being used for intramyocardial injections in a *Large White* pig model with induced acute myocardial infarction to study the effects of CDCs over postinfarction arrhythmias. Magnetic resonance imaging is programmed for weeks 5 and 18 after injection to determine cardiac functionality, and histopathology studies will be made after euthanasia.

Our well-characterized porcine CDCs constitute a proper adult cardiac stem cell line for preclinical studies in a large animal model. These preclinical trials may allow the optimization of cell dosage and delivery strategy for further *in vivo* studies. In summary, the similarities between these cells and human CDCs make them optimal to find out new strategies for the application of stem cell therapy in cardiovascular affections.

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Benefits of intracoronary delivery of pCSCs in a swine model of acute myocardial infarction

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Purpose: The aim of this study was to evaluate in a swine model of acute myocardial infarction the functional benefits obtained by intracoronary delivery of two different porcine cardiac stem cell (pCSC) doses.

Methods: Intracoronary injection of either vehicle (n=5), 25×10^6 (n=5) or 50×10^6 pCSCs (n=5) was performed 1 week after endovascular infarct creation using a microcatheter placed in the mid left anterior descending artery. Blood samples for troponin I (TpnI) determination were taken before and 24 h after infusion. Left ventricular ejection fraction (LVEF), left ventricular end diastolic (LVEDV) and systolic volumes (LVESV) were evaluated by magnetic resonance at baseline, 1 week after in-

farction, and 1 and 10 weeks after cell/vehicle injection. After euthanasia hearts were explanted for pathology.

Results: No significant increase in TpnI was observed after cell/vehicle delivery. At the end of the study higher LVEF and lower LVEDV and LVESV values were observed in the 25×10^6 ($51.48 \pm 4.37\%$; 81.77 ± 12.13 ml and 39.98 ± 9.01 ml) and 50×10^6 pCSC-treated animals ($51.06 \pm 4.74\%$; 86.83 ± 13.48 ml and 42.85 ± 9.68 ml) compared to the control pigs ($39.78 \pm 10.97\%$; 98.66 ± 21.10 and 60.73 ± 21.96). Histology revealed more severe lesions in the tissue samples belonging to the control animals. The 50×10^6 pCSCs samples showed the lowest degree of injury.

Conclusion: Intracoronary administration of both evaluated cell doses appeared to be safe as demonstrated by the small increase in TpnI. Although differences between groups were not statistically significant, there was a trend towards improvement of cardiac function in the cell-treated subjects. Histological results suggested that this trend was greater when the higher cell dose was injected.

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Autophagy activation by hydrogen sulfide protects cardiomyocytes of death by starvation.

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Hydrogen sulfide (H₂S) together to nitric oxide and carbon monoxide is an endogenous gaseous mediator. Different studies have shown that H₂S has protective effects on myocardial ischemia-induced cell apoptosis. In addition to oxygen deprivation ischemia also creates a nutrient deficiency that also will be responsible, at least in part, of cell death. In this study, we investigated the effects of a H₂S donor GYY4137 on acute starvation-induced apoptosis on HL-1 cardiac myocytes. Exposure to no-glucose, no-amino acid media for 4 hours resulted in the induction of apoptosis by $41.6\% \pm 1.01\%$, which was attenuated by pretreatment with H₂S donor GYY4137. Further investigation of the protective mechanisms in the cells demonstrated that pretreatment H₂S donor GYY4137 activates autophagy, reduce ATP content and induce starvation signalling in HL-1 cells.

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Intracerebral administration of lentiviral vectors in juvenile non-human primates: a biodistribution study

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Metachromatic leukodystrophy (MLD) is a rapidly progressing demyelinating disease caused by a genetic deficiency in the lysosomal arylsulfatase A (ARSA). We have shown that a single injection of lentiviral vectors (LV) coding for the ARSA gene into the external capsule (EC) of MLD mice results in rapid, widespread and long-lasting ARSA activity in CNS tissues,

highlighting a major rationale for LV application to intracerebral gene therapy (GT). However, before considering a potential clinical application, scaling-up issues should be addressed in large animals. Thus, we applied convection enhanced delivery to inject LV.GFP or LV.hARSA in interconnected white matter (WM) regions (EC, corona radiata) and in the thalamus (2 unilateral injections; 2.5×10^7 TU/site) of normal juvenile non-human primates (NHP; n=8), evaluating vector and transgene expression/distribution at 1 and 3 months post-treatment. The procedure was tolerated without any adverse events or change in neurobehavioral examination. Histopathology revealed a minimal to severe neuroinflammation close to the injection areas in 6 out of 8 NHP. Analysis of CNS tissues from LV.GFP-injected NHP (n=2) showed efficient transduction and moderate LV diffusion (<3 cm) in the injected hemisphere. Of note, robust GFP expression was detected in multiple brain areas along the rostral-to-caudal axis, in both hemispheres, up to the cervical spinal cord. These data strongly suggest the potential of the proposed GT platform to effectively treat large brains. We are currently analysing CNS tissues from LV.hARSA-treated NHP (n=6), in which we expect similar or enhanced biodistribution of hARSA due to enzyme secretion, CSF transport and cross-correction.

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Protection and repair of the dopaminergic nigrostriatal pathway by striatal xenografts of in vitro expanded CB cells in a chronic Parkinson's disease model.

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Intra-striatal carotid body (CB) grafts induce trophic protection and restoration of the dopaminergic nigrostriatal pathway in rodent and primate models of Parkinson's disease (PD). This trophic action seems to be mediated, at least in part, by the high levels of glial cell line-derived neurotrophic factor (GDNF) produced by the CB transplants. Pilot clinical trials have also demonstrated that CB autotransplantation can improve motor symptoms in PD patients although the effectiveness of CB cell therapy is affected by the scarcity of grafted CB tissue. In this work, we analyzed the antiparkinsonian efficacy of in vitro expanded CB tissue using stem cell-derived neurospheres. The neurospheres contained a core of nestin progenitors and blebs of TH glomus cells. We have performed xenografts of rat CB neurospheres using a long-term immunosuppressive protocol compatible with a chronic 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) mouse PD model. Using this experimental procedure we show that intra-striatal grafts of in vitro expanded CB cells protect ipsilateral dopaminergic nigral neurons from degeneration. In addition, grafts performed at the end of the neurotoxic treatment produced a repair, by axonal sprouting, of dopaminergic striatal terminals. Interestingly, both neuroprotective and reparative effects induced by in vitro expanded CB cells are similar to those produced by the graft of rat CB tissue. This action can be explained because in vitro derived CB cells produce similar amounts of GDNF than native CB tissue. This study provides the first evidence that in vitro expanded CB cells could be a clinical option for cell therapy in PD.

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Cleavage of amyloid- β by the Nuclear Inclusion a (NIa) protease of turnip mosaic virus

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The nuclear inclusion a (NIa) protease of turnip mosaic virus is responsible for the processing of the viral polyprotein into functional proteins. NIa has a strict substrate specificity with a preference for Val-Xaa-His-Gln, with the scissile bond located after Gln. We found that NIa efficiently cleaved the oligomeric as well as monomeric amyloid- β ($A\beta$) peptide, which contains the sequence Val-His-His-Gln in the vicinity of the cleavage site by α -secretase. The overexpression of NIa in B103 neuroblastoma cells resulted in a significant reduction in cell death caused by both intracellularly generated and exogenously added $A\beta$. Moreover, lentiviral-mediated expression of NIa in APPsw/PS1 transgenic mice significantly reduced the levels of $A\beta$ and plaques in the brain. Behavioral tests were also performed. Lentivirus-NIa significantly restored the behavioral deficits of APPsw/PS1 mice as assessed by water maze, passive avoidance, and marble burying tests. Oligomeric $A\beta$ -induced cytotoxicity and mitochondrial dysfunction were significantly ameliorated by NIa. Endocytosed fluorescently labeled $A\beta$ localized to mitochondria, which was significantly reduced by NIa. These data suggest that NIa exerts its protective roles by degrading $A\beta$ in the cytosol and thus preventing mitochondrial deposition of $A\beta$. Collectively, our study shows that NIa provides a unique therapeutic opportunity for Alzheimer's disease.

P200

Bonemarrow-derived mesenchymal stem cells protect degenerating cells in the dorsal root ganglia of ataxic mice: a step forward towards a clinical application

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Mesenchymal stem cells (MSC) are a promising tool for the treatment of various diseases, including neurodegenerative disorders. This is partly due to their ability to secrete various trophic factors, inducing regeneration and/or protection in the surrounding tissue. Previous works in our lab have shown that MSC-conditioned medium was capable of protecting in vitro dorsal root ganglia (DRG) cells isolated from Friedreich's ataxia (FA) mice when submitted to oxidative stress. The DRGs of these mice degenerate and are very susceptible to oxidative stress. As a result, the DRG cells survived thanks to various trophic factors present in the MSC-conditioned medium. Also, no differences were observed using MSC isolated from either wildtype or FA mice.

In this present work, we transplanted MSC in the subarachnoidal space of the spinal cord of FA mice, in order to confirm in vivo the results observed in vitro. The MSC migrated to the DRG, where they integrated into the tissue, observed by MRI, and released the same trophic factors observed in vitro. This resulted in the mice improving in various behavior tests performed, as well as increased cell survival markers, and improved oxidative stress-related gene expression. Also, frataxin expression, which is the target gene for the disease, was increased. As observed in vitro, no differences were detected using MSCs from wildtype or FA mice.

The results shown here prove the feasibility of using stem cell transplantation in protecting the DRGs from degeneration in an FA mouse model, making it a step forward towards a possible therapeutic application.

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Selective neuronal targeting by self-complementary AAV9 or AAV10 via intrathecal delivery in nonhuman primate: application for chronic pain or motor neurons diseases

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Adeno-associated viral (AAV) gene therapy has shown hope for clinical treatment of neurodegenerative diseases, however one limit of direct brain injection of AAV vectors is the lack of widespread gene expression in the central nervous system (CNS). Therefore, diffuse vector delivery covering large CNS areas remains a critical issue for future gene therapy trials. The aim of this study was to test the feasibility and the efficiency of AAV delivery into the cerebrospinal fluid (CSF) of non-human primates (PNH) using intrathecal (IT) or intracerebroventricular (ICV) injections. The device, volume and flow rate of IT and ICV deliveries were first optimized with CSF monitoring, neurobehavioral and MRI-follow up. Thereafter, we investigated the transduction profiles of AAV9 and AAV10 CSF gene transfer. IT delivery of GFP-expressing AAV10 vector resulted in transgene expression in sensitive neurons of the dorsal root ganglions. In contrast, a single IT administration of AAV9 led to efficient and widespread transduction of spinal motor neurons from cervical to lumbar regions. Remarkably, a large proportion of MN from the cervical to the lumbar spinal cord was also transduced after AAV9 ICV delivery. Results of this study could have considerable impact on defining clinical trials applied to human neurodegenerative diseases.

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Somatic transgenesis of the central nervous system for disease modelling and treatment

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Perinatal asphyxia, termed Hypoxic Ischemic Encephalopathy (HIE), occurs in approximately 3–5% of human live births. Un-

fortunately, only 25% of affected newborns survive and suffer from severe neurological disorders. A hallmark of HIE is the occurrence of reactive astrogliosis, where astrocytes become activated and proliferate. During this process astrocytes express more of the intermediate filament, glial fibrillary acidic protein (GFAP).

Perinatal gene transfer using viral vectors is a useful tool and promising therapy in the field of neuroscience. Light emitting transgenic mice, where luciferase expression is controlled by a surrogate promoter, are used to provide an *in vivo* readout of disease processes. In this study, we aimed to deliver a GFAP promoter controlling luciferase to brains of neonatal mice as a form of assessing the amount of astrogliosis in a mouse model of HIE.

A rodent GFAP promoter (GfaABC1D) was cloned into a lentivirus vector upstream of the genes encoding a codon-optimised firefly luciferase and green fluorescent protein. This vector was injected intra-cranially into wild-type neonatal (P0) mice, and luciferase expression continuously monitored by whole body bioluminescence imaging.

We observed long-term expression of firefly luciferase under the control of the GFAP promoter; expression was restricted to the CNS including both brain hemispheres.

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Recombinant AAV9 vectors to silence the mutant SOD1 gene in Amyotrophic Lateral Sclerosis

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Amyotrophic Lateral Sclerosis (ALS) is an incurable motor neuron (MN) disease characterized by MNs death leading to muscle atrophy, paralysis and premature death. Approximately 20% of the familial ALS forms (fALS) are related to toxic gain of function mutations in the SOD1 gene.

Recently, we and others have identified self-complementary (sc) AAV9 as a vector of choice to target the central nervous system after systemic injection in mammals and showed its tremendous therapeutic value in spinal muscular atrophy mouse models. We now wish to use scAAV9 vectors to establish a new gene therapy approach for the SOD1-linked fALS, by expressing *artificial* MicroRNA to human SOD1 (hSOD1-miRNA) in SOD1^{G93A} mice, a mouse model for fALS. Indeed, the intravenous injection of a hSOD1-miRNA expressing scAAV9 could reduce the levels of mutant SOD1 in both MNs and other cell types such as glial cells or muscle fibers that may also be affected in ALS.

First the silencing efficacy of the hSOD1-miRNA versus a non-targeting miRNA (CTL-miRNA) was verified in transfected HEK-293T cells. We found that hSOD1-miRNA induced 50% and 70% reduction of the hSOD1 protein and mRNA, respectively. We also found that the hSOD1-miRNA significantly reduced the number of intracytoplasmic aggregates in a cellular model of fALS (NSC34 cells overexpressing mutant hSOD1).

We then produced recombinant scAAV9 carrying either the hSOD1-miRNA or the CTL-miRNA, together with EGFP, regulated by the CMV or PGK promoters. The potential therapeutic effect of intravenous scAAV9-hSOD1-miRNA are currently investigated in neonatal (presymptomatic) and 90 day-old (onset of symptoms) SOD1^{G93A} mice.

P205

miRNA-mediated restriction of myotubularin expression to the skeletal muscle corrects pathology and prevents cardiac toxicity in a murine model of X-linked myotubular myopathy

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GENETHON

Gene therapy of muscular disorders that involve the skeletal musculature but not the heart remains a challenge, in part because commonly used viral vectors are cardiotoxic. Myotubular myopathy (XLMTM) is a severe congenital disease that affects skeletal muscles, which contain small myofibers with frequent occurrence of central nuclei. The disease is due to mutations in the Mtm1 gene encoding a phosphoinositide phosphatase named myotubularin. We have previously demonstrated the efficacy of local administration of adeno-associated virus (AAV) vectors carrying the Mtm1 cDNA to treat the disease in a mouse model, and have more recently extended these studies to the whole body. We found that intravenous delivery of a serotype 8 AAV vector that contains the Mtm1 cDNA under the potent desmin promoter leads to high levels of myotubularin in muscles throughout the body including heart, where lesions were observed in Mtm1-treated knockout mice. Therefore, to restrict transgene expression to skeletal muscles, we constructed various AAV8-Mtm1 vectors that contain miRNA208a target sequences for cardiac detargeting. We show that a single target sequence in the 3'UTR of Mtm1 mRNA is sufficient to reduce specifically myotubularin level in the heart. Systemic administration of this vector results in phenotype correction with prolonged survival and no toxicity. Altogether our results show that this strategy is important for gene therapy of disorders in which cardiac transgene expression is not required and describe a novel vector that represents a strong candidate for clinical trials in XLMTM patients.

P206

Expansion of differentiated GDNF-producing carotid body cells by reversible lentiviral gene transfer. Potential use in cell therapy

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Previous studies have shown that intrastriatal carotid body (CB) transplants induce trophic protection and restoration of the dopaminergic nigrostriatal pathway in rodent and primate models of Parkinson's disease (PD). This action of CB transplants seems to be due to a trophic effect of the grafts on nigrostriatal neurons, which survival is strictly dependent on the production of glial cell line-derived neurotrophic factor (GDNF). Indeed, *in situ* and transplanted CB glomus cells are known to produce high levels of GDNF. Application of CB transplantation to the clinical setting is limited by the scarcity of grafted tissue due to the small size of the CB. We have attempted reversible expansion of CB tissue prior transplantation. HLOX lentiviral backbone for CRE-dependent transgene excision was used. Lentivector-mediated transfer of immortalizing

genes (*Bmi1* and SV40 large T antigen) was performed in primary cultures of adult rat and mouse CB cells. Cells were left to grow for a month and thereafter clones were obtained by single cell deposition. In selected clones with high tyrosine hydroxylase (TH) and low nestin expression, there were cells with variable level of GDNF as determined by ELISA and quantitative RT-PCR. Low and high GDNF-producing cells were intrastrially transplanted in adult mice (100.000–300.000 cells per graft) after CRE-mediated deimmortalization. Experiments performed so far indicate that high GDNF-expressing cells seem to survive in the brain for up to 3 months without signs of proliferation. Interestingly, low GDNF-expressing cells frequently generate tumour masses after transplantation. Further experiments should be performed to investigate the cause of tumourigenesis. The neuroprotective action of these transplants is currently under evaluation.

P207

Towards improved predictability in pre-clinical research: Human dopaminergic 3D in vitro model for development of gene delivery strategies

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The development of novel and efficient therapies for central nervous system (CNS) disorders, such as Parkinson's disease (PD), remains a formidable challenge. Advances in gene therapy, which could greatly impact PD treatment by acting directly on the causes rather than symptoms, have been hindered by the lack of robust CNS models. Human cells have great potential as complementary tools in pre-clinical research, bridging the gap between human clinical studies and animal models. Here we describe the development of a human 3D neural in vitro model, making use of human midbrain-derived neural progenitor cells (hmNPCs).

hmNPCs were differentiated as neurospheres under dynamic culture systems. Global gene expression analysis revealed the activation of neurogenesis developmental pathways during 3D differentiation, resulting in neurospheres enriched in neurons, astrocytes and oligodendrocytes. 1H-NMR metabolic profiling revealed a decrease in the reliance on glycolytic metabolism, typical of differentiating cultures. Differentiated cells presented dense filopodia, critical in axonal development, guidance and synapse formation. Moreover, an increase in the expression of the dopaminergic (DA) markers tyrosine hydroxylase (TH) and *Nurr1*, along with an increase in synaptic functionality and ability to synthesize and release dopamine, strongly indicated the generation of DA neurons. Furthermore, gene transfer using helper-dependent canine adenovirus vectors in differentiated neurospheres led to stable transgene expression and low toxicity.

The 3D model developed herein, along with a flexible toolbox of characterization methods, represents a powerful complementary tool to enhance the understanding of the mechanisms of action of new therapeutics.

P208

Feasibility of selective intra-arterial infusions of mesenchymal stem cells in children with Rasmussen EncephalitisV Cantarín-Extremera¹, A Frieria-Reyes², G Melen³, I de Prada⁴, C Fournier⁵, MA Pérez-Jiménez⁶, ML Ruíz-Falcó¹, M Ramírez³¹Neuropediatría, Hospital Universitario Niño Jesús; ²Radiodiagnóstico, Hospital Universitario La Princesa, Madrid; ³Oncohematología, Hospital Universitario Niño Jesús, Madrid; ⁴Anatomía Patológica, Hospital Universitario Niño Jesús; ⁵Neuropsicología, Hospital Universitario Niño Jesús; ⁶Neurofisiología, Hospital Universitario Niño Jesús

Rasmussen encephalitis (RE) is a devastating pediatric syndrome of unknown etiology and characterized by a progressive loss of neurological capacities in the presence of intractable focal epilepsy. Cytotoxic T lymphocytes have an active role in the pathogenic process of RE. The patients with RE receive symptomatic therapies with antiepileptic and immunosuppressant drugs, intravenous immunoglobulins, and eventually undergo functional hemispherectomy as the only treatment to achieve complete control of epileptic seizures. New therapies are needed to improve the prognosis of this rare disease. We are evaluating the feasibility of mesenchymal stem cells (MSCs) for patients with RE, based on the known immunomodulatory and regenerative potential of these cells. A 12 years-old female diagnosed of RE presented *epilepsia partialis continua* with generalized seizure episodes. She received antiepileptic drugs, steroids and immunoglobulin. After informed consent, autologous MSCs were cultured from a marrow aspirate, and infused through catheterization of the carotid right system. The patient received 4 doses of MSCs in the 4 months before hemispherectomy. The administrations were well-tolerated and no adverse effects were seen after each infusion. A decrease in the frequency of generalized seizure episodes was found in the first 2 weeks after each infusion. Connors' Continuous Performance Test (CPT II), a task-oriented computerized assessment of attention disorders and neurological functioning, significantly improved after cell therapy. Though preliminary, this strategy appears to be safe and feasible in patients with otherwise no curative options, and should be evaluated in additional children with RE.

P209

Oligodendrocyte progenitor cell activation and remyelination mediated by mesenchymal stem cell transplantation in chronically demyelinated mice

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Mesenchymal stem cell transplantation has been proven to have beneficial effects in various degenerative diseases, including demyelinating disorders. The aim of this work is to investigate the therapeutic potential of mesenchymal stem cells (MSC) in a chronically demyelinated mouse model. To this end, bone marrow-derived MSC were pre-incubated in vitro with iron nanoparticles and stereotaxically injected into both lateral ventricles of mice fed with cuprizone for 12 weeks, inducing an irreversible demyelinating state. After transplantation, all the animals were analyzed by MRI at different time points (0–90 days). The MRI images were processed to quantify myelin in the corpus callosum using image analysis software. Also, several mice were sacrificed at the same time points to perform immunohistochemistry

analysis and corroborate the data obtained by MRI. In this regard, myelin density as well as several immature and mature oligodendrocyte markers were analyzed. As a result, the grafted stem cells were detected near the injection site as well as in several areas of the demyelinated corpus callosum. Oligodendrocyte progenitor cells were detected near the stem cells as early as 1 month after transplantation. Furthermore, increased myelin content was detected in the corpus callosum two months after treatment. In conclusion, the findings of this study revealed that MSC transplantation activates remyelinating processes throughout the corpus callosum of cuprizone-treated mice. This work might have major implications for the development of future therapeutic strategies for chronic demyelinating disorders.

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P210

Beneficial role of astrogliosis in promotion of functional recovery of completely transected spinal cord following transplantation of hESC-derived oligodendrocyte and motoneuron progenitors

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Spinal cord injury (SCI) results in neural loss and consequently motor and sensory deficit below the injury. Neural progenitors derived from hESC and iPS cells neural induces locomotor improvement following their transplantation into the animal models of SCI, but little is known about the effects and the underlying mechanism of these grafted cells on local tissue and endogenous neural stem cells. Recently, we have reported the regenerative effects and significant improvement of locomotor function in complete transection rat model of SCI following transplantation of oligodendrocyte progenitors cells (OPC) and motoneuron progenitors (MP) derived from hESC. In the present

study, we further analyzed the underlying cell and tissue mechanisms of functional recovery after cell transplantation of OPC and MP hypothesized that beneficial effect is mediated by regenerative signalling pathways activated in the host tissue by transplanted cells. Here we show that transplantation of hESC-derived OPC and MP promote astrogliosis, thorough activation of jagged1-dependent Notch and Jak/STAT signalling supporting axonal survival.

P211

Effects of the mesenchymal stem cell administration in an animal model of subcortical cerebral infarct

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Introduction: Despite its high incidence (15–22% of all ischemic strokes) subcortical cerebral infarct has not been deeply investigated, for this reason, no data is currently available on its response to the treatment.

Objective: To analyze the effect on functional recovery of the mesenchymal stem cell (MSC) administration in an animal model of subcortical cerebral infarct.

Methods: Subcortical cerebral infarct was induced in the internal capsule of rats by Endothelin-1 injection (0.25 ug/uL). 24 h after stroke the animals were subjected to 2×10^6 MSC administration (iv) (treated group) or saline injection (control group). Lesion size (MRI) and motor functional evaluation (Walking-beam, rotarod, Rogers) were analyzed during 7 days after treatment. Histological studies of proliferation (Ki-67), cell death (TUNEL), immunofluorescence and western blot were performed after they were sacrificed (day 7th).

Results: MSC-treated animals showed a functional deficit reduction (Walking-beam, $p < 0.05$) compared to controls 72 h after treatment. Furthermore, at 7 d the lesion area ($p < 0.05$) and the cell death ($p < 0.05$) was reduced compared to the control animals. Thus in treated group we observed an increase of cell proliferation ($p < 0.05$) and higher levels of cerebral repair markers as VEGF ($p < 0.001$) and LINGO-1 ($p < 0.05$) compared to control group 7 d after treatment.

Conclusion: In an animal model of subcortical cerebral infarct MSC therapy was effective on functional recovery, lesion area and cell death reduction. Furthermore, this treatment enhanced brain repair processes.

P212

Comparison between xenogenic and allogenic adipose tissue mesenchymal stem cells in treatment of acute cerebral infarct Concept proof in rats

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Background: It has been suggested that mesenchymal stem cells (MSC) are appropriate for stroke treatment, demonstrating safety, feasibility and efficacy. However, more information re-

garding appropriate cell type is needed from animal model. Therefore, it would be interesting to perform a concept proof: to administer the human Adipose Tissue-derived-MSC (hAD-MSC) (xenogenic administration) to demonstrate the safety itself in the animal model. Aims: To study the safety and the of acute intravenous (i.v.) xenogenic administration of hAD-MSC or allogenic rat Adipose Tissue-derived-MSC (rAD-MSC) on functional evaluation in rat model of permanent Middle Cerebral Artery Occlusion (pMCAO).

Methods: Model of pMCAO in rats in 4 groups (n=10): a) Sham; b) Control with Infarct; c) hAD-MSC: surgery infarct i.v hAD-MSC (2×10^6 cells); d) rAD-MSC: surgery infarct i.v rAD-MSC (2×10^6 cells). We analyzed: functional evaluation; infarct volume by Magnetic Resonance Imaging (MRI) and Hematoxylin-Eosin; cell implantation by MRI and immunohistochemistry. All these parameters were analyzed at 24h and 14 days. Cell death by TUNEL at 14 days and tumor formation at 3 months.

Results: Compared to Control with Infarct group, a significant improvement in recovery was found at 24h and continued at 14 d after i.v. administration of either hAD-MSC or rAD-MSC ($p < 0.05$). No reduction in infarct volume or any migration/implantation of cells into the damaged brain were observed in the treatment groups. Nevertheless, cell death was reduced significantly in both treatment groups with respect to the Control with Infarct group ($p < 0.05$). We did not observe tumor formation at 3 months in none treatment groups.

Conclusion: The administration of hAD-MSC or rAD-MSC demonstrated equal efficacy on functional recovery and decreased ischemic brain damage (reduction cell death). Besides, both treatment groups have demonstrated safety without side effects nor tumor formation.

P213

Intravenous mesenchymal cells therapy in the acute phase after traumatic brain injury improves neurological recovery

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Objective: The aim of this study was to evaluate the effects of intravenously (IV) administered allogeneic mesenchymal stem cells (MSC), in the acute period after a traumatic brain injury (TBI), in improving short term functional recovery.

Methods: MSCs were isolated from peritoneal fat of healthy rats, expanded in vitro, and labeled with a fluorescent protein GFP. Groups of 6 Sprague-Dawley rats received physiological saline, a single dose of two million MSCs or three doses of two million MSCs respectively, within the first 24–72 hours after receiving a moderate, unilateral, controlled cortical impact. Histological examination and immunohistochemistry were used to identify cell distribution. Motor and cognitive behavioural testing (Rota rod, stickytape and modified Roger's test) were performed to evaluate functional recovery.

Results: No adverse effects were observed during or after the administration of MSCs. MSCs were found in the perilesional area 24 hours and 14 days after the IV infusion. There was higher punctuation of all three cognitive behavioural tests in the

multidoses treatment group compared to single dose group and to placebo. These differences were statistically significant in the Roger's test.

Conclusions: IV infusions of multidoses of MSCs immediately after a TBI were well tolerated. The multidoses treatment resulted in better recovery of motor and cognitive functions compared to single dose. This cellular therapy might be considered for patients suffering TBI.

P214

FGF8 and disease: a new role for an old friend

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Fibroblast growth factor 8 (FGF8) is an intercellular signaling molecule which plays vital roles during embryonic development of the central nervous system (CNS). Specifically, this growth factor regulates neural induction, survival, proliferation, differentiation and axon path-finding. Several studies have demonstrated the importance of Fgf8 in the treatment of neurodegenerative disorders such as Parkinson's disease, however, our knowledge concerning the role of Fgf8 in CNS pathologies remains poorly understood.

In this work, we have observed a new possible role of this factor in progenitor cell activation. In vitro, neural stem cells (NSC) and oligodendrocyte progenitor cells (OPC) were cultured with differentiating medium and in the presence of various concentrations of FGF8. Differentiation and proliferation studies were performed by immunocytochemistry, conventional and quantitative PCR. Also, migration studies were performed in matrigel, where NSC or OPC were placed at a certain distance of a FGF8-soaked heparin bead. In this case, outgrowths from the progenitor cell clusters were observed, indicating either a migratory or proliferation effect mediated by the growth factor. To discern which effect was activated, migration and proliferation markers were analyzed in the matrigel cultures. The results shown in this work demonstrate that FGF8 is capable of activating progenitor cells (both NSC and OPC) in culture. This effect may be interesting for neurodegenerative diseases, as FGF8 may be injected either into the cerebral-spinal fluid or directly into the region of interest to activate nearby NSC and OPC to induce regeneration. Future work will be focused on this aspect using neurodegenerative animal models.

P215

A new cell therapy approach: endogenous stem cell mobilization against amyotrophic lateral sclerosis

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ALS is the most common adult-onset motor neuron disease affecting humans. It is characterized by gradual degeneration of

upper and lower motor neurons as well as a progressive damage of skeletal muscle. Unfortunately, there is not an appropriated therapy for treating this disease. Here we propose the use of Pegfilgramstin, a pegylated form of the Granulocyte Colony Stimulation Factor pegylated analog Filgrastim as a potential treatment for Motorneuron disease. Our hypothesis is that hematopoietic stem cell (HSC) mobilization could be beneficial in the treatment of neurodegenerative diseases such as Amyotrophic Lateral Sclerosis. To this aim, SOD1G93A mice were treated with 300 ug/kg Neulasta[®], subcutaneously, once a week beginning at the age of 70 days. Then, behavioral tests, as well as flow cytometry, qRT-PCR and Western Blot techniques were carried out to evaluate the effect of Neulasta[®] treatment. Mobilization of endogenous stem cells by Neulasta[®] showed a beneficial effect on locomotor performance and prolongs the survival of SOD1G9A mice. Neulasta[®] treatments prolonged on time the HSC increase on peripheral blood. Moreover, the genetic expression profile of the muscle may indicate that under Neulasta[®] treatment, the NMJ stabilization and muscle metabolic restoration can be favored in the SOD1G93A mice model of ALS. These results suggest that mobilizing stem cells, facilitating their transformation and assisting in their recruitment by damaged tissue could be a potential therapeutic approach. Further studies will be needed to determinate its molecular mechanism of action.

P216

Delivery of the 135 Kb human frataxin genomic DNA locus gives rise to different frataxin isoforms by correct alternative splicing

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Friedreich's ataxia (FA) is the most common form of hereditary ataxia among the Caucasian population and is caused by recessive mutations in the *FRDA* (*FXN*) gene which encodes for a protein referred to as frataxin.

Frataxin was thought to be mainly localized to mitochondria. However recent results have indicated the presence of different isoforms produced by alternative splicing. Thus, in addition to the canonical *FXN* I isoform (which is localized to mitochondria), two novel isoforms have been characterized: *FXN* II which is mainly found at the cytoplasm and *FXN* III which localizes into cell nuclei. Interestingly these novel isoforms seem to be expressed in tissues such as cerebellum and heart which are severely affected in FA.

Our previous studies have demonstrated some potential advantages of using high capacity herpes simplex virus type 1 (HSV-1) amplicon vectors containing the entire *FXN* genomic locus as gene-delivery vehicles capable of ensuring physiologically-regulated and long-term persistence of *FXN* gene expression.

Here we describe how expression from the 135 Kb human *FXN* genomic locus renders all frataxin isoforms both in cultured neuronal cells and also *in vivo* after the intracranial injection of HSV vectors into the adult mouse cerebellum. Moreover, we have also observed the correct expression of all frataxin isoforms in patient-derived cells after the delivery of the 135 Kb human *FXN* genomic locus.

In a more general way, these results support the potential of using high-capacity vectors containing entire genomic loci whose expression is mediated by complex posttranscriptional mechanisms for gene therapy applications.

P217

Astrocyte-specific silencing with a tetracycline-regulated lentiviral vector

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Astrocytes represent the most abundant cell type of the brain and are indispensable for neuronal function. They are implicated in neuronal dysfunction and death observed in many neurodegenerative diseases, thus making them potential targets for gene delivery systems. Among these systems, viral vectors and especially lentiviral vectors (LV) offer the possibility to overexpress a transgene in the CNS. We have previously developed an LV targeting specifically astrocytes by combining a Mokola G (MOK/LV) pseudotyping and miRNA detargeting (miRT) strategy (Colin *et al.* 2009). However, this system is not suitable for astrocyte-specific silencing due to the maturation and cleavage of miR-embedded shRNA. To overcome this limitation, we used four strategies: pseudotyping of LV with Mokola G protein (MOK/LV), expression of miRNA-embedded shRNA under a Tetracycline promoter, regulation of the transactivator using astrocyte specific glutamine synthetase promoter (GS) and miRT detargeting. The efficacy of a vector encoding an shRNA targeting the GFP was evaluated in BAC GLT1-GFP transgenic mice expressing the reporter gene exclusively in astrocytes. To demonstrate the specificity of the silencing, we used a bicistronic LV expressing both GFP and mCherry into neurons. The mCherry/GFP ratio was used to quantify potential residual silencing in neurons. Our results demonstrate that astrocyte-specific gene silencing can be achieved by an inducible system regulated by an astrocytic promoter and miRT detargeting. We are currently using this astrocytic LV to specifically silence mutant huntingtin in astrocytes in order to evaluate the contribution of astrocytes in HD

P218

Changes in DNA methylation and gene expression in human periodontal ligament stem cells from healthy and Friedreich's ataxia patients throughout passages and under oxidative stress

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Friedreich's ataxia (FA) is the most common hereditary ataxic disorder, caused by an expanded GAA repeat in the first intron of the frataxin (FXN) gene. This repeat expansion is the cause for decreased FXN protein in the cells, and ultimately in gene silencing. The most affected cells are the cardiomyocytes and proprioceptive neurons in the dorsal root ganglia of the spinal cord. Several studies have demonstrated the existence of FXN gene methylation patterns in human tissues from brain, heart and blood, although with varied results due to the heterogeneity of the cell populations. Thus, in our work, we strived to analyze

DNA methylation patterns and transcriptional expression in primary cultures of periodontal ligament cells isolated from healthy and FA patients. This cell type derives from the neural crest, and expresses both neural and mesenchymal stem cell markers. Previous studies, both in our lab and others, have demonstrated that cells isolated from FA patients are sensitive to oxidative stress, causing premature death, and could be rescued by trophic factors, mainly BDNF, present in adipose tissue-derived stem cell-conditioned medium. In this work we compared periodontal ligament cells both at different passages (as an aging model) as well as under oxidative stress. Also, 5-aza-2 deoxycytidine, a DNA methylation inhibitor, was applied to the cultures to study the possible reactivation of aberrantly epigenetically silenced genes in FA. Finally, besides FXN, other genes related to oxidative stress, trophic factors, epigenetics and cell cycle were analyzed. The results of this study may help understand how FXN is regulated as well as allow us to search for new therapeutic pathways for FA.

P219

Restriction of Calpain 3 expression to the skeletal muscle prevents cardiac toxicity and corrects pathology in a murine model of Limb-Girdle Muscular Dystrophy

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Background: Genetic defects in calpain 3 (*CAPN3*) leads to Limb-Girdle Muscular Dystrophy type 2A (LGMD2A), a disease of the skeletal muscle that affects predominantly the proximal limb muscles. We previously demonstrated the potential of Adeno-Associated Virus (AAV)-mediated transfer of the *CAPN3* gene to correct the pathological signs in a murine model for LGMD2A after intramuscular and loco-regional administrations. **Methods and Results:** Here, we showed that intravenous injection of calpain 3-expressing vector in mice can induce mortality in a dose-dependent manner. An anatomic-pathological investigation revealed large areas of fibrosis in the heart that we related to unregulated proteolytic activity of calpain 3. To circumvent this toxicity, we developed new AAV vectors with skeletal muscle-restricted expression by using new muscle-specific promoters that include the *CAPN3* promoter itself and by introducing a target sequence of the cardiac-specific microRNA-208a in the cassette. Our results show that *CAPN3* transgene expression can be successfully suppressed in the cardiac tissue, preventing the cardiac toxicity, while expression of the transgene in skeletal muscle reverts the pathological signs of calpain 3 deficiency. **Conclusion:** The molecular strategies used in this study may be useful for any gene transfer strategy with potential toxicity in the heart.

P220

Murine muscle engineered from dermal precursors: an in vitro model for skeletal muscle generation, degeneration and fatty infiltration

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Skeletal muscle can be engineered by converting dermal precursors into muscle progenitors and differentiated myocytes. However, the efficiency of muscle development remains relatively low and it is currently unclear if this is due to poor characterization of the myogenic precursors, the protocols used for cell differentiation, or a combination of both. In this study, we characterized myogenic precursors present in murine dermospheres, and evaluated mature myotubes grown in a novel 3D culture system. After 5–7 days of differentiation we observed isolated, twitching myotubes followed by spontaneous contractions of the entire tissue-engineered muscle construct on extracellular matrix (ECM). In vitro engineered myofibers expressed canonical muscle markers and exhibited skeletal (not cardiac) muscle ultrastructure, with numerous striations and presence of aligned, enlarged mitochondria, intertwined with sarcoplasmic reticula (SR). Engineered myofibers exhibited Na and Ca²⁺ dependent inward currents upon acetylcholine (ACh) stimulation and tetrodotoxin-sensitive spontaneous action potentials. Moreover, ACh, nicotine and caffeine elicited cytosolic Ca²⁺ transients; fiber contraction coupled to these Ca²⁺ transients suggest Ca²⁺ entry is activating calcium-induced calcium release from the SR. Blockade by d-tubocurarine of ACh-elicited inward currents and Ca²⁺ transients suggests nicotinic receptor involvement. Interestingly, after one-month engineered muscle constructs showed progressive degradation of the myofibers concomitant with fatty infiltration, paralleling the natural course of muscular degeneration. We conclude that mature myofibers may be differentiated on ECM from myogenic precursor cells present in murine dermospheres, in an in vitro system that mimics some characteristics found in aging and muscular degeneration.

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The hybrid promoter providing the high level expression in myoblasts

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Our laboratory has been working on the project aimed at the development of a muscle-specific viral vector for use in gene therapy of muscle specific genetic diseases such as DMD or EDMD. One of the employed strategies is to design a muscle-specific, highly active expression cassette containing hybrid

promoter for such virus. High efficiency and long term expression of a genetic drug is the crucial, limiting factor for designing the effective therapy for muscular dystrophies. We designed the hybrid promoter, composed of various, muscle specific regulatory elements, that provides a high level of a gene expression in myoblasts. It contains control elements responsible for obtaining a stable, specific and high-level expression of a therapeutic gene needed for a correction of a genetic defect in the muscle tissue. It consists of elements such as enhancers, core promoter and intron with small intronic enhancer. They originate from murine, muscle specific genes chosen and modified basing on their expression profiles, previous research data and in silico analysis. The studies on the activity of the hybrid promoter was performed by analyses of expression of a reporter gene—secretory luciferase using luminescence. Our data shows, that developed promoter provides several folds higher level of the expression in C2C12 cell line that promoter of desmin. The specificity of the promoter was also examined using different cell lines: H9C2, NIH-3T3, Hek 293, HepG2. The influence of the particular components of the hybrid promoter on the expression level in muscular and non-muscular cell lines was also investigated.

P222

Serum miRNA profiling of GRMD dog identified novel cardiomyopathy biomarker candidates for DMD

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Duchenne Muscular Dystrophy (DMD) is a fatal, X-linked neuromuscular disease that affects 1 boy in 3500. The GRMD dog (Golden Retriever Muscular Dystrophy) is the best clinically relevant DMD animal model. In the present study we evaluated some newly identified circulating miRNA biomarkers for muscular dystrophy in the GRMD model. Additionally we screened, using both RT-QPCR arrays and High Throughput Sequencing, for new candidate circulating miRNA biomarkers. We confirmed the dysregulation of the previously described dystromiRs, miR-1, miR-133 miR-206 and miR-378, and identified a new candidate, miRNA that might be classified in the same dystromiR group. We identified two other classes of dysregulated serum miRNAs in muscular dystrophy. The first are miRNAs associated with cardiac pathology. The second are miRNAs belonging to the largest known miRNA cluster that resides in the imprinting DLK1-DIO3 genomic region. Finally we have confirmed a dysregulation of the miRNAs associated with cardiac pathology also in a small cohort of DMD patients.

This is the first report characterizing seric miRNA biomarkers in GRMD, a highly useful preclinical DMD model. Given the strong interspecies conservation of miRNAs, and our preliminary data in DMD patients, these newly identified dysregulated miRNAs in GRMD are strong candidate biomarkers for DMD.

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Bone marrow engraftments in hind limb muscles of ALS mouse model improves motor function and biological markers of neuromuscular integrity

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Amyotrophic lateral sclerosis (ALS) is a motoneuron degenerative disease, characterized by degeneration of upper and lower motor neurons; which leads to progressive paralysis and death from respiratory failure within 3–5 years of symptoms onset. In this work, SOD1 mutant mice were used to study the potential neurotrophic effect of bone marrow cells grafted into Quadriceps Femoris muscle.

Bone Marrow cells were grafted into *Quadriceps Femoris* of SOD1 mice and behaviour, trophic factors, muscle genetic markers and lifespan were studied. Intramuscular engraftments resulted in increased longevity with improved motor function and decreased motoneuron degeneration in the spinal cord. Moreover, certain muscle disease-specific markers, which are alternated in SOD1 mutant mouse, and may serve as molecular biomarkers for the early detection of ALS in patients, have been studied.

This work demonstrated that stem cell transplantation in the muscle increased motoneuron survival and showed the correction of muscular biomarkers of disease progression.

P228

In vitro study of MSCs human cultures differentiated into osteoblasts and albumin scaffolds to repair bone defects: preliminary results.

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Introduction: Bone healing is often impaired in clinical situations in which loss of bone is caused by disease, trauma or tumour resection. The treatment with autologous bone grafting presents some restrictions. Mesenchymal Stem Cells (MSCs) constitute a cell type with promising possibilities in bone regenerative medicine based on their high pro-angiogenic and pro-osteogenic properties. In other hand, a cross-linked plasma-based matrix is a biodegradable scaffold which have been used successfully in several tissue engineering approaches. Combination of these two components could mimic the advantages of autologous bone grafting while avoiding its main limitations.

Objective: The aim of this study was to investigate the microstructure and biocompatibility of an albumin scaffold developed from human serum and human MSCs differentiated into osteoblasts.

Material and Methods: MSCs isolated from human adipose tissue were cultured in the noncalcified protein scaffold prepared with plasmatic albumin crossed with a glutaraldehyde-type agent. Measurement of the differentiation marker alkaline

phosphatase were performed after 30 days of osteogenic differentiation. These scaffolds were processed to be analysed at *Scanning Electron Microscope* (SEM).

Results: Results showed that differentiated cells adhered to the scaffold and these cells kept their cellular viability. Cultured MSCs differentiated into osteoblasts showed comparable phenotypic profiles and expressed alkaline phosphatase in albumin scaffold. The microanalyses showed the presence of calcium in the samples of scaffolds with bone differentiated cells.

Conclusion: The results indicate that this scaffold is adequate for the growth and differentiation of MSCs into osteoblasts and a promising material for bone tissue engineering.

P229

In vitro effects of hyaluronate on adipose tissue-derived mesenchymal stem cells

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Local delivery of mesenchymal stem cells (MSC) could be a useful treatment option for knee osteoarthritis (KOA), a degenerative disease of the joint with no current completely effective treatment. Hyaluronate (HA) is injected within the joint as a common therapy with a lubricant effect that decreases pain. Recent clinical trials for KOA tested intraarticular injection of MSC, with promising benefits in reducing pain and preventing cartilage degeneration. We evaluated if HA has any effect on MSC in the likelihood of a combined treatment for KOA. Human ASC were maintained quiescent for 1 week under serum deprivation \pm HA (1 mg/ml). Cell proliferation was evaluated at day 7 with CCK-8 assay. Total RNA was extracted, cDNA synthesized, and expression of CD44 (HA receptor), collagen type 2, and anti-inflammatory cytokines was evaluated by QPCR. ASC proliferation significantly increased after 7 days with HA ($158 \pm 39\%$, $p < 0.05$), with no change in the level of expression of CD44. Hyaluronate treatment did not induce chondrogenic differentiation (undetectable COL2) in ASC, nor change in TGFbeta expression. However, ASC with HA showed a 2-fold increase in IL-10 expression and a 4-fold decrease in IL-6 expression. Hyaluronate effects on ASC could increase the benefits in a combined intraarticular injection for KOA, reducing synovial inflammation that causes pain and disability.

P230

Unexpected impaired autophagy in a model of premature aging based on hMSCs

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The accumulation of immature forms of key component of the nuclear lamina, the Lamin A proteins (prolamin A and progerin), leads to the development of laminopathies. Among these disorders are included the premature aging human syndromes such as mandibuloacral dysplasia, LMNA-linked lipodystrophies and Hutchinson-Gilford Progeria syndrome, which mainly affect mesenchymal lineages. To date, the role of nuclear envelope abnormalities in the physiological aging is

unclear although recent studies have demonstrated an accumulation of prelamin A and progerin in normal aging. This result supports the idea that studies on *in vitro* progeroid cell models are useful tools for studying the normal human aging process.

Autophagy is a basic mechanism involved in degradation of unnecessary or dysfunctional proteins and organelles. A common characteristic of aging cells is the accumulation of these damaged proteins and organelles, in part due to a decrease in their autophagic activity. However, the mechanisms by which autophagy could be impaired in aging (pathological or physiological) remain unclear.

Here we show that hMSCs which demonstrate a premature aging phenotype due to prelamin A accumulation are more susceptible to stress conditions. Surprisingly, these cells show an enhanced mTOR pathway inhibition and autophagy activation. Gene expression analysis suggests that a specific transcription factor could account for this phenotype. The silencing of this transcription factor in hMSCs results in the induction of senescence and activation of autophagy suggesting that its dysregulation could account in part for the premature aging phenotype observed in hMSCs which accumulate prelamin A.

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Study of the osteoconductive and angiogenic potential of Electron Beam Melting Titanium (EBMT)

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According with the increasing incidence of bone diseases with great loss of tissue, it is necessary to employ new biomaterials with capacity to host osteoprogenitor and angioprogenitor cells to repair this bone tissue lack.

Otherwise, bone injuries are not the same in form and size in every patient, so EBMT allows to obtain different kind of implants according with the injury's size and form on the way of the personalized medicine.

In order to obtain a completed bone regeneration it is necessary to employ bone progenitor cells seeded on an appropriated scaffold to proliferate and differentiate refilling the bone injury. But it is also necessary to use angiogenic progenitor cells to obtain blood vessels to ensure the viability of the repaired tissue. So, on this work both kind of cells were seeded over EBMT pieces to study the capacity of this biomaterial for become a right support for these cells and favor their proliferation and differentiation.

Different seeding situations were employed on this work to evaluate the cell behaviour over EBMT implants: bone and endothelial monoculture and bone-endothelial co-culture over implants with and without fibronectin coating. Also an specific endothelial inflammatory assay was included on this work. The results were observed with confocal microscopy techniques using specific trackers and antibodies during 21 days of culture. In conclusion, EBMT seems to be a nice scaffold for bone and endothelial cells even without a fibronectin coating, no endothelial inflammatory response was observed and cell proliferation and differentiation were observed over the implants including the beginning of blood vessels formation in co-culture groups.

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Bone marrow mesenchymal stem cells, collagen scaffold and BMP-2 for rat spinal fusion

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The use of autograft for posterolateral spinal fusion, continue being considered the gold standard for the treatment of spine pathologies. However, due to complications such as donor site morbidity, increased operating time, and limited supply, the use of allograft has become an acceptable practice especially in multisegment arthrodesis or in patients with previous graft harvests. Since their use involves the risk of immune response or disease transmission and fusion rates are not as good as with autogenous bone, a variety of bone graft substitutes are being studied to obtain a better alternative. Osteoinductive growth factors, which initiate the molecular cascade of bone formation and play a key role in the development and regeneration of the skeletal system, have been shown to be effective in numerous animal studies. These molecules must be used in combination with a biomaterial to avoid their dispersion from the application site. On the other hand, it is well known that cultured bone marrow cells, harvested from adult bone marrow, may contribute to the regeneration of bone. Thus, hybrid constructs can be used as alternatives to autologous and allogenic grafts.

In this study, we have evaluated different combination of cultured bone marrow cells with recombinant human osteoinductive growth factors, all of them in combination with a natural polymeric carrier, for the promotion of posterolateral spinal fusion in rats.

P233

Characterisation of a collagen-targeted RGD biomimetic peptide for bone regeneration

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Osteogenesis is a complex process in which many different signals are involved. Although bone morphogenetic proteins (BMPs) are the most potent inducers of osteoblastic differentiation used in the clinics, very high doses of these pleiotropic growth factors are needed, in combination with collagen type I formulations, to achieve successful bone healing. To date, integrin-binding Arginine-Glycine-Aspartate (RGD) peptides have shown some promising abilities to promote the attachment of cells to biomaterials and to direct their differentiation. However, functionalization of the biomaterials with these peptides implies chemical linking steps. In this work we describe the design and characterization of a synthetic RGD biomimetic peptide (CBD-RGD) formed of a collagen-binding domain derived from the von Willebrand and the integrin-binding RGD sequence from fibronectin. This peptide was demonstrated to bind stable to absorbable

collagen type I sponges (ACs) without chemical manipulations, and to induce the differentiation of preosteoblasts and bone marrow-derived mesenchymal stem cells, as well as enhancing matrix mineralization, without triggering apoptotic responses. Furthermore, ectopic bone formation experiments in rats showed that ACs functionalized with this peptide and loaded with a subfunctional dose of BMP-2 gave rise to ectopic bone, while non-functionalized sponges loaded only with the same dose of BMP-2 did not. These results indicate that the combination of this biomimetic peptide with the currently used collagen BMP system might be a promising approach to improve osteogenesis and to reduce the doses of BMPs needed in clinical orthopaedics.

P234

Improvements in gait function after mesenchymal stem cell therapy for knee osteoarthritis

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Introduction: Mesenchymal stem cell therapy (MSCT) is a promising approach in regenerative medicine. Quantitative analyses of the effects will help in evaluating its potential. Knee osteoarthritis (KO) associates gait dysfunctions. Pain and stiffness are the main findings of the clinical examination, but clinical gait analysis using 3D motion capture systems could offer a more in detail evaluation. We present here the effects of MSCT on gait function.

Patient & Methods: A 70 years old male patient with severe KO received 3 doses of autologous adipose-derived MSCs. Therapy was administered locally, with at least 1 month interval. A 3D Gait Analysis was performed before and 1 month after each administration. We used an 8 infra-red camera system (Smart-D from BTS BioEngineering) for kinematic acquisition. A correction of the conventional gait model using a quad test for adjustment was done.

Results: We studied some critical kinematic values of the hip and knee joints (maximum and minimum flexion and extension) during stance phase expressed as a percentage of normal values. In order to obtain a global index for gait modifications, Gait Deviation Index (GDI) was calculated before and after therapy. A normalization of all the values studied was observed. Values pre-therapy were in the 30%-40% (control 100%), raised to 50%-70% after 1 dose, and up to 80%-90% after the last dose. We also observed a moderate improvement of the GDI (less than 5 points in the right side and more than 5 in the left one).

Conclusions and Clinical Applications: The normalization of knee extension in stance and the improvement observed at the hip joint during gait were the main benefits found after cell therapy. These preliminary results are promising, and show that the effects of MSCT in patients with KO can be evaluated quantitatively.

P235

MPLSM Imaging of VEGF-induced vascular permeability

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Vascular permeability has shown to reflect changes in the barrier function of the endothelium and its interendothelial junctions. Molecular mechanisms regulating the permeability of the endothelium are still obscure. Known common mediators of vascular permeability and intercellular junction regulation are growth factors, such as vascular endothelium growth factors (VEGFs). Previously, therapeutic angiogenesis, utilizing VEGF-induced vascular growth, has shown to induce blood vessel growth and skeletal muscle perfusion. At present, the main hurdle in Ad-mediated VEGF therapy is the increased plasma protein extravasation and vascular hyperpermeability, leading to tissue edema and instability of newly formed vessels. In this study, vascular permeability caused by different VEGF family members was studied *ex vivo* in mice using multiphoton laser scanning microscopy (MPLSM). Amount of the extravasated fluorescent microspheres, used as a detection marker for vascular hyperpermeability, was shown to be dependent on the VEGF type, whereas no leakage was detected in the negative control without the permeability agents. Permeability response was also shown to vary between VEGFs using the same VEGF receptor. Knowledge of the cellular mediators causing VEGF-mediated hyperpermeability *in vivo* is needed for further optimization of the AdVEGF therapy.

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Differences between methodological approaches and animal species on the whole liver decellularization process, for tissue engineering

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Liver diseases, ranging from congenital metabolic viral hepatitis, to the injuries from alcohol abuse, often result in the need for liver transplantation. Insufficient supply of suitable organs for transplantation has limited the ability to cure many cases of these diseases.

Three techniques to make artificial organs have been described: (I) To make obtaining a collagen scaffold with the form of an organ that will be covered by the patient's cells, (II) developing to make cell arrays, by "tattooing" cell groups and so forming a body structure mounted layer by layer and (III) removing to deplete completely all the cells of an organ and then recellularizing it with the patient's cells by regular techniques as the ones tested here.

In the technique discussed here, we will use a donor organ -in this case a liver- and we will remove all the cells, leaving only the scaffold. This is achieved by 'washing' with enzymatic detergents. Then, by infusion, the liver will be repopulated with the patient's new cells using the original structure, but obtaining a compatible organ that could be implanted onto the patient.

In this work, we show how previously described the techniques previously described for organ decellularization, showed by several groups, do not show the same efficiency level, at least for liver tissue. Moreover, the animal specie of study we have been observed to be that an important issue when obtaining to obtain a total or partial decellularization would be the animal

specie of study. Therefore, we conclude that the correct specie of study and technique employed are essential for the success of the total decellularization process.

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Lentivirus-mediated silencing of prothymosin α decreases the incidence and severity of cigarette smoke-induced emphysema in mice

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Emphysema, the major consequence of chronic obstructive pulmonary disease (COPD), is characterized by permanent air-flow restriction resulting from enlargement of alveolar airspace and loss of lung elasticity. Cigarette smoking is associated with the severity of emphysema, which also correlates with the degree of progressive pulmonary inflammation. Although cigarette smoking is the main risk factor, only about 25% of smokers develop emphysema. Therefore, there may be unidentified genetic or host factors that predispose individuals to emphysema. Prothymosin α (ProT) possesses diverse functions that are involved in proliferation, apoptosis, oxidative stress, immunomodulation, and acetylation. We have generated ProT transgenic mice that exhibit an emphysema phenotype, but the pathophysiological role of ProT in emphysema remains unclear. Here we show that ProT contributes to the pathogenesis of emphysema by increasing acetylation of histones and NF- κ B, particularly upon cigarette smoke (CS) exposure. We find a positive correlation between ProT levels and the severity of emphysema in ProT transgenic mice. ProT overexpression increases susceptibility to CS-induced emphysema, and CS exposure further enhances ProT expression. To further support the physiological association of ProT with the development of emphysema, we delivered lentiviral vectors expressing ProT short hairpin RNA (shRNA) into the lung of wild-type FVB mice to knockdown the endogenous ProT expression. This results in decreases in the incidence and severity of emphysema after CS exposure. We further show that ProT inhibits the association of histone deacetylases (HDACs) with histones and NF- κ B, and that ProT overexpression increases expression of NF- κ B-dependent matrix metalloproteinase 2 (MMP2) and MMP9. Taken together, our findings elucidate the pathophysiological role of ProT and identify a potential novel molecular mechanism in the pathogenesis of COPD.

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Role of mesenchymal stem cells in equine laminitis

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Laminitis is the most serious disease of the equine hoof and causes dramatic loss of function. It consists in a failure of the

attachment between the distal phalanx and the inner hoof wall, causing pain and strong lameness in horses (1). Many therapeutic options have been investigated and used, but to date, a definitive treatment does not exist. Regenerative medicine, in particular stem cells, has been suggested as a possible treatment option for this disease (2). The aim of this study is to investigate the possible implication of mesenchymal stem cells (MSCs) in the equine laminitis. MSCs have been isolated for the first time from coronet and lamellae of the dorsal hoof wall by enzymatic digestion. To characterize the isolated cells, we have studied the expression of different surface markers by Flow Cytometry and qRT-PCR, and their tri-lineage differentiation capacity. Cells obtained from hoof met the criteria for defining MSCs established by the ISCT (3). Proliferation and differentiation ability of MSCs obtained from hoof (HF-MSCs) were compared with the bone marrow MSCs (BM-MSCs). Comparisons between HF-MSCs from healthy and laminitic horses were carried out in order to better understand the possible role of this type of cells in the hoof growth. The present study could allow us to develop effective treatments for this devastating disease.

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Molecular imaging of the mesenchymal stem cells migratory properties towards different pathologies

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Bone marrow-derived mesenchymal stem cells (MSCs) are multipotent adult stem cells from mesodermal origin that are present within the bone marrow stroma. MSCs are capable of multilineage differentiation under appropriate stimuli. Due to their ease isolation and their pluripotent nature, MSCs are an ideal source for clinical and regenerative medicine in a great variety of applications. One characteristic of MSCs is their capacity to migrate and to proliferate within areas of inflammation and tumors, as part of the tissue remodeling process.

In this report, we demonstrated that when labeled mesenchymal stem cells are injected systemically and simultaneously into animals with different pathologies, they tend to migrate naturally to the damaged tissue independently of the pathology.

To prove this hypothesis, we performed *in vitro* and *in vivo* imaging techniques by using different reporter genes (GFP, hNIS and R-Luc) and non-invasive techniques (PET, BLI or fluorescence). As expected, MSCs engrafted tumors, targeted the pancreas (for diabetes) or went to the skin (for injury/wound healing), as those were the expected target organs. We were not able to detect almost signaling in any other organs at the different time points, but changes in the intensity of the signal along the experiment were observed, suggesting that MSCs possess migratory capacity to damaged areas independently of the pathology and that they could be used to enhance tissue repair.

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Combination of low oxygen tension and HIF-1 α overexpression in mesenchymal stem cells induces secretion of exosomes loaded with anti-fibrotic and pro-angiogenic microRNAs

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Background: Mesenchymal stem cells (MSCs) are effective for treating ischaemic diseases and we have recently demonstrated that HIF-1 α overexpression in MSC (MSC-HIF) potentiates their therapeutic effects. It is believed that paracrine mechanisms are mediated at least in part by membrane vesicles. Among them, exosomes are spherical particles that are released to the outside of the cells and are capable of interact with a variety of cell types. In this work we analyzed the changes in composition of MSC and MSC-HIF derived-exosomes.

Methods: MSC or MSC-HIF were cultured in normoxia or hypoxia and exosomes were isolated from the culture medium. Exosome microRNA content was analyzed by qPCR.

Results: Exosomes released by hypoxia cultured MSC were loaded with hypoxia-regulated microRNAs. Exosomes derived from normoxia cultured MSC-HIF, but not MSC, contained miR-93, a pro-angiogenic microRNA and miR-193 that shows proliferative effects on MSC, as well as miR-106b and miR-29b that have been associated with HIF-1 α regulation in hibernating species. Moreover, culture of MSC-HIF induced exosomes containing miR-138, a negative regulator of apoptosis, miR-29c, an anti-fibrotic microRNA that directly targets a large number of extracellular matrix genes and reduces interstitial fibrosis, and miR-424, a pro-angiogenic microRNA also induced by hypoxia in endothelial cells.

Conclusion: The combination of HIF-1 α overexpression in MSC with culture in low oxygen tension induce the production of exosomes loaded with anti-fibrotic and pro-angiogenic microRNAs that could be used as therapeutic tools in regenerative therapies.

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Tracing the hallmarks of aging in an hMSC experimental model

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Aging, defined as time-dependent functional decline, is a natural consequence for every living organism. At least nine phenotypic hallmarks have been proposed to establish the aging phenotype. In the human aging process there is a clear correlation between age and the gradual loss of functions of many organs. Indeed, the process of aging is associated with an intrinsic deficiency of mesenchymal stem cells (MSC) to regenerate tissues and organs, as is the case with patients with premature aging syndrome (e.g. progeria), which could be due to a premature depletion of MSC reservoirs.

Alterations in the lamin A/C gene are responsible for the diseases called laminopathies, among them progeria and LMNA-linked lipodystrophy are characterized by the accumulation of progerin or the immature precursor prelamin A, respectively. Both disorders are accompanied by different metabolic complications with an increased risk of premature atherosclerosis and coronary disease, besides progeroid features, all characteristics of aging. Furthermore studies have revealed an accumulation of low levels of prelamin A and progerin in normal aging.

In an attempt to investigate the hallmarks of aging (normal or pathological) and the characteristics commonly involved in aging we used a cellular model based on human MSC, generated in our laboratory. This study demonstrated that prelamin A accumulation in human MSC is responsible for the characteristic phenotype of aging. Moreover, it shows that this experimental model could be an essential tool for the study of human aging and to develop therapeutic strategies to increase the disease-free time of life or healthspan.

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Double RNA *trans*-splicing induced gene repair of COL7A1

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In autosomal recessive dystrophic epidermolysis bullosa (RDEB), a severe inherited skin disease, mutations are located within the gene *COL7A1* encoding for type VII collagen. In this work we concentrate on the correction of an internal exon of *COL7A1* by RNA *trans*-splicing. To overcome the size-associated limitations in gene therapy we are using the combination of 5' and 3' *trans*-splicing to exchange a short *COL7A1* gene region. A double RNA *trans*-splicing molecule (RTM) includes two binding domains (BD) binding up- and downstream of the mutated gene region. We have established a fluorescence reporter-based screening system to identify the molecules with the highest *trans*-splicing efficiencies by co-transfection experiments in HEK293 cells.

In this setup the reporter molecule acGFP was split up into three parts. The internal part was cloned into the RTM whereas the 5' and 3' end of the reporter were part of an artificial target molecule containing the mutated gene region. In our double *trans*-splicing experiments we were able to achieve a GFP expression in up to 71% of all analyzed cells. The most efficient RTM can then be selected for further experiments in RDEB patient cells. Prior RTM transduction in this more relevant endogenous model system the internal acGFP part will be replaced with the wildtype sequence of *COL7A1*. We assume that double RNA *trans*-splicing is a promising tool to correct hot spot mutations in the gene *COL7A1* involved in the severe blistering skin disease epidermolysis bullosa.

P246

Fate and function of adipose-derived mesenchymal stem cells (ADMSC) in bioengineered skin equivalents *in vitro* and *in vivo*S Crespo Garcia^{1,2}, G R Malik¹, R M Moro², M Del Rio¹, J Luis Jorcano^{1,2}, F Larcher^{1,2}¹Área de Ingeniería Tisular y Medicina Regenerativa (TERMeG), Departamento de Bioingeniería e Ingeniería Aeroespacial, Universidad Carlos III de Madrid, Leganés, 28911, Spain; ²División de Biomedicina Epitelial, Departamento Investigación Básica, CIEMAT, Madrid, 28040, Spain

Skin equivalents have been effectively tested therapeutically using human fibroblasts embedded in a fibrin gel and seeded with keratinocytes. These skin substitutes can be used to cover areas of damaged skin in patients with various diseases, such as chronic ulcers, as well as patients with severe burns. However, as native human skin is composed of numerous mesenchymal cell types and adnexa, Adipose-derived mesenchymal stem cells (ADMSC) have turned into a prime candidate for replacing fibroblasts in current models. To assess the potential benefits of ADMSCs for transient and permanent skin regeneration, our team has analyzed both *in vitro* and *in vivo* the persistence of "stemness" and the regenerative performance of ADMSCs in comparison with that of human dermal fibroblasts. Studies were performed both on bioengineered skins before (*in vitro*) and after engraftment of bioengineered skins in immunodeficient mice (*in vivo*). The studies included the analysis of dermal matrix remodelling, the effects on keratinocyte maturation, the degree of angiogenesis promotion and the ability of ADMSCs to maintain their differentiation abilities towards adipose and osteocyte lineages upon recovery from engrafted tissue at different time points. Overall, our results indicate that ADMSCs maintain their stem-cell nature and show enhanced performance than dermal fibroblasts in all tests. Further studies are required to determine whether ADMSCs, forming part of either autologous or allogenic bioengineered skins, are beneficial under challenging clinical situations involving sustained cutaneous inflammation with concomitant impaired wound healing.

P247

Development of bioengineered skin-humanized mouse models for inflammatory skin diseasesM Carretero¹, S Guerrero-Aspiza², N Illera¹, B Duarte¹, A Holguin¹, L Retamosa¹, M Navarro³, F García⁴, J Dopazo⁴, F Larcher¹, M del Rio²¹Epithelial Biomedicine Division, Basic Research Department, Centro de Investigaciones Energéticas, Medioambientales y Tecnológicas (CIEMAT) and Centre for Biomedical Research on Rare Diseases (CIBERER) U714, Madrid, Spain; ²Department of Bioengineering, Universidad Carlos III de Madrid (UC3M), Madrid, Spain; ³Molecular Oncology Unit, Centro de Investigaciones Energéticas, Medioambientales y Tecnológicas (CIEMAT), Madrid, Spain; ⁴Computational Medicine Prince Felipe Research Centre and Centre for Biomedical Research on Rare Diseases (CIBERER) U715, Valencia, Spain

The objective of the present study was to develop a skin-humanized model for the two most common forms of inflammatory skin disease, atopic dermatitis and psoriasis. We have previously developed in our laboratory a bioengineered-based

skin-humanized mouse model for psoriasis. In this model, we used skin biopsies and blood samples derived either from psoriatic patients or from unrelated healthy donors. After engraftment in immunodeficient mice, phenotype was fully developed by intradermal injection in regenerated human skin of *in vitro* differentiated Th1/Th17 lymphocyte subpopulations/cytokines together with removal of stratum corneum barrier by tape-stripping. Following the same experimental design, intradermal injection of *in vitro* derived-Th2 lymphocyte subpopulations together with tape-stripping was used to develop a skin-humanized mouse model for atopic dermatitis. In both models, major hallmarks of psoriasis and atopic dermatitis were confirmed by the evaluation of specific epidermal markers either by immunohistochemistry or by RNA expression analyses. The characterization of the dermal component (angiogenesis and infiltrate) was also performed. Inflammatory and autoimmune cutaneous disorders constitute a major health and social problem around the world. Establishing reliable animal models for inflammatory cutaneous pathologies will allow for a deeper understanding of the basic mechanisms underlying the epidermal-immune cell interactions, and more importantly, will provide a useful platform for the evaluation of potential therapeutic strategies.

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A screening system accelerates the design of RNA trans-splicing molecules for skin cancer therapyU Koller¹, C Gruber¹, E M. Murauer¹, S Hainzl¹, C Hüttner¹, T Kocher¹, A P. South², H Hintner¹, J W. Bauer¹¹Division of Experimental Dermatology and EB House Austria, Department of Dermatology, Paracelsus Medical University, Salzburg, Austria; ²Division of Cancer Research, Medical Research Institute, Ninewells Hospital and Medical School, University of Dundee, United Kingdom

The possibility to target tumor marker genes by RNA trans-splicing molecules (RTMs) is a promising way to induce cell death in a tumor cell specific manner. Thereby a new genetic information (e.g. suicide genes) can be specifically expressed in tumor cells using the cell's own splicing machinery. The trans-splicing process is induced by designed RNA trans-splicing molecules leading to the recombination of the tumor marker gene and the suicide gene on RNA level. The resulting chimeric protein consists of the endogenous target mRNA and the coding sequence of a toxin and exhibits suicide activity. In order to improve the specificity of RTMs specific for the tumor marker gene solute carrier organic anion transporter family member 1B3 (SLCO1B3) overexpressed in recessive dystrophic epidermolysis bullosa (RDEB)-associated squamous cell carcinoma (SCC) we have established a HSV thymidine kinase-based RTM suicide model system. Different SLCO1B3-specific RTMs were created including thymidine kinase from herpes simplex virus (*HSV-tk*) and analyzed in HEK293AD cells co-transfected with a SLCO1B3 minigene (SLCO1B3-MG) for their trans-splicing efficiency. Expression of the trans-spliced SLCO1B3-*tk* fusion molecule was detected by semi-quantitative RT-PCR and Western blot analysis on RNA and protein level, respectively. Additionally, MTT and TUNEL assays showed the ability of the constructed RTMs to induce toxin-mediated apoptosis in SLCO1B3-MG expressing cells. Our inducible cell-death system provides a means to identify optimal RTMs prior to their use in suicide gene therapy approaches for RDEB-SCC.

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Blocking cis-splicing elements leads to increased RNA trans-splicing in the COL7A1 gene

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Mutations in the COL7A1 gene result in the dystrophic form of the blistering skin disease epidermolysis bullosa (DEB). Due to the huge size of the COL7A1 transcript with over 9 kb and the present packaging limitations of commonly used viral vector systems for gene therapeutic applications we are using RNA trans-splicing (SMaRT) as a potential tool to exchange mutated gene regions of COL7A1 with its wildtype version on RNA level.

However, the trans-splicing efficiency is generally low. To improve the trans-splicing potential of highly functional RTMs specific for COL7A1 we therefore used antisense sequence-mediated blocking of competitive 3' splice sites of a rational designed COL7A1 target molecule. We performed co-transfection experiments in HEK293 with RTM and COL7A1 target molecule expressing plasmids together with different concentrations of randomly designed antisense molecules (AM) specific for the target intron/exon region. Dose dependent treatment of HEK 293 cells with an AM concentration ranging from 0,5 – 5µg revealed an up to 3 fold increase of trans-splicing efficiency verified by SQRT-PCR, western blot analysis and flow cytometry. The ability of the antisense molecule to enhance the trans-splicing rate in the nucleus will now be further tested in COL7A1 deficient patient cells.

P250

A stable fluorescence-based COL7A1 mini gene expression cell line accelerates the design of efficient RNA trans-splicing repair molecules (RTMs)

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Functional defects in type VII collagen are responsible for the skin disease dystrophic epidermolysis bullosa (DEB). The size of the COL7A1 transcript impedes the application of successful gene-therapeutic approaches in DEB. RNA trans-splicing may provide an alternative gene repair strategy on pre-mRNA level. The trans-splicing reaction is achieved by RNA trans-splicing molecules (RTMs) that bind to a target pre-mRNA thereby inducing the gene correction. Using a screening system we are able to identify efficient RNA-trans-splicing molecules for the target intron 46 of COL7A1. This system features a COL7A1 minigene carrying intron 46 and the 5' part of GFP and individual RTMs including a specific binding domain for intron 46 and the missing 3'GFP part. Transfection into HEK293 cells resulted in the fusion of both GFP parts by trans-splicing, leading to the expression of full-length GFP.

To avoid varying COL7A1 mini gene concentrations the COL7A1 minigene was stably integrated into the genome of HEK293 cells using the PiggyBac transposon system to mimic an endogenous target pre-mRNA expression at a constant level. We treated the COL7A1 minigene cell line with RTMs carrying

different binding domains for the target region and analyzed their trans-splicing efficiency by flow cytometry. The most efficient RTM induced a GFP expression in about 95% of treated cells. The identification of the most functional RTM will improve trans-splicing in the COL7A1 gene in order to lay the basis for gene therapy of DEB patients.

P251

Role of mitochondria in the pathogenesis of Kindler SyndromeE Zapatero-Solana¹, J Luis Garcia⁵, M Garcia⁶, S Guerrero-Aspizua⁶, A Toll³, E Baselga², J.M Garcia-Verdugo⁴, C Conti⁶, F Larcher¹, F Pallardó⁵, M del Rio Nechaevsky⁶¹*Bioepithelial Medicine Division CIEMAT-CIBERER Spain* ²*Dermatology, Hospital Sant Pau, Barcelona, Spain;* ³*Dermatology, Hospital del Mar, Barcelona, Spain;* ⁴*Electronic Microscopy Service, Medicine University, Valencia, Spain;* ⁵*Physiology, Medicine University, Valencia, Spain;* ⁶*Bioengineering, Carlos III University, Madrid, Spain*

Kindler Syndrome (KS) (OMIM 173650) is an autosomal recessive skin disorder with a phenotype that has long intrigued dermatologists. Skin blistering, photosensitivity, premature aging and propensity to skin cancer are phenotypes usually reported in patients affected by KS. Through a European collaborative effort, we have recently provided an overview of the genotypic-phenotypic correlations in this disease (Hum Mutat 00:1-9, 2011). However, neither the pathognomonic signs nor the genotype-phenotype correlations suffice to fully understand the pleiotropic nature and clinical variability of this genodermatosis. Mitochondrial oxidative stress has long been implicated in both aging and cancer. Mitochondria contain the enzymes for energy production, via oxidative phosphorylation. During this process, reactive oxygen species (ROS) are generated as by-products. Progressive defects in mitochondrial function during aging lead to the increased production of ROS, resulting in accumulated DNA damage and an increase of susceptibility towards cancer. Thus, aging, mitochondrial dysfunction and cancer seems all inextricably linked. We decided, therefore, to explore the potential role of mitochondria-linked oxidative stress on the pathogenesis of KS. Patient-derived keratinocytes showed an increase in ROS levels (observed by confocal microscopy (JC-1), glutathione levels and redox biosensors experiments). Moreover, electron microscopic studies of skin biopsies and cells from the same patients showed morphological alterations in KS keratinocyte's mitochondria, including cristae disorganization. Together, *in vitro* and *in vivo* alterations of mitochondrial architecture and function are consistent with a pro-oxidant state as a hallmark in KS. To our knowledge, this study provides first link between mitochondrial dysfunction and the pathogenesis of this genodermatosis.

P252

Pre-screening of trans-splicing molecules improves COL7A1 mRNA repair

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The repair of defective pre-mRNAs by RNA *trans*-splicing has become an emerging alternative to conventional gene therapy in the treatment of genetic disorders involving large genes. Previous studies have made clear that the design of binding domains (BDs) of mRNA-pre-*trans*-splicing molecules (RTMs) is crucial for the *trans*-splicing efficiency. Even minor changes in length and position can have vast effects on their functionality. We developed a fluorescence-based screening system, which allows selecting most efficient BDs from randomly generated variants utilizing FACS. The applicability of pre-screening reporter RTMs was validated in the repair of the *COL7A1* mRNA in which mutations are the cause of the skin disease dystrophic epidermolysis bullosa (DEB). Comparison of single RTMs containing different BDs hybridizing to *COL7A1* intron 64/exon 65 revealed different *trans*-splicing efficiencies. Three BDs highly variable in length, binding position and *trans*-splicing efficiency were selected and adapted for endogenous *trans*-splicing in a recessive DEB (RDEB) keratinocytes cell-line expressing reduced levels of *COL7A1* mRNA. Retroviral transduction with the respective RTM resulted in variable increases of *COL7A1* mRNA expression levels compared to the mock transduced RDEB cell-line. High levels of endogenous *COL7A1* mRNA repair were seen with RTMs identified as being highly functional in our pre-screening system. Thus, by using our reporter-based test system, the efficiency of *trans*-splicing can be demonstrated easily prior to testing RTMs in endogenous model systems, which marks an important step forward towards optimization of our splice-modulating therapy for use in a clinical context.

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Long-term survival of type XVII collagen revertant cells in an animal model of revertant cell therapy

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Revertant mosaicism (RM) is the co-existence of mutant cells carrying germline mutations and revertant cells that have spontaneously corrected the germline mutation by a somatic mutation. RM has been reported for several hereditary diseases, including the genetic skin blistering disorder epidermolysis bullosa. Here, we investigated the feasibility of using the naturally corrected keratinocytes harvested from a revertant, healthy skin area for autologous cell therapy. A 6-mm punch biopsy was taken of a revertant non-blistering patch of the forearm of a patient with epidermolysis bullosa due to mutations in the *COL17A1* gene. Subsequently, keratinocytes were cultured for several passages. Stainings showed 40% and 25% revertant cells after passage 1 and 2, respectively. When cultured longer the percentage of revertant cells dropped further to 15%, 1% and <1% after passage 3, 4 and 5, respectively. From cultured patient's fibroblasts and passage 2 keratinocytes, a 75 cm² bioengineered skin equivalent was produced. A side sample taken from this skin equivalent revealed 20% of revertant cells. Four

immunodeficient nu/nu mice were transplanted, and biopsies from the engrafted skin after 10 and 16 weeks showed a similar percentage of 20%. Our data demonstrate that after a marked decrease during in-vitro expansion on plastic, the percentage of revertant keratinocytes stabilizes during skin equivalent production and remains stable in-vivo. Further, we show long-term survival of revertant keratinocytes because of transplantation of revertant epidermal stem cells, thereby demonstrating the feasibility of revertant cell therapy for patients with epidermolysis bullosa.

P254

Adenovirus-relaxin gene therapy on keloids: Attenuated proliferative response and collagen degradation

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A keloid or hypertrophic scar is pathologic proliferations of the skin dermal layer resulting from excessive collagen deposition. The pleiotropic hormone relaxin (RLX) inhibits the collagen synthesis and expression in stimulated fibroblasts; however RLX does not affect basal expression levels. We generated a replication-incompetent adenovirus expressing RLX (dE1-RGD/lacZ/RLX) to investigate the effects of RLX on the expression of collagen and matrix metalloproteinase (MMP) on the keloid fibroblasts (KFs). Here, we showed that β -galactosidase expression confirmed the efficient transduction of dE1-RGD/lacZ/RLX into HDFs and KFs, and high level of RLX expression was induced at 100 MOI on KFs. The dE1-RGD/lacZ/RLX adenovirus decreased mRNA levels of type I and III collagen as well as those of MMP-1 and MMP-3 in infected KFs. Moreover, the expression of collagen types I and III, elastin, and fibronectin were significantly decreased in keloid spheroids transduced with dE1-RGD/lacZ/RLX. Taken together, gene therapy with relaxin-expressing adenovirus attenuated collagen synthesis and decreased MMP-1 and MMP-3 expression in KFs and, therefore, functions to prevent KFs invasion of peripheral normal area after keloid excision.

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Amniotic membrane promotes epithelialisation of massive post-traumatic wounds

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Introduction: Large-surface or deep wounds often become senescent in the inflammatory or proliferation stages and cannot

progress to re-epithelialisation. In this stage the best current treatment is autologous skin graft, or alternative therapies, such as allogenic or autologous skin substitutes and synthetic dressings. Amniotic Membrane (AM) is a tissue of particular interest as a biological dressing due to its special structure and biological properties: low immunogenicity, anti-inflammatory, anti-fibrotic, anti-microbial, and re-epithelialisation effects. We have used AM to dress the large wound in animal assays and in 5 patients on compassionate use with the aim to promote wound epithelialisation. As a consequence of the good preliminary results in re-epithelialisation of wounds, we have initiated a prospective clinical trial to test the feasibility and safety of AM. Here we present the safety data obtained in the first patient included in this clinical assay.

Methods: Inclusion criteria were: patient >18 years old, with diagnosis of massive post-traumatic wounds more than 100 cm in granulation stage. All patients have to sign the Informed Consent before participating in the study. In total we will include ten patients who provide enough guarantees to adhere protocol. The protocol implies the application of AM each week during six weeks. Primary endpoint is: a) Absence of severe treatment-related adverse events (AE), graded according to the CTCAE. b) Non inflammatory changes around the wound.

Results: One patient has reached the application period of 6 weeks of AM. During this time he has not shown severe and non-severe treatment-related AE. He has neither developed inflammatory changes around the wound. The wound has re-epithelialize almost completely with normal skin in 6 weeks, and this result will be showed graphically.

Conclusion: The preliminary results obtained in this patient evidence that AM used as a biological wound dressing was safe and did not produce any adverse side effects. These results are in concordance with those obtained in patients on compassionate use.

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Comparison between Mesenchymal stem cells and amniotic membrane in treatment of chronic wounds

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Background: Chronic leg ulcers often heal poorly necessitating the search for different lines of treatment such as skin grafting and induction of angiogenesis. Amniotic membrane (AM) graft can be used as it reduces fibrosis through down-regulation of TGF- β and its receptor. Bone marrow derived mesenchymal stem cells (MSCs) are capable of differentiation into structures found in skin tissue. This work aims to compare the use of both (AM) graft and autologous MSCs separately and in combination in chronic ulcers.

Methods: 37 chronic leg ulcers were included, 11 controls treated conventionally, 14 treated by AM graft, 6 by MSCs injected into ulcer bed and edges, and 6 by both MSCs and AM. Patients were followed up for 2 months using reduction in ulcer size, rate of healing, ulcer characteristics and pain score.

Results: Reduction in ulcer size (cm²/day): control group showed zero reduction in size, group II showed complete reduction in ulcer size with healing rate of 0.064-2.22 cm²/day with mean of 0.896 \pm 0.646, group III showed 50.4% to 100% reduction in ulcer size with mean of 83.9% \pm 24.9% and healing rate of 0.084-0.787 cm²/day with mean of 0.303 \pm 0.254. Group IV showed 46.5%-100% reduction in ulcer size but with higher healing rate than Group III, of 0.359-1.23 cm²/day with mean of 0.759 \pm 0.361. Reduction in ulcer size shows significant difference between group I in comparison to group II (p=0.001), Group III (p=0.001), and Group IV (p=0.001), and significant difference between group II in comparison to group III (p=0.020), and Group IV (p=0.003). Healing rate shows significant difference between group I and group II (p=0.001), and significant difference between group I and group IV (p=0.002), also there is significant difference between group II and group III (p=0.009).

Conclusion: Both MSCs and AM promote wound healing, however in chronic wounds the combination of both modules gives better results. As chronicity increases, the use of MSCs alone becomes less effective.

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Non-viral gene delivery by Sleeping Beauty transposon vectors for gene therapy of Gaucher disease

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Gaucher disease (GD) is the most common lysosomal storage disease caused by inherited mutations in the GBA gene that result in a deficiency of the enzyme β -glucocerebrosidase (GCCase), an important component of the sphingolipid metabolism pathway. The disorder is characterized by a wide spectrum of clinical manifestations and ranges from adult asymptomatic cases to severe neurological forms in infants. At present, there is no treatment available that could be successfully applied to GD patients. A gene therapy approach based on the genetic correction of autologous hematopoietic stem cells (HSCs) potentially offers a unique possibility to treat disease symptoms by correcting their actual genetic cause. The proposed preclinical gene therapy approach aims at genetic modification of murine enzyme-deficient HSCs with a therapeutic construct delivered *ex vivo* by a non-viral *Sleeping Beauty* (SB) transposon system. Subsequent transplantation of the modified cells into murine Gaucher recipients envisions sustained enzyme expression, secretion and targeting to the central nervous system as a prerequisite to a successful therapeutic intervention. For that purpose, a protocol of stable, SB-mediated gene delivery was established and optimized for hard-to-transfect murine HSCs. Therapeutic SB/GCCase construct was created and successfully validated *in vitro* in terms of vector excision/integration ability, transient and persistent gene expression, and protein secretion. Enzyme activity and inhibition studies performed in GCCase-deficient human fibroblasts and murine HSCs confirmed the functionality of the exogenous protein. Further implementation of the SB/GCCase system *in vivo* utilizing two GD mouse models is expected to estimate efficacy (genetic and phenotypic analysis) and safety (vector genomic integration profile) of our gene therapy approach.

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Preliminary safety data from phase I clinical trial in acute intermittent porphyriaE Lopez-Franco¹, C Olagüe¹, D D'Avola², AIPGENE in Europe Project's Consortium^{1,2,3,4,5,6,7,8}

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Background: Acute Intermittent Porphyria (AIP) is a rare genetic disease, where mutations in the *porphobilinogen deaminase* (PBGD) gene lead to insufficient activity of a protein necessary for heme synthesis. This leads to the accumulation of toxic intermediate metabolites that produce a wide variety of clinical symptoms, including acute and severe abdominal pain, muscle weakness and psychiatric and neurological illnesses, decreasing patient's quality of life.

Methods and aim: This is a Phase I trial aimed to determine the safety of the investigational GT product for the treatment of AIP. We administered a single dose of a recombinant adeno-associated serotype 2/5 vector expressing a codon-optimized human PBGD transgene under the control of a liver-specific promoter (rAAV2/5-PBGD) by peripheral infusion. Eight AIP patients of both sexes are being sequentially enrolled in a dose escalation design constituted by four cohorts with two participants in each. Participants are going to be followed for 12 months. Humoral and cellular immune responses against the transgene and the viral capsid proteins were analysed by ELISA and ELISPOT and viral shedding was determined by quantitative PCR.

Results: As expected, we observed an increase in the levels of total and neutralizing antibodies against AAV capsid, no total antibodies against PBGD protein were detected. No T-cell-mediated immune responses against AAV capsid proteins or transgene were observed. Patients treated with lowest vector doses, showed a complete disappearance of vector genome copies of all body fluids after two weeks. However patients treated with highest doses, showed a longer permanence of virus genome copies that disappeared four weeks after treatment.

Conclusions: No immune responses against the vector or the transgene have been detected in a phase I clinical trial for the treatment of AIP after peripheral-vein infusion of rAAV2/5-PBGD.

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Life-long liver-specific AAV-mediated gene therapy in a Crigler-Najjar mouse modelG Bortolussi¹, L Zentilin¹, J Vaníková², E Vianello³, A Mancarella³, L Bočkor¹, C Bellarosa³, C Tiribelli³, L Vitek², M Giacca¹, A F Muro¹

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Crigler-Najjar syndrome type I (CNSI) is a rare genetic disorder characterized by the inability to conjugate bilirubin due to UGT1a1 deficiency. As a consequence, babies have high levels of unconjugated bilirubin and are at constant risk of developing neurological damage unless phototherapy (PT) is applied since birth. However, during puberty PT becomes less effective, and liver transplantation is required.

To find alternative therapies to permanently correct the genetic defect we generated a mouse model of CNSI that shows neonatal lethality and all major features of CNSI. We previously showed that neonatal gene transfer of the CMV-hUgt1a1 gene rescued all the mutant mice by skeletal muscle expression of the therapeutic protein. To promote liver-specific expression we here used an AAV8 vector carrying the enhancer element of ApoE gene and the minimal promoter region of alpha1antitrypsin (AAT). Mutant mice were treated at P4 with a single injection of the therapeutic gene. Bilirubin levels were 70–80% lower than untreated controls at 1 and 2 months after injection. At 17 months post-injection, injected mice showed serum bilirubin level 50% lower compared to untreated controls. We compared the liver-specific gene therapy of ATT-hUGT1a1 with that of the CMV-hUGT1a1. Despite liver (AAT) expressed 25 times less hUGT1a1 compared to skeletal muscle (CMV), bilirubin levels were significantly lower (3.1 ± 1.5 and 6.4 ± 2.0 , respectively). Western blot analysis revealed that less than 5% of liver expression of hUGT1a1, as compared to wt, is sufficient to maintain lifelong low levels of plasma bilirubin. We speculate that the remarkable difference in bilirubin levels is related to the expression in liver, but not in muscle, of the Mrp2 transporter, which accounts for the biliary excretion of conjugated bilirubin.

These data confirm that liver is the appropriate target organ for long-term CNSI gene therapy, ruling out the possibility of using extra-hepatic tissues.

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Hematopoietic stem cell gene therapy for the treatment of Type I Mucopolysaccharidosis: towards clinical applicationI. Visigalli¹, S. Delai¹, F. Ferro¹, F. Cecere¹, F. Sanvito³, P. Cristofori⁴, L. Naldini^{1,2}, A. Biffi^{1,2}

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Type 1 I Mucopolysaccharidosis (MPS-I) is an inherited lysosomal storage disorder caused by the deficiency of α -L-iduronidase (IDUA), characterized by accumulation of mucopolysaccharides within the lysosomes in different tissues, leading to multisystemic impairment and mental delay. Available treatments such as enzyme replacement therapy and hematopoietic stem cell transplantation (HSCT), are poorly effective on skeletal and brain disease manifestations. To improve the therapeutic efficacy of HSCT, we developed a gene therapy (GT) strategy based on lentiviral vector (LV) for HSC transduction aimed at inducing supra-physiological enzyme production by HSCs and their tissue-infiltrating progeny. HSCGT showed a high efficacy in preventing disease onset and/or in correcting neurological and skeletal defects of the MPS-I murine model. A Based on these results, a clinical development plan for moving this approach to clinical testing has

started, including safety studies conducted in Good Laboratory Practice. For studying the long-term toxic and tumorigenic potential of the IDUA.LV transduced HSCs, murine HSCs have been transplanted in MPS-I mice. The study will last 12 months, including both primary and secondary transplants. IDUA.LV-transduced HSCs efficiently engrafted in primary recipients, resulting in enzyme over-expression in peripheral blood cells, without any macroscopic abnormality. For studying the efficacy and safety of IDUA gene transfer by clinical-grade LV into human HSCs and their biodistribution properties, of human HSCs transduced with a clinical-grade IDUA.LV, preliminary *in-vitro* and *in-vivo* experiments have been performed. Transduced Bone marrow-derived hHSCs were efficiently transduced and consequently over-expressed IDUA up to 30 fold above the normal healthy donor's level, and maintained their clonogenic potential without any functional consequence. When Indeed, cells showed a normal differentiation and growth potential *in vitro* and, when transplanted into sub-lethally irradiated immunodeficient mice, these cells showed demonstrated a normal long-term repopulation and differentiation potential as compared to untransduced HSCs, maintaining IDUA over-expression *in vivo*.

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Myeloid driven stem cell gene therapy corrects a mouse model of Mucopolysaccharidosis IIIA

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Mucopolysaccharide (MPS) type IIIA (MPSIIIA, Sanfilippo A) is a paediatric lysosomal storage disease caused by mutations in N-sulphoglucosamine sulphohydrolase (SGSH). SGSH deficiency results in heparan sulphate storage and a severe progressive neurodegenerative disease. Enzyme replacement therapy is potentially feasible, but fails because enzyme cannot cross the blood-brain barrier. Haematopoietic stem cell transplantation (HSCT) circumvents this via monocyte trafficking and engraftment in the brain as microglia. HSCT is curative for the similar HS storage disease MPSI but is ineffectual for MPSIIIA which has no treatments.

We previously used lentiviral mediated gene therapy to augment SGSH enzyme in WT HSCT, which improves neuropathology and corrects behaviour of MPSIIIA mice. However, correction could not be achieved with lentiviral transduced MPSIIIA cells.

To improve expression and brain specificity, lentiviral vectors expressing eGFP under ubiquitous PGK, or myeloid-specific CD11b promoters were compared in transplanted HSCs. CD11b gave significantly higher monocyte and B-cell eGFP expression than PGK after 6 months. Subsequently, MPSIIIA HSCs were transduced with PGK or CD11b lentiviral vectors expressing codon-optimised SGSH and transplanted into MPSIIIA mice.

Eight months after HSCT, PGK lentiviral vectors produced more bone marrow SGSH (576% normal activity) than CD11b (473%), but CD11b was more brain specific (11% vs 7%). CD11b lentiviral vectors fully corrected MPSIIIA behaviour, brain heparan sulphate, GM gangliosides and neuroinflammation to WT levels, whilst PGK lentiviral vectors partly corrected neuropathology but not behaviour. These data provide strong evidence of pre-clinical neurological disease correction using an autologous myeloid-specific lentiviral HSCT approach in the MPSIIIA mouse model.

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In vivo AAV-mediated genetic engineering of white and brown adipose tissue in adult mice

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Obesity and type 2 diabetes (T2D) are very strongly associated and are a major health problem because of their alarmingly growing prevalence worldwide. Deregulation of metabolic and endocrine functions of white adipose tissue (WAT), as well as impaired brown adipose tissue (BAT) activity and/or decreased mass, are considered among the main contributors to obesity, insulin resistance and T2D both in experimental animal models and in humans. To fully understand the metabolic and molecular mechanism(s) involved in adipose dysfunction, *in vivo* genetic modification of adipocytes holds great potential. Here, we demonstrate that adeno-associated viral (AAV) vectors, especially serotypes 8 and 9, mediated efficient transduction of white (WAT) and brown adipose tissue (BAT) in adult lean and obese-diabetic mice. The use of short versions of the aP2 or UCP1 promoters or miRNA target sequences enabled highly specific, long-term AAV-mediated transgene expression in white or brown adipocytes. As proof of concept, delivery of AAV vectors encoding for hexokinase or vascular endothelial growth factor to WAT or BAT resulted in increased glucose uptake or increased vessel density in targeted depots. This gene transfer methodology also enabled the secretion of stable high levels of the alkaline phosphatase marker protein into the bloodstream by transduced WAT. Therefore, AAV-mediated genetic engineering of adipose tissue represents a useful tool for the study of adipose pathophysiology and, likely, for the future development of new therapeutic strategies for obesity and diabetes.

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Long-term efficacy following intra-CSF administration of AAV9-sulfamidase in the treatment of MPSIIIA

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Mucopolysaccharidosis Type IIIA (MPSIIIA) or Sanfilippo A syndrome is an autosomal recessive Lysosomal Storage Disease (LSD) caused by deficiency in sulfamidase, a sulfatase involved in the stepwise degradation of the glycosaminoglycan (GAG) heparan sulfate (HS). As a consequence of enzyme deficiency, HS accumulates in the lysosomes of cells, causing cell dysfunction and, eventually, cell death, in tissues and organs of the whole body, but affecting predominantly the CNS. We previously demonstrated in MPSIIIA mice that intracisternal (IC) delivery to the cerebrospinal fluid (CSF) of sulfamidase-encoding AAV9 vectors mediated whole-body disease correction through transgene expression throughout CNS and liver as soon as four months after vector administration. Here, we provide evidence for the long-term efficacy of the approach in MPSIIIA mice, including data on functional and behavioral correction. Moreover, we also demonstrate that in healthy Beagle dogs, a single intra-CSF administration of AAV9 vectors encoding for canine sulfamidase at the therapeutic dose resulted in a long-term stable increase in sulfamidase activity in the CSF, in the absence of any safety concerns. Overall, these results support the clinical translation of this approach for the treatment of MPSIIIA and other LSD with CNS involvement.

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Helper-dependent canine adenovirus corrects neuropathology and behaviour in mucopolysaccharidosis type VII mice

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Lysosomal storage disorders (LSD), a group of inherited metabolic diseases, stand for the most common cause of neurodegeneration in children. Among LSD, Mucopolysaccharidosis (MPS) VII or Sly syndrome, is caused by β -glucuronidase deficiency. This primary defect triggers glycosaminoglycan accumulation into enlarged vesicles in peripheral and CNS tissues resulting in peripheral and neuronal dysfunction and death. No effective treatment is currently available for patients with MPS VII. Gene transfer with viral vectors conferring stable, long-term correction could provide sustained therapy if a sufficient level of enzyme was secreted. Canine adenovirus type 2 vectors (CAV-2) appear particularly promising for CNS gene therapy due to their neuronal tropism and a very efficient long-distance targeting, via axonal transport. In addition, no neutralizing antibodies against CAV-2 vectors seem to be present in human population.

To test the therapeutic capacity of CAV-2 vectors, we injected eight-week old MPS VII mice in the striatum with helper-dependent CAV-2 vectors (HDCAV-2) expressing β -glucuronidase (HDGusB). β -glucuronidase activity was observed in these animals six and sixteen weeks after injection of HDGusB. Transduced mice showed a significant decrease of β -hexosaminidase activity and glycosaminoglycans content consistent with global

biochemical correction. Semi-thin sections showed complete correction of lysosomal accumulation both in striatum and cortex and in more distal areas, at short and long-term after transduction. Behavioural tests performed in mice six weeks after the treatment exhibited significant improvements in learning and long-term memory.

Thus, a single intrastriatal injection of helper dependent CAV-2 could result in a global and sustained expression and may have implication for a brain gene therapy in patients with MPSVII or other lysosomal storage diseases.

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Polyinosinic acid blocks adeno-associated virus macrophage endocytosis *in vitro* and enhances adeno-associated virus liver directed gene therapy *in vivo*

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AAV8 is an effective and safe vector for liver directed gene therapy. Hepatocytes are the main target, but there is loss of AAV8 due to uptake by macrophages. Reducing this would increase the efficacy of AAV8 gene therapy. The receptor mediating AAV8 uptake by macrophages has not been identified, therefore we looked if the scavenger receptor A (SR-A) can mediate AAV8 endocytosis. Expression of SR-A in CHO-cells induced endocytosis of scAAV8 3 fold. The efficient endocytosis of AAV8 by the human macrophage cell line THP1 and primary human macrophages was significantly reduced by blocking SR-A with poly[i]. Since these data indicate a role of SR-A in AAV8 uptake by macrophages we decided to study its role *in vivo*. The Gunn rat is the relevant model for Crigler-Najjar, inherited severe hyperbilirubinemia due to lack of UGT1A1 activity. In male rats a low (1×10^{11} gc/kg) and high (3×10^{11} gc/kg) dose of scAAV8-LP1-UGT1A1 significantly corrected hyperbilirubinemia. Pre-administration of poly[i] increased the efficacy of the low vector dose ($p < 0.05$). In female rats the correction of serum bilirubin by scAAV8-LP1-UGT1A1 was less pronounced. Blocking SR-A with poly[i] significantly increased the correction provided by both vector doses ($p < 0.05$). This effect was more pronounced in the group treated with the low vector dose in which it also resulted in a significant increase of bilirubin glucuronides in bile. In conclusion, our data show that SR-A mediates the endocytosis of AAV8 and that blocking it improves the efficacy of scAAV8 liver directed gene therapy in the Gunn rat.

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In utero gene therapy rescue of an acute neonatal lethal mouse model of neuropathic Gaucher Disease

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Background: Type II Neuropathic Gaucher Disease (nGD) is caused by mutation of the glucocerebrosidase gene. Patients exhibit aggressive neurodegeneration and die before two years of age; no treatment exists. A neonatal lethal mouse model presents the symptoms and neurodegeneration and dies before 14 days of age. We conducted a spatio-temporal analysis of brain pathology. We used this data to measure the efficacy of *in utero* gene therapy to rescue this mouse model.

Methods: Brains from control and knockout mice were collected at 1 day of age (P1), P9 (pre-symptomatic) and P12 (symptomatic). Microglial activation, astrogliosis, lysosomal content and neurodegeneration were measured.

AAV9 carrying therapeutic glucocerebrosidase gene was administered to the brains of fetal mice by *in utero* intracranial injection. Survival, behavior and brain pathology were analysed.

Results: Pathology was observed in the brain stem of KO mice at P1. Global and progressive spread of pathology was observed at P9 and P12, although certain regions were more affected i.e. the brain stem, VPL/VPM and cortex. This was accompanied by rapid neurodegeneration.

In utero injection of AAV9 vector dramatically reduced pathology and extended the lifespan of treated KO mice by tenfold (day 131, n=5, p<0.005). As treated mice aged, behavioral abnormalities and weight loss became apparent. We are investigating if this is nGD related or a novel phenotype.

Conclusion: *In utero* gene therapy is successful in rescuing an early neonatal lethal mouse model of nGD. To date, this is the most severe model of a neurodegenerative lysosomal storage disorder to be rescued by gene therapy.

P270

Lentiviral vectors for gene therapy of type I Gaucher disease

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Background: Gaucher Disease (GD) is a metabolic disorder which is effectively treated by enzyme replacement therapy. However treatment must be administered fortnightly throughout the life of the patient and is not always effective in treating skeletal complications. Gene therapy and autologous

transplantation of haematopoietic stem cells could provide a method of treating Type I GD which overcomes these barriers.

Methods: We have created a number of lentiviral vectors carrying the glucocerebrosidase (GBA) gene some of which contain fusions of the GBA gene with various protein transduction domains including that of the HIV-1 TAT protein to provide a method of uptake for GBA secreted by transduced cells that is independent of the mannose receptor pathway.

Results: We show that all vectors express GBA enzyme by means of functional assays and Western blot. We demonstrate that when human embryonic kidney cells are transduced with a high multiplicity of infection active protein is secreted (p = <0.005) and that secreted enzyme can be used to correct untransduced patient cells (<50% increase in GBA positive cells after culture in conditioned medium). This presents an opportunity for the cross correction of tissues other than the haematopoietic system.

Conclusions: We have shown that lentiviral vectors can be used to treat not only the directly transduced cells but also the neighbouring cells of other systems. In the future we plan to test these vectors further using a range of cell types and *in vivo*.

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Safe lentiviral vectors using cellular and tissue-specific promoters to drive glucosylceramidase expression corrects type1 Gaucher disease in mice

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Gaucher disease (GD), the most common of the lysosomal storage disorders, is an autosomal recessive disorder affecting the enzyme glucosylceramidase (GCCase). In patients, the mutated GBA gene encoding GCCase gives rise to a deficient enzyme unable to degrade the cell membrane lipid glucosylceramide (GluCer), leading to a progressive build-up of GluCer in macrophages. Macrophages laden with unprocessed lipids give rise to hepatosplenomegaly, cytopenias and bone disease in patients. Our conditional mouse model of type 1 GD displaying key clinical symptoms can be cured with gene therapy using oncoretroviral vectors with a strong viral promoter (Enquist *et al*, PNAS 2006). To investigate whether safer vectors can correct the enzyme deficiency we utilized SIN lentiviral vectors harboring the human GBA transgene under the control of human phosphoglycerate kinase (PGK) and myeloid specific (CD68) promoters, respectively, for correction of disease symptoms in the type 1 GD mice. Here we show correction of manifest symptoms after transplantation of gene corrected cells, resulting in enzyme restoration of 25–44% of wild type levels in bone marrow. Five months post transplant treated mice exhibit a clearance of GluCer from tissues, reversal of splenomegaly and Gaucher cell infiltration and a restoration in hematological parameters. The findings demonstrate the feasibility of developing safe clinical vectors with cellular/tissue specific promoters for the correction of symptoms in type 1 Gaucher patients. Furthermore, we demonstrate that GBA-deficient mice with 6% normal macrophages are symptom-free, suggesting that 6% gene transfer may be sufficient to cure type 1 Gaucher disease.

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A therapeutic strategy for MNGIE based on systemic clearance of dThd and dUrd mediated by keratinocytes genetically modified to produce high levels of TP

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MNGIE (mitochondrial encephalomyopathy neurogastrointestinal, OMIM: 603041) is a rare devastating autosomal recessive disease caused by mutations in the TYMP gene encoding thymidine phosphorylase (TP), a key enzyme in the degradation of thymidine (dThd) and deoxyuridine (dUrd). TP deficiency leads to the accumulation of dThd and dUrd reaching micromolar concentrations, both in plasma and tissues, which interferes with the correct mtDNA replication resulting in mitochondrial dysfunction.

We propose a sink therapeutic approach through the use of skin cells producing high levels of TP able to reduce circulating levels of toxic nucleosides. Here we have carried out in vitro and in vivo studies with normal and Tymp/dUrdP deficient mouse keratinocytes (MNGIE mK) modified with lentiviral vectors containing the TP gene. We have analyzed the metabolism of dThd and dUrd in supernatants of transduced cells and after transplantation of TP overexpressing keratinocytes into immunodeficient mice and analysis of serum clearance of nucleosides. Western blot analysis showed overexpression of TP both in MNGIE mK cells. Noteworthy, normal keratinocytes expressed detectable levels of TP. Clearance of nucleosides in vitro was highly efficient particularly in TP overexpressing MNGIE mK cells.

Preliminary feasibility experiments demonstrated that, after transplantation in immunodeficient mice, TP overexpressing MNGIE mK were capable of regenerating a full epithelial tissue with skin adnexa. New in vivo experiments will soon be conducted with MNGIE mice to test the efficacy of the approach.

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Thymidine phosphorylase is both a therapeutic and a suicidal gene in a murine model of Mitochondrial Neurogastrointestinal Encephalomyopathy (MNGIE)

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Suicide gene therapy (SGT) constitutes a promising strategy for treating cancer. In this work, we show that thymidine phosphorylase (TP) deficiency, the underlying genetic defect in mitochondrial neurogastrointestinal encephalomyopathy (MNGIE), provides an opportunity for SGT using capecitabine, a prodrug that is administered orally and that is converted to 5-fluorouracil (5-FU) by this enzyme. Using an immortalized lymphoblastoid B-cell line from a MNGIE patient, the tumorigenic murine EL-4 cell line, lentiviral vectors encoding TP and a double KO

Tymp^{-/-} Upp1^{-/-} (dKO) murine model, we found that EL-4 cell-derived TP tumours were exquisitely sensitive to capecitabine, especially in the dKO mice, in which the TP tumour cells are the only ones that express the prodrug-metabolizing enzyme, whereas in these animals the TP⁺ tumours were resistant to the prodrug therapy. In addition, we found that there is a significant direct, but not an indirect bystander effect. In addition, despite the fact that the EL-4 cell line and the mice used shared the same MHC, we detected cytolytic anti-tumour immune responses in a significant fraction of the animals surviving for more than 20 days after the therapy was stopped. These results have implications in gene therapy for MNGIE. As the use of some gene therapy vectors is associated with a risk of insertional oncogenicity, malignancies that can develop in MNGIE patients as a complication of this therapy will represent a unique setting in which the potential derived tumours, as they will necessarily derive from the gene-modified cells, will be selectively targeted by the prodrug.

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Metabolic characterisation of a LMNA-linked lipodystrophic model based on human adult stem cells

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Human lipodystrophies are heterogeneous disorders characterized by adipose tissue loss with a variable range of metabolic complications, such as lipid profile disturbances (hypertriglyceridemia and low high-density lipoprotein (HDL) cholesterol), glucose intolerance and insulin resistance, among others. Lamin A (LMNA)-linked lipodystrophies belong to a group of clinical syndromes called laminopathies which exhibit an altered nuclear envelope. Both congenital (associated with LMNA mutations) and acquired (associated with the use of human immunodeficiency virus protease inhibitors (HIV-IP)) lipodystrophies share a non-physiological accumulation of the lamin A precursor, prelamin A.

Given that affected tissues in lipodystrophies are mainly mesenchymal in origin, we previously developed a LMNA-linked lipodystrophy experimental model using human mesenchymal stem cells (hMSC) that accumulated prelamin A. This experimental human model was able to recapitulate the different phenotypes observed in LMNA-linked lipodystrophies. In fact, this model has allowed us to identify a mechanism responsible, at least in part, of the lipodystrophic phenotype as a consequence of prelamin A accumulation. We have performed further characterization of the metabolic phenotype on our lipodystrophy model based on hMSCs. In order to achieve this goal, proteomic profiling was performed with 2D-PAGE followed by MALDI TOF/TOF, and endogenous lipid profiling was analysed using UPLC-MS. Complementary ultra-structural studies and measures of lipolytic activity were assessed.

Taken together, the results confirm the usefulness of our human cell-based model to study metabolic diseases, such as lipodystrophies, and to extend the knowledge about the homeostasis of adipocyte derived from adult human stem cells.

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A cutaneous gene therapy approach for the correction of congenital generalized lipodystrophy

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Congenital generalized lipodystrophy is a rare autosomal recessive disorder characterized by marked lack of body fat. Patients develop insulin resistance, dyslipidemia, hepatic steatosis, acanthosis nigricans, polycystic ovarian disease and hypertension. These complications arise from low serum levels of leptin and adiponectin, chemokines produced and secreted by subcutaneous and visceral fat. Mutations in the AGPAT2 gene, a key player in triacylglycerol and phospholipid biosynthesis, lead to lipodystrophy on one hand by triacylglycerol synthesis inhibition and on the other by interfering with signal transduction leading to adipogenesis, resulting in absence of functional, chemokine-secreting adipocytes. Our aim is to develop cellular and gene therapy standardized protocols to obtain functional adipogenic differentiation of dermal fibroblasts from lipodystrophic patients.

In an attempt to make both lipodystrophic (LD) and normal dermal fibroblasts (HDF) responsive to adipogenic stimuli, we overexpressed Peroxisome Proliferator-Activated Receptor gamma (PPAR γ , isoform 1), a master regulator of adipogenesis by means of retrovirus transduction. As predicted, PPAR γ 1-overexpressing HDF grown under adipogenic conditions, efficiently differentiated to functional adipocytes. Surprisingly, the same was observed for AGPAT2 null fibroblasts. Analysis of *in vitro*-differentiated adipocytes for several early and late adipogenesis markers revealed that PPAR γ 1-overexpressing cells induced aP2 and C/EBP β , transcription factors involved in the activation of adipogenic differentiation and were capable of expressing leptin and adiponectin. Our results indicate that basal expression of PPAR γ 1, observed in pre-adipocytes but not in HDF (or LD fibroblasts) is a key determinant overcoming AGPAT2 deficiency. We believe that our model of PPAR γ 1-expressing lipodystrophic LD can be a promising approach to combat this disease.

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Pancreatic expression profiles of vasoactive intestinal peptide and its receptors in mouse model of type 2 diabetes

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Vasoactive Intestinal Peptide (VIP) stimulates post-prandial insulin secretion and acts as an anti-inflammatory factor by virtue of inducing immune tolerance. These features make this molecule a promising gene therapy agent for the treatment of Type 2 Diabetes. The expression pattern of VIP and its cognate receptors (VPAC1 and VPAC2) were investigated in Diet Induced Obesity (DIO) coupled with STZ-induced Type 2 Diabetes (T2DM) in C57BL/6J mice. VIP, VPAC1 and VPAC2 content of the pancreas including insulin and glucagon were revealed by immunohistochemistry. VIP expression was

slightly reduced in mice fed with HFD compared to control mice fed with standard diet (SD). Upon STZ injection, a significant decrease in islet VIP content was observed in SD-fed lean mice. No such change was observed in HFD-fed obese animals with or without STZ injection. SD-fed STZ injected lean animals displayed reduced VPAC1 expression in both pancreatic islets and acinar cells while STZ injection did not alter VPAC1 expression in HFD-fed obese animals. Islet specific VPAC2 expression was not different between HFD-fed obese and SD-fed lean animals while acinar VPAC2 expression was higher in HFD-fed obese animals versus SD-fed lean animals. Although STZ injection did not change islet VPAC2 expression in HFD-fed obese versus SD-fed lean mice, acinar VPAC2 expression was decreased in SD-fed lean mice but increased in HFD-fed obese mice.

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GLP-1 and GLP-1-receptor expression profiles in pancreatic islets of Diet Induced Obese (DIO) Mice

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Glucagon-like peptide-1 (GLP-1) is a gut-derived incretin hormone expressed from intestinal L cells located in distal jejunum, ileum and colon with glucoregulatory actions. Its functions include but not limited to glucose-enhanced insulin secretion, suppression of glucagon secretion, delay in gastric emptying, and reduction in appetite and food intake causing weight loss. Enhancement of insulin gene expression and its biosynthesis, stimulation of beta cell proliferation and differentiation, and inhibition of beta cell apoptosis are the other known beneficial effects of GLP-1. Despite all these beneficial effects, how high fat diet and hyperglycemia influence pancreatic expression profile of GLP-1 and its receptor remains to be clarified. An animal model of Type 2 Diabetes (T2DM) was generated using C57BL/6J mice to study the alteration in GLP-1 expression profile induced by high fat diet and hyperglycemia. Obese mice fed with high fat diet (HFD) exhibited insulin resistance and glucose intolerance compared to lean mice fed with standard diet (SD). Hyperglycemia was induced by low dose streptozotocin (STZ) injection. Immunohistochemistry analysis of pancreatic tissues demonstrated low levels of GLP-1 and GLP-1R expressions in pancreatic islets of both obese and lean mice. Intriguingly, proglucagon cross-reactivity of anti GLP-1 antibodies resulted in strong staining of pancreatic alpha cells in both group. Future experiments are underway to reveal alterations of GLP-1 and GLP-1 receptor expression profile in HFD fed-STZ induced diabetic mice.

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Efficient Sleeping Beauty-mediated transposition of primary pigment epithelial cells with PEDF delivered by plasmids free of antibiotic resistance markers (pFARs)

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Age-related macular degeneration is a major cause of blindness in the elderly. Its neovascular form is characterized by increased expression of the pro-angiogenic vascular endothelial growth factor (VEGF). The current treatment, based on often monthly and life-long intravitreal injections of anti-VEGF antibodies, improves vision in about 30% of patients, but may be accompanied by severe side effects and non-compliance. We have postulated that subretinal transplantation of pigment epithelial cells genetically modified to stably overexpress pigment epithelium-derived factor (PEDF), a VEGF inhibitor, may be more effective. To deliver the transgene to pigment epithelial cells, we used the Sleeping Beauty (SB100X) transposon system and cloned both the transposase and the PEDF genes into pFAR4-plasmids, an expression vector free of antibiotic resistance markers. Pigment epithelial cells were electroporated with pFAR4-plasmids encoding the SB100X transposase and the PEDF gene at ratios ranging from 1:12 to 1:28. Consistency of PEDF secretion was analysed by immunoblotting and ELISA. The PEDF transgene was efficiently delivered into as few as 10,000 primary pigment epithelial cells. Recombinant PEDF was continuously secreted for the 5 months that cells were followed, at stable levels of up to 0.64 ± 0.021 ng PEDF/h/104 cells. Merging of the SB100X and pFAR4 technologies led to sustained PEDF expression and improved safety by avoiding the transfer and potential integration of antibiotic resistance genes. Furthermore, this study demonstrates the feasibility of our approach based on ex vivo transfection of a low number of primary autologous pigment epithelial cells followed by subretinal transplantation into the same patient.

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Multigenic lentiviral vectors for anti-angiogenic treatment of age-related macular degeneration (AMD)

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Ocular angiogenesis is the primary cause of severe vision loss in the western world. The balance between pro- and anti-angiogenic factors in the eye regulates angiogenesis, and overexpression of the pro-angiogenic factor VEGF has been found to lead to aberrant neovessel formation in the case of AMD. Anti-VEGF therapy has gained widespread acceptance as a

treatment for angiogenic diseases, but since VEGF is required for maintenance of the subretinal vasculature, this leaves the optimal balance between pro- and anti-angiogenic factors delicate. To improve efficacy and biosafety in ocular gene therapy, we have designed versatile lentiviral (LV) vectors with expression of both multiple miRNAs and proteins for long-term suppression of VEGF. We have evaluated the capacity of LV-encoded miRNAs to down-regulate VEGF gene expression in a dual luciferase assay in vitro, and mature miRNA expression and processing was verified by northern blot analysis. In these vectors, we combine RNAi with overexpression of an anti-angiogenic inhibitor and neuroprotective factor PEDF, as verified by immunostaining and Western blotting of LV-transduced cells. The effect of miRNAs and PEDF on tube formation has been evaluated in vitro. Furthermore, an RPE-specific promoter (VMD2) was included to limit transgene expression to RPE cells, the cells responsible for overexpression of VEGF in AMD patients. The transduction profile and efficiency of LV vectors incorporating the VMD2 promoter was evaluated in mice following subretinal injection, and reporter gene expression demonstrated efficient gene transfer limited to RPE cells. In conclusion, our results clearly show co-expression of therapeutic anti-VEGF-miRNAs and PEDF, demonstrating the potential applicability of our multigene LV vectors in ocular anti-VEGF gene therapy, including treatment of AMD.

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Evaluation of EncorStat®, a highly effective Equine Infectious Anaemia Virus-based lentiviral gene therapy, in a rabbit model of corneal rejection

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Corneal transplantation is one of the most successful transplant procedures due mainly to the relatively immune-privileged status of the eye and the fact that the cornea is largely avascular. However there is a 14% failure rate in the first year and in high rejection risk patients (such as those with failed grafts) it is considerably higher (50–90%) due to vascularisation of the recipient corneal bed, and regrafting is consequently a significant indication for corneal transplantation in many centres. The prognosis in these patients can be so poor that they are not offered a replacement transplant and are left blind. The most common reason for graft failure in patients is irreversible immunological rejection so it is unsurprising that neovascularisation (both pre- and post-grafting) is a significant risk factor for subsequent graft failure. It is logical therefore to target neovascularisation to prevent corneal graft rejection. EncorStat® is a novel process to engineer the human donor corneal tissue prior to transplant to prevent rejection by suppressing neovascularisation. Modified corneas are engineered by the ex vivo delivery of the genes encoding secretable forms of the angiostatic human proteins, endostatin and angiostatin. The ex vivo gene therapy product used to modify the cornea is a non-replicating, recombinant lentiviral vector product derived from the Equine Infectious Anaemia Virus (EIAV). Here we present dose-ranging pre-clinical efficacy data to support the EncorStat® programme in a rabbit model which closely mirrors the clinical setting.

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pEPito-based vectors enable long-term expression in mouse models of diabetic retinopathy

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Vector loss and silencing are major limitations to the success of non-viral gene therapy. Our previous work focused on vector, containing a scaffold/matrix attachment region sequence in the backbone and reduced methylated CpGs, that allow the maintenance of the vector as an episome and lesser susceptibility to silencing effects, respectively. This vector, named pEPito, displayed mitotic stability and prolonged gene expression both in vitro and in vivo in retinal cells. Based on these, we decided to modify our system, by cloning the anti-inflammatory and neuroprotective Pigment Epithelium-Derived Factor (PEDF) to be used therapeutically in models of diabetic retinopathy.

The expression of PEDF was confirmed by immunocytochemistry and western blot analysis of the supernatant of D407 cells transfected with pEPito-hCMV-PEDF. This vectors was injected intravitreally and electroporated in diabetic *Ins2^{Akita}* mice and PEDF expression monitored up to 2 months post-injection. The anti-inflammatory potential of PEDF was verified by immunohistochemistry for Iba1, which is specific for microglia and macrophages.

Immunohistochemistry of the sectioned eyes showed that after 2 months of injection, PEDF detection was more intense for injected than for non-injected contralateral eye. PEDF protein expression was restored in the retina of the *Ins2^{Akita}* mice to levels similar to C57Bl6 WT mice. Our results show that in non-injected retina, active microglia morphology is more pronounced than the injected retina of diabetic *Ins2^{Akita}* mice.

In this study we show that pEPito vectors combined with electroporation can efficiently be used as therapeutic vehicles for retinal gene therapy.

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Adeno-Associated Viral vector serotypes for gene transfer to corneal endothelial cells: a comparison of serotypes

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Purpose: The corneal endothelium (EC) is an ideal target for gene therapy. This is due to its anatomical location on the inner surface of the cornea and due to its monolayer layout. The aim of this study was to examine the efficacy and efficiency of recombinant adeno-associated viral vectors (rAAV) for gene transfer to EC.

Therefore, different rAAV pseudotypes were studied to compare the expression and kinetic patterns of a reporter gene (GFP).

Methods: A comparison of GFP expression and kinetics after EC transduction using a AAV 2/1, 2/2, 2/5, 2/8, 2/9, 2/10 was performed on both murine EC (Balb/C) and human EC (corneas, cell line and primary cells). In addition, the effects of different vector concentrations ($3 \times 10^3/10^4, 10^5, 10^6, 10^7, 10^8$ IU/ μ l) were investigated. Analyses were performed using laser scanning microscopy and flow cytometry.

Results: We detected significant differences between human and murine EC as well as primary and immortalized EC. Whereas in murine corneas transduction of EC with AAV2/2, 2/5 and 2/9 resulted in considerable protein expression, AAV 2/5 did not show considerable expression in human corneas. We also detected a slow onset of GFP expression both in immortalized and primary EC, leading to peak and stable expression around 2-3 weeks. However, peak expression was reached earlier in primary EC and immortalized murine EC compared to immortalized human EC. In addition, the overall plateau of expression was highest in primary EC (~80%).

Conclusions: Recombinant AAV pseudotypes differ in their tropism and transduction efficiency between murine and human corneas. In general high levels of gene expression can be obtained with several of these pseudotypes. Characteristic slow-onset kinetics of protein expression have to be taken into account when applying AAVs in translational applications, e.g. in corneal storage in eye banks.

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Glucose and H₂O₂ modulate the expression of VEGF isoforms in human retinal cells

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Retinal diseases such as diabetic retinopathy are still among the main cause of blindness. Hyperglycemia in diabetes produces reactive oxygen species (ROS) and activates angiogenic cascades. Vascular endothelial growth factors (VEGF) are implicated in angiogenesis. By contrast, VEGF_{xxx}b isoforms have an anti-angiogenic effect. In pathological conditions the effect of angiogenic isoforms predominates over the anti-angiogenics. Our aim was to evaluate the effect of glucose and H₂O₂ upon VEGF isoforms in retinal cells. D407 cells, a human retinal pigment epithelial cell line, were exposed to increasing glucose and H₂O₂ concentrations. We found that VEGF and VEGF_{165b} are present in the cytoplasm of D407 cells. Glucose up-regulated VEGF and VEGF_{165b} and caused an increase in the production of ROS by overexpression of p22^{phox} and catalase. Sub-micromolar H₂O₂ concentrations increased VEGF_{165b} and slightly decreased VEGF. In contrast, micromolar H₂O₂ concentrations increased VEGF expression while the expression of VEGF_{165b} was not affected. Our results showed that the angiogenic and anti-angiogenic VEGF isoforms are co-localized in D407 cells and are differentially regulated by H₂O₂. It is suggested that H₂O₂ at low levels may act as a signalling molecule promoting the activation of anti-angiogenic factors. In addition, the glucose-induced ROS production may have a role in the altered expression of VEGF isoforms. Altogether, these results contribute to elucidate the role of glucose and oxidative stress in retinal cells, particularly in the

differentiated expression of pro- and anti-angiogenic factors normally involved in retinal diseases.

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Carotenoid-based lipoplexes efficiently transfect retinal pigment epithelium cells

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The eye is a desirable target for gene therapy due to its small size, low immune and inflammatory responses and minimal diffusion of drug to the systemic circulation. For cationic lipids, one of the most studied nonviral vectors, usually possess either rigid or non-rigid hydrophobic chains, leaving a gap in the effect of chain rigidity in transfection efficiency to investigate. Our objective is to evaluate the efficiency of DNA delivery to human Retinal Pigmented epithelium (RPE) cells by novel lipoplexes, designated C30-20, C20-20 and C20-18, that possess a highly unsaturated, conjugated, rigid polyene chain plus a non-rigid saturated alkyl C20:0 or C18:0 chain.

Lipoplexes, formulated by solvent evaporation of ethanolic mixtures of the new carotenoid lipids with the co-lipids DOPE or cholesterol, and complexed with DNA, were physically characterized by size, zeta potential, and DNA complexation capacity. Their biocompatibility and transfection efficiency were evaluated using RPE cells. We observed all lipid formulations formed stable lipoplexes displaying characteristics suitable for gene transfer. The C20-20/DOPE and C20-18/DOPE formulations had transfection efficiencies up to 8%, comparable to the efficiency of commercial reagents in RPE cells. These results demonstrate that these new vectors are promising for ocular gene therapy.

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The Rabbit as a model for *in vivo* preclinical experimentation in limbal deficiency: Optimization of primary cultures of rabbit limbal cells

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With the aim of establishing an animal model for preclinical transplantation of limbo-corneal epithelial cells for the treatment of limbal deficiency, we have tried to optimize the *in vitro* primary culture to maintain the undifferentiated phenotype of cells.

Stemness was measured in terms of number of cell colonies with holoclone morphology as well as immunocytochemical staining for cytokeratin 3. Duplication number and viability of cells was also analyzed in each cell passage.

We studied the culture of limbal explants versus enzyme-digested cells from limbal rings. Four different enzymatic treatments were evaluated. Several substrates were used for culture, such as a mitomycin-inactivated feeder-layer of 3T3 murine fibroblast, amniotic membrane (AM), AM upon a 3T3 feeder-layer or cell-treated plastic. In addition, two different culture medium were assayed, a hormone-based medium with 10% FBS and the serum-free and low-calcium medium KSFM.

Our results show that the best conditions for *ex vivo* expansion of limbo-corneal epithelial cells for transplantation consist in enzymatic digestion of rings with dispase and trypsin, followed by cell culture onto a mitomycin-inactivated feeder-layer of 3T3 murine fibroblast with the serum-free and low-calcium medium KSFM. AM favors holoclon formation but doesn't allow cell passaging. For that reason, we prefer the use of other substrates for cell expansion although it could be a very useful substrate for cell transplantation.

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Mesenchymal Stem Cells (MSCs) in the treatment of canine indolent ulcers

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Indolent ulcers (also known as recurrent epithelial erosions or SCCED) are common in middle to older aged dogs of all breeds. Pathogenesis is thought to involve a defect in stromal-epithelial adhesion, with resultant superficial ulceration and the inability of the epithelium to adhere to the underlying stroma results in abortive and prolonged healing. Although the exact mechanism of this defect has not been fully resolved but one possible mechanism could be the loss of regenerative potential of Mesenchymal Stem Cells (MSCs). Current treatment recommendations consist of identifying and treating the underlying cause and performing procedures that stimulate epithelialization and adhesion of the corneal epithelium. MSCs are being widely studied as new tools in the treatment of a great number of pathologies in both human and animal species. We isolated MSCs from multiple origins like bone marrow, adipose tissue and limbus. In our preliminary studies Limbal Stem Cells (LSCs) showed the highest regenerative potential. We treated four cases of canine indolent ulcers, in which the conventional treatment was failed. The treatment is based on the application of contact lens with allogeneic LSCs in the damaged eye. LSCs were obtained from the same animal and distributed homogeneously on contact lens. After two weeks, indolent ulcers were totally regenerated. The fluorescein stain was negative and

scars were not present. Our results show the regenerative effect of LSCs on the corneal epithelium versus no response obtained with the current treatments. These promising results could help us to better understand the pathogenesis and to find new treatments for this pathology which nowadays lacks an adequate treatment.

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Modulation of ATP: adenosine balance by exosome and RAIN (Recombinant Anti-Inflammatory fusion) delivery of CD39 and CD73 is effective in reducing pro-inflammatory cytokine and chemokine production

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The conversion of ATP to adenosine is an important mechanism of immune suppression by Tregs, and this is done by expression of ENTPD1 (CD39) and 5NTE (CD73). CD39 is a membrane bound ATPase that converts ATP and ADP to AMP, whereas CD73 is a membrane bound ecto-nucleotidase that converts AMP to adenosine.

We have developed two novel systems for the delivery of CD39 and CD73. The first is a Recombinant Anti-Inflammatory fusion protein (RAIN) that consists of soluble CD73 fused to soluble CD39 by a flexible linker. This standalone molecule can be expressed from a plasmid/vector or used directly as a protein therapeutic and retains both CD39 and CD73 activity. The second approach uses exosomes containing CD39 and CD73. These exosomes are harvested from HEK 293T cells over-expressing CD39 and CD73. An advantage of the exosome delivery system is the increased specific activity of membrane bound CD39 compared with soluble CD39 (~10 fold).

RAIN and exosomes demonstrated high specific activity and were potent in reducing pro-inflammatory cytokine and/or chemokine (IL-6, CCL2) expression in a THP-1 (human monocyte cell line) based *in vitro* inflammation assay (CCL2 EC50: RAIN 44 pM±1.13, exosomes 12.4 pM±1.27; IL-6 EC50: RAIN 29.3 pM±1.1, exosomes 5.9 pM±1.2). RAIN and CD39-CD73 exosomes were also effective in reducing IL-1 β secretion in a whole blood inflammasome activation assay (IL-1 β EC50: RAIN ~4000 pM, exosome ~100 pM).

We have developed two novel biological therapeutics, RAIN and exosomes expressing CD39 and CD73 that are able to simultaneously decrease a potent pro-inflammatory molecule while increasing an anti-inflammatory molecule. The use of CD39 and CD73 to modulate inflammation should be applicable to broad spectrum of acute and chronic inflammatory disorders.

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Antigen-specific myeloid-derived suppressor cells generated during retroviral transduction of murine bone marrow ameliorate experimental autoimmune encephalomyelitis

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Our previous work showed that transferring bone marrow (BM) cells transduced with the MOG40-55 autoantigen into non-myeloablated mice with experimental autoimmune encephalomyelitis (EAE) improved the disease. We also reported that the vast majority of cells that are generated in the standard retroviral transduction cultures of murine BM cells consisted of myeloid cell populations (CD11b Gr-1^{low}) and (CD11b Gr-1^{high}), which correspond to the phenotypes of the monocyte-like and the granulocyte-like myeloid-derived suppressor cells (mMDSCs and gMDSCs). *In vitro*, these MDSCs expressed arginase-1 and iNOS, produced reactive oxygen species and suppressed splenocyte proliferation from EAE mice. Besides these well known suppressive mechanisms, both MDSCs subtypes expressed PD-L1 (Programmed Death Ligand-1). With this background, to further investigate the contribution of these cells to the observed therapeutic effect we performed *in vivo* studies using either transduced unfractionated BM or purified MDSCs. In a preventive approach, in the EAE model, a single *i.v.* infusion of 5×10^5 MOG-specific MDSCs into non-myeloablated animals significantly ameliorated the clinical course of the disease to a similar extent as total MOG-specific BM cells, indicating that MDSCs are contributing to the therapeutic effect. Remarkably, this effect was absent in mice receiving sham-transduced cells, indicating that antigen-specific mechanisms play a role in the tolerogenic effect. Furthermore, the spleens of these mice contained significantly less activated T cells (CD3 CD4 CD25 FoxP3⁺) and more B regulatory cells (CD45 B220 CD5 CD1d) than those of their counterparts. Adoptive cell therapy using *ex-vivo* generated antigen-specific MDSCs constitute a useful approach to induce tolerance in the context of autoimmune diseases.

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The TSG-6 promoter is a promising candidate for disease-induced expression of biological drugs in the joints of rheumatoid arthritis patients

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Local and regulated expression of biological drugs in arthritic joint for treatment of rheumatoid arthritis (RA) can minimize side effects encountered with conventional drug administration. Our approach is to obtain expression in an offer-meets-demand fashion using disease-inducible promoters. Microarray analysis of mice with experimental collagen-induced arthritis (CIA) revealed a correlation between disease severity and expression of TSG-6, an RA-associated gene that was also upregulated ~6-fold in 20 human end-point RA synovium samples, compared to 6 non-arthritic synovium samples ($P=0.01$).

We studied the disease-inducibility of the TSG-6 promoter by developing a lentiviral construct in which the isolated TSG-6 promoter controls the expression of a luciferase reporter transgene (TSG6-luc). Expression of the TSG6-luc reporter in mice peaked at day 7 after Streptococcal cell wall induced arthritis 19-fold compared to naive mice. Next, we transduced murine 3T3 cells with the LV TSG6-luc construct and incubated the cells with serum from CIA mice with increasing severity. The response of the promoter correlated significantly with the macroscopically assessed disease

severity (R^2 0.47, $P < 0.0001$) and with the circulating levels of the neutrophil chemokine KC (R^2 0.31, $P = 0.002$). Serum from human RA patients also showed a significantly higher activation of the TSG6-luc construct, compared to serum from healthy patients ($P = 0.001$).

An important characteristic of a successful disease-inducible promoter is a reduced activation after successful treatment. We tested this by comparing the response to serum from diseased arthritic mice to serum from mice that were successfully treated with the biological drug Enbrel. Serum from Enbrel treated mice showed a significantly lower activation of the TSG-6 promoter, compared to serum from untreated mice ($P = 0.001$). These results show that the TSG-6 promoter is disease-responsive in both mouse and man and therefore a promising candidate for transcriptional targeted gene therapy in RA patients

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Transgenic expression of IL-10 by lentiviral vectors for increasing the anti-inflammatory efficacy of mesenchymal stromal cells

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Introduction: The potent anti-inflammatory activities of mesenchymal stromal cells (MSCs) coupled with their mesodermal-differentiation potential make this cell type the ideal candidate for regenerative medicine applications, including GVHD, arthritis and inflammatory bowel diseases. Nonetheless, clinical results so far have been contradictory. Possible reasons include the functional plasticity of MSCs and the still poorly understood mechanisms by which MSCs exert their therapeutic effects.

Aim: To develop a gene-therapy approach for increasing the efficacy of MSC therapy.

Results: By using two sources, bone marrow and adipose tissue, we found that the antiproliferative effects of MSCs on T cells require cell-cell contact and the presence of antigen-presenting cells (APCs), and is maximal after pre-stimulation with IFN- γ . These properties were maintained after expanding MSCs with platelet-rich plasma lysate. Importantly, IFN- γ pre-stimulated MSCs did not directly produce the potent anti-inflammatory cytokine IL-10, suggesting an indirect licensing role of APCs. To by-pass the need for APCs, we have forced IL-10 expression by transducing MSCs with a lentiviral vector (LV) carrying a bidirectional promoter. High transduction efficiency (50–70%) was reached at MOIs that did not interfere with MSC vitality and expansion. Since genetically modifying MSCs carries unpredictable genotoxic risks, we have co-expressed the inducible caspase-9 suicide gene and demonstrated fast (2–4 hrs) and complete MSC ablation through a chemical-inducer of dimerization.

Conclusions: The transgenic expression of IL-10 has the potential to dramatically increase the anti-inflammatory efficacy of MSCs. Co-expressing a non-immunogenic suicide gene is an efficient antidotal measure in case of potentially associated toxicities.

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miR-335 expression levels in human mesenchymal stem cells correlate with age, and regulate cell differentiation and immunoregulation through modulation of AP-1 activity

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miR-335 is the most prominent miRNAs whose down-regulation in human mesenchymal stem cells (hMSCs) is critical for response to regulatory signals and cell differentiation. Sustained expression of this miRNA dysfavours proliferation, migration and differentiation of hMSC, rendering them less responsive to activation stimuli (pro-inflammatory and/or pro-differentiation signals) and more prompted to aging/senescence. Analysis of miR-335 expression levels along the ex vivo culture process demonstrated a significant progressive increase that is paralleled by a decline in the therapeutic properties of the cells. hMSCs forced to express this miRNA undergo early senescent-like alterations in comparison with naïve hMSC, including: augmented SA- β -gal and cyclin D1 expression, reduction of SOD2 expression, displaying of a senescent-associated secretory phenotype (SASP), but no significant changes in p21 or p53 expression. In addition, miR-335 also negatively controls hMSC differentiation and immunoregulatory capacities. In particular, exogenous overexpression of miR-335 abolished their in vivo chondro-osseous potential, as well as their protective effect in a model of lethal endotoxemia. These effects were accompanied by a severely reduced capacity for cell migration, and seemed to be mediated by the very pronounced reduction measured in AP-1 activity. Finally, analysis of miR-335 expression from donors in different age groups demonstrated a strong direct correlation between the endogenous levels of miR-335 and aging.

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Intralymphatic administration of adipose mesenchymal stem cells shows therapeutic effects in experimental models of inflammation

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Mesenchymal stem cells (MSCs) of allogeneic origin have been reported to reduce inflammatory processes and could therefore have therapeutic effects in immune disorders. The aim of our work was to determine potential anti-inflammatory and therapeutic effects and biodistribution of human adipose-derived MSCs (hASCs) in well-established models of rheumatoid arthritis and inflammatory bowel disease. In this

study, we did compare the intralymphatic (IL) route of administration, an innovative route for ASC administration developed by us, with other more classical routes like intraperitoneal (IP) and intravenous (IV). We administered hASCs in the inguinal lymph nodes of mice and compared the distribution of Luciferase-expressing hASCs in healthy and diseased mice (colitic) using IL, IV and IP routes of administration. We examined the therapeutic action of hASCs (using IV and IL routes) in arthritis induced by administration of chicken collagen II in immunocompetent mice. Efficacy was determined by clinical signs of the disease, inflammatory markers and imaging techniques. For the first time to our knowledge, we report here that IL administration of hASCs ameliorates the severity of arthritis. Distribution of hASCs varied depending on the route of administration and the inflammatory status, and a different mobilization throughout the lymphatic system and main organs was observed depending on the experimental conditions. Our results show that the direct administration of adult human ASCs to the lymphatic system is an effective alternative for cell-based therapy for the treatment of inflammatory and autoimmune disorders.

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Immunomodulatory effect of human adipose-stromal cells is related to the administration route

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Human adipose-derived stromal cells (hASCs) have a large potential for cell therapy in inflammatory and autoimmune diseases thanks to their immunomodulatory properties. In this study, we evaluate, in an *in vivo* model of colitis, the efficacy of hASCs to modulate inflammation when hASCs are administered through intralymphatic administration (IL). This route may have major benefits for the clinical use of the hASCs since equal or even superior therapeutic effect to conventional routes have been found. Colitis is induced by intrarectal administration of TNBS in C57BL/6 mice. A single dose of 3×10^5 hASCs is administered either IP, the most commonly used in animal models of IBD, or IL and the score of colitis (weight of mice, stools, histology and general aspect of mice) is monitored. Human ASCs infused through both routes of administration, IL and IP, were able to modulate the acute inflammatory response induced by TNBS. The responder mice within the IP group had very mild signs of colitis both at 24 h and at 48 h suggesting a protective effect of the hASCs. When hASCs were administered IL, the responder mice had similar degree of colitis than the TNBS-treated group at 24 h, but, interestingly, at 48 h responder mice nearly recovered their initial weight. These results indicate that it is feasible to administer hASCs through the IL route and that the mechanism of immunomodulation may be different according to the route of administration.

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Characterisation of the cellular responses involved in the therapeutic effects of hASCs in an *in vivo* model of rheumatoid arthritis

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Human adipose-derived stromal cells (hASCs) are proposed as a new treatment for rheumatoid arthritis (RA) due to their immunoregulatory and regenerative properties. In this study, collagen-induced arthritis (CIA) is induced into DBA/1 mice and hASCs are administered intravenously (day 0) once the onset of the disease reach between 2 to 4 score of arthritis. Progression of the disease and cellular responses by FACS are determined for 14 days. A single infusion of hASCs significantly delays the progression of the arthritis. From day 3, among the 32 mice treated with hASCs, 8 mice consistently have an attenuated degree of arthritis (Responders) along the 14 days follow up than their corresponding counterparts (Less Responders) and compared to mice within the CIA group. An inverse correlation in the frequency of Tregs and the arthritis score of the CIA group is observed. Upon infusion of the hASCs, the frequency of Tregs in the hASCs-treated mice remained constant along the experiment and is similar to the healthy mice (11.5%). CIA mice have increased frequencies of CD11b Gr1 myeloid cells (55%) compared to healthy mice (22%). An inverse correlation in the frequency of CD11b Gr1 cells and the arthritis score of the CIA group is observed. At day 3, CIA mice treated with hASCs have increase frequencies of CD11b Gr1 (72%) in the Responders and 60% in the Less Responders suggesting a direct interaction between hASCs and monocytes. In summary, therapeutic use of hASCs leads to a delay in the progression of the disease. These results suggest that hASCs are responsible for the maintenance of the Tregs in circulation and may induce mobilization of cells within the myeloid lineage.

P301

Snail as a regulator of cadherin-11 expression and a potential therapeutic target for rheumatoid arthritis

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Cadherin-11, a molecule responsible for cell-cell adhesion, is expressed in rheumatoid arthritis synovial fibroblasts (RASf) and contributes to the pathogenic mechanisms involved in

inflammation and cartilage erosion of rheumatoid joints. Identifications of critical processes that regulate cadherin-11 expression and lead to the transformation of SF within rheumatoid joints would be of importance to the development of therapeutic intervention targeting SF in RA. We hypothesized that snail may regulate an epithelial-mesenchymal transition (EMT)-like process and contribute to the transition of quiescent SF into an invasive mesenchymal phenotype. We studied the pathogenic role of snail in EMT-like changes and regulating cadherin-11 in SF from RA patients and rats with collagen-induced arthritis (CIA). We showed that SF from the synovial lining undergo an EMT-like process and acquire mesenchymal characteristics during arthritis progression. By modulation of snail expression via lentivirus-mediated gene transfer in CIA rats, we demonstrate the attenuation of SF-induced inflammation and joint destruction through snail-specific shRNA, as well as down-regulation of cadherin-11 through induction of a mesenchymal-epithelial transition (MET)-like process in CIA rats. Taken together, these findings clearly indicate that regulation of snail can modulate cadherin-11 expression and alter the patterns of cell morphology and gene expression between epithelial and mesenchymal phenotypes in SF. Our results also implicate that snail-induced EMT-like process may serve as a novel therapeutic target for RA.

P302

sTRAIL treatment induces beta cell proliferation

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Treatments that replenish functional beta cell mass in diabetic patients are of great interest since both type-1 and type-2 diabetes involve loss of beta cell mass and function. TRAIL (TNF-Related Apoptosis-Inducing Ligand), which selectively induces apoptosis in a wide variety of tumor cells, has also proven to promote survival and proliferation in various cell types, such as vascular smooth muscle cells. Furthermore, TRAIL is claimed to protect pancreatic beta cells against cytokine-related harm. We hypothesized that TRAIL could also induce proliferation of beta cells. To investigate this, we dispersed freshly isolated Wistar rat pancreatic islets into single cells. Our cultures consisted of 73.3%±2.3 beta cells (insulin), 16.3%±1.6 alpha cells (glucagon), and 10.4%±0.9 other cell types (only DAPI). Dissociated islet cells were treated with different concentrations of recombinant human sTRAIL (0, 1, 10 ng/ml) for 48 h. AnnexinV/PI staining was performed to determine whether TRAIL induced apoptosis in islet cells. No significant difference was evident between TRAIL-treated and untreated group. The viability of islet cells tended to increase in the TRAIL-treated group compared to the untreated group, as examined by WST-1 assay. Beta cell proliferation was tested by insulin-Ki67 staining. The percent of proliferating beta cells was significantly higher in 1 ng/ml sTRAIL-treated group (0.41%±0.04) compared to the untreated group (0.28%±0.04)(P<0.05). Thus, sTRAIL does not induce apoptosis in primary Wistar rat pancreatic beta cells, while increasing their viability and proliferation. In conclusion, TRAIL may be a candidate molecule with a potential to prevent beta cell loss and/or replenish beta cell mass.

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Development of a new murine model for autoimmune hepatitis useful for pathogenetic studies and preclinical trials

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Background and aims: Autoimmune hepatitis (AIH) consists of an immune-mediated destruction of hepatocytes of unknown etiology. Understanding the underlying molecular mechanism is essential for the development of new effective therapies, so animal models able to resemble human AIH are required. This work aims to develop a murine model able to reproduce these characteristics.

Methods: An adenoassociated virus hepatospecifically expressing murine IL-12 (AAVIL-12) was injected in C57BL/6, IFN- γ R^{-/-}, CD4- and CD8-depleted mice. At different time points, serum transaminases, cytokines and auto-antibodies were analyzed. Liver histopathology and FACS analysis of intrahepatic lymphocytic (IHL) populations were performed 30 and 60 days post-treatment. For adoptive transfers, syngeneic naïve mice received IHL from IL12-treated mice and were sacrificed 7 days post-transfer.

Results: AAVIL-12 induced increased IFN- γ and transaminases serum levels and, animals treated for 60 days, also showed anti-nuclear and anti-smooth muscle antibodies, but no anti-mitochondrial or anti-LKM. Liver histopathology presented dense inflammatory infiltrates and, 60 days post-injection, livers showed portal inflammation and fibrosis with severe interface hepatitis. We also observed increased number and activation of CD8 and CD4 T cells; depletion experiments showed that both populations were essential for the development of the disease, as well as IFN- γ responsiveness. Adoptive transfers showed that IHL from IL12-treated mice were able to significantly proliferate in naïve syngeneic recipients.

Conclusions: AAV-mediated IL-12 expression breaks the immunological tolerance to liver antigens giving rise to an active immune-mediated hepatic destruction. This scenario mimics human type I AIH and constitutes the first reported model without the use of transgenic animals.

P304

Unraveling hFOXP3 function in physiological and pathological lymphoid development

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Immune Dysregulation, Polyendocrinopathy, Enteropathy, X-linked (IPEX) syndrome is a monogenic autoimmune disease caused by mutations in the FOXP3 gene. Although FOXP3 mutations lead primarily to dysfunction of T regulatory (Treg) cells, novel findings suggest that other lymphoid subsets can be dysregulated and involved in IPEX pathogenesis. We have previously shown that both wild type and patient conventional CD4 T cells can be efficiently converted

into functional Treg by lentiviral vector (LV)-mediated constitutive overexpression of FOXP3, thus suggesting that gene-transfer based therapies can be envisaged for IPEX Syndrome.

Aim of this work is to investigate the role of FOXP3 in shaping human lymphoid cell development, in order to define the best gene transfer strategy to be adopted to restore immune tolerance. We have established *in vitro* and *in vivo* models to study lymphoid development of HSC transduced by LV expressing FOXP3 or anti-FOXP3 shRNA. *In vitro* models, including OP9-DL1 co-culture, showed that FOXP3 constitutive expression affects HSC differentiation and proliferation at early stages of hematopoiesis and skews CD4/CD8 differentiation at later stages. Preliminary data from *in vivo* systems, including NOD/SCID common γ -chain^{-/-} (NSG) mice-based humanized models, confirm *in vitro* findings and further suggest a wide role for FOXP3 in T cell lineage commitment.

Overall, our results will help understanding IPEX pathogenesis and assessing the feasibility of HSC-based therapeutic strategies for this disease, including HSC gene therapy and gene correction approaches. Moreover, the development of this disease model might serve as a prototype pre-clinical model for several autoimmune and lympho-proliferative disorders.

P305

Using an adeno-associated virus to generate *PTPN22*-deficient Jurkat T cell line

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Introduction: Protein tyrosine phosphatase *PTPN22* is encoded by the *PTPN22* gene. Polymorphisms in this gene are associated with several autoimmune diseases such as type I diabetes and lupus erythematosus, indicating that this phosphatase could have a role in modulating immune response.

Objectives: To generate a *PTPN22*-knockout in the human T cell line Jurkat, by gene targeting using an adeno-associated virus (AAV2)

Materials and methods: Homology arms were amplified by PCR. The pAAV-*PTPN22* vector was generated containing the respective homologous regions of *PTPN22*, with an in-frame introduced STOP codon 12 bp downstream of the ATG start site of the *PTPN22* sequence and a blasticidin resistance cassette flanked by two loxP sites. The viral particles were produced by co-transfection of the recombinant plasmid, together with pRC and pHelper in AAV293 cells. The viral titre was quantified by qPCR. Jurkat cells were infected with virions at an MOI of 1000, and heterozygous Jurkat for *PTPN22* were obtained which carry one mutant and one normal allele. The recombinant protein 6xHis-TAT-NLS-Cre was used to remove the blasticidin resistance cassette and again Jurkat cells were infected to obtain *PTPN22* knockout cells. The absence of *PTPN22* protein was confirmed by Western-blotting.

Results and Conclusions: rAAV virions were produced to target *PTPN22* gene, and *PTPN22* knockout Jurkat cells were successfully obtained. The mean frequency of gene targeting was 2% in four independent experiments. This study describes the generation of a new tool in order to better assess the relevance of *PTPN22* in T cell activation and function.

P306

BB02 improves therapeutic effectiveness of extracorporeal photopheresis with 8-MOP in a murine model of graft-versus-host disease

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Introduction: Extracorporeal photopheresis (ECP) is a cell-based immunomodulatory therapy involving the separation of peripheral blood autologous mononuclear cells followed by ex-vivo administration of 8-methoxypsoralen (8-MOP) and UVA radiation before reinfusion. ECP is efficient for the treatment of immune-mediated diseases such as graft-versus-host disease (GVHD), the major complication after allogeneic bone marrow transplantation. Our aim in the present work was to compare the therapeutic effectiveness of 8-MOP with other two new compounds (BB01 and BB02) in a murine model of GVHD.

Methods: Murine GVHD was induced after transplanting bone marrow cells and splenocytes from donor Balb/c mice into C57Bl6J recipients previously conditioned with a lethal dose of 10 Gy. To investigate the therapeutic effectiveness of ECP with either 8-MOP, BB01 or BB02, splenocytes from separate cohorts of C57Bl6J with GVHD were isolated 12 days after transplantation, incubated with the different compounds, irradiated with UVA light and injected intravenously once a week for four weeks. Survival after transplantation was monitored daily and clinical GVHD was graded using a previously described score analyzing weight loss, posture, mobility, skin integrity and fur texture.

Results: Mice treated weekly with BB02 showed a significant higher survival than those treated with 8-MOP ($p=0.038$), while BB01 had a similar effect to that of 8-MOP. Mice treated with either compound improved their clinical GVHD score compared to untreated mice, being significantly lower with BB02 than with the others ($p=0.023$).

Conclusions: BB02 was more efficient than 8-MOP in the reversal of murine GVHD, while BB01 showed the same therapeutic effectiveness than 8-MOP.

P309

The dystrophic environment widens the activation and functional contribution of immune cells after rAAV gene transfer in muscle

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Recombinant AAV2/1 vectors (rAAV2/1) have emerged as strong candidates for therapeutic gene transfer for various genetic diseases. However, preclinical evidence in muscular dystrophy models suggests that the development of immune responses may limit the efficacy of treatment and prevent long-term expression of the transgene in muscle. To explore the impact of the skeletal muscle environment on the rejection of gene-modified cells, we compared the activation and functional contribution of immune cell subsets in dystrophic mice devoid of alpha-sarcoglycan or in healthy C57Bl/6 mice. In both

models, the intramuscular delivery of a tagged transgene with rAAV2/1 induced strong transgene-specific CD4- and CD8 T cell-mediated immune responses in lymphoid organs, associated with marked infiltrations of CD3 T cell and CD11b myelomonocytic cell in muscles. Using various approaches for selective cell subset depletion in the models, we found that CD4 T cells were essential for transgene rejection in all strains, but macrophages and CD8 T cells also contributed as effectors of transgene rejection in dystrophic mice. To investigate how CD4 T cells become activated following antigenic presentation, we used mir142T-regulated rAAV2/1 vectors to control the endogenous presentation of the transgene by Antigen-Presenting Cells. The use of such approach was sufficient to fully circumvent CD4 and CD8 T cell activation in normal mice, but antigenic presentation was only delayed and still occurred in dystrophic mice leading to secondary CD4 T cell activation. Therefore, the dystrophic context modifies the cellular immune response mechanisms with severe outcome in terms of immune response against a gene therapy treatment.

P310

Immunological ignorance allows long-term gene expression following perinatal rAAV-mediated gene transfer to murine airways

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Introduction: Transduction of actively dividing tissue, such as the airway epithelium, with a non-integrating rAAV vector requires repeated vector administration(s) to achieve long-term gene correction. Fetal or neonatal vector delivery resorts to the immaturity of the immune system preventing the mounting of an effective immune response to the viral vector and allowing readministration in adult life.

Results: rAAV2/5 encoding the reporter genes Fluc or β -gal was administered perinatally, by intra-amniotic injection to mouse fetuses or by intranasal instillation to neonatal pups resulting in $6.6 \pm 1.1\%$ positive cells in the lungs of fetally and $8.9 \pm 1.5\%$ for neonatally treated animals. Although efficient, transduction was transient, underscoring the need for vector readministration to ensure long-term gene expression. Administration of a second vector dose increased reporter activity in the lungs 5- to 6-fold in both groups that remained stable up to 7 months at the time of sacrifice. Only after the readministration, neutralizing antibodies against rAAV capsid were detected in the perinatally treated group indicating that a primary immune response was elicited only after administration of the second vector dose, but not after initial perinatal gene transfer. However, this immunological ignorance was lost after readministration in adult life counteracting a successful third administration.

Conclusions: We demonstrated long-term gene expression in murine airways by readministration of rAAV2/5 after initial perinatal gene delivery, resulting in efficient and stable gene transfer up to 7 months without evidence for a decrease in gene expression.

P311

Transient depletion of specific immune cell populations to improve adenovirus-mediated transgene expression in the liver

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Background: Adenoviral (Ad) vectors are currently one of the most efficient tools for *in vivo* gene transfer to the liver. However, anti-Ad immune responses limit the safety and efficacy of these vectors. The initial inflammatory reaction is a concern in terms of toxicity, and it favors the development of cellular and humoral responses leading to short transgene persistence and inefficient vector re-administrations. Therefore, safe and simple ways to interfere with these processes are needed.

Aims: Study ways to deplete specific immune cell populations and their impact on liver-directed gene transfer.

Methods: First-generation Ad vectors encoding reporter genes (luciferase or β -galactosidase) were injected intravenously into Balb/c mice. Kupffer cells and splenic macrophages were depleted by intravenous administration of clodronate liposomes. B lymphocytes, CD4 or CD8 T lymphocytes were depleted by intraperitoneal injection of anti-M plus anti-D, anti-CD4 or anti-CD8 monoclonal antibodies (mAbs), respectively. Long-term evolution of luciferase expression in the liver was monitored by bioluminescence imaging.

Results: The anti-CD4 mAb impaired cellular and humoral anti-Ad immune responses, leading to efficient vector readministration. Clodronate liposomes had no impact on humoral responses but caused a 100–1000 fold increase in liver transduction, stabilized transgene expression, reduced the concentration of inflammatory cytokines, and inhibited lymphocyte activation.

Conclusions: Transient CD4 T cell depletion using mAbs is a clinically feasible procedure that allows efficient Ad redosing. Systemic administration of clodronate liposomes may further increase the safety and efficacy of vectors.

P312

Expression of the M2-type cytokines IL4 and IL13 prevents mesenchymal stem cell graft infiltration by microglia/macrophages in the central nervous system of immune competent mice

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Cell grafting in the central nervous system (CNS) is a promising therapy for numerous neurological injuries and diseases. However, it is well recognized that this approach is limited by strong glial immune responses triggered by cellular

grafts. In a model of mesenchymal stem cell (MSC) grafting in the CNS of immune competent mice, we previously described that MSC grafts become highly surrounded and invaded by Iba1 microglia. Here, we further characterized microglial responses following grafting of blue fluorescent protein-expressing MSC in the CNS of CX3CR1-eGFP transgenic mice (where all microglia express eGFP). Our results indicate a significant distribution of Iba1 CX3CR1 microglia surrounding and Iba1 CX3CR1- microglia (or monocytes/macrophages) invading the graft. Currently, bone marrow transplantation experiments are performed to confirm the peripheral origin of graft-infiltrating Iba1 myeloid cells. As we previously also described that graft-infiltrating Iba1 cells display an M1-activated phenotype (i.e. CD11b, MHCII and NOS2), we aimed to modulate the M1 phenotype of graft-infiltrating microglia/macrophages towards the neuroprotective M2 phenotype. First, we evaluated the potential of interleukin (IL)4-, IL10- and IL13-expressing MSC to inhibit *in vitro* activation of BV2 cells, determined by absence of TNF α -secretion following LPS/IFN γ -stimulation. While all cytokines displayed immunomodulating features, *in vivo* only IL4- and IL13-secreting, but not IL10-secreting MSC, could inhibit microglia/macrophage infiltration of MSC grafts and direct graft-surrounding Iba1 microglia towards the arginase-expressing M2 phenotype. In conclusion, we demonstrate the potential of IL4 and IL13 to modulate the occurrence and/or phenotype of MSC graft-infiltrating and -surrounding microglia and/or macrophages in the CNS of immune competent mice.

P313

Mannose Binding Protein: A therapeutic target for childhood recurrent infections?

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Introduction: Mannose Binding Protein (MBP), a member of the collectin superfamily, is a C-type serum protein produced by hepatocytes, which plays an important role in the innate immune defence. The human MBP gene is located on chromosome 10 at 10q11.2-q21. MBP is thought to play an important role in defence against infections during childhood. The low serum concentrations of MBP, which can lead to an opsonic defect, may be related to a polymorphism at base 230 of exon1, causing a change of codon 54 from GGC to GAC (AA homozygote). This results in the substitution of aspartic acid by glycine in the translated protein.

Materials and Methods: Correlation between this polymorphism and serum MBP levels were studied in children with recurrent infections. Forty children between the ages of 6–36 months with recurrent infections, in addition to 50 healthy individuals up to 16 years of age were included in this study. Blood MBP levels were measured by nephelometric methods in serum. Genomic DNA was isolated from peripheral whole blood, and polymorphism analysis was performed by PCR-RFLP.

Results: The results of our study indicated that the AA homozygous genotype of codon 54 in MBP gene was significantly associated with low serum MBP levels in recurrent infections.

Conclusion: This finding confirms a relationship between the mentioned polymorphism and recurrent infections. Although it

needs further investigation whether it could be a marker for propensity for recurrent infections, this information might be useful in the design of novel gene therapy modalities targeting childhood recurrent infections.

P314

Adipose mesenchymal stromal cell function is not affected by treatment with methotrexate and azathioprine

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Adipose mesenchymal stem cells (ASC) have been used in recent years, given their capacity to modulate the immune response, as therapeutic tools for the treatment of chronic inflammatory and autoimmune diseases in preclinical and clinical studies. Patients enrolled in such clinical trials are often treated with concomitant immunomodulatory drugs such as Methotrexate (MTX) or Azathioprine (AZA). Therefore it is needed to investigate the possible impact of these drugs on the ASC function. ASC were cultured *in vitro* in the absence or presence of meaningful physiologic concentrations of MTX or AZA and the effects on the following features of ASC were studied: i) viability and proliferation (MTT assay), ii) immunomodulatory properties *in vitro* (IDO activity, inhibition of lymphocyte proliferation and generation of regulatory T cells), and iii) immunogenic features (expression of HLA-I, HLA-II, CD80 and CD86 and cytolytic capacity of allogeneic NK and CD8 T cells). Drugs did not affect the viability and proliferative capacity of ASC. When the drugs and the ASC were concomitantly used to inhibit lymphocyte proliferation, no synergistic or antagonizing inhibitory effects were found. MTX and AZA did not impair the capacity of ASC to induce the generation of Treg cells *in vitro*, confirming that the immunomodulating features of ASC are fully functional after exposure or treatment with these drugs. Whereas MTX did not affect the capacity of NK or CD8 T cells to lyse allogeneic ASC *in vitro*, AZA significantly did affect the capacity of NK cells, likely by modulating the NK activation status.

P315

Efficient monitoring of the T cell receptor repertoire in different immunological conditions by deep sequencing

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Dissection of T cell receptor (TCR) repertoire diversity at the nucleotide level can provide important insights into immune competence and ongoing immune reactions. We established RNA-based quantitative TCR-LAM direct sequencing of the α and β TCR loci.

From the TCR sequencing analysis of 6 healthy non related individuals we defined the occurrence of non random events in the combinatorial and junctional diversity, the positive selection of defined TCR specificities (up to 17%) by convergent recombination and the presence of shared interindividual sequences (on average 1.1% for α -chain and 0.4% for β -chain).

To demonstrate efficient monitoring of immunological responses, we restimulated *in vitro* PBMC from 2 CMV seropositive donors at day 0, day 9 and day 15 with peptide pools of 2 highly immunodominant viral proteins. CMV-specific TCR molecules emerged already 9 days after restimulation. Less than 1% of the T cells at day 0 were found to be reactive against the peptides used and their identification was possible despite their very low frequency contribution (down to 0.01%).

The potential of our approach as a diagnostic tool was shown by the TCR repertoire analysis in 10 patients affected by different forms of Sézary syndrome. We observed a dominating TCR clonotype in the severe disease form and a less restricted oligoclonal repertoire in the mild disease form.

Our results highlight the robustness of TCR-LAM direct sequencing in performing a detailed non invasive monitoring of immune reactions in humans and in mouse models and in dissecting the TCR repertoire in different immunological conditions.

P316

Development of genetic engineering strategies to prevent the effects of antibody and complement on xenogeneic chondrocytes

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The use of xenogeneic chondrocytes may benefit the development of clinical tissue-engineering applications for cartilage repair. Particularly, porcine cartilage is rejected by humoral and cellular mechanisms that could be overcome by identifying key molecules triggering rejection and developing genetic-engineering strategies to counteract them. Accordingly, high expression of α 1,2-fucosyltransferase (HT) in xenogeneic cartilage protects from the galactose α 1,3-galactose (Gal)-mediated antibody response. Now, we studied whether the combination of HT with a complement inhibitor provides further protection. To this end, we isolated porcine articular chondrocytes (PAC) from non-transgenic, single and double transgenic pigs expressing HT and human CD59 (hCD59) and assessed their response to human serum. The effect of high recombinant expression of human complement regulatory molecules hCD59 and hDAF attained by retroviral transduction of PAC, and inhibiting C5a with mAb was also studied. Exposure of control PAC to 20% human serum for 24 hours mainly triggered the release of the pro-inflammatory cytokines IL-6 and IL-8 (determined by ELISA in supernatants). This effect was only partially reduced by the transgenic expression of HT and hCD59, even in combination. However, high hCD59 expression produced a higher protection that was further enhanced by inhibiting anti-Gal antibody deposition or C5a-mediated effects. On the contrary, high hDAF expression alone attained the most dramatic reduction in IL-6/IL-8 secretion and the additional inhibition of anti-Gal antibodies or C5a did not provide

further improvement. In conclusion, our study shows that complement activation contributes to rejection of xenogeneic cartilage and provides valuable information for selecting approaches to prevent humoral rejection.

P318

Genotoxicity following AAV gene therapy for Methylmalonic Acidemia (MMA) in mice

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Numerous toxicology studies have demonstrated the safety of AAV vectors. However, Donsante *et al.* have reported an increased incidence of hepatic cellular carcinoma (HCC) in neonatal mice treated with AAV and they have implicated insertional mutagenesis of the vector at the RIAN locus as a causative factor. We have reported several pre-clinical AAV gene therapy studies for MMA and demonstrated the efficacy of this approach in a neonatal lethal murine model of this disease. Although evaluating long-term genotoxicity was not the intended focus of our studies, 75% (n=48) AAV8-CBA-Mut treated mice developed HCC between 12 and 21 months, conversely only 2% (n=41) uninjected mice developed HCC. In addition, 50% of the mice treated with AAV8-CBA-GFP developed HCCs, indicating that overexpression of the Mut transgene is not solely responsible for the development of HCC. In a separate study, we performed an AAV dose escalation study that showed increasing the AAV8 dose from 7×10¹¹-12 GC/kg (n=16) to approximately 1×10¹⁴ GC/kg (n=19) caused a corresponding increase in the occurrence of HCC from 12% to 84%, respectively. As previously observed by Donsante *et al.*, our preliminary data indicates that insertional mutagenesis of the vector at the RIAN locus may be a causative factor. Determining why AAV gene delivery in neonatal mice is infrequently associated with increased tumorigenesis and if such toxicity is relevant to humans will lead to improved safety in AAV gene delivery. Alternatively, site-specific integration vectors may offer greater efficacy and safety for gene delivery in the neonatal period.

P319

Identification of genes involved in the resistance to targeted anti-cancer therapies by lentiviral vector-based insertional mutagenesis

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Insertional mutagenesis screens with retroviruses and transposons have been extensively used in forward genetics screenings for cancer gene discovery. We have recently demonstrated the potential of modified lentiviral vectors (LVs) for the discovery of cancer genes in solid tissues. Now, we show that the new LV-based insertional mutagenesis platform, besides cell transformation, can be successfully used for the identification of the genetic culprits of resistance to targeted anti-cancer drugs, one of the most daunting hurdles in clinical oncology. An LV specifically tailored to deregulate genes upon

integration was used to transduce at escalating doses different HER2 breast cancer cell lines, which were then exposed to the clinically approved HER2-targeting drug *lapatinib*. LV transduction induced, in a significative and dose-dependent fashion, the emergence of resistant cell clones. Vector-genome junctions were retrieved from resistant cells and untreated controls and deeply sequenced with the Illumina technology. We identified common integration sites specific of resistant cells, which harbor both known and novel putative *lapatinib*-resistance genes, such as *PIK3CA*, *MET*, *PIK3CB* and *RPS6KA5*, whose validation in clinical samples is ongoing. Additionally, we identified molecular circuits and novel genes which may play a relevant role in shaping tumor response to *lapatinib* and other HER2-targeted drugs. This forward genetics platform was applied to other tumor types and compounds, leading to the identification of different candidate drug resistance genes. The new acquired knowledge might be seminal for the development of novel targeted combinatorial therapies to overcome the occurrence of resistance in human tumors.

P320

Gene therapy models of X-linked severe combined immunodeficiency and Wiskott-Aldrich Syndrome using foamy virus vectors

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Vectors based on Simian Foamy virus (FV) have recently been used to correct a number of genetic diseases. Over the conventional gammaretrovirus vectors, FV vectors have several advantages including non-pathogenicity, stability in quiescent cells, and the lack of insertional oncogenesis in nonhuman primate natural hosts. The occurrence of leukemia in the clinical trials for X-linked severe combined immunodeficiency (SCID-X1) and Wiskott-Aldrich Syndrome (WAS) substantiates the need for development of vectors alternative to gammaretrovirus vectors. In this study, we have developed the FV-based vectors for SCID-X1 and WAS, and assessed the use of FV vectors as a gene transfer system for the primary immunodeficiency. Gamma chain (gc) knock out mice (SCID-X1 mice) were treated with FV vectors containing gc cDNA and the development of T and B lymphocytes was observed in transplanted mice. Increased T cell response to cytokines and the significantly elevated serum levels of immunoglobulin indicated the functional reconstitution of immune system by FV vectors.

Was knock out mice were also treated with FV vectors with high transduction efficiency, and transplanted animal showed functional multi-lineage reconstitution of hematopoietic cells (T cells, B cells, dendritic cells and platelets). Analysis of provirus integration site in spleen and bone marrow cells revealed the lower likelihood of being located near the transcriptional start sites and inside the gene transcriptional units. These data may imply that FV vectors are less prone to insertional oncogenesis due to the transactivation or destruction of the cellular genes, as compared to gammaretrovirus and lentivirus vectors.

P321

In vivo genotoxicity profile of a chimeric promoter driven lentiviral vector for X-CGD in mice

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Gene therapy by retroviral vectors has proven its potential to ameliorate the health status of patients suffering from inherited blood disorders or even offer a final cure for these diseases. Earlier experiences in treating chronic granulomatous disease (X-CGD) with LTR-driven gammaretroviral vectors highlighted the importance of the vector design on the clinical outcome. Therapeutic benefits were undermined by silencing of the viral promoter and disturbed hematopoiesis due to insertional mutagenesis. To overcome some of the observed drawbacks, a lentiviral vector was developed, using a synthetic chimeric promoter (CatG/Cfes) favouring high-levels of gp91phox expression in the myeloid lineage. This promoter is less prone to silencing and the insertional profile of lentiviral vectors is believed to be safer regarding the distance to transcriptional start sites and other regulatory elements of active genes. We analyzed the genotoxic potential of this vector in mice and compared it either to an LTR-driven gammaretroviral vector (RSF91) or a lentiviral vector with an internal SFFV promoter/enhancer. In total, we transplanted 47 mice with a low and high vector dosage and monitored the animals for 26 weeks. We analyzed engraftment, body weight, blood parameters and contribution of transgene positive cells over time. Insertion site analysis by LAM-PCR and 454 pyrosequencing suggested a considerable level of clonal fluctuation in all mice. Verification of overrepresented sequences by locus specific PCR (n=61) illustrated the challenges associated with clonality assessment by deep sequencing. Both lentiviral vectors, however, showed a favourable safety profile in mice with polyclonal hematopoietic repopulation in comparison to RSF91.

P322

Distribution of lentiviral vector integration sites in gene therapy for X-Adrenoleukodystrophy

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Here, we report on an integration site (IS) analysis performed on patient samples from the first clinical trial to treat a monogenetic cerebral disease using autologous hematopoietic stem cell transplantation with lentiviral SIN-vector, initiated more than six years ago. The correction of hematopoietic stem cell has not been accompanied by signs of clonal dominance

or even premalignant disproportional distribution of cellular contributions in the 4 treated patients. Our large scale IS analysis performed on *ex vivo* transduced cells prior to re-infusion and on engrafted cells by linear amplification mediated PCR (LAM-PCR) and next generation sequencing (454 pyrosequencing and Illumina MiSeq) revealed a polyclonal hematopoietic reconstitution. More than 30.000 unique exactly mappable IS have been identified from distinct time points and sorted cell fractions, suggesting a highly diverse clonal repertoire. Our data revealed the characteristic integration profile as described for lentiviral vectors, showing a preferred integration within genes (P1: 73.1%; P2: 73.0%; P3: 71.5%; P4: 71.4%), a favored integration on chromosomes harboring gene dense regions and in genomic sites described as lentiviral hot spots, such as KDM2A, PACS1 and HLA genes. The landscape of ALD vector integration was very similar in all four patients analyzed. A successful *ex vivo* transduction of early hematopoietic progenitors is indicated by the detection of identical IS identified in myeloid and lymphoid lineages as well as in CD34 cells in P1, P2 and P3 (analysis is ongoing for P4). Lentiviral gene therapy shows to be safe and effective, the cerebral disease has been stabilized in 3 out of 4 patients.

P323

Self-inactivating alpharetroviral vectors have a low genotoxic integration profile in human CD34⁺ hematopoietic progenitor cells

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Gammaretroviral and lentiviral vectors currently used in gene therapy trials display a non-random integration pattern targeting either regulatory regions or transcribed portion of expressed genes and have the potential to deregulate gene expression at the transcriptional and post-transcriptional level, respectively. A recently developed self-inactivating (SIN) alpharetroviral vector is able to sustain long-term transgene expression and shows a more favorable integration pattern compared to SIN gammaretroviral and lentiviral vectors in murine hematopoietic stem cells (Suerth *et al.* 2012).

In this study, we performed a high-throughput analysis of >6,000 SIN-alpharetroviral vector integration sites in human CD34 hematopoietic stem/progenitor cells and compared them to previously generated datasets of SIN-gammaretroviral and lentiviral integration sites. We showed that SIN-alpharetroviral vectors have no bias for integration inside transcription units or in proximity to transcription start sites, enhancer and promoter regions (marked by H3K4me3 and H3K4me1 histone modifications), PolII binding sites and silenced regions marked by H3K27me3. Interestingly, SIN-alpharetroviral integrations do not cluster in specific genomic regions and hit at a very low frequency MLV highly targeted loci, including the genes involved in most of the severe adverse events observed in clinical trials, i.e., LMO2. Therefore, alpharetroviral vectors, showed a lower genotoxic potential compared to gammaretroviral and lentiviral vectors, in human clinically relevant cells.

P324

The genotoxic potential of lentiviral vector integration is modulated by the interplay between vector design and mouse genotype

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Retroviral vectors with self-inactivating (SIN) Long-Terminal-Repeats (LTRs) have a superior safety profile than vectors with active LTRs. However, weaker mechanisms of insertional mutagenesis, could still pose a significant risk in clinical applications when SIN.LTR vectors are used. We developed novel in-vivo genotoxicity assays based on systemic vector injection into newborn tumor-prone *Cdkn2a*^{-/-} and *Cdkn2a* *-* mice. Injection of transgene-expressing SIN Lentiviral-Vectors (LVs) harboring strong (SF, *Cdkn2a*^{-/-} N=33, *Cdkn2a* *-* N=17) or moderate (human-PGK, *Cdkn2a*^{-/-} N=31) enhancer/promoters in internal position caused a significant acceleration in hematopoietic tumor onset with respect to control mice (p<0.05). The genotoxicity of these SIN.LVs was significantly lower than an LV with the SF within the LTRs (LV.SF.LTR) (N=43 in both mouse strains p<0.0001). Injection of a SINLV without any open-reading-frame downstream the internal SF (ORF-less, N=9) caused a dramatic acceleration of tumor onset (p<0.0001). Analysis of >8500 integration sites from vector-induced tumors confirmed that oncogenesis was caused by insertional mutagenesis. The highly genotoxic LV.SF.LTR and ORF-less-LVs induced oncogenesis in both mouse strains mainly by *Braf*-activation throughout a read-through/splicing-capture mechanism. The moderately genotoxic SINLVs, being unable to activate *Braf*, activated other oncogenes or disrupted tumor-suppressor genes through enhancer-mediated and/or aberrant splicing mechanisms (*Map3k8* or *Pten* in *Cdkn2a*^{-/-} mice of *Sfi1* in *Cdkn2a* *-*). Therefore, the cancer genes deregulated by vector integrations in tumors were dependent on a complex interplay among vector design and mouse genotype. Hence, our approach shed light on the molecular mechanisms and host genetic factors modulating the genotoxicity of SIN.LVs with different backbone designs and internal promoters.

P325

“Coloured” barcodes for clonal tracking

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Introduction: We recently introduced RGB marking – a method based on combined expression of fluorescent proteins by

integrating retroviral vectors – for investigating the clonal fate of gene-modified cells. An alternative, expression-independent method for assessing clonality relies on the introduction of short, specific sequence tags (“barcodes”) into target cells. Barcodes can be readily amplified by PCR and quantified by next-generation sequencing (NGS). We here combined the virtues of both marking techniques by barcoding RGB vectors.

Methods: We designed four different barcodes, each specific for one fluorescent protein. All barcodes consisted of eight pairs of random nucleotides intersected by triplets of fixed nucleotides. RGB-barcode vectors were used for transduction of different cells, including murine primary hepatocytes and haematopoietic stem cells prior to transplantation into uPA/SCID and conditioned C57Bl/6 mice, respectively.

Results: We estimated the complexity of plasmid and high-titre lentiviral-vector libraries by NGS to be in the range of 5×10^5 barcodes. Single-cell PCR revealed barcode combinations corresponding to the RGB colours of individually picked RGB-marked cells (in vitro). Barcodes could also be retrieved from single cells isolated by laser-microdissection from regenerated RGB-marked liver tissue. Finally, barcode marking allowed us to perform clonal follow-up studies in our murine bone marrow transplantation model.

Conclusion: Genetic cell marking with barcoded vectors facilitates quantitative analysis of organ regeneration, e.g., of haematopoiesis and liver. The use of barcodes ciphering for different colours permits to verify genetic identity of RGB-marked cells. RGB-barcode vectors thus provide a powerful tool for long-term clonal cell tracking.

P326

***In vivo* safety evaluation of a novel insulated SIN LV reveals escape-mechanisms leading to genotoxicity**

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We used a sensitive *in vivo* genotoxicity assay, based on systemic vector administration into newborn tumor-prone *Cdkn2a*^{-/-} mice, to test whether the inclusion of a Chromatin Insulator (CI) into the Self-Inactivating Long Terminal Repeats (SIN.LTRs) of a lentiviral vector with a strong internal enhancer-promoter (INS.SIN.LV.SFFV) could improve its safety profile *in vivo*.

Cdkn2a^{-/-} mice treated with INS.SIN.LV.SFFV (N=22) or its uninsulated counterpart (SIN.LV.SFFV, N=17) developed hematopoietic tumors significantly earlier than mock controls (p=0.006 and p=0.0095, respectively). Therefore INS.SIN.LV.SFFV, although to a lower extent with respect to SIN.LV.SFFV (median survival 203 days vs. 186 days), was still genotoxic.

To unravel the molecular mechanisms underlying genotoxicity we retrieved and mapped 4678 integrations from tumor-infiltrated tissues and identified the Common Insertion Sites (CIS), hallmarks of insertional mutagenesis. The comparison between the CIS found in the two treatment groups showed remarkable differences: in tumors induced by SIN.LV.SFFV the most relevant CIS was represented by *Map3k8* (targeted by 19 integrations), that was activated by a mechanism involving enhancer-mediated overexpression and transcript-truncation. In INS.SIN.LV.SFFV marked tumors we observed a significant reduction of integrations targeting *Map3k8* (only 2 integrations, p=0.001) accompanied by a remarkable skewing towards tu-

mors harboring inactivating integrations targeting *Pten* (11 integrations). Therefore the genotoxicity of INS.SIN.LV.SFFV is mainly caused by an “escape mechanism” represented by the inactivation of a tumor suppressor gene.

Despite in our model the inactivation of tumor suppressor genes, on which insulators cannot act, negatively impacted the mouse survival, our results indicate that this CI is able to significantly reduce enhancer-mediated activation *in vivo*.

P327

***In-vivo* selection and integration analysis after lentiviral O⁶-methylguanine DNA methyltransferase (MGMT^{P140K}) expression in a human xenograft model**

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MGMT^{P140K} mediated myeloprotection and *in-vivo* selection of transduced cells have been demonstrated in numerous animal models and recently also in a clinical study. However, with this strategy the genotoxic risk of integrating vectors may be augmented by cytotoxic-agent induced lesions and the proliferative stress during selection. Thus, we investigated MGMT^{P140K} *in-vivo* selection by transplanting SIN lentiviral vector (EFS1 α internal promoter) transduced human cord blood-derived CD34 cells into NOD.SCID.IL2R γ c^{-/-} mice. Chemotherapy (CTX) comprised two doses of combined O⁶-benzylguanine (BG; 20 mg/kg) plus 1,3-Bis-(2-chlorethyl)-1-nitroso-urea (BCNU; 5-10 mg/kg) given one week apart. While at highest BCNU doses animals succumbed to myelosuppression, intermediate doses (BCNU: 7.5&5 mg/kg, 5&5 mg/kg) yielded significant enrichment of transgenic human cells in the peripheral blood and bone marrow (BM; 1.7±1.1% untreated versus 54.5±13.7% and 52.8±15.6% both P≤0.05; n=3-6). Significant enrichment upon CTX was observed in BM myeloid, B and primitive cells. Specifically, significant expansion of transduced cells was observed in the immature myeloid compartment (0.64±0.33×10⁵ versus 3.4±1.1×10⁵ and 3.4±1.0×10⁵; P≤0.05, n=3-6). Clonal inventory analysis utilizing LAM-PCR followed by high throughput sequencing revealed a characteristic lentiviral integration profile with 74.2% of insertions in genes (54.9% intronic). Among the BM insertions 16.6% (treated; 13/78) and 8.6% (non-treated; 2/23) overlapped with previous MGMT studies whereas 20.5% (treated; 16/78) and 26% (non-treated; 6/23) genes were common to cancer databases without significant differences between the groups. Functional annotation of hit genes using Panther Database did not yield significant enrichment for specific gene-classes. These data indicate efficient *in-vivo* selection without clonal imbalance using this SIN lentiviral vector for MGMT^{P140K} expression.

P328

A quantitative method for vector integration site retrieval based on genomic DNA sonication and Illumina next generation sequencing

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Vector integration in the host genome allows the stable and robust expression of a therapeutic transgene in transduced cells. However in some hematopoietic stem cell (HSC)-based clinical trials, vector integration caused mutations triggering cell transformation. To address these safety issues, PCR-based methods selectively amplifying the vector/host genome junctions from patients' cells and massive sequencing are required. Since integration sites (ISs) are unique genetic marks specific for each transduced cell and its progeny, it is possible to monitor patients' blood and bone marrow cells during time and follow the fate of thousands vector-marked cell clones in a dynamic fashion. However, the most sensitive methods for IS retrieval generate DNA fragments heterogeneous in size, whose abundance does not precisely correlate to the abundance of vector-marked cell clones. We developed a novel method for quantitative IS retrieval named Sheared DNA Linker and Linear Amplification Mediated (SDLLAM)-PCR. This approach, based on genomic DNA sonication and Illumina sequencing technology, can be used on relatively small amounts of genomic DNA (150 ng). ISs are quantified by counting the different genomic shear sites, getting rid of product size-related biases. Our data on test samples with known vector copy number suggest that this approach allows to accurately determine relative abundance of ISs. Moreover, the sequencing depth granted by the Illumina platform dramatically increase the number of IS retrieved (18×10^6 reads in a single run), strengthening the power of the safety studies and allowing to study the dynamics of hematopoiesis in HSC gene therapy trials.

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Modelling viral insertion sites via network based frameworks

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The non-random character of retroviral integration profiles allows to determine the functional and mutagenetic potential of the targeted regions. While the formal common insertion sites (CIS) analysis is based on a rigid definition that takes into account the number of IS in a fixed window, previous studies have shown that this definition is outdated, returning an excess of false positive CIS.

Here we address the challenge to find 1) a definition of CIS that is constraint-free independent from the IS database size based on a representation of IS based on graph, 2) return a representation of CIS that is immediate and easy to manage and 3) discover new potential genes that might be involved in tumorigenesis.

In this work we propose a new graph based framework to model common insertion sites and is implemented as a plugin for Cytoscape (2.x) in order to simplify the CIS analysis and to integrate directly the data in the context of system biology networks.

Five dataset were used to test our framework. The the x-linked adrenoleukodystrophy (lenti-virus, LV) clinical trial, the Wiskott-Aldrich syndrome (γ -retro-virus, GV) clinical trial, a HIV study, a human preclinical unpublished study (LV) and the Retrovirus and Transposon tagged Cancer Gene Database (RTCGD, GV).

P330

Accurate deep-sequencing of vector integration sites using mutually dependent sequencing barcodes

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Stable integration of retroviral vector DNA into the host genome is a mutagenic event that may directly contribute to unwanted side effects. A comprehensive and accurate analysis of the integrome is indispensable to study clonality in transduced cells obtained from gene therapy patients. We have explored the use of the Illumina MiSeq Personal Sequencer platform to sequence vector integration sites (IS) amplified by LAM-PCR. The MiSeq sequencing technology provides much higher read numbers (up to 15 million) compared to established 454 pyrosequencing technology (up to 1 million), thus enabling a more profound representation of the IS repertoire. However, deep-sequencing of LAM-PCR products by the MiSeq platform showed a much higher degree of collisions – identical IS detected in unrelated samples – compared to 454 pyrosequencing. This was especially evident in samples with low numbers of IS that have been sequenced in parallel with samples containing high number of IS. To overcome high frequency of false-positive IS observed in MiSeq sequenced LAM-PCR products, we used a double barcoding strategy to filter raw sequences after deep-sequencing. Only sequences that matched the unique combination of both barcodes were considered for downstream analyses. Using double barcoding strategy, contaminating sequences were efficiently removed. Similarly, removing sequence reads with wrong combination of barcodes eliminated identification of contaminating IS in untransduced samples.

Our data show that reliable identification of IS by the MiSeq system is feasible by barcode labeling of PCR products *ab initio* and applying filtering of raw reads based on double barcode usage.

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Bioinformatic framework for vector integration sites analysis and clonal abundance estimation

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The study of chromosomal vector integration sites (ISs) in vector marked cells from hematopoietic stem cell gene therapy patients is instrumental to assess the safety and efficacy of the treatment and studying stem cell biology and hematopoiesis. Exploiting PCR-based methods, DNA fragments containing the vector/genomic junction were amplified and sequenced using the Illumina MiSeq platform. Currently up to 18 million PCR products are sequenced in a single run. The huge amount of data generated required a new bioinformatic framework for IS mapping and data mining. We also set-up algorithms to analyze the Shear Sites (SS) of sequences obtained by a novel non-restrictive PCR method for IS retrieval developed in our laboratory and perform clonal abundance estimates.

The framework is composed by: (1) raw sequence data processing and SS quantification for each IS; (2) IS mapping and datamining (3) SS saturation assessment and for each IS to estimate the relative abundance of cell clones in a given sample.

To fine tune and validate both the new technology and the bioinformatic framework we analyzed a number of test DNA samples composed by a highly polyclonal pool of vector marked cells (JY cells) mixed with a clone with 6 known integrations serially diluted in the DNA of JY cells.

Testing the SS relative quantification approach in the experimental environment, allowed to correctly estimate and quantify clonal abundance, supporting the development of this promising technology.

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Therapeutic gene delivery to glioblastomas by enhanced retroviral vectors eradicate brain tumors and promotes survival

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The conventional replication competent retroviruses (RCR) have significant therapeutic effects in glioma cancer model. However, their *in vivo* applications are restricted by the one vector amphotropic murine leukemia virus (MuLV) system. Furthermore, so far, the therapeutic genes that can be delivered by this system are limited to relatively small-size genes, such as cytosine deaminase (CD). We constructed a semi-murine leukemia virus-based, gibbon ape leukemia virus envelope-pseudotyped replication competent retrovirus (spRCR) system that circumvents these limitations. In this study, we found that a spRCR vector spread more efficiently than a semi-Murine leukemia virus-based RCR (sRCR) in human glioblastoma cells *in vitro*. Furthermore, injections of equal numbers of infectious units into pre-established intracranial U-87 MG human glioblastomas in nude mice demonstrated that the spRCR vector had significantly enhanced transduction efficiency and also expressed the therapeutic genes more rapidly than the sRCR vector. Finally, intratumoral injection of spRCR successfully delivered the herpes simplex virus 1-thymidine kinase (HSV-TK) gene into intracranial U-87 MG glioblastomas and resulted in both tumor eradication and prolonged survival upon administration of ganciclovir (GCV). Our data suggest that the semi-GaLV-Env-pseudotyped RCR system provides significant advances for delivery of therapeutic genes into cancerous tumors.

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Tissue-specific regulation of adenoviruses by incorporation of miR target sites controlling the late-phase fiber protein

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Adenovirus antitumor activity is based on their ability to lyse infected cells during viral release. However, adenoviruses do not have a natural tropism towards tumor cells, so it is necessary to

provide them with tumor selectivity. Commonly, selectivity has been approached by controlling viral entry to tumor cells or by focusing on the control of early expressed regulatory proteins to achieve conditional viral replication. In this work, we have studied the feasibility of providing replication-competent adenoviruses with tissue-specific selectivity through the regulation of the late structural fiber protein. Since late proteins are all transcribed from the common MLP, we have engineered a 3'UTR containing microRNA binding sites to provide tumor selectivity to the adenoviral fiber (Ad-Fiber-miRT). The results were compared to an engineered virus containing the same miRNA binding sites controlling the early E1A protein (Ad-E1A-miRT). We present *in vitro* and *in vivo* evidences of a miRNA dose-dependent regulation of Ad-Fiber-miRT. Such regulation was specific to Fiber-miRT expression and no effect was observed on other early or late viral proteins such as E1A or hexon. As a consequence, viral release of infective particles was impaired in miRNA expressing cells. Despite intracellular viral genomes were only reduced in Ad-E1A-miRT, production of infective viral particles was similarly observed in cells infected with Ad-E1A-miRT, and Ad-Fiber-miRT.

Thus, miRNA-control of late proteins like the fiber constitutes a novel strategy for providing specificity to adenoviruses. Besides, the combination of late proteins control with already proven selective strategies for *early* proteins, may confer oncolytic adenoviruses enhanced selectivity.

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Advanced two-step transcriptional amplification as a novel method for cancer-specific gene expression and imaging

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The two-step transcriptional amplification (TSTA) system was previously reported to enhance the tissue-specific gene expression driven by weak promoters, but the enhancement of the gene expression is limited to use in *in vitro* and *in vivo* experimental situations. To achieve robust tissue-specific gene expression using the TSTA system, we developed an advanced TSTA system which includes polyglutamines and rat glucocorticoid receptor sequences between the GAL4 and VP16 sequences in the region of the first step of transcription. We herein evaluated the advanced TSTA system as a method to enhance the human telomerase reverse transcriptase (hTERT) promoter-driving cancer-specific transcription in various cancer cell lines. We also demonstrated the utility of the advanced TSTA system for cancer-specific gene expression and imaging, we constructed a luciferase gene encoding adeno-associated virus (AAV) vector in which the hTERT promoter-mediated expression was driven by the advanced TSTA systems. In an orthotopic liver tumor model, mice were treated with the vector via tail vein injection. An optical imaging device was used to visualize the *in vivo* luciferase expression in the orthotopic tumor. The advanced TSTA system significantly enhanced the luciferase expression compared with the one-step and conventional TSTA systems (18.0±1.0- and 15.9±0.85-fold gain, respectively). The advanced TSTA method is expected to become a valuable tool enabling *in vivo* site-specific targeting in the field of gene therapy and molecular imaging.

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iRGD (tumor-penetrating peptide)-modified oncolytic adenovirus shows enhanced antitumor efficacyC. Puig-Saus¹, E. Laborda¹, A. Figueras¹, L. A. Rojas¹, R. Alba², C. Fillat³, and R. Alemany¹¹Translational Research Laboratory, Institut Català d'Oncologia-Institut d'Investigació Biomèdica de Bellvitge (ICO-IDIBELL)-, L'Hospitalet de Llobregat, Barcelona, Spain; ²Nanotherapix S.L. Parc Empresarial Can Sant Joan. Sant Cugat del Vallès, Barcelona, Spain; ³Institut d'Investigacions Biomèdiques August Pi i Sunyer (IDIBAPS), Barcelona, Spain

Systemic tumor targeting with oncolytic viruses is limited by poor extravasation. Cyclic peptide iRGD (CRGDKGPDC) binds to integrins on tumor vasculature and, upon cleavage by proteases, binds to neuropilin-1 (NRP-1) to promote vascular extravasation and tumor penetration. We inserted iRGD in the fiber of an oncolytic adenovirus to increase tumor targeting and penetration. *In vitro*, NRP-1 interaction improved binding and internalization of the virus in different cancer cells over-expressing integrins and NRP-1. *In vivo*, iRGD enhanced tumor transduction, intratumoral dissemination, and antitumor efficacy in several tumor models. iRGD insertion into the Adenovirus 5 fiber C-terminus is useful to improve the therapeutic outcome of oncolytic adenoviruses.

In conclusion, this GFAP biosensor appears suitable for achieving prolonged somatic transgenesis and astrocyte-selective. We now plan to use this construct in an HIE model, which would locate areas of perinatal brain injury and provide regions for delivering therapeutic transgenes.

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Bacterium-based microrobot for visualization of tumor targeting and drug deliveryS-H Park¹, S J Park², D-M Kim¹, J-O Park², S Park², and J-J Min¹¹Department of Nuclear Medicine, Chonnam National University Medical School, Gwangju, 501-746, South Korea; ²School of Mechanical Systems Engineering, Chonnam National University, Gwangju, 500-757, South Korea

Despite recent advances in drug delivery systems (DDS), drug delivery to cancer sites remains difficult because DDS lack active motility and because physical barriers, are encountered in solid tumors. The present study describes a bacterium-based micro-robot (bacteriobot) in which the avirulent *S. typhimurium* is attached to a microstructure enabling the microrobot swim toward tumors; the microstructure acts as a therapeutic molecule containing high amounts of drugs and/or imaging signals and the bacterium acts as a combination of microsensor and micro-actuator. We used a high-motility strain of attenuated *S. typhimurium* that expresses bacterial luciferase (*lux*) or green fluorescent protein (*gfp*). Strong attachment of the bacterium to the microstructure was achieved by exploiting the high-affinity interaction between biotin and streptavidin. The biotin-labeled bacteria were then attached to microstructures (PerCP-Cy5.5). The Bacteribots showed higher average velocity when migrating toward tumor cell lysates or spheroids than when migrating toward normal cell lysates or spheroids. To validate tumor targeting *in vivo*, bacteribots were injected systemically into CT-26 tumor-bearing mice via tail veins. Bioluminescence was detected in the tumors from the bacteria- and bacteriobot-injected animals, but not in the tumors from the microbead-injected control

animals. Subsequently, Cy5.5 fluorescence was observed in tumors from the bacteriobot-injected animals, but not in the tumors from the bacteria- or microbead-injected control animals. The present study suggests that the bacteriobot concept will be of great influence in the development of biomedical theranostic microrobots that can carry on versatile functions such as the detection and eradication of incurable malignancies.

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Cytotoxicity of replication-competent adenoviruses are enhanced by co-transduced p53 geneM Tagawa^{1,2}, S Yang^{1,2}, K Kawamura¹, S Okamoto^{1,4}, S Kubo³, Y Tada⁴, K Hiroshima⁵, and H Shimada⁶¹Division of Pathology and Cell Therapy, Chiba Cancer Center Research Institute; ²Department of Molecular Biology and Oncology, Graduate School of Medicine, Chiba University; ³Department of Genetics, Hyogo College of Medicine; ⁴Department of Respiriology, Graduate School of Medicine, Chiba University; ⁵Department of Pathology, Tokyo Women's Medical University Yachiyo Medical Center; ⁶Department of Surgery, School of Medicine, Toho University

Human tumors are often resistant to type 5 adenoviruses (Ad5)-mediated gene transfer. We examined cytotoxicity of replication-competent Ad5 of which the receptor-binding site was replaced with that of serotype 35 (AdF35) and investigated possible combinatory effects of the AdF35 and Ad5 expressing the wild-type p53 gene (Ad5/p53). We firstly tested AdF35 infectivity to 9 kinds of human esophageal carcinoma with flow cytometry and found that AdF35 infected better than Ad5. We then examined cytotoxicity of replication-competent AdF35 powered by the *midkine* or the *survivin* transcriptional regulatory region. Replication-competent AdF35 in general achieved greater cytotoxic effects to esophageal carcinoma cells with a low expression level of coxsackie adenovirus receptor than the corresponding replication-competent Ad5. Cytotoxicity of the AdF35 was accompanied by cleavage of caspase-8, -9 and -3, but was not linked with autophagy-linked pathways. Cell cycle analyses demonstrated hyperploidy fractions followed by increased sub-G1 populations in the Ad infected cells. Transduction of esophageal cells with Ad5/p53 in combination with replication-competent AdF35 further enhanced the AdF35-mediated cytotoxicity in a synergistic manner. We also demonstrated the combinatory effects of Ad5/p53 and replication-competent AdF35 in an animal model. Co-transduction with Ad5/53 was rather inhibitory to production of replication-competent AdF35 progenies, but the AdF35 infection augmented p53 expression levels and the phosphorylation. These data suggests that combination of replication-competent AdF35 and Ad5/p53 achieved synergistic cytotoxicity due to enhanced p53-mediated apoptotic pathways.

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A new Oct3/4-activated oncolytic adenovirus mediated by hypoxia exert enhanced antitumor activity in bladder cancersJ-L Hsieh¹, P-C Shen¹, A-L Shiau², and C-L Wu³¹Department of Nursing, Chung Hwa University of Medical Technology; ²Department of Orthopedic Surgery, Tainan Hospital, Department of Health, Executive Yuan; ³Department of Microbiology and Immunology, National Cheng Kung University Medical College; ⁴Department of Biochemistry and Molecular Biology, National Cheng Kung University Medical College

Most solid tumors experience hypoxia to lead to rapid cell division, metastasis and cancer stem cells (CSC) formation. The viral replication and antitumor activity of oncolytic adenovirus can be hindered due to the deprivation of oxygen. There is a need to develop more potent virus that could exert the antitumor effect under hypoxia. We previously developed a targeted oncolytic adenovirus, Ad9OC that specifically kill tumor cells over-expressing Oct-3/4. A new hypoxic-activated oncolytic adenovirus, AdLCY, was created by inserting a hypoxia-response element, HIF-2 α , in the upstream of Ad9OC promoter. The expression of HIF-2 α under hypoxia in bladder cancer cell lines was analyzed by Western blotting. CSCs targeting of AdLCY was examined by Flow cytometry and immunohistochemistry. The cytolytic and antitumor activity of AdLCY on bladder cancer cells were evaluated *in vitro* and *in vivo*. HIF-2 α and Oct-3/4 were overexpressed in bladder cancer cells under hypoxia. AdLCY exhibited better cytolytic effects than Ad9OC after hypoxia induction whereas spared normal cells. More CD44 and CD133 positive tumor cells can be targeted by AdLCY than Ad9OC under hypoxia. Efficient replication of AdLCY was detected in bladder cancer cell lines and tumor tissues. Treatment of AdLCY could effectively retard tumor growth in subcutaneously bladder tumor model. Therefore, under hypoxia, AdLCY was more effective for treating Oct-3/4-expressing tumors, especially for targeting CSCs within bladder tumor mass. The newly modified Oct-3/4-activated oncolytic adenovirus provides more potential ways to treat bladder cancers.

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Evaluation of the efficacy of different oncolytic vaccinia viruses on primary canine mammary tumour cells

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Over the last fifteen years, oncolytic viruses have emerged as promising therapeutic agents against cancer due to their selectivity for replication in cancer cells and their limited off-target toxicities. Amongst them, vaccinia viruses (VV) are currently in clinical trial.

Classical research and development experiments, involving *in vitro* testing in human established cell lines as well as *in vivo* xenografts in nude mice, have shown convincingly that oncolytic VV have anti-tumour activities against breast cancer. However, the development of these oncolytic viruses in humans, in the context of breast cancer, is hampered by the competition with other new treatments (small molecules or antibodies).

In this context, in order to emphasise the potential of VV against breast cancer and to strengthen the case for early-phase clinical trials with these oncolytic viruses, we have used primary cells obtained from canine mammary tumours to demonstrate the efficacy of VV against this cancer.

For this purpose, we have benefited from a collaboration with a company, OncoBioTek, that specialises in collecting samples from canine tumours and we have characterised the samples obtained using classical markers used to classify human tumours. For each sample, primary cancer cells have been cultivated, dose-response curves assessing the potency of alternative

VV designs have been established and viral replication has been monitored.

Our data show that different sub-types of breast cancer respond differently to vaccinia viruses and emphasize the relevance of primary canine mammary cancer cells to predict the efficacy of cancer agents.

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Treatment of soft-tissue sarcoma with a combination of chemotherapy and oncolytic adenovirus coding for GM-CSF

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Oncolytic viruses represent a novel approach to the treatment of cancer types that lack effective therapies. Yet, their efficacy alone might not be sufficient to cure cancer. Efficacy can be improved by using them in conjunction with conventional chemotherapy. We have studied the antitumor efficacy and immunological parameters of the capsid-modified and GM-CSF-coding conditionally replicating adenovirus Ad5/3-D24-GMCSF in combination with the chemotherapeutic agents doxorubicin and ifosfamide which are the most common first line option for the treatment of soft-tissue sarcoma (STS). *In vitro* experiments have focused on the immunogenicity of cell death (ie. calreticulin exposure, HMGB1 release, ATP release) induced by adenovirus and drug combinations. Coinfection of the human fibrosarcoma cell line HT-1080 with adenovirus and doxorubicin resulted in increased levels of extracellular HMGB1 and ATP when compared to single agents. Antitumor efficacy of the combination approach was evaluated in a semipermissive and immunocompetent Syrian hamster model for STS. Doxorubicin and ifosfamide administered together with Ad5/3-D24-GMCSF had highly synergistic antitumor activity and significantly prolonged the survival of combination treated animals over mock (p=0.002, Kaplan-Meier log-rank test), virus only (p=0.002) and chemotherapy only (p=0.003) treated animals. Furthermore, increased serum levels of HMGB1 were measured from combination treated hamsters compared to other treatment groups. These results suggest that immune response is an important characteristic of the synergistic antitumor effect produced by the combination of oncolytic virotherapy and conventional chemotherapy.

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AduPARE1A oncolytic adenovirus targets pancreatic cancer stem cells

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Pancreatic adenocarcinoma is usually diagnosed in an advanced state where there are little or no effective therapies. Emerging evidences suggest that the capability of a tumor to

grow and propagate is dependent on a small subset of tumor cells, termed tumor-initiating cells or cancer stem cells. The identification of therapies that can be effective on this cell population might provide with more effective treatments. In the current work we have evaluated the potential of oncolytic adenoviruses to act against pancreatic cancer stem cells (PCSC) and compared to gemcitabine treatment. PCSC from two patient-derived xenograft models were isolated and characterized based on the cell surface marker combination CD24, CD44, ESA and the CD133 marker, plus the capacity of tumor cells to grow as tumorspheres. Mice bearing orthotopic pancreatic tumors were treated with saline, gemcitabine or the oncolytic adenovirus AduPARE1A. Antitumor effects were observed in gemcitabine treated tumors, although stronger responses were observed in mice receiving a single dose of AduPARE1A intravenously. PCSC analysis in treated tumors revealed an enrichment in the PCSC population expressing CD133, and a marked enhancement of tumorspheres formation capacity in the gemcitabine group. In contrast AduPARE1A treatment did not result in an enrichment on tumor spheres neither on stemness markers. These results are in line with the elevated cytotoxicity observed *in vitro* in neoplastic sphere-forming cells exposed to AduPARE1A infection, indicating the susceptibility of PCSC to adenoviral-mediated killing. Therefore, our data provides evidences of the relevance of uPAR-controlled oncolytic adenoviruses for the elimination of PCSC.

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Development of replication-competent adenovirus for gemcitabine-resistant bladder cancer

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Background: Gemcitabine is a commonly-used chemotherapeutic agent for bladder cancer patient, but many of them experiences relapse after treatment. Survivin is a member of inhibitors of apoptosis protein (IAP) family. For the development of new therapeutic agent for gemcitabine-resistant patient, we constructed the recombinant adenovirus of which replication was under the control of survivin promoter by replacing adenoviral E1a and E4 promoters.

Materials and Methods: First of all, we established gemcitabine-resistant bladder cancer cell. Survivin promoter was engineered to control adenoviral early genes *E1a* and *E4*. The adenovirus-mediated killing efficacies in gemcitabine-resistant bladder cancer cell were evaluated in comparison with surviving-negative L132 cell as a negative control. Finally, we tested whether AdE1a/psurvivinE4 would suppress the tumor growth using gemcitabine-resistant Ku19-19/Luc xenograft models subcutaneously induced in nude mice.

Results: As reported previously, gemcitabine-resistant and -sensitive cells turned out positive for survivin expression. Such expression pattern implicated the regulatory element mediating survivin protein expression could be utilized for discriminating cancer from normal cells. As expected from abundant survivin expression in gemcitabine-resistant bladder cancer cell, AdE1a/psurvivinE4 actively replicated in gemcitabine-resistant bladder

cancer cell as green fluorescence intensity gradually increased with more days post infection. Consistent with replication ability, Ad5/35E1a/psurvivinE4 exerted the greater cytotoxic effect on all bladder cancer cells tested, but not in L132 cells. Importantly, Ad5/35E1a/psurvivinE4 inhibited the growth of gemcitabine-resistant Ku19-19/Luc orthotopic xenograft in nude mice.

Conclusion: AdE1a/psurvivinE4 implicates that survivin promoter could be utilized for developing the replication-competent adenovirus to target gemcitabine-resistant bladder cancers.

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Exploring the use of new oncolytic adenoviruses for the treatment of head and neck squamous cell carcinomas in Fanconi Anemia patients

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Patients with Fanconi Anemia (FA) are at high risk for developing hematological and solid cancers. Squamous cell carcinomas of the head and the neck (HNSCCs) are the most commonly diagnosed solid tumors in FA patients. Surgery is not an option for multifocal lesions and chemo and radiotherapies are highly toxic for these patients, so new therapeutic approaches are needed. We have explored the feasibility of using virotherapy for the treatment of HNSCCs in FA patients. ICOVIR17 and ICOVIR17K are 2 oncolytic adenoviruses with the RGD domain (higher expression for the K version), and with replication restricted to cells with an activated RB pathway, a hallmark of cancer cells. For this study we used the following cell lines: VU1131 (FA-C derived HNSCC), VU1365 (FA-A derived HNSCC), HN6 and HN12 (non FA derived HNSCC), and HN12-FANCA^{-/-}. To determine the susceptibility to ICO17 and ICO17K, we infected cells at different MOIs (0–500) and cell viability was studied by flow cytometry and by crystal violet staining at different timepoints. We observed a mild susceptibility of FA-HNSCC to ICOVIR oncolysis *in vitro*, while non-FA cell lines presented mortality even at low MOIs. To explain these differences, we compared the permissiveness to virus infection and the levels of E2F1 expression of FA-HNSCC versus FA-HNSCC. We also analyzed the effects of virotherapy on cancer-initiating cells (C-IC), and observed a high susceptibility of C-IC from FA-HNSCC obtaining 50% died (7AAD annex) cells at MOI 50. We suggest that oncolytic adenoviruses may be an alternative therapy in FA patients with solid tumors, with especial tropism to C-IC.

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Targeted gene therapy of colon cancer using *Clostridium perfringens* enterotoxin (CPE)

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For the treatment of therapy-refractory tumors use of bacterial toxins represents an applicable option. The most extensively used toxins are Diphtheria toxin (*Corynebacterium diphtheriae*) and Pseudomonas exotoxin A (*Pseudomonas aeruginosa*), which both inhibit protein synthesis. Alternatively, the pore-forming bacterial toxins streptolysin O (*Streptococcus pyrogenes*) and *Clostridium perfringens* enterotoxin (CPE) came into focus as alternative cancer therapeutics.

The CPE, produced by *Clostridium perfringens* bacterial type A strain is an enterotoxin, that binds to claudin-3 and -4 tight junction transmembrane proteins, which were shown to be highly upregulated in several human epithelial cancers, including colon, breast, ovarian, pancreatic carcinoma. Binding of CPE to claudin-3 and -4 initiates formation of multiprotein membrane pore complex, resulting in rapid cell lysis. In our approach we used the CPE cDNA expressing vector for targeted gene therapy of claudin-3 and -4 overexpressing human colon cancer cell lines and for patient-derived tumor models (PDX) *in vitro*. Transfection of the CPE-expressing vector caused rapid cytotoxic effects by membrane disruption in all claudin-3 and -4 overexpressing cell lines, but did not affect claudin-negative control cells. The CPE-mediated cytotoxic effect correlated well with claudin-3 and/or -4 expression level in the colon carcinoma cells. Furthermore, the CPE-gene transfer was also associated with a "bystander effect", potentiating the CPE-mediated cytotoxicity. Therefore, this novel approach demonstrates, that CPE gene transfer can be employed for a targeted suicide gene therapy of claudin-3 and -4 overexpressing colon carcinomas, leading to the rapid and efficient tumor cell killing.

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Combination of temozolomide chemotherapeutic drug and promoter of the glucose-regulated protein 78 improves bacteriophage-mediated cancer gene therapy

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Glioblastoma multiform (GBM) is the most common and deadly primary brain cancer in adults, affecting ~4500 patients every year in the United Kingdom alone. Despite continuous improvement of conventional treatments, patients have a median survival of about 15 months even after aggressive surgery, chemotherapy and radiotherapy. Novel therapeutic approaches are therefore urgently needed. Combination of chemotherapy and gene therapy is a promising practice in cancer therapy. Temozolomide (TMZ) is a DNA-methylating chemotherapy drug used as a standard treatment for glioblastoma multiform. It has been shown that treatment of tumour cells with TMZ induces expression of the glucose-regulated protein 78 (Grp78) gene which is associated with glioblastoma chemoresistance to this drug. In this study, we aimed at combining temozolomide with cancer gene therapy driven by the Grp78 promoter by using a novel tumor-targeted bacteriophage vector able to target therapeutic gene expression in tumors specifically.

Our findings showed, dose-dependent chemotherapeutic drug induction of gene expression from bacteriophage in glioblastoma cells. Subsequently, we found dramatic increase of tumor cell killing when temozolomide was combined with bacteriophage expressing a cytotoxic gene under the control of Grp78 promoter.

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Systemic epidermal growth factor receptor-targeted sodium iodide symporter (NIS) gene therapy in a genetically engineered mouse model of pancreatic ductal adenocarcinoma

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A genetically engineered mouse model (GEMM) of pancreatic ductal adenocarcinoma (PDAC) is induced by activation of constitutively active Kras^{G12D} in combination with a deletion of p53, which shows the typical changes of human disease.

In previous proof-of-principle studies using xenograft mouse models the sodium iodide symporter (NIS) as well characterized theranostic gene allowed detailed molecular imaging of transgene expression and highly effective application of therapeutic radionuclides. As a next step towards clinical application, here we investigated tumor specificity and transduction efficiency of tumor-targeted polyplexes as systemic NIS gene delivery vehicles in the advanced GEMM of PDAC. Therefore, we used tumor-targeted polyplexes based on linear polyethylenimine (LPEI), polyethylene glycol (PEG), and the synthetic peptide GE11 as an epidermal growth factor receptor (EGFR)-specific ligand (LPEI-PEG-GE11) to target a NIS-expressing plasmid to the high EGFR-expressing PDAC.

In vitro iodide uptake studies with cell explants derived from murine EGFR-positive and EGFR-knockout PDAC lesions demonstrated high transduction efficiency and EGFR-specificity of LPEI-PEG-GE11/NIS. *In vivo* ¹²³I γ -camera-imaging and 3-dimensional high-resolution ¹²⁴I-PET-imaging experiments showed significant tumor-specific accumulation of radioiodine. These results were further confirmed by NIS-specific qPCR analysis and immunohistochemistry. A first series of therapy studies indicates that the tumoral accumulation is high enough for a dramatic therapeutic effect of ¹³¹I as demonstrated by significant reduction of tumor volume measured by magnetic resonance imaging.

In conclusion, our preclinical data in an advanced GEMM of PDAC clearly demonstrate the enormous potential of EGFR-targeted synthetic polymers for systemic NIS gene delivery allowing for targeted radionuclide therapy of non-thyroidal cancers.

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Enhancing hybrid bacteriophage-mediated gene therapy for cancer treatment through extracellular matrix modulation

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The adeno-associated virus/phage (AAVP) is a novel systemic gene delivery vector for use in cancer gene therapy. It is a hybrid of a mammalian AAV transgene cassette contained within an fd filamentous bacteriophage capsid. Its effectiveness in targeting

the delivery of suicide transgenes to cancer cells specifically, both in vitro and in vivo, has been shown in numerous studies. Although it is superior in safety and economy of production compared to mammalian viruses, due to non-mammalian cell tropism, it has still to catch up on benchmarks of successful transduction rates. However, many physiological barriers hinder such efforts. We hypothesized that the extracellular matrix (ECM) presents an obstacle for AAVP transduction in applications of phage cancer therapy. Our findings demonstrate that collagenase and hyaluronidase-mediated degradation of the ECM significantly increases AAVP transduction efficiency up to 6.7-fold. We show that this enhanced efficacy can be translated into tangible cell-killing, eliminating up to 98% of tumour cells through suicide gene therapy, from the transduction of the herpes simplex virus-thymidine kinase and co-application of ganciclovir. Moreover, we proved that ECM modulation is relevant in clinically applicable settings by using 3D multicellular tumour spheroids, simulating in vivo environments more accurately. In addition, we substantiated an alternative non-enzymatic method to clear the ECM collagen component with losartan, a clinically approved drug, and subsequently observed significant improvements of up to 2.4-fold in transduction rates. This combination therapy of AAVP-mediated anticancer treatment and ECM depletion approach represents a powerful strategy that could advance targeted bacteriophage-mediated cancer gene therapy.

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Increased miR135a induced by Helicobacter pylori suppresses cancer cell invasion and migration through down-regulation of ROCK1 in gastric cancer

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Introduction MicroRNAs are known to play a role in the development and progression of gastric cancer. Several studies reported that *Helicobacter pylori* (*H. pylori*) status is associated with good prognostic factor in gastric cancer patients. However, its mechanism is unknown. We investigated the role of *H. pylori*, miR-135a and its potential target, ROCK1 in gastric cancer metastasis. Methods The expression of miRNAs in gastric cancer by *H. pylori* (G27 and ATCC43504) infection was analyzed by microarray and qRT-PCR. Mimic-miR-135a and anti-miR-135a were transfected into gastric cancer cells. Luciferase assay with point mutation, cell migration and invasion assays were performed. The expression of miR-135a and ROCK1 were detected in 160 early gastric cancers and their corresponding non-tumorous tissues by qRT-PCR and western blot. Results *H. pylori* infection resulted in increased expression of miR-135a and decreased expression of ROCK1, and suppressed cell migration and invasion abilities. Mimic miR-135a treatment reduced the protein level of ROCK1, and decreased cell migration and invasion in gastric cancer cells and anti-miR-135a vice versa. miR-135a directly bound to the 3'UTR of ROCK1. An inverse correlation was found between the expressions of miR-135a and ROCK1 in gastric cancer patient tissues ($r = -0.789$, $P < 0.001$). Downregulated miR-135a and increased ROCK1 protein expression were associated with numbers of lymph node metastasis. Conclusion miR-135a functions as a lymph node metastasis suppressor in gastric cancer. *H. pylori* may act as a migration inhibitor by upregulating miR-135a through down-regulation of ROCK1 expression in gastric cancer cells.

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Advanced proteomic and transcriptomic analyses to identify diagnostic biomarkers predictive and/or indicative of response to gene therapy treatment in patients with pancreatic cancer

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We have recently completed the first phase I gene therapy trial for patients diagnosed with advanced and metastatic pancreatic cancer using non-viral vectors to deliver chemo-sensitizing genes. We found that the intra-tumoral injection of the gene therapy product was safe and feasible and resulted in the presence and the expression of the therapeutic genes in the tumors. Consequently, tumor progression was inhibited and we speculate that patients with locally advanced PDAC at the time of diagnosis may clinically benefit from this approach. The identification of any biomarkers that helps refine our therapeutic decision making would be immensely helpful and represents a worthy goal. If validated, getting such a test into routine clinical practice for patients with locally advanced PDAC entering anticancer gene therapy clinical trial would still, undoubtedly, represent a formidable challenge, but one very much worth tackling. Consequently, we carried out several advanced proteomic analyses to identify diagnostic biomarkers predictive and/or indicative of treatment response and follow-up, to select clinical trial participants and/or to tailor therapy for individual patient. At present, it is unlikely that a single class of biomarkers will be sufficient to reach our objective. Consequently, we also scrutinized other class of clinical biomarkers such as circulating miRNAs that have the potential to revolutionize diagnosis and treatment of pancreatic cancer. We already identified several biomarkers of clinical interest using either nanoHPLC-Chip-MS/MS (nanoHPLC-microfluidic chip interfaced to mass spectrometry) or high throughput q(RT)PCR for peptide and miRNA identification, respectively. The data collected herein that will be presented during this meeting will offer the great opportunity to identify patient subpopulations with differential response to the treatment to create a clear prescription path for gene therapy in forthcoming clinical trials.

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Molecular and cellular mechanism of tumor dissemination in the peritoneal cavity

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Peritonitis carcinomatosa is an advanced and intractable state of gastrointestinal and ovarian cancers. Because this pathology remains highly resistant to the current standard therapeutics and shows high mortality ratio, its underlying mechanism requires elucidation. Although organ distribution of peritoneal dissemination seems not to be completely random, the mechanism of homing of cancer cells to the dissemination favor sites has been largely unknown.

Here we demonstrate the molecular and cellular mechanism of tumor dissemination. Surprisingly, we first identified hematopoietic progenitors and stem-like cells in the peritoneal cavity lavage fluid (PCLF), and these cells existed on SCF /CXCL12 cell niches at the border area of mesenteric perivascular adipose tissue. Of

interest, PLCF dramatically facilitated the process of the spheroid formation of CT26 cancer cells with mediation by peritoneal collagen type IV and plasma fibronectin, and spheres large enough to be grossly visible within several hours and were promptly directed to SCF/CXCL12 cell niches in a CXCR4-dependent manner. Importantly, spheroidal expression of CXCR4 was induced by sphere-formation itself via the transcription factor Sp1. These results are the first demonstration of the overall process of directional tumor dissemination, and provide clues to the metastatic processes of malignancies that could assist in treatment development.

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The overexpression of a RUNX1 truncated protein generated by a novel t(1;21)(p32;q22) chromosomal translocation impairs the proliferation and differentiation program of human hematopoietic progenitors

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RUNX1 gene chromosomal translocations and mutations are frequent in acute myeloid leukaemia (AML), many times resulting in the aberrant expression of C-terminal-truncated *RUNX1* proteins lacking the transactivation domain (TAD). Interestingly, some AML-M2 patients anomalously over-express a *RUNX1a* splice variant with the same TAD deficit. We performed an in-depth *in vitro* study of the role of TAD-defective *RUNX1* proteins in AML development in human hematopoietic/progenitor stem cells. Transformation ability, maturation phenotype and differentiation/proliferation effects were assessed. Short *RUNX1* protein expression seemed to increase both proliferation and self-renewal ability, disrupting the differentiation program and interfering with wild-type *RUNX1b* protein, in a dominant-negative, dose-dependent manner. Expression microarray analysis showed that short *RUNX1* and *RUNX1/ETO* models had similar aberrant expression patterns. Genes known to be broadly involved in self-renewal and leukemogenesis were found to be altered; specifically observed were overexpression of homeobox genes, downregulation of primitive erythroid genes and alterations in leukemogenic transcription factors. The Wnt/ β -catenin and RhoA pathways were also observed altered, whereas short *RUNX1* proteins appeared not to affect the Notch1 pathway, which is altered in the *RUNX1/ETO* model. Therefore, we propose that TAD-defective *RUNX1* proteins may contribute to leukemogenesis and could form the basis of a new genetic category of AML.

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Nucleus-targeted maspin as genetic drug for gene therapy of breast cancer

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Maspin (Mammary Serine Protease Inhibitor) is classified as class II tumor suppressor. Maspin demonstrates antiapoptotic, antimetastatic and antiangiogenic properties but its expression is downregulated in malignant breast cancer and in most breast

cancer cell lines. Maspin subcellular localization in cancer may be cytoplasmic, nuclear or mixed. Our recent study on archived breast cancer specimens from patients indicated positive correlation between the level of maspin in cell nuclei and survivability of patients and negative correlation with Ki-67 protein expression in breast cancer tissue.

In order to evaluate the use of nucleus – targeted maspin for breast cancer gene therapy we studied the effect of maspin localization and expression level on proliferation of breast cancer cells. Three breast cancer cell lines: MCF-7, MDA-MB-231, SKBR-3, and normal epithelial cell line from breast - MCF10A, were transfected with plasmid encoding maspin – EGFP (fusion protein locates in cytoplasm) and maspin-NLS-EGFP (fusion protein with nuclear localization signal locates in nucleus), and Ki-67 protein expression was evaluated. We observed the inhibitory effect of maspin located in nucleus on cell proliferation in breast cancer cell lines. A statistically significant correlation was found between nuclear maspin and loss of Ki-67 protein in breast cancer cell lines. There was no significant effect of nuclear maspin on cell proliferation in MCF10A cells. The anti-proliferative effect of nuclear maspin on breast cancer cells was also statistically significant in comparison to cytoplasmic maspin. Thus the use of exogenous nucleus - targeted maspin may have a strong potential in gene therapy against breast cancer.

P355

BCR-JAK2 gene fusion drives a myeloproliferative neoplasm in transplanted mice

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Chromosomal translocations in tumors frequently produce fusion genes encoding for chimeric proteins with a key role in oncogenesis. Recent reports described the presence of *BCR-JAK2* fusion gene in patients with atypic chronic or acute myeloid leukemia, however its role in leukemogenesis has not been demonstrated. *BCR-JAK2* encodes for a protein containing the BCR oligomerization domain fused to the JAK2 tyrosine-kinase domain. We have recently described the functional behavior of this chimeric protein, consisting of a constitutively phosphorylated IL-3-independent tyrosine-kinase located at the cytoplasm and signaling downstream via STAT5, thus eliciting the expression of genes related to proliferation and survival such as *Osm*, *Socs2* and *Bcl-XL*. The expression of *BCR-JAK2* in mouse hematopoietic progenitors (*Lin*⁻) increased the *in vitro* proliferation and survival capacity of these cells. We also demonstrated the ability of a highly selective JAK2 inhibitor, TG101209, to abrogate this signaling and trigger apoptosis in *BCR-JAK2* expressing cells. *BCR-JAK2* expressing Ba/F3 cells were also proved to induce tumorigenesis in an immunodeficient mouse model. Finally, *in vivo* experiments in which *BCR-JAK2* *Lin*⁻ expressing cells were transplanted into lethally irradiated mice lead to a myeloproliferative syndrome characterized by various malignant features including splenomegaly, leukocytosis and presence of immature cells in peripheral blood. Immunophenotypic analysis also revealed expansion of the myeloid lineage. Some mice showed dyserythropoiesis with

defective development of erythrocytes and anisocytosis. Integration of the retroviral vector as well as BCR-JAK2 expression were confirmed in different hematopoietic tissues. Taken together these findings support the oncogenic properties of BCR-JAK2 both *in vitro* and *in vivo*, and suggest that JAK2 inhibitors should be a good therapeutic approach for these patients.

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Off-tumor expression of the target antigen does not predict CAR-T cell killing: a foundation for the safety of CD44v6-targeted T cells

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Introduction: Off-tumor expression of the target antigen raises justified safety concerns about newly designed chimeric-antigen receptors (CARs). We have recently developed a CAR targeting CD44v6 and demonstrated potent antitumor effects against acute myeloid leukemia and multiple myeloma. Since CD44v6 immunotoxins have caused severe adverse events, it is crucial to pre-clinically evaluate the potential off-tumor toxicities of CD44v6-targeted T cells.

Aim: To profile the off-tumor expression of CD44v6 and to verify the susceptibility of expressing cells to CAR-T cell killing.

Results: Quantitative PCR analysis on a wide panel of cDNA from normal tissues revealed low-level CD44v6 expression only on flat stratified epithelia, e.g. skin, oral mucosa. Of the different cells of the hematopoietic system analyzed, including CD34 hematopoietic stem cells (HSCs), only circulating CD14 monocytes expressed CD44v6. Interestingly, tissue-resident monocyte-derived cells analyzed by immunohistochemistry, e.g. lymph-node macrophages, brain microglia and liver Kupffer cells, were completely CD44v6-negative. In co-culture experiments, at the E:T ratios that result in the complete elimination of tumor cells and monocytes by CD44v6-targeted T cells, keratinocytes were strikingly completely spared. Interestingly, CD44v6-targeted T cells also failed to produce cytokines in response to keratinocyte, suggesting non-productive CAR-mediated recognition. Comparative analysis of accessory molecules, showed that, differently from tumor cells and monocytes, keratinocytes expressed significant lower levels of adhesion/costimulatory molecules (ICAM-1, LFA-3, CD86), but higher levels of the critical check-point molecule PD-L1.

Conclusions: Differently from CD44v6 immunotoxins, therapeutic doses of CD44v6-targeted T cells may be at low risk for causing skin toxicity due to lack of costimulation/PD-L1 expression on keratinocytes.

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Development and preclinical evaluation of a lentiviral based anti-HIV therapeutic vaccine

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Theravectys, spin-off the Pasteur Institute, develops a new generation of prophylactic and therapeutic vaccines using

optimized lentiviral vectors. It's most advanced product, a therapeutic anti-HIV vaccine treatment, has entered clinical Phase I/II. This vaccination should allow seropositive patients to gain an immunological status identical to the so-called "Functional Cured" patients who develop an efficient immunological response capable of controlling the infection without therapy.

Vaccine candidates are integrative and self-inactivated live-recombinant lentiviral vectors. They encode an HIV antigen, under the regulation of a patented promoter that is preferentially induced in APC (generating of a strong, specific and long lasting T-cell immune response), and showing a basal level expression in all cells (allowing their elimination by the settled immune response). Furthermore, Theravectys developed a vaccination regimen based on iterative immunizations with lentivectors encoding the same HIV transgene, relying on different VSV-G serotypes for pseudotyping without generating cross-neutralizing antibodies. These vaccine candidates are classified as "Live recombinant vectored vaccines" (EMA, 2011).

Theravectys set up an innovative manufacturing process combining high production yields, impurity profiles compatible with direct injections into humans and high immunogenicity. Pilot and GMP batches have been manufactured and GLP pre-clinical studies (amongst which biodistribution, shedding and toxicity) performed, that showed the restricted diffusion of the vaccine candidates after injection and their fast disappearance within few weeks, correlated with an absence of macroscopic and microscopic toxicity.

These data allowed the settlement of an anti-HIV therapeutic Phase I/II clinical trial that has received the authorizations of the French and Belgium regulatory agencies in 2012. This trial will be held in France and Belgium and is enrolling of 36 HIV-1 infected patients. Theravectys' anti-HIV vaccine treatment will be assessed at three doses and safety, tolerability and immunogenicity compared to a placebo group. Results are expected by 2014 with intermediary analyses in November 2013.

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Serotype 5/3 chimeric oncolytic adenovirus coding for GM-CSF for treatment of melanoma: results *in vitro*, in rodents and humans

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Metastatic melanoma can be highly aggressive and refractory to irradiation and chemotherapy. Consequently, approaches such as ipilimumab and vemurafenib have gained attention recently. Malignant melanoma patients could benefit from oncolytic immunotherapy, since melanoma is classically an

“immunogenic tumor” and oncolytic virus replication is an immunogenic phenomenon.

This study evaluated the effect of the oncolytic adenovirus Ad5/3-D24-GMCSF (CGTG-102) on a panel of melanoma cell lines and animal models, and summarized the available melanoma specific human data from the Advanced Therapy Access Program (ATAP), in preparation for further clinical development.

Efficacy was seen in all the human melanoma cell lines analyzed. Furthermore, due to the chimeric capsid, coxsackie-adenovirus receptor (CAR) deficiency of primary melanoma cells such as low-passage pMelL, that renders them resistant to viruses with Ad5wt capsids, is overcome.

Ad5/3-D24-GMCSF displayed effective antitumor activity in nude mice bearing subcutaneous SK-MEL-28 melanoma xenografts, particularly when combined with low-dose cyclophosphamide. A total of 9 patients with treatment refractory melanoma were treated in the ATAP. The treatment appeared safe and well-tolerated. In 4 patients, the radiological benefit was evaluated by RECIST, detecting minor response in one case, two cases of stable disease and one of progressive disease. All patients were progressing and refractory to other forms of therapy prior to treatment with Ad5/3-D24-GMCSF. Interestingly, 2 patients are still alive at 1501 and 559 days after virus treatment.

Thus, Ad5/3-D24-GMCSF appears to be promising for treatment of melanoma. Further clinical testing is important to support and expand the data in more uniform patient populations. Additional studies are currently ongoing to investigate the immunostimulatory effects of GM-CSF in this context.

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Adeno-associated virus vectors expressing IFN- β induction pathway activating elements as alternative antiviral treatment

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Detection of pathogens by cells is a key event of defense against infections. RIG-I like receptors (RLRs) detect specific RNAs produced by virus replication and activate a signaling cascade that results in the production of interferon beta (IFN- β) as well as several other antiviral and proinflammatory cytokines. Conventional type I IFN treatments are based on administration of recombinant purified protein or administration of different vectors that can produce type I IFN. Despite its proven antiviral and antitumoral effects, many individuals do not respond to administration of such type I IFNs. This is particularly relevant in the treatment of patients with chronic hepatic infections and those with certain type of tumors. We hypothesize that triggering of RLR pathways instead of direct IFN administration is a valid alternative for the induction of antiviral, antiproliferative and proinflammatory genes. We have developed adeno-associated virus (AAV) vectors expressing different elements of the RLR dependent pathway. We have observed an efficient IFN- β induction by the constructs generated in a broad spectrum of cells from different species. Those vectors have been tested for their ability to induce IFN- β , creating an antiviral state in different *in vitro* and *in vivo* models and to synergize with the host immune system to combat viral infections. We propose the use of our strategy as an alternative of malignancies currently treated with

recombinant type I IFN as well as those that are refractory to such type I IFN treatment like some IFN-treated resistant chronic viral infections.

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Immunomodulation and anti-cancer activity of REIC/Dkk-3 protein

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The REIC/Dkk-3 gene has been reported to be a tumor suppressor gene and the expression is significantly downregulated in a broad range of cancer cell types. Our previous study has revealed a cytokine-like role of REIC/Dkk-3 protein in monocyte differentiation to dendritic cells and it is likely that REIC/Dkk-3 possesses anti-cancer immunomodulatory function. We herein investigated the anti-tumor effects and immunological aspects of recombinant REIC/Dkk-3 protein in an orthotopic murine renal cancer (RENCA) model with pre-established lung metastases. As a result, significant inhibition of tumor growth was observed in the high-dose (100 microgram i.p. \times 13 times) treatment group in comparison to the PBS and low-dose (10 microgram i.p. \times 13) treated groups. In the flow cytometric analysis, the percentage of dendritic cells (DC), active cytotoxic T lymphocytes (CTL) in the blood of high-dose group was significantly increased in comparison to those in PBS and low-dose treatment groups. Interestingly, the percentage of myeloid derived suppressor cells (MDSC) in the high-dose group was significantly down-regulated in comparison to that in PBS group. These results indicate that recombinant REIC/Dkk-3 protein serves as novel protein therapeutics of cancer, activating systemic anti-cancer immunity. Currently, a clinical trial for prostate cancer using adenoviral vector encoding REIC/Dkk-3 gene is on-going in our institution and some definite therapeutic effects have been observed. As well as the utility of REIC/Dkk-3 based gene therapy, the protein therapy with recombinant REIC/Dkk-3 protein could be useful in the treatment of some types of cancer diseases.

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AAV/RNAi-mediated improvement of anti-malaria whole-organism vaccination strategies

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Malaria, caused by *Plasmodium* parasites, remains one of the most prevalent infectious diseases due to the lack of an efficient and safe vaccine. In mice, genetically-attenuated parasites (GAP) that arrest in the liver have been shown to induce sterile immunity upon triple administration. To better understand the host

factors controlling the *Plasmodium* liver stage, we profiled hepatic miRNAs in mice after inoculation with wildtype (wt) or GAP sporozoites. Strikingly, microarray and qRT-PCR data revealed a dysregulation of 31 miRNAs. The strongest upregulation was noted for miR-155, a miRNA known to exert critical functions in mammalian immunity. Using AAV8 vectors, we could artificially inhibit or over-express host miR155 *in vivo* to study its effect on GAP-mediated protective immunity. Interestingly, treating mice with a miR-155 sponge vector reduced GAP-induced immunity. Vice versa, we found that GAP vaccination capacity could be significantly improved by miR-155 over-expression, as in these mice single GAP priming already sufficed to confer complete protection against wt challenge. To dissect the underlying mechanisms of this protection, we investigated miR-155 targets with negative regulatory functions in immunity. Remarkably we found that *in vivo* suppression of these targets closely recapitulated the results of miR-155 over-expression. Our work provides the first *in vivo* evidence for a substantial involvement of the mammalian miRNA/RNAi machinery in the *Plasmodium* liver stage. The use of well established AAV vectors to regulate miRNA or target gene expression is a new approach to improve the protective capacity of attenuated parasites in an anti-malarial vaccination regime.

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Short-term intratumoral IL-12 expressed from an alphaviral vector is sufficient to induce an efficient antitumoral response against spontaneous hepatocellular carcinomas

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Interleukin-12 (IL-12) is an immuno-stimulatory cytokine that has shown strong antitumor effects in animal models of liver cancer. In order to overcome the severe toxicity associated with its systemic administration, we have previously assayed strategies based on IL-12 gene transfer to tumor cells or to the surrounding liver tissue. Promising results were obtained with both a recombinant Semliki Forest virus (SFV) vector expressing high levels of IL-12 (SFV-IL-12) after intratumoral injection, and with a plasmid vector (pTonL2(T)-mIL12) that allows liver-specific and inducible IL-12 expression. The aim of the present study was to compare the antitumor responses induced by both systems in a clinically relevant animal model of hepatocellular carcinoma (HCC) developed in *L-PK/c-myc* transgenic mice. These animals overexpress *c-myc* oncogene in the liver, giving rise to spontaneous hepatic tumors with latency, histopathology, and genetic characteristics similar to human HCCs. We observed that intratumoral inoculation of SFV-IL-12 induced growth arrest in most tumors providing 100% survival rate, in contrast to no survival in control animals. Similar results were obtained with hydrodynamic injection of pTonL2(T)-mIL12 and long-term induction of IL-12 expression in the liver. However, tumor arrest was less evident in plasmid-treated mice and the survival rate was slightly lower, despite higher and more sustained levels of IL-12 and IFN-gamma in serum. The fact that SFV-IL-12 was able to induce apoptosis of tumor cells and a type-I IFN response specifically in the tumor could explain why short-term IL-12 expression from this vector was sufficient to mediate an antitu-

moral response as strong as that induced by long-term IL-12 expression provided by pTonL2(T)-mIL12. Since SFV-IL-12 could reduce the possible toxicity associated to long-term IL-12 expression we believe that this vector could have potential application for HCC gene therapy.

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Enhanced proliferation and tyrosine kinase pathway engagement in 4-1BB domain-containing CAR T cells

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Chimeric antigen receptor (CAR)-expressing T cells incorporating a 4-1BB signaling domain in the CAR have impressive efficacy in patients with B cell leukemia. We have compared a third generation (3G) CAR including CD3-zeta, CD28 and 4-1BB signaling domains with an otherwise identical CAR that lacks 4-1BB (2nd generation CAR; 2G). CAR T cells targeting CD19 were generated from healthy donors and patients with chronic lymphocytic leukemia by retroviral transduction. CAR expression, cytotoxicity and phenotype were unaffected by incorporation of 4-1BB. T cells made from healthy individuals reacted equivalently to CD19 target cells with proliferation and reduced tumor growth in mice equally well. However, when the 3G CAR T cells were derived from leukemic patients they showed better proliferation than 2G CAR T cells. To compare the activation status of intracellular signaling pathways in 3G and 2G CARs, the CAR T cells were stimulated with CD19 targets and analyzed with a tyrosine kinase array (PamGene). 3G CAR T cells had less activation of tyrosine kinases at 1 hrs than 2G cells. Since both types of CAR T cells were normalized at 3 hrs post stimulation, the difference at 1 hrs may represent a faster reset of the tyrosine kinase pathways in the 3G cells. Signaling molecules, including ZAP70, LAT and Jak1, were phosphorylated in both groups while LCK, and Jak2 were more active in 2G. Conversely, calmodulin was phosphorylated in 3G. Taken together, these data show that 4-1BB signaling gives a proliferative benefit as well as a modulated tyrosine kinase response in T cells from leukemia patients.

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Development of novel chimeric antigen receptors containing intracellular signaling domain of GITR

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Adoptive immunotherapy using Chimeric Antigen Receptor (CAR) gene modified T cells, which can recognize antigen in HLA independent manner, is a promising strategy to treat cancer

and autoimmune diseases. First-generation CARs consist of the intracellular domain (ICD) of CD3z, while second-generation CARs have an additional ICD from various co-stimulatory protein receptors (e.g. CD28, 41BB, ICOS) to enhance effector functions and prolong *in vivo* survival of CAR expressing T cells.

Glucocorticoid-induced TNF-receptor (GITR), a member of the TNF receptor superfamily, is known to inhibit the suppressive activity of regulatory T cells and extend the survival of T-lymphocytes. In order to extend T cell survival and inhibit the suppressive activity of regulatory T cells, we have developed our novel second and third generation CARs containing GITR ICD. We found that the position of GITR ICD in the signaling domains was crucial for proper expression of CARs, as our second generation CARs having a signaling domain "CD3z-GITR" were efficiently expressed, while expression of CARs with "GITR-CD3z" was extremely low. With a comprehensive evaluation of the CAR constructs, we have optimized the construction of CAR, and our novel second and third generation CARs containing GITR ICD were highly expressed with antigen specific cytokine secretion *in vitro*. And we have examined the persistence and tumor suppression activities of the second and third generation CAR's gene modified T cells in tumor-bearing NOD/SCID (non-obese diabetic/severe combined immune-deficient) mice. We believe our novel CARs containing GITR ICD may be promising for CAR gene therapy.

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Targeting tumor HLA expression to increase the immunogenicity of cancer cells

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Cell surface expression of HLA class I molecules (heavy chain with beta2-microglobulin) in tumor cells is required for the presentation of tumor-associated peptides to cytotoxic T cells. Hence, the elimination of tumor cells by the immune system and the success of cancer immunotherapy depend on the proper expression of HLA class I molecules. However, frequently, tumor cells lose HLA class I expression and escape immune recognition leading to dissemination of HLA-negative tumor cell variants with underlying structural genetic defects. We have previously demonstrated that generation of progressing post-immunotherapy metastases in melanoma patients or local tumor recurrence in bladder carcinoma patients after Bacillus Calmette-Guerin (BCG) therapy correlates with alterations in tumor HLA class I expression. More profound structural alterations in HLA class I expression were found in post-BCG recurrent tumors than in pre-BCG lesions. These results suggest that immunotherapy stimulates elimination of HLA-positive tumor cells and immune selection of HLA-negative cells with underlying genetic alterations in HLA genes. Therefore, the concept of recovering the

tumor HLA class I expression using gene therapy has emerged as an attractive new strategy. In particular, this approach could be successfully applied to increase the efficacy of BCG immunotherapy in bladder cancer, since it involves local treatment in a form of intravesicular instillations. In addition, selection of an appropriate vector, as well as *in vitro* bioassays characterizing vector transduction efficiency are becoming increasingly important. Here we present pre-clinical data demonstrating the efficacy of adenoviral vector coding for b2m protein and adeno-associated vectors to restore tumor HLA class I expression in various types of malignancy with the focus on bladder carcinoma. We believe that upregulation of tumor HLA class I expression by gene therapy in combination with immunotherapy and chemotherapy, is a promising approach in fighting cancer.

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Assessing the anti-tumoral and immunological responses to oncolytic vaccinia virus armed with CD40-ligand

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Oncolytic vaccinia viruses have many appealing characteristics as cancer therapeutics. Furthermore, arming the virus with immunomodulatory proteins can activate the immune system at the tumor site. CD40 ligand can suppress tumor cell proliferation, induce apoptosis of tumor cells and triggers several immune mechanisms. CD40 is expressed on CD4 T-cells, macrophages and dendritic cells and binds to the CD40 receptor on the membrane of antigen-presenting cells. Interactions between CD40L and its receptor CD40 provide critical co-stimulatory signals that trigger T-lymphocyte expansion and increase IL-12 production which is required for the engagement of cytotoxic T lymphocytes in the anti-tumor immune response. Therefore, we constructed a double-deleted oncolytic vaccinia virus expressing human soluble CD40L (vvdd-hCD40L). We also sought to assess the induction of immune responses in a immunocompetent syngeneic mouse model. For this purpose we also constructed vvdd-mCD40L which is based on the same backbone virus but expressing murine CD40L. For the detection and imaging of the virus also tdTomato fluorochrome was cloned into the virus construct.

We show effective expression of functional CD40L both *in vitro* and *in vivo*. In a xenograph model of bladder carcinoma, we show that tumor growth was significantly inhibited by the oncolysis and apoptosis. We also show that the vvdd-CD40L promotes more immunogenic form of cell death compared to the control virus. To gain a more in-depth understanding of the mechanisms of CD40L in modulating the immune response, we analyzed tumors for different markers of the immune system in a immunocompetent murine melanoma model.

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Treatment with Ad5/3-D24-hGMCSF results in redistribution of T-cell subsets in a murine melanoma model

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Background and hypothesis: Oncolytic virotherapy is the use of tumor cell specific, conditionally replicative viruses in the treatment of cancer. Besides lysing tumor cells, viruses trigger immune responses that may participate in tumor destruction. We have observed transient lymphopenia (i.e. decrease of lymphocyte levels in blood) in cancer patients treated with Ad5/3-D24-hGMCSF, a 5/3 capsid modified adenovirus coding for granulocyte macrophage colony-stimulating factor (GMCSF). We hypothesize that oncolytic virus infection can induce recruitment of T cells from blood to the tumor.

Methods: We compared T cell distribution in immunocompetent C57/BL6 mice harboring subcutaneous B16.OVA melanoma tumors treated with six consecutive daily injections of Ad5/3-D24-hGMCSF to control mice treated with saline. We assessed kinetics of both total lymphocytes as well as tumor (OVA)-specific CD8 T cells in blood, spleen and tumor by FACS at three time points post treatment.

Results: We observed that virus induced accumulation of OVA specific CD8 T cells at the tumor site over time, concomitant with a low count of these cells in blood and a transient spike in the spleen. Moreover, the total amount of CD3, CD8 and CD19 immune cells increased in the adenovirus treated tumors at later time points.

Conclusions: Intratumoral adenovirus infection can result in redistribution of immune cell subsets and thus induce anti-tumor responses in our animal model. Whether this phenomenon is linked to the transient blood lymphopenia seen in cancer patients remains to be studied.

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In vitro generation of regulatory T Lymphocytes is improved at physiological oxygen levels

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Regulatory T cells (Tregs) are central element of cell-mediated immune tolerance. In this regard, CD4 CD25 Foxp3 Tregs could be used for immunotherapy, as well as for immune tolerance to therapeutic proteins expressed by means of gene transfer. Such cells can be obtained *in vitro* in mice by stimulation of naïve CD4 T cells with anti-CD3/CD28 beads, in the presence of TGF- β and IL-2. It has been reported that the oxygen levels used for the culture of primary lymphocytes can impact on their proliferation rate and function, and modulate the activity of some proteins involved in the maintenance of Treg cell compartment. This led us to investigate if oxygen levels (atmospheric (20%) or physiological (5%) oxygen levels), could influence the efficiency of Treg induction in a culture system. Using bead-stimulated splenic CD4 T cells from Foxp3 KI-GFP TCR transgenic mice, we found that greater yields of induced-Tregs were obtained under physiological oxygenation, resulting not from better viability but from a higher proliferative rate of the committed Tregs, which could be facilitated by a higher expression at the cell membrane of Glut1. These results have important implications in developing *in vitro* protocols for optimization of the induction of Treg lymphocytes from naïve T cells.

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Oncolytic adenovirus expressing IL-23 and p35 elicits IFN- γ - and TNF- α -co-producing T cell-mediated antitumor immunity

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Cytokine immunogene therapy is a promising strategy for cancer treatment. Interleukin (IL)-12 boosts potent antitumor immunity by inducing Th 1 cell differentiation and stimulating cytotoxic T lymphocyte and natural killer cell cytotoxicity. IL-23 has been proposed to have similar but not overlapping functions with IL-12 in inducing Th1 cell differentiation and antitumor immunity. However, the therapeutic effects of intratumoral co-expression of IL-12 and IL-23 in a cancer model have yet to be investigated. Therefore, we investigated for the first time an effective cancer immunogene therapy of syngeneic tumors via intratumoral inoculation of oncolytic adenovirus co-expressing IL-23 and p35, RdB/IL23/p35. Intratumoral administration of RdB/IL23/p35 elicited strong antitumor effects and increased survival in a murine B16-F10 syngeneic tumor model. The levels of IL-12, IL-23, interferon- γ (IFN- γ), and tumor necrosis factor- α (TNF- α) were elevated in RdB/IL23/p35-treated tumors. Moreover, the proportion of regulatory T cells was markedly decreased in mice treated with RdB/IL23/p35. Consistent with these data, mice injected with RdB/IL23/p35 showed massive infiltration of CD4 and CD8 T cells into the tumor as well as enhanced induction of tumor-specific immunity. Importantly, therapeutic mechanism of antitumor immunity mediated by RdB/IL23/p35 is associated with the generation and recruitment of IFN- γ - and TNF- α -co-producing T cells in tumor microenvironment. These results provide a new insight into therapeutic mechanisms of IL-12 plus IL-23 and provide a potential clinical cancer immunotherapeutic agent for improved antitumor immunity.

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Natural killer cell production under good manufacturing practice conditions to treat cancer patients

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Interest in natural killer (NK) cell based cancer immunotherapy has emerged since biotechnology allows purification and expansion of large number of clinical grade cells.

NK cell selection from leukaphereses and NK cell expansion from peripheral blood mononuclear cells remain the most common cell procedure. In the present study, we demonstrate the feasibility of both methods for the large-scale NK cell production under good manufacturing practice (GMP) conditions. Fourteen NK cell products were obtained and infused, 8 from unstimulated healthy donor leukaphereses using immunomagnetic CD3 T-cell depletion, followed by a CD56 cell enrichment step and overnight ex vivo IL-15 activation, and 6 from 2 weeks co culture of myeloma patients peripheral blood mononuclear cells (PBMCs) with irradiated GMP K562mb-IL15-41BBL cell line. The immunomagnetic process resulted in a median number of $1,15 \times 10^9$ cells with both purity and viability of 94% and 85%, respectively. The T cell population was lower than 1×10^3 . The expansion and activation process resulted in a median number of $1,02 \times 10^9$ cells with both purity and viability higher than 93% and 86%, respectively. The T cell population was 11×10^6 . *In vitro* cytotoxic activity against various cancer cell lines was enhanced respecting non-tumour cells in both processes. *In vivo* antitumor

effect was also observed in tumour xenograft NSG mice models with both processes. No toxicities were observed during infusion either autologous neither allogeneic setting. In conclusion, anti-tumour activated NK cells can be obtained in a GMP-compliant facility and may be infused without any side effects.

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RGD sequence beside Mda-7 protein for tumor targeting; not always works?!

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Background and Aims: Based on previous achievements for mda-7 therapy as an antitumor cytokine, to improve its bystander effect we decided to construct and evaluate vectors including Mda-7 conjugated with two types of RGD peptide at the end.

Materials and methods: Three sequences including Mda-7 alone, Mda-RGD full and Mda-RGD truncate were amplified by PCR and cloned into TA-cloning vector. Thereafter three resultant genes were sub-cloned into expression vector then evaluated by colony PCR, restriction digestion analysis and sequencing. They were transfected into Hep-G2 cell then their expression evaluated by RT-PCR and immunofluorescent assay. The survival rate of cells was first measured using MTT assay. Apoptosis was analyzed by flow cytometry using PI/Annexin staining kit. The expression of BAX and MCL-I mRNA also were evaluated by Real time-PCR as final step.

Results and conclusion: Suitable Expression of three genes were confirmed by RT-PCR and IF. The IF result showed the coincidence expression of Mda-7 in those transfected cells which were shined in green due to plasmid GFP expression. Results from the MTT assay showed significant increase of proliferation inhibition by plasmid expressing Mda-7 and those with truncated RGD. Surprisingly flow cytometry analysis showed significant apoptosis induction by Mda-7 gene but not for those RGD tagged Mda-7 containing plasmids. The expression level of BAX was increased again in Mda-7 and Mda-RGD truncated but it was not the case in Mda-RGD full and in opposite MCL-I mRNA level was similar in all gene indicating it, s relative suppression by all test groups. Conclusively opposite to our expectation RGD full and even truncated form didn't improve our Mda-7 protein anti-apoptosis property demonstrating further need to precise design of new constructs again.

Key words: Mda-7, RGD peptide, apoptosis.

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Modulating co-stimulation during antigen presentation to enhance cancer immunotherapy

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Dendritic cells (DCs) are the most potent professional antigen presenting cells (APCs) of the immune system. DCs provide three different signals to activate effector T cells during antigen presentation. Signal 1 is delivered by the binding of the T cell receptor (TCR) to the peptide antigen complexed to MHC molecules, which is exposed on the surface of DCs. Signal 2 is provided by the integration of positive and negative co-stimulatory interactions on the surfaces of DCs and T cells. In order to direct the differentiation of T cells into different subtypes a third signal is induced by the secretion of cytokines by the DCs.

To enhance cancer immunotherapy we have developed a lentivector, which simultaneously modulates these three signals. First, an antigen of interest is included in the lentivector to provide signal 1. Second, a PD-L1-targeted shRNA that eliminates PD-L1 expression on DCs reinforces positive co-stimulation (signal 2). And finally a cytokine array to modulate signal 3 is expressed to induce the desired anti-cancer effector T cell response.

Using this lentivector expression system, we expressed a collection of Th1-, Th2-, Th17-, and Treg-inducing cytokines and evaluated their antigenicity in cultured DCs. We also evaluated these lentivectors by vaccination in mice and found that we could induce distinct T cell profiles combined with low Treg numbers. Finally, we have data demonstrating anti-tumour activities of some of these constructs in a mouse model for melanoma.

Concluding, we have developed a lentivector system that is capable of activating as well as fine-tuning adoptive T cell responses according to the disease in question and that induces *in vivo* anti-tumour responses in mice.

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Interference with PD-L1/PD-1 co-stimulation during antigen presentation enhances the multifunctionality of antigen-specific T cells

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The release of cytokines by T cells strongly defines their functional activity *in vivo*. The ability to produce multiple cytokines has been associated with beneficial immune responses in cancer and infectious diseases, while their progressive loss is associated to T cell exhaustion, senescence and anergy. Consequently, strategies that enhance the multifunctional status of T cells are key for immunotherapy. Dendritic cells (DCs) are professional antigen presenting cells that regulate T cell functions by providing positive and negative co-stimulatory signals. A key negative regulator of T cell activity is provided by binding of programmed death-1 (PD-1) receptor on activated T cells, to its ligand PD-L1, expressed on DCs. We investigated the impact of interfering with PD-L1/PD-1 co-stimulation on the multifunctionality of T cells, by expression of the soluble extracellular part of PD-1 (sPD-1) or PD-L1 (sPD-L1) in human monocyte-derived DCs during antigen presentation. Expression, secretion and binding of these soluble molecules after mRNA electroporation were demonstrated. Modification of DCs with sPD-1 or sPD-L1 mRNA resulted in increased levels of the co-stimulatory molecule CD80 and a distinct cytokine

profile, characterized by the secretion of IL-10 and TNF- α , respectively. Co-expression in DCs of sPD-1 and sPD-L1 with influenza virus nuclear protein 1 (Flu NP1) stimulated Flu NP1 memory T cells, with a significantly higher number of multifunctional T cells and increased cytokine secretion, while it did not induce regulatory T cells. These data provide a rationale for the inclusion of interfering sPD-1 or sPD-L1 in DC-based immunotherapeutic strategies

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Long-term expression of interferon alpha fused toapolipoprotein A-I allows the development of vaccine-induced adaptive immuneresponses

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Interferon alpha (IFN α) is a key cytokine for the development of adoptive immune response due to its role as signal 3 in CD8 activation. However, the high and frequent doses used for the treatment of chronic viral hepatitis and cancer subvert the immunomodulatory capacity of IFN α , boosting the NK cell activities and abrogating the CD8-mediated immune responses.

In this work, we modeled the long-term treatment with IFN α expressing in the liver of mice this cytokine by a recombinant adenoassociated virus.

The sustained production of IFN α exerted a potent antiviral effect in hepatitis B transgenic mice and in mice infected by a lethal dose of encephalomyocarditis virus. However, these antiviral effects were associated with a profound hematological toxicity, and with a chronically activated immune system that presented an exhausted phenotype.

Interestingly, the long term expression of IFN α fused to apolipoprotein A-I exerted a similar antiviral effect but minimized the hematological toxicity and the exhaustion of the immune system. In this case, the activity of vaccines designed to boost the adaptive immune responses was preserved.

In conclusion, IFN α fused to apolipoprotein A-I maintains the potent antiviral activity of IFN α and the capacity of the adaptive immune system to respond to other immunotherapies designed to boost cytotoxic T cell responses able to eradicate established tumors or chronic viral infections.

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Pre-T cell receptor for improved expansion of TCR α disrupted T cells

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Recent data have emerged from adoptive T-cell therapies where exogenous expression of a chimeric antigen receptor (CAR) has been shown to confer cancer recognition on autologous T cells. However, the ability to apply this technology in an allogeneic setting would permit the generation of universal "off the shelf" T cells that would overcome many current technical and logistic hurdles to the practical application of adoptive im-

munotherapies. Transcription Activator-Like Effector Nucleases (TALENTM) can be used to inactivate the T cell receptor (TCR) alpha gene, eliminating the TCR and thus the potential of graft versus host disease (GVHD), one of the major obstacles towards an allogeneic approach. However, TCR disruption also results in the elimination of the CD3 signaling complex from the T-cell surface, and thus may alter the cells' capacity for expansion and/or survival.

The pre-T cell receptor (pre-TCR) is expressed by immature thymocytes and is crucial for T cell development. Pre-TCR consists of an invariant pre-T alpha chain, variable rearranged TCR beta chains and CD3 signaling components. In contrast to the TCR, which requires interaction with peptide-loaded major histocompatibility complexes to initiate T cell signaling, the pre-TCR is thought to signal through a ligand-independent mechanism that occludes TCR surfaces required for MHC interaction. Here we demonstrate that the expression of the invariant pre-T alpha chain, in the absence of TCR alpha, results in the restoration of CD3 at the cell surface in association with a pre-TCR. Cells with preTCR/CD3 complexes have an improved life span, and can be expanded ex vivo through standard CD3/CD28-based bead methods. Application of this technology in association with allogeneic CAR modified T cells will also be presented.

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Targeted disruption of endogenous TCR expression by transcription activator-like effector nucleases

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The introduction of high avidity T-cell receptor (TCR) genes into T-cells for adoptive T-cell therapy holds a lot of promises for the clinics, but still comprises certain challenges. The parallel expression of endogenous and exogenous TCR-chains provokes competition for surface expression, thereby reducing the avidity for their respective antigens. Furthermore, mispairing of endogenous and exogenous α - and β -chains may occur and result in the generation of TCR with unpredictable specificities, potentially leading to autoimmunity. Designernucleases like transcription activator-like effector nucleases (TALEN) can be used for targeted genome editing. Upon dimerization of a TALEN-pair, specific DNA double strand breaks (DSB) are introduced, potentially resulting in permanent gene knockout during DSB repair. To disrupt endogenous TCR expression, we assembled seven TALEN monomers targeting the constant regions of the TCR α -chain (*TRAC*) and the TCR β -chain (*TRBC1* and *TRBC2*), respectively. The TALEN monomers were designed for possible dimer combinations with 12 bp- and 15 bp-spacers. In order to minimize unspecific off-target activity, obligate heterodimeric TALEN were included. TALEN activity at the target sites was examined in 293T, K562 and Jurkat cells using Cel1-Assay and deep-sequencing. Here we show the specific DSB induction by homodimeric and obligate heterodimeric TALEN with mutation frequencies of up to 22%. Indeed, only TALEN separated by a 15bp-spacer were active. TALEN specificity was examined by the identification of integrase-deficient lentiviral vector (IDLV)-marked TALEN-induced DSB by LAM-PCR. TALEN that show high efficiency and specificity to their target sequence will be used for safe and effective TCR knockout in primary T-cells.

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Lymphatic regeneration and de novo antigen-specific human T and B cell responses in humanized mice after dendritic cell-mediated gene therapy

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We explored dendritic cells (DC) to address a major problem in hematopoietic stem cell transplantation (HSCT), namely several weeks of profound immune deficiency and lack of functional adaptive immune responses. We performed human HSCT with CD34 cells from G-CSF mobilized peripheral blood into NO-D.Rag1-/-IL2r gamma-/- (NRG) mice. These humanized immune system NRG mice ("HIS-NRG") show incomplete hematopoiesis, lack of lymph nodes (LN) and scarce adaptive immune responses. Since DC are pivotal for the organization of LN and activation of naïve T and B cells, we therefore infected autologous human monocytes with integrase-defective lentiviral vectors expressing huGM-CSF, huIFN-alpha and the human cytomegalovirus (CMV) antigen pp65 protein, to generate highly viable DC (Self-differentiated myeloid-derived lentivirus-induced Smyle DCSM). Remarkably, ten weeks after SmyleDC immunization, HIS-NRG mice showed developed peripheral lymph nodes (LN) and recovered lymphatic flow. SmyleDC immunization was further associated with several relevant findings: 1. Several human cytokines in mouse plasma; 2. Significantly higher frequencies of human T cells in PBL and spleens; 3. Accumulation of human effector memory T cells in PBL, spleens and LN; 4. Presence of human terminally differentiated plasma B cells and follicular T helper cells in spleens and LN; 5. Functional human T cell responses against pp65, and 6. Human IgM and IgG reactive to pp65. Our results demonstrated the important role of potent and long-lasting human DC functions after HSCT for peripheral lymph node and lymphatic flow regeneration in order to activate, mobilize, and finally mature lymphocytes towards full immune function in the HIS-NRG mouse model.

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Antigen-presenting cell subset specific targeting has immunological consequences

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Antigen-presenting cells (APCs) represent a heterogeneous population characterized by their functional specialization. Consequently, targeting specific APC-subsets offers opportunities to induce particular immune responses. To validate this, we targeted lentivectors (LVs) specifically to APC-subsets using the nanobody (Nb) display technology. This approach enables pseudotyping of LVs by combining a binding-defective but fu-

rogenic form of VSV.G with a membrane-bound APC-specific Nb. Three Nbs were selected: DC1.8, R3_13 and DC2.1. The respective LVs are referred to as DC1.8-, R3_13- and DC2.1-LVs. Flow cytometry performed on cells obtained from *in vivo* transduced murine or *ex vivo* transduced human lymph node cells demonstrated that DC1.8-LVs and R3_13-LVs specifically transduced murine and human myeloid dendritic cells (DCs) respectively. In contrast, DC2.1-LVs transduced both human and murine macrophages, plasmacytoid and myeloid DCs. Next we examined the immune response elicited by intranodal immunization of mice with ovalbumin (OVA) encoding DC1.8- or DC2.1-LVs. DC2.1-LVs outperformed DC1.8-LVs in expansion of functional OVA-specific CD8 T cells. Whereas the expansion of CD4 T cells was comparable upon DC1.8- and DC2.1-LV immunization, the cytokine profile of these CD4 T cells revealed that DC1.8-LVs primarily induced the secretion of IL-17 and IFN- γ while DC2.1-LVs primarily induced the production of IFN- γ and IL-2. This difference in CD4 and CD8 T cell response was reflected in therapy, demonstrating lower efficacy of DC1.8-LVs when compared to DC2.1-LVs. In conclusion, these findings open the perspective of a vaccination strategy that induces very particular immune responses by targeting a specific DC-subset hence enables full exploitation of the immune system.

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Plasmid vector design and technologies for DNA vaccine development

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Nucleic-acid based DNA vaccines represent a novel class of biodrugs with great therapeutic potential as competitive alternative approach to conventional protein vaccines both as prophylactic and therapeutic treatment of infectious diseases, cancer and allergy. Despite safety concerns have been overcome, low immunogenicity profiles of DNA vaccines has hindered their progress in humans. DNA vaccines need to make up for this limitation by altering plasmid construction through complementary vector design innovations that, in combination with improved delivery platform, may enhance DNA vaccine performance and clinical outcomes. DNA vaccination platform takes advantage of *in vivo* processes and has the potential to harness the full power of the immune system, through engagement of multiples routes to activate both branch of the immune system (i.e. innate immunity as well as adaptive immunity). Current knowledge of the molecular and immunological mechanisms by which DNA vaccines work can be used to bring about improvements in their efficacy. Advanced technologies such as immunoinformatics (i.e. *in silico* prediction of potential T cell epitopes), antigen/epitope optimisation and expression, provision of CD4 T cell help, intracellular antigen targeting ensuring efficient MHC I and MHC II compartment addressing, inclusion

of genetic adjuvants have been applied to improve the efficacy of DNA vaccines. In order to translate these approaches into a therapeutic strategy, we have developed a series of modular anti-idiotypic DNA vaccines and have assessed the induction of antitumor immunity in an aggressive murine B-cell lymphoma model. Here we report that the DNA vaccine variants, in combination with electroporation delivery platform, are suitable to engage both humoral and cellular immune responses, thus resulting in efficacious DNA vaccines performance.

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AdCD40L immunostimulatory gene therapy shifts the MDSC- and macrophage profiles and promotes T cell infiltration in the tumor microenvironment

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Immunotherapy is becoming an important new treatment option for cancer. CD40 ligand (CD40L) is a potent stimulator of tumor immunity via its activation of dendritic cells, which initiate T cell activation. However, T cells are effectively suppressed by myeloid-derived suppressor cells (MDSCs), which contribute to tumor immune evasion. We hypothesized that CD40L may create a positive T cell milieu including an effect on MDSCs because of its broad effects as an immune activator and this was investigated in the experimental murine bladder cancer model MB49/C57BL6. Upon adenoviral CD40L (AdCD40L) gene therapy, MDSC tumor infiltration was significantly reduced. In vitro, CD40L-expressing MB49 cells could shift the MDSC subpopulations in favor of the granulocytic MDSCs instead of the highly suppressive monocytic MDSCs. Further, MB49 tumor cells expanded macrophage-like cells in splenocyte cocultures. However, MB49 cells expressing CD40L shifted the macrophages to M1-like cells. T cells from transgenic pmel mice were utilized to demonstrate that T cells indeed infiltrate better into B16 tumors treated with AdCD40L. Hence, these data supports that AdCD40L creates a favorable milieu in tumors and can be an interesting partner to combine with other immunotherapies such as adoptive T cell therapy.

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Correlation between the early expression of *ctla4* and *foxp3* genes and the failure of the anti-tumour response employing genetically modified tumor cell vaccines

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Introduction: Currently it is known that the failure of the anti-tumour response of antitumor vaccines may be due to the expression of immunosuppressive genes as *ctla4* and *foxp3* gene expression. The objective of this study is evaluating the relation

between tumor development and *ctla4*, *foxp3* and *cd25* expression in mice vaccinated with B16 genetically modified cells in a murine melanoma model.

Methodology: C57BL6 mice were vaccinated with 2×10^5 irradiated B16 cells genetically modified to produce GM-CSF and then, B16 tumor was transplanted. The survival mice to this antitumor vaccine were injected with 10^5 B16 mouse melanoma cells six months after vaccination. Blood samples were collected on days 0, 2, 5, 14 and 21 after tumor injection. The RNA of blood cells was isolated. The *ctla4*, *foxp3* and *cd25* RNA expression was measured by RT and qPCR. The tumor development was controlled.

Results: In mice that developed the tumor a peak of *ctla4*, *foxp3* and *cd25* mRNA expression appeared between 2 and 5 days after tumor injection whereas in mice that did not develop the tumor the peak of this mRNA gene expression was observed on day 21 after tumor injection.

Conclusions: The early mRNA expression (days 2–5) of *ctla4*, *foxp3* and *cd25* genes appears to be related with the tolerance to tumor growth, while the late expression (day 21) seems to be linked to the success of the antitumor immune response and protection against tumor. Partially supported by SAF 2011-27002.

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Soluble PD1 enhances anti-immunity in orthotopic bladder tumors by strengthening CD8 T cell response

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Background: Adenoviruses harboring the HSV thymidine kinase (HSVtk) gene under the regulation of a *trans*-splicing ribozyme targeting human telomerase (hTERT-TR) show marked and specific antitumor activity. In addition to inducing tumor cell death by direct cytotoxicity, it is becoming clear that HSVtk also induces anti-tumor immunity. PD-L1 expressed on tumor cell surfaces mediates tumor-induced immunoresistance by inhibiting PD1-expressing tumor-infiltrating T cells.

Materials and Methods: In order to explore whether a soluble form of PD1 (sPD1-Ig), which blocks PD-L1, could synergize with TERT-TR-regulated HSVtk to enhance the adenoviral therapeutic efficacy, we constructed adenovirus expressing both HSVtk and sPD1-Ig. Then we characterized its cytotoxicity to bladder cancer cell and its tumor suppressive activity in orthotopic MBT-2/Luc syngeneic mouse model.

Result: Adenovirus expressing HSVtk and sPD1-Ig was demonstrated to exert cytotoxic effects on mouse bladder cancer cell by inducing cell cycle arrest and apoptosis. Regression of MBT-2/Luc murine subcutaneous and orthotopic tumors was markedly enhanced when sPD1-Ig was incorporated into the adenovirus compared with a single-module adenovirus expressing only HSVtk. This effect was abolished by CD8 T cell depletion. Additionally, secondary tumor challenge at a distal site with no treatment was completely suppressed in mice, when primary site was treated with a dual-module adenovirus.

Conclusion: These results suggest that a dual-targeting strategy to elicit both direct tumor cell lysis and breakage of tumor-induced immune-tolerance could be utilized for immunotherapeutic agent for bladder cancer.

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Interference with PD-L1/PD-1 co-stimulation results in effective anti-tumour immunity by preventing ligand-induced T cell receptor down-modulation in effector CD8 T cells

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T cell receptor (TCR) down-modulation after antigen presentation is a fundamental process that regulates TCR signal transduction. Current understanding of this process is that intrinsic TCR/CD28 signal transduction leads to TCR down-modulation. We demonstrate a novel mechanism whereby the interaction between programmed cell death 1 ligand 1 (PD-L1) on dendritic cells (DCs) and programmed death 1 (PD-1) on CD8 T cells triggers ligand-induced TCR down-modulation. This occurred via Cbl-b E3 up-regulation in CD8 T cells. PD-L1 silencing in DCs with lentiviral vectors delivering microRNAs markedly inhibited TCR down-modulation. PD-L1 silencing hyper-activated CD8 T cells as assessed in vitro and in vivo in an arthritis model. Anti-tumour immune responses (lymphoma and melanoma) were accelerated and DC anti-tumour capacities potentiated, when combined with mitogen-activated kinase (MAPK) modulators that promote DC activation, or with cytokine priming.

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CMV-specific and EBV-specific T cells for adoptive immunotherapy in viral diseases resistant to conventional therapies

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In transplanted patients, CMV, EBV or other viruses' re-activations may occur, needing treatment with antiviral drugs with adverse side effects. In recent years the use cytotoxic T lymphocytes (CTLs) specific for different pathogens allows to consider adoptive immunotherapy.

We have used the IFN- γ Capture Secreting Cells for isolating CMV and EBV specific stimulated T cells under GMP conditions. This method allows obtaining CTLs for clinical use in short time and minimum HLA restriction. 35 CMV-positive donors were studied and 10 selected for isolating CTLs because their CMV-CTLs frequency were³ 0.3%. We assayed different methods of stimulation. The best results were obtained using specific CMV peptides and a short time of stimulation (6 h) with a median purity of 90% of IFN γ producing cells, 78.3% CD8 and 7.2% CD4 (n=3). Also contaminating non-T cells were fewer than with other conditions. Only 3 donors out of 17 EBV-positive donors were selected because their EBV-CTLs frequency was³ 0.05%. In this case the final product (n=3) had a median purity of 63.3% of IFN γ producing cells, 38.3% CD8 and 5.5% CD4 cells.

CMV-specific CTLs have been used for compassionate use in 3 hematopoietic transplanted patients with positive effect, one of them with well documented analytical and clinical disease control. In our experience, the isolation of CMV-specific CTLs works

better using peptides and a short stimulation. Under these same conditions, EBV-specific CTLs selection did not reach the same quality. The use CMV specific CTLs could have a beneficial effect in immunosuppressed patients.

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Nucleotide sequence optimization of TRIM5a rhesus-human chimeric gene enhances HIV-1 antiviral activity of the gene

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The host restriction factor TRIM5 α provides intrinsic defense against retroviral infections in mammalian cells in a species-specific manner. It was shown that rhesus macaque TRIM5 α protein, but not its human ortholog, blocks HIV-1 production through rapid degradation of HIV-1 Gag polyproteins. Another study reported generation a chimeric human-rhesus TRIM5 α in which an 11-amino acid was replaced with the rhesus 13-amino acid. The expression of this chimera gene in cells conferred resistance to HIV infection as well as a selective survival advantage on HIV-1 challenge. We optimized nucleotide sequence coding for Trim5 α chimera protein by increasing GC-content. We have shown that codon optimization significantly improved the expression level of TRIM5 α chimeric gene and enhanced its antiviral activity in MT4 cell line.

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Alternative splicing variants of STING present a differential ability inhibiting human wild type STING

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The innate immune system provides a defense system based on the detection of pathogens followed by the activation of a coordinated response culminating in the suppression of infection. Chordates present a complex detection system of pathogen associated molecular patterns (PAMPs) that leads to the activation of interferon (IFN). Stimulator of interferon genes (STING) is a critical protein involved in IFN- β induction in response to some DNA and RNA pathogens infection, including herpes simplex virus-1 (HSV-1), *Listeria monocytogenes* and vesicular stomatitis virus (VSV). We have found that in addition to the previously described Wt STING several other species are expressed. In the present work we have cloned and sequenced alternative splicing variants of STING. Preliminary experiments revealed the inability of alternative spliced variants to induce IFN- β . However, they are able to inhibit Wt STING mediated IFN- β induction with different strength. We are exploring the effect of shRNA delivery to target alternative spliced STING isoforms to derepress such proteins effect on Wt STING activity. Eliminating this repression could be of interest for the treatment of some chronic viral infections. In particular, we have found that STING mRNA is less expressed in the liver of patients with chronic viral hepatitis B or C than in the liver of healthy individuals. In such context, the inhibition of STING alternative spliced isoforms could restore some of Wt STING activity, including its antiviral potential during chronic liver infections.

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Breaking T cell tolerance against viral antigens in an animal model of chronic HBV infection by genetic transfer of interferon-alpha and interleukin-15 to the liver

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Chronic HBV infection is characterized by a weak and exhausted T cell response. To promote sustained viral clearance it is necessary to boost a viral specific immune response of the host. Hepatitis B virus (HBV) transgenic mice are immunologically tolerant to HBV antigens, representing a good model to test strategies aiming at breaking T cell tolerance. We now provide a novel strategy to induce functional T cell in HBV transgenic mouse model based on the genetic transfer of immunostimulatory cytokines. Sustained liver specific interleukin-15 (IL-15) expression expands the population of HBV-specific CD8 T cells in the liver. However, IL-15 expanded T cells failed to kill peptide loaded cells indicating that they remained functionally silent. However, when IL-15 expression is combined with interferon-alpha (IFN- α) the HBV specific expanded T cells are able to kill in vivo antigen loaded cells. Furthermore, a synergistic antiviral effect was observed in animals receiving both cytokines. Interestingly we found that IL-15 liver expression induces a significant increase of PD-1 expression and of PD-L1 in the liver, and treatment with IFN- α reduced the expression of both molecules licensing the HBV-specific T cells to kill the infected cells. Thus, our study defines a potent new approach to break the tolerance in a model of chronic viral infection by (1) expanding virus-specific CD8 T cell with IL-15 and (2) blocking negative signals with IFN- α . Our study defines a potent new approach to break immune tolerance with broad applications in chronic viral infections and neoplastic conditions.

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Mimicry of anti-HIV retrotransposition events by TALEN mediated Cyclophilin insertion into the human TRIM5 locus

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Strategies against HIV include gene therapy based approaches to transfer HIV-1 restriction factors to immune cells. Certain primate species have demonstrated a resistance to HIV-1 infection mediated by the expression of the restriction factor TRIM5-Cyclophilin A (TRIM5Cyp), a fusion protein formed by retrotransposition of Cyp cDNA into the TRIM5 gene.

Previously we have generated humanised versions of TRIM5Cyp and showed that they elicit potent anti-HIV-1 effects when delivered to human lymphocytes using lentiviral vectors. As an

alternative to gene-addition, we are now investigating transcription activator-like effector nuclease (TALEN) mediated integration of Cyp DNA into the TRIM5 gene at one of two possible loci. Firstly, integration of a Cyp cDNA into exon 7 of TRIM5, which would closely mimic primate retrotransposition events and secondly, insertion of a full length TRIM5Cyp gene downstream of the TRIM5 transcriptional start site. Both strategies would potentially allow regulated, interferon inducible expression of TRIM5Cyp under control of the endogenous TRIM5 promoter.

Plasmids encoding TALEN sequences targeting either of the two loci in the human TRIM5 gene were delivered to cell lines by transfection. Cleavage at both of the target sites was confirmed by surveyor nuclease assay and by direct sequencing. Delivery of an engineered donor template encoding either TRIM5Cyp or Cyp cDNA flanked by TRIM5 homology arms allowed site directed insertion into the TRIM5 gene. Investigations are now underway to characterise human cells modified to express targeted TRIM5Cyp sequences, and to assess whether the resistance to HIV can be conferred through this approach.

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Genome-wide landscape of NHEJ-repair activity and DNA double strand breaks in the human genome

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The specificity and toxicity of ZFNs, TALENs, and RNA-guided DNA endonucleases is being explored extensively. However, genomic factors and intervals influencing the frequency of DSB and DNA repair activity are not fully evaluated in this context. Therefore, we hypothesize that generating a genome wide map of DSB and DNA repair activity will enable the identification of genomic vulnerabilities influencing both fidelity and safety of DSB induction and repair for genome editing. DNA templates serving as molecular tags which are incorporated via NHEJ were used to stably trap DSB in surviving cells or were applied for enzymatic DSB labeling *in situ* in real-time. Accordingly, we have induced genome wide DSB by radiation and sequenced >10.000 unique repaired NHEJ sites in A549, PC3 and U87 tumor cell lines and primary cells. Distribution of DSB and NHEJ-repair activity is not enriched in RefSeq genes and not associated to mRNA transcriptional activity. The majority of targeted genes (54%) are transcriptional inactive, 25%, 13% and 8% show low, medium and high transcriptional activity, respectively. Interestingly, in irradiated A549 and PC3 cell lines as well as primary human fibroblasts small genomic intervals were detected showing massive and significant accumulation ($p < 0.0001$) of induced and trapped DSB with up to 200 trapped breaks in a 200 kb linear genomic window. These sites potentially resemble regions of increased DNA repair activity or fragility. We are able to clonally track these repaired breaks in the genome of living cells and measure NHEJ activity in correlation to genomic intervals influencing safety and stability of genome editing in different cell types and diverse differentiation stages. With the established genomic landscape of NHEJ-activity, we are identifying novel potential "safe-harbors" with an increased probability of DNA repair and homologous recombination and may dissect cell type intrinsic factors influencing off-target activity of designer nucleases.

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Long term persistence of TK-cells and dynamics of memory T cells in patients treated with suicide gene therapy for hematological malignancies

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Suicide gene therapy applied to allogeneic hematopoietic stem cell transplantation is one of the widest clinical applications of gene therapy. By the infusion of donor lymphocytes transduced to express the Herpes Simplex Virus Thymidine Kinase (TK) suicide gene, patients achieve a rapid immune reconstitution and substantial protection against tumor recurrence. TK-cells are promptly eliminated in case of graft versus

host disease, with complete resolution of the adverse reaction. In the present work we studied the long term fate of TK-cells, and exploited TK gene marking to shed light on memory T cell dynamics.

We analysed 14 patients in complete remission after receiving post-transplant TK-cell infusions for hematological malignancies. At a median follow-up of 8,7 years (range 3–16), a complete recovery of the T-cell compartment, comprising of physiological levels of circulating naïve and memory CD4 and CD8 cells is observed. TK-cells are detected in the majority of analyzed patients (88%), at low levels (median=0,43%±6,9%). The number of TK cells/ml detectable at the longest follow-up does not correlate with the number of infused cells, but instead with the peak of TK cells observed within the first months after infusion, suggesting a role for antigen recognition. While infused cells displayed a predominant effector memory phenotype, TK-cells persisting long-term are enriched for central memory (CD45RA-CD62L) and stem memory (CD45RA CD62L CD95) T cells. Ganciclovir sensitivity is preserved in persisting TK-cells, independently from their phenotype. Further studies on T cell repertoire and vector integrations will elucidate the *in vivo* dynamics of infused memory T cells.

Additional Abstracts

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G-CSF+Plerixafor-mobilized blood represents an optimal graft source for gene therapy of thalassemia

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The therapeutic effect of gene therapy (GT) in β -thalassemia largely depends on the infusion of high numbers of genetically modified hematopoietic stem cells (HSCs). We investigated the Lin-sca-1+c-kit+ cell (LSK) and CFU-GM yields after mobilization with G-CSF, Plerixafor or Plerixafor+G-CSF in thalassemic (Hbbth3/45.2+) mice and the engraftment kinetics of the differently mobilized cells after transplantation. In competitive transplantation experiments, mobilized peripheral blood (PB) cells from Hbbth3/45.2+ donors were infused into lethally irradiated PepBoy (45.1+) recipients, along with an equal number of competitor bone marrow (BM) cells. Six months after primary transplantation, BM cells from competitively engrafted mice

were injected into lethally-irradiated secondary recipients in a noncompetitive manner. The Plerixafor+G-CSF combination resulted in significantly higher yields of LSKs and CFU-GM, compared to G-CSF- or Plerixafor-alone ($p \leq 0.004$). Plerixafor+G-CSF-mobilized cells achieved faster hematologic reconstitution and higher engraftment levels when transplanted at a 3:1 (donor:recipient) ratio to PepBoy recipients ($p \leq 0.05$). Although this could simply reflect the higher LSK and CFU-GM frequencies in the Plerixafor+G-CSF grafts, the superiority in engraftment was maintained when equal number of LSKs (1×10^4) were transplanted in a competitive manner again. Furthermore, secondary Plerixafor+G-CSF recipients presented similar or even higher donor chimerism levels ($p \leq 0.05$) compared to G-CSF- or Plerixafor-alone, implying an enhanced self-renewal activity. The qualitative superiority of Plerixafor+G-CSF-mobilized cells was not associated with differences in surface CXCR4 and CD26 expression or cell cycle status, however, Plerixafor+G-CSF-mobilized c-kit+ cells showed higher migration capacity towards SDF-1 as compared to G-CSF- or Plerixafor-mobilized progenitors ($p \leq 0.0002$). In conclusion, Plerixafor+G-CSF-mobilized HSCs potentially represent an optimal graft source for GT of thalassemia because of the higher HSC yields, the faster hematological recovery and the superiority in long-term engraftment over single-agent-mobilized cells.