Revertant cell therapy for epidermolysis bullosa

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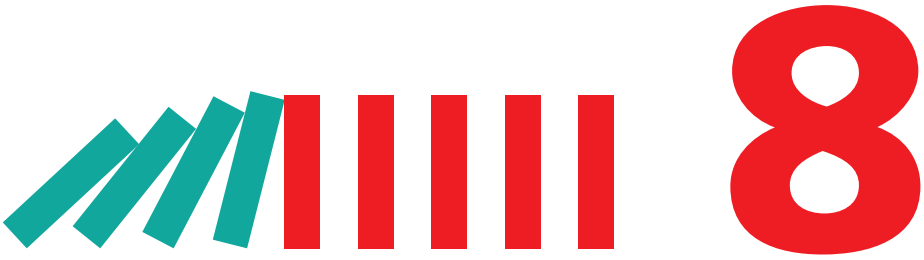

SUMMARY

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This thesis describes a quest to understand and use the phenomenon of revertant mosaicism for treatment of the heritable skin disease called epidermolysis bullosa (EB). EB is caused by mutations in the dermal and epidermal proteins and is characterised by blistering of the skin and mucosa.

In **Chapter 1** of this thesis we describe the pathological mechanism behind EB, we introduce the phenomenon of revertant mosaicism and discuss current approaches to treat EB. At the moment there are 18 genes identified in which mutations may cause EB. The recent consensus divides EB in four major types and more than 30 subtypes depending on the level of blister formation, affected protein, disease severity and presence of extra cutaneous manifestations. Moreover, a detailed overview of function of three proteins involved in the dermal epidermal junction, type XVII collagen (Col17), laminin-332 (lam-332) and type VII collagen (Col7) is given. Mutations in genes encoding Col17 and lam-332 cause junctional epidermolysis bullosa (JEB), while mutations in type VII collagen are responsible for the dystrophic variant of EB. At the moment no curative therapy is available for EB and the current approach to treat EB can be divided in three main groups: gene therapy, protein replacement and cell therapy. Gene therapy focuses on correction of the affected gene; protein replacement therapy aims at supplying the wild-type version of the affected protein, whereas cell therapy uses autologous or allogeneic cells to change the course of disease. The current state-of-the-art of those therapies is discussed in more detail in **Chapter 1**.

This thesis focuses on a novel approach to treat EB using revertant mosaicism. Revertant mosaicism (RM) is a phenomenon in which there is a co-existence of affected cells containing a disease-causing mutation (mutant), and cells in which the mutation is naturally corrected to the wild-type phenotype (revertant) within one individual . That is why RM is often addressed to as 'natural gene therapy'. Since the first description of RM in a patient affected with Lesch-Nyhan syndrome in 1988, this phenomenon has been found in many other diseases, like Wiskott-Aldrich Syndrom (WAS), Fanconi anemia and EB. There are different mechanisms described that can cause RM, like single base pair substitution or a second-side mutation. The full overview of those mechanisms can be found in **Chapter 1**. Within one individual many different correcting mechanisms can be found, for example 38 different reversions were identified in a patient affected with WAS.

In the skin RM was first described in 1995 in a patient affected by the JEB subtype caused by mutations in the *COL17A1* gene coding for the Col17 protein. Between affected skin areas on the patient's arms healthy looking skin patches were found. Immunofluorescence staining of the skin biopsy taken from a skin patch showed presence of Col17, whereas the biopsy taken from the affected skin was negative for the protein. The molecular mechanism behind RM in this patient was found in 1997. In cells with normal Col17 expression one of the original mutations causing the disease disappeared due to a gene conversion. The presence of healthy skin patches

was named revertant mosaicism and healthy cells were called revertant. Since 1997, RM has been found in many JEB and DEB patients and it is believed that all patients affected by mutations in *COL17A1* have revertant skin patches. Presence of the healthy cells within an affected body is a unique opportunity for autologous cell therapy. Different methods of skin transplantation have already been used to treat burn wounds and chronic wounds. Transplantation of revertant keratinocyte to expand the revertant skin area is the aim of revertant cell therapy.
In **Chapter 2** we describe an attempt to increase revertant skin area in a Col17 deficient, JEB generalized intermediate (JEB-gen-intermed) patient. The patient was a compound homozygous for a maternal frame shift mutation in exon 18 (c.1601delA) and paternal nonsense mutation in exon 51 (c.3676C>T). This patient had multiple revertant skin patches and in some of them the following correction mechanism was identified, i.e. part of the paternal allele of *COL17A1* containing a healthy copy of exon 18 was moved to the maternal allele and covered the c.1601delA mutation resulting in one allele with a paternal mutation and one wild-type. From such a naturally corrected revertant skin patch a skin biopsy was taken and cultured *in vitro* to acquire two 6x7 cm epidermal sheets. The wound bed was prepared by adhesive tape stripping – an innovative method that uses EB's pathological mechanism of reduced adhesion in the lamina lucida. Briefly, an adhesive tape was placed on the patient's thigh and small incisions were made around it. The lack of adhesion due to the mutations in *COL17A1* resulted in removal of the epidermis when tape was pulled off. Skin grafts were placed on the prepared wound bed and the healing process was uneventful and successful. Unfortunately, the functional test showed no reversion of EB phenotype and thus no expansion of revertant skin area. Analysis of the biopsy, cultured cells and epidermal grafts revealed that the percentage of revertant cells decreased from 50% in the biopsy to less than 3% in the graft. At that moment the reason for such a decrease was unknown.
Chapter 3 describes the animal model of the revertant cell therapy for EB. In this chapter we looked into survival of revertant cells during cell isolation, graft production and engraftment on immunodeficient mice. A biopsy from the same patient taken from the same revertant patch as in experiments described in **Chapter 2** was used. We isolated keratinocytes and fibroblasts and assessed the percentage of the revertant cells after the first passage to be 40%. This percentage dropped to 25% and 20% after the second passage and in the cultured skin equivalent, respectively. We grafted skin equivalents containing 20% of revertant cells on the immunodeficient mice and assessed the percentage of revertant area after 10 and 16 weeks. On both time-points 20% of the cells was revertant meaning that long-term survival of revertant keratinocytes *in vivo* is possible. In **Chapter 3** we also looked into the colony forming potential of revertant keratinocytes. We showed that revertant keratinocytes have a high ability to form colonies, but revertant colonies were smaller than mutant ones. This, together with the recently published work describing influence of Col17 on immunomodulation and NF-κB levels, gave us a basis to

formulate a theory about decrease of percentage of Col17 revertant cells during *in vitro* expansion. This theory is discussed in more detail in **Chapter 7**.

Chapter 4 describes a different approach to revertant cell therapy, where *in vitro* expansion of revertant cells is omitted and naturally corrected skin is transplanted directly onto the acceptor site. We successfully treated a then 69-year-old male patient affected by JEB-gen-intermed due to homozygous c.628G>A mutations in the *LAMB3* gene with revertant punch biopsy grafting. Multiple revertant patches on this patient's body were earlier identified. Immunofluorescence on a biopsy from his mutant skin showed strongly reduced staining for laminin-332, while normal levels of laminin-332 were observed in the revertant patches. In 2012 this patient presented with multiple, persistent (>1 year) ulcers. We used an earlier identified revertant skin patch on his right shoulder as a donor site and harvested 73 punch biopsy specimens that were then placed in the wounds. All biopsies were accepted and wounds healed within 2 weeks. During the course of 18 months no blistering or ulcerations were observed in the treated areas. Skin biopsies from both donor and acceptor site showed re-epithelialisation with revertant epidermis expressing normal levels of laminin-322. Thus, a successful expansion of revertant skin area and application of revertant cells in therapy of EB were shown.

Revertant mosaicism is a source of naturally corrected cells for therapy as well as an interesting phenomenon that allows studying two genetically different cell populations within one body. In **Chapter 5** we investigated why revertant skin patches in patients with revertant mosaicism and mutations in *COL17A1* are hyperpigmented in contrast to their mutant skin. In a population of 13 patients with EB and revertant mosaicism with mutations in *COL17A1* (n=8), *LAMB3* (n=2) and *COL7A1* (n= 3) the phenotype of affected (=mutant) and healthy (=revertant) skin was compared. The amount of pigment and density of melanocytes were identified in biopsies taken from mutant and revertant skin. There was a clinical difference in pigmentation in the *COL17A1* group, which was not present in the other two groups. Further, more pigment in the Fontana-Masson staining and a significantly higher melanocyte density was found in Col17 revertant skin versus Col17 mutant skin. In contrast, patients with mutations in *LAMB3* showed a lower melanocyte density and amount of pigment in both revertant and mutant skin. Mutations in *COL7A1* did not to have a correlation with the amount of pigment or density of melanocytes. In the study presented in **Chapter 5** we concluded that pigmentation depends on Col17 and that lam-332 might have a negative influence on proliferation of melanocytes and therefore on pigmentation. How the proteins of the dermal-epidermal junction regulate the pigmentation and density of melanocytes is still unknown. Col17 seems however to have an important role in the inflammatory pathways, cell signalling and survival of melanocyte stem cells, while lam-332 might influence the melanocytes proliferation. These theories will have to be investigated in the future.

Many other hypotheses and possible improvements to expand the revertant area of the skin were investigated during work on this thesis. In **Chapter 6** three selected experiments, that were perhaps not a direct step forward on the path to revertant cell therapy but are worth mentioning, are presented. First, an approach to select Col17 revertant keratinocytes involving differences in adhesion between Col17 positive and negative cells is described. Unfortunately, not only Col17 is responsible for adhesion to plastic and coatings such as lam-332 or type I collagen in keratinocytes and therefore we could not separate keratinocytes based on Col17 expression. Next, the successful sorting of living revertant and mutant Col17 cells with flow cytometry is shown using the 233 monoclonal antibody. Non-enzymatic detachment of keratinocytes was used in this method to preserve the extracellular domain of Col17 and allow immunofluorescence staining. This method allowed further *in vitro* culture of sorted cells. This is, however, not possible to apply in the clinical setting at the moment due to safety regulations.

In the second part of **Chapter 6** we describe an attempt to establish a murine model for Col7 revertant cell therapy based on a similar approach as presented in **Chapter 3**. A recessive dystrophic EB patient due to mutations in the *COL7A1* gene had a revertant patch on her arm. From this patch a biopsy was taken, cells were isolated and skin equivalents were cultured. In the side samples only less then 10% of cells were revertant while in the biopsy taken previously from this patch 85% of the epidermis showed presence of Col7. Skin grafts were placed on the immunodeficient mice and analysed with immunofluorescence after 10 and 16 weeks. No revertant cells could be found in the grafts meaning that we did not succeed in transplantation of healthy cells. Low numbers of naturally corrected cells in the original biopsy taken for cell isolation was a probable cause of the unsuccessful experiment. Col7 revertant cell therapy should be further studied with a biopsy containing a higher percentage of revertant keratinocytes.

The last experiment presented in **Chapter 6** involves the same patient as described in **Chapter 4**. He underwent transplantation of non-cultured epidermal cells in suspension harvested from his revertant skin patch to treat a chronic leg ulcer. Impaired wound healing due to age and arterial obstruction had a negative influence on the procedure and complete re-epithelialization was not reached. We expected that some revertant cells were engrafted with this procedure, because a bridge dividing the ulcer in two smaller wounds was seen 6 weeks after the procedure and remained stable during the seven-month follow-up. A study of transplantation of non-cultured revertant cells in suspension with a controlled, well prepared acceptor site, will validate this technique for future usage.

Chapter 7 of this thesis summarizes the findings of **Chapters 2-6** and discusses advantages and disadvantages of revertant cell therapy as an approach to cure EB. Currently, treatment of limited skin areas with punch grafting is possible. In the near future we expect to use new techniques to transplant revertant cells with and without *in vitro* expansion, depending on the needs of the

patient. **Chapter 7** also discusses the future use of induced pluripotent stem cell technology to produce a population of naturally corrected stem cells that could be used to treat EB locally (with skin grafts) and systemically (through infusion).

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