Exploring the regeneration potential of salivary glands using organoids as a model

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CHAPTER 8

SUMMARY & DISCUSSION

SUMMARY

Irreversible hyposalivation and xerostomia (also known as dry mouth syndrome) are the main consequences of salivary gland dysfunction. Radiation-induced hyposalivation may lead to xerostomia and can be caused by radiotherapy of head and neck cancer patients. Inclusion of the salivary glands in the field of radiation during radiotherapy may lead to the disruption of the salivary gland cellular niches and to a progressive and irreversible decline of the progenitor-like acinar cells leading to a subsequential loss of homeostasis and ultimately to the permanent loss of acinar tissue responsible for saliva production and secretion. While xerostomia *per se* is not a life-threatening condition, of the 500,000 patients diagnosed every year with head and neck cancer 40% of those who receive radiotherapy, even the most modern forms, will experience a severe decrease in their quality of life. Although in the past two decades there has been a significant amount of research on the development of a means to overcome xerostomia, current clinical strategies approved by the Food and Drug Administration (FDA) provide only temporary relief from the discomfort caused by xerostomia-related symptoms. Therefore, novel approaches such as those derived from regenerative medicine are needed. Understanding the regenerative potential of the adult salivary gland is essential for progressing our knowledge on salivary gland biology and for the development of new regenerative therapeutic strategies aimed at a long-term restoration of the damaged salivary gland. Although it has been shown that salivary glands retain, to a certain extent, the ability to regenerate, current regenerative approaches for radiation-induced salivary gland dysfunction have been curtailed by the lack of identity/knowledge of both the key cellular players and the signalling pathways regulating the tissue integrity of salivary glands.

By connecting preclinical and clinical research, the work described in this thesis shows how combining murine-injury models with both mouse and human salivary gland-derived organoid technologies can be used to identify cell sources relevant for salivary gland homeostasis and regeneration as well as the underlying regulatory mechanisms. Thereby this work aids the development of potential clinically relevant regenerative therapy approaches to treat radiation-induced hyposalivation.

Chapter 2 reflects on how over the last 30 years changes in adult stem cell definition have influenced the way salivary gland tissue integrity has been investigated and perceived. The hardwired professional stem cell paradigm based on the hematopoietic

stem cell system is introduced and by providing an overview of the available data on salivary gland stem/progenitor cells, the applicability of such a paradigm as a template for salivary gland biology (and other rapidly dividing tissues) is questioned. Next it is discussed how the stem cell function in the salivary gland can be executed by cells of different nature either under homeostasis or during repair of damaged tissue. It is speculated that more than a well-defined, rare, quiescent cell that resides at the apex of a hierarchical tree, dynamic mechanisms of plasticity within the gland epithelium might be responsible for the renewal of salivary glands. Furthermore, it is hypothesised that in order to replace lost or damaged tissue it is the environment, in terms of the signals provided by the niche, rather than cell specific surface phenotypic characteristics (such as cell surface marker expression), which shapes a stem cell-like function. Speculation is cast on how understanding the dynamic of cell-cell interactions through time and taking advantage of integrative multi-omic approaches could be a unique opportunity to unravel which pathways regulate cell-fate specification in salivary gland regeneration. Finally, how the knowledge from these integrative multi-omic approaches could open up possibilities for new therapeutic strategies to rescue radiation-induced hyposalivation is discussed.

In **Chapter 3**, the identification of the Wnt pathway as an essential component of the complex signalling network that regulates salivary gland tissue integrity during homeostasis and regeneration is described. Immunofluorescent co-staining for the general ductal epithelial marker EpCAM and the Wnt-reporter gene β-catenin was used to visualize Wnt-responsive cells within the salivary gland epithelium. EpCAM+ cells were isolated using Fluorescent Activate Cell Sorting (FACS), and subsequently cultured in Matrigel and media supplemented with Wnt3a and Rspo1, both well-known stimulators of the Wnt pathway. Only $EpCAM^{high}$ cells under Wnt stimulation generated organoid cultures capable of long-term expansion while maintaining the ability to give rise to all salivary gland cell lineages. Notably, the addition of Wnt inhibitors to EpCAM-derived organoid cultures led to a reduction in organoid formation efficiency confirming the essentiality of ectopic Wnt source in the renewal of salivary gland-derived organoids. Finally, transplantation of cells derived from Wnt-driven salivary gland derived organoids into locally irradiated mouse salivary glands was shown to rescue the radiation-induced hyposalivation phenotype to a greater extent than using the cells obtained from organoids cultured in medium devoid of Wnt.

In **Chapter 4**, evidence is provided that the Hippo pathway regulator YAP functions as a sensor of tissue integrity in response to salivary gland damage. Following severe local damage, induced *in vivo* via ligation of the salivary gland, the normally quiescent ductal compartment activates a YAP-driven tissue response characterized by high levels of nuclear YAP and increased proliferation of ductal cells in close proximity to the damage site, suggesting that YAP nuclear activity is required during regeneration. This local-injury response indicates that differentiated epithelial cells can function as a source of stem-like cells for tissue regeneration. Using a well-established salivary gland organoid culture system, chemical and genetic modulation of YAP nuclear activity was shown to impact the self-renewal ability of both mouse and human salivary gland-derived cells. Inhibition of YAP nuclear activity led to a reduction in organoid formation efficiency, while stimulation of YAP nuclear translocation increased organoid forming efficiency and promoted long-term expansion of salivary gland-derived cells. Finally, using organoids as a regenerative model, pharmacological inhibition of Mst1/2 kinases, and consequent activation of YAP nuclear activity after irradiation, was demonstrated to significantly improve the regenerative response after irradiation of human salivary gland-derived cells.

In **Chapter 5**, it is reported that autophagy is a key pathway for self-renewal activation of salivary gland organoids and that its pharmacological manipulation has the potential to promote tissue regeneration. Utilising organoid formation as a regeneration process, an increase in autophagy flux within the stem-like cell population, reflected by the ability to form secondary and tertiary organoids, is identified. Evidence that such self-renewal ability requires a constant “fuel of energy” from the autophagy machinery is provided. Furthermore, inhibition of both early and late steps of autophagy, as well as the knockout of the autophagy related gene 5 (*Atg5*), led to a significant reduction of self-renewal ability and therefore regenerative capacity of salivary gland-derived cells. Furthermore, it was shown that the levels of LC3, p62 and ATG16L1, all of which reflect autophagic activity, remain constantly low in the acinar compartment before and after injury, while they significantly increase in the normally quiescent ductal compartment in response to injury. Based on the expression of surface markers, stem-like cells ($CD24^{hi}/CD29^{hi}$) and a progenitor-like cell population ($CD24^{med/hi}/CD29^{med/hi}$) that reside in the ductal compartment were isolated and their basal autophagy activities were compared. Interestingly, the stem-like cell population showed a low autophagic flux compared to progenitor-like cells, reflecting the difference in the basal

autophagic activity of these cells *in vivo* as well as their potentially different roles during homeostasis and regeneration.

In Chapter 6, the potential mechanisms that could drive cell fate specification during human salivary gland regeneration were investigated. Using submandibular salivary gland-derived organoids as a model for regeneration and an unbiased molecular network-based analysis approach, gene activity patterns, which indicate differential cell states, were identified. Gene co-expression analysis revealed that passage 1 (P1) salivary gland-derived organoids are characterized by a transcriptomic profile that closely resembles the SOX2-driven acinar development responsible for homeostasis maintenance in the mouse sublingual gland. This transcriptomic profile is downregulated in human salivary gland-derived organoids enriched for stem cells by treatment with the GSK3 inhibitor and Wnt pathway activator, CHIR, and the histone deacetylase inhibitor, valproic acid. The co-expression gene pattern of these enriched organoids points towards the integration of several biological pathways to maintain a proliferative, non-differentiated phenotype resembling a potentially more pluripotent cell-like state. Interestingly, the high expression of peroxisome proliferator activated receptor δ (PPARδ), a nutrient sensor and enzyme involved in lipid metabolism and fatty acid oxidation (FAO), indicate a potential role for a PPARδ-driven FAO program in the formation of organoids. This was emphasised by an increased organoid forming efficiency of human salivary gland-derived cells with PPARδ overexpression. Finally, when looking at the pathways and genes active during long-term culture, it was revealed that maintenance of salivary gland organoids could be dependent on integrin levels and potentially on the activation of the *ITGA3-SRC-RAC2* axis leading to a LATS1/2-independent YAP-activation program. This gene expression pattern could be responsible for the transition from a stem-like cell state (P1) to a more proliferative transient amplifying (TA)-like state (P2-P4). The expression of the fatty acid elongase ELOVL1 in adult human salivary gland biopsies could confirm a role of metabolic pathways in salivary gland organoid self-renewal, a hypothesis which is strengthened by an increased organoid formation efficiency upon overexpression of ELOVL1. The transcriptomic information gained in this chapter will allow for the exploration and understanding of new biological mechanisms potentially involved in maintaining the balance between tissue homeostasis and regeneration in adult salivary gland.

In Chapter 7, the development of a good manufacturing practice (GMP)-compliant protocol for the isolation and the expansion of human-derived salivary gland organoids is described. This protocol is potentially suitable for an autologous cell-based therapy to regenerate the lost functionality of the tissue after radiotherapy. The proposed GMP-compliant protocol allows for the isolation and the expansion of human salivary gland-derived cells from patient biopsies maintaining an efficiency that is comparable to the current non-GMP research-based protocol. The viability and the maintained commitment towards salivary gland function displayed by the cells after cryopreservation allows this procedure to be adapted to the patient's radiotherapy treatment schedule. Finally, the cells obtained are genetically stable and displayed encouraging results when transplanted into a murine *in vivo* regenerative model for salivary gland. By placing our newly developed protocol in the context of currently available treatment options for radiation-induced hyposalivation, the strengths, limitations, and future challenges of such an approach are highlighted.

DISCUSSION AND FUTURE PERSPECTIVES

Despite the recent progress in the salivary gland regeneration field, there is still little known about the processes regulating tissue homeostasis and regeneration. The work described in this thesis focused on two main aspects. Firstly, we aimed to bridge the gap in knowledge of regenerative processes gained recently with the use of both *in vivo* and *in vitro* mouse, and human *in vitro* models. We explored the potential of human salivary gland organoids as a model of regeneration and provide future perspectives that could help the design and development of new organoid-based experimental approaches. Secondly, we aimed to utilize the human salivary gland-derived organoid system to develop a GMP-compliant protocol with the goal of taking the research from "bench to the bedside" and open up to new cell-based therapies for the long-term treatment of radiation-induced hyposalivation. Furthermore, we examined the advances made, and consider potential future developments in the field.

Modelling salivary gland regeneration using organoids

In the salivary glands, as well as other adult tissues, homeostasis is the result of a tightly controlled signalling process that maintains the balance between proliferation and differentiation. Decades of research in embryonic salivary gland development, as well as the development of genetic lineage-tracing models, have set a basis to explore homeostasis and regenerative processes in adult salivary glands. The emerging model for salivary gland maintenance, discussed in Chapter 2, is that salivary glands rely on lineage-restricted progenitor cells for homeostatic-renewal while showing an intrinsic degree of plasticity upon damage that confers a temporary stem-like state to mature differentiated and post-mitotic cells. The stemness of salivary gland cells therefore appears to be a bidirectional dynamic cell state[1,2] that is imposed on cells by the microenvironment where the cells reside through a spatial and temporal regulation[3]. This intrinsic plasticity of salivary gland cells can be recapitulated *in vitro* when exogenous Wnt and R-spondin signalling are provided (Chapter 3). The ability of Wnt-responsive cells to generate asymmetric, self-organized 3D structures (known as organoids) containing all salivary gland cell lineages (Chapter 3) recapitulates the self-organization and branching morphogenesis of salivary gland epithelial cells[4]. This *in vitro* behaviour indicates a context-dependent functionality of salivary gland-derived cells that can potentially be adopted in response to changes within the microenvironment to fulfil regenerative purposes. It appears therefore that, similar to

what has been shown for intestinal organoids, salivary gland-derived organoid formation recapitulates a cycle of repair and homeostasis typical of the regeneration process[5,6] and therefore can be used to model salivary gland regeneration. While the derivation of certain organoids (such as those derived from pluripotent stem cells) relies uniquely on endogenous signalling, it is demonstrated in Chapter 3 that salivary gland-derived organoids require specific exogenous signals from the niche to drive a first cell expansion phase. This is then followed by a second self-patterning and morphogenetic rearrangement phase that leads to the final organoid architecture, similar to what has been shown in intestinal organoids[7]. While it is shown that Wnt stimulation is fundamental to orchestrate the generation of "mature", self-organized organoids, it is established in Chapter 4 that the activation of nuclear YAP rather than Wnt seems to be the signal required to drive the first phase in both salivary gland regeneration response upon injury *in vivo* and salivary gland-derived organoid formation. Sensing morphogens (such as Wnt) and tissue integrity via cellular mechanosensors (such as YAP) are two of the many ways a cell can sense the microenvironment. In the last decade, a growing body of evidence has highlighted how the central dogma of molecular biology, from DNA to protein, has evolved and how cell metabolism, before thought to be a passive player, has gained a central role in regulating cell fate decision[6,8,9]. The results obtained in Chapter 5 and Chapter 6, shed light on the importance of "metabolic reprogramming" and the energy substrates provided by the environment, such as fatty acids and long chain fatty acids, in regulating salivary gland-derived cell self-renewal ability (Chapter 6). This, together with the role of the autophagy pathway described in Chapter 5, highlights the importance of metabolic plasticity, as well as catabolic processes, in choosing and maintaining the appropriate metabolite levels to support the bioenergetic needs required by specific cells, such as those able to exert plasticity to respond to changes in homeostasis.

While the "deconstructive approach" taken in Chapters 3, 4 and 5 aimed at dissecting the niche signalling has led to the recognition of Wnt, YAP and autophagy as important key players during the regeneration process and potential targets for regenerative-based therapy, individual elements of a much bigger picture were studied in isolation. Starting from the assumption of Aristotle that "the whole is greater than the sum of its parts", the complexity of the salivary gland regenerative process itself cannot be completely explained by the sum of each single pathway without taking into

consideration the possible interplay between them, as well as the cell variability generated through cell collective behaviour (cell-cell interactions and feedback loops). The cellular state enrichment approach, combined with a co-expression gene analysis described in Chapter 6, moves towards this direction: understanding the system as a whole instead of reporting a list of individual parts, providing an unbiased, broad overview of the complexity of the regulatory network responsible for human salivary gland organoid formation and expansion. Chapter 6 shows how exposure to different environments (different morphogens in this case) increases the propensity of a cell towards a specific organoid state. This indicates that the initial conditions, in terms of morphogens, extrinsic physical cues and metabolic flux, to which the single cells are subjected affect the final characteristics of the organoids and, as a direct consequence, their range of applicability. The multicellular dynamics interaction which governs salivary gland regeneration processes is perhaps "the elephant in the room". Therefore, how can the transient spatiotemporal activation of these factors, which cells sense and dynamically respond to in order to orchestrate the regenerative response, be mapped? Considering the interplay between YAP with Wnt and YAP and autophagy[10], can YAP therefore be the hub for integration of other signalling pathways within the salivary gland regeneration process and thus responsible for the switch between the regenerative and homeostatic state?

To answer these questions an approach similar to the one recently taken in intestinal organoids[5,6] and new born skin organoids[11] could pave the way to unravel the molecular mechanisms and the morphological dynamics of salivary gland organoid formation and potentially open up new regenerative strategies. Using technologies that allow resolution of the transcriptome (single cell sequencing) and the proteome (image-based technology) at a single cell level and taking advantage of computational modelling to integrate spatiotemporal information of cells within its "society" will allow researchers to unravel cell-fate decisions of salivary gland cells and the pathways by which they are specified. Finally, it would be interesting to validate if the knowledge gained from the proposed approach could be used to manipulate the *in vivo* phases of salivary gland tissue regeneration, as well as to answer the remaining unanswered questions in the salivary gland field. What are the sources of the morphogens required for epithelial tissue maintenance and their plasticity activation upon (radiation-induced) injury? Can we extend the concept of plasticity to the salivary gland niche? Or is it the loss of niche plasticity upon damage that impedes (or drastically reduces) the

activation of mechanisms responsible in guiding epithelial plasticity and therefore affect regeneration?

While the time may be ripe for the salivary gland (organoids) field to embrace this approach, some challenging points remain. 1) The scarcity of reliable landmark genes that identify specific cell types within salivary gland-derived organoids will potentially pose an issue in mapping single cell RNA sequencing (scRNAseq)-derived transcriptomic data onto spatial reference maps[12].This step is fundamental to maintain the spatial context of the single cells in terms of local environment and cell-to-cell interactions, that otherwise would be lost considering that scRNAseq approaches require the dissociation of organoids to single cells. Combining scRNAseq with laser microdissection[13] or the use of photoactivable reporter in combination with scRNAseq[14] could potentially allow the identification of a salivary gland landmark gene landscape. Once obtained, the use of multiplex cell approaches that combine scRNAseq data with sequential high-throughput imaging techniques and segmentation algorithm, will allow an understanding of how gene expression and the subcellular distribution of the proteome are responsible for cell fate determination[6,15,16]. 2) The phenotypic variability of salivary gland organoid culture could pose an issue in terms of reproducibility of spatial organization of landmark genes. 3) Beside the transcriptome, proteome and metabolome, there is increased evidence highlighting how perturbation of the microenvironment can lead to changes at the chromatin level and how these changes could be responsible for the transcriptomic switches that determine cell fate decisions[9]. Therefore, epigenetic marks, such as DNA methylation or histone modifications, should be traced through space and time, and integrated within the multiscale approach. 4) While organoids have already reached a certain degree of complexity, both in terms of cell heterogeneity and spatial self-organization, when compared to the complex macro physiology of their tissue counterparts, they lack important aspects such as a vasculature system, a parasympathetic nervous system and an immune system. Recent advances have enabled the vascularisation of liver[17] organoids, which improved their maturation and graft survival upon transplantation. Furthermore, the co-culturing of organoids with immune cells possessing the potential to recapitulate the tumour immune microenvironment has opened up new avenues for immuno-oncology and auto-immune disease research[18]. However, the degree of complexity of

the organoid system should be chosen and weighted based on the research question at hand.

Resolving the salivary gland organoid formation process and its spatiotemporal dynamics as a whole will offer unprecedented opportunities to map the regeneration process and potentially establish a blueprint for targeted control of cell fate. Can we find a “switch point” within the self-organization process that can be targeted, or stabilized to increase the regeneration potential? In this way, we could use intrinsic cellular properties to develop drugs that will not be targeting a single specific gene, but rather complex multicellular processes, such as self-organization as a means that leads to a specific cellular state and not to a cell type.

Salivary gland regeneration: the road ahead

As the population rapidly ages, the natural decay of tissues and organs together with an increased propensity for infection and cancer have placed the need for developing regenerative medicine strategies as high priority. Head and neck cancers affect worldwide 500,000 patients annually, with 40% of the patients receiving radiation treatment facing “the challenges of living beyond”: the consequences of the radiotherapy treatment and the chronic and progressive decline of salivary gland function, ultimately leading to the onset of xerostomia[19]. Despite increased investments in cell-based preclinical research and clinical trials for xerostomia treatment, the number of proven therapies is relatively small (7%)[20].

Clinical trials for salivary gland regenerative therapies, based on the replacement of lost epithelial tissue with exogenous cells may need to consider three main points: 1) a large-scale production of salivary gland committed cells, 2) a strategy to increase the survival and the engraftment of the transplanted cells, and 3) a means to induce proliferation of transplanted cells *in vivo*. In Chapter 7, we have described the potential of a GMP-compliant protocol for the isolation and the expansion of human salivary gland organoid-derived cells to be used in the treatment of radiation-induced hyposalivation. Heterologous transplantation of human salivary gland organoid-derived cells has proven the potential, safety, and beneficial effect of this approach in *in vivo* pre-clinical studies providing significant rescue of saliva production compared to irradiated non-transplanted control animals[21]. The preserved functionality and the commitment towards salivary gland mature lineage of cryopreserved human salivary gland-derived cells, as well as their genetic stability, make the proposed GMP-compliant protocol a suitable, and currently unique, autologous organoid-based

treatment for xerostomia. Currently among internationally registered clinical trials there are none which focus on the potential of adult salivary gland tissue-resident stem cells. The formulation of a robust mechanistic hypothesis (such as cell replacement and/or paracrine effects of the transplanted cells) and the selection of the most appropriate cell source are only the first two major steps to ensure the success of future clinical trials of salivary gland cell-based regenerative therapies. The first one strongly influencing the second one and therefore the design of the trial itself.

Establishing well-defined standards for *ex vivo* processing that guarantee the quality, reproducibility, and potency of the cell source, required for international regulation and implementation[22] is essential for any proposed cell-based regenerative therapy. In Chapter 2, we have documented the growing evidence that plasticity mechanisms, rather than a single defined cell entity, are potentially responsible for salivary gland renewal. Therefore, instead of looking for potential predictive stem cell surface markers as identifier for salivary gland stem-like cells, we should look at the response patterns that characterize the newly acquired stem-like cell state (or regenerative state), and which are thus able to drive at first a regenerative response and subsequently reinstating tissue homeostasis[5,6]. The transcriptomic approach conducted in Chapter 6 suggests that cells giving rise to P1 organoids could possess these characteristics and therefore could be used as potential cell source for the Phase I/II clinical trial planned to be performed at the University Medical Center Groningen (UMCG). In this way the source of cells for clinical application could be chosen based on their therapeutic (or regenerative) potential rather than on a single characteristic, such as the presence of a surface marker. A surface marker-based selection could lead to “false positives and false negatives” in the identification of optimal cell source candidates since different cells within the tissue with diverse potentials could present the same surface markers.

Besides understanding the signals which regulate cell fate specification to allow for the selection and expansion of the optimal cell source for transplantation, it is also necessary to understand how radiation can affect the recipient tissue. An increased understanding of how the recipient environment is altered upon injury could have an impact on the success of any transplantation, both in terms of promoting endogenous cell repair and efficient engraftment of transplanted cells. Cellular senescence has recently been suggested as a contributor to the development of radiation-induced hyposalivation[23], as well as an early feature of primary Sjogren’s syndrome[24], an

autoimmune disease that causes dry mouth. It remains unclear as to whether cellular senescence is the driver of the pathology or solely a consequence of radiation-induced DNA damage. However, the presence of senescent cells within the acinar[23] and ductal compartments[24,25], as well as their SASP expression profile, could be responsible for the deterioration of the microenvironment[26] also in irradiated salivary glands. This, in turn, could potentially not only lead to an impaired endogenous regeneration response of salivary glands, but also create a hostile microenvironment for the engraftment and proliferation of transplanted cells. Recently, studies on aged mouse models showed that the genetic and pharmacological removal of senescent cells significantly improved the age-related phenotype[27] and mitigated the radiation-induced senescent phenotype in hematopoietic stem cells[28] and in salivary glands[25] . Thus, there is increasing numbers of early phase clinical trials addressing safety, target engagement and efficacy of senolytics in disease treatment. Furthermore, the improved secretory functionality of irradiated glands upon senolytic treatment[25] could open up to the use of senolytics

as a “priming” (or preconditioning) strategy to improve the engraftment potential of transplanted cells and consequent repopulation of the salivary gland epithelium. Moreover, the evidence showing that YAP functions as a sensor of salivary gland tissue integrity *in vivo* and promotes the initiation of a regenerative response similar to what has been described in intestinal organoids, reported in Chapter 4[6], may provide new potential targets within the Hippo signaling cascade to promote proliferation of transplanted cells.

At the point of diagnosis and treatment, head and neck cancer patients are often of a progressed age and thus may already exhibit underlying xerostomia due to age-related microenvironmental changes. The additive effect of age-induced xerostomia and radiation-induced xerostomia could further inhibit the repair ability of salivary gland. While the success of mouse transplantation of salivary gland organoid-derived cells from young donors into young recipients (described in Chapter 3) is relevant as a proof of principle and emphasizes the potential of such an approach, the age of patients necessitates proof of successful applicability in aged mouse models. Transplantation of salivary gland organoid-derived cells from old mice into young recipient mice showed that the young microenvironment, although injured, sustained the engraftment and proliferation of old-transplanted salivary gland-derived cells[29]. Future experiments should include transplantation of patient-derived cells into aged

irradiated salivary glands in order to highlight potential differences, pitfall and variables in response to transplantation that could be crucial for the development and the improvement of new cell-based regenerative strategies for older patients.

The bench-to-bedside approach proposed here will benefit from a combined reverse translational research approach (bedside-to-bench approach). Currently the primary endpoints to describe/quantify the success of transplanted salivary gland-derived cells in treating radiation-induced hyposalivation include increased saliva production. The post-mortem analysis of the transplanted gland via scRNA seq could be used to identify predictive markers of successful graft outcome. Such markers and the involved cues could be used to improve the *in vitro* generation and selection of salivary gland-derived organoids with an increased capacity for engraftment, which subsequently could increase the efficiency of the transplant and the success of a clinical trial. Furthermore, the use of imaging technique such as magnetic resonance imaging (MRI) or positron emission tomography/computed tomography (PET/CT) to map the irradiated gland in combination with labelling and tracing of transplanted cells, for example with superparamagnetic iron oxide nanoparticles[30,31] or prostate specific membrane antigen ligand[32,33], could help to monitor in real time the behavior of the transplanted cells. The identification of a migratory path, as well as a homing preference, of the transplanted cells could be a unique opportunity to detect a receptive niche within the irradiated gland that may be better suitable for the transplanted cells. The characterization of the molecular signature of these regions could pave the way to the discovery of biomarkers that could be used to predict the responsiveness of the tissue and therefore the patient transplantation outcome. It should be kept in mind that perhaps the presence of transplanted cells itself could not be enough to produce a functional therapeutic outcome, but that a receptive environment should also be present.

While the GMP protocol, developed in Chapter 7, will be used to test the safety and feasibility of salivary gland organoid-derived cells in a clinical setting, it is necessary to keep in mind that other mechanistic hypotheses beyond long-term cell replacement, such as the recently suggested paracrine effect of transplanted salivary gland-derived cells[21,34,35] could be (co)-responsible for tissue regeneration. This hypothesis will lead to new potential therapeutic approaches and to clinical trials aimed at leveraging the effect of exogenously supplied cells to initiate the activation of endogenous regeneration responses of surviving cells (if still present) that will ultimately lead to

improved functionality of the tissue. Consequently, different standards for the characterization of the cell source should be considered. In contrast to the selection of cells for long-term replacement strategies based on the self-renewal and the regenerative cellular state that support appropriate cell fate decisions, the focus of choosing cells for paracrine stimulation should be based on the secretory profiles of the cells. Therefore, characterization of the secretome of human salivary gland organoids by stable isotope-labeling with amino acids in cell culture-coupled mass spectrometry (SILAC-MS) would potentially provide insights in the secreted factors that are likely to contribute to the regeneration process and organoid formation. Furthermore, the targets of the secreted factors should be considered. Are the secreted factors targeted towards epithelial cells or other components of the salivary gland niche? Do they contribute to the induction of intrinsic cell plasticity of salivary gland epithelial cells or are they acting on the reactivation of the receptive niche?

We could therefore speculate that the “best” therapeutic options to rescue radiation-induced hyposalivation would be a combination of priming strategies to allow better engraftment, transplantation of cells with regenerative properties for long term tissue replacement and transplantation of cells with paracrine stimulatory functions that could support both the endogenous receptive niche and the transplanted cells for an enhanced and prolonged regeneration and subsequent functionality.

REFERENCES

1 Ninche, N., Kwak, M. & Ghazizadeh, S. Diverse epithelial cell populations contribute to the regeneration of secretory units in injured salivary glands. *Development* **147**, doi:10.1242/dev.192807 (2020).

2 Weng, P. L., Aure, M. H., Maruyama, T. & Ovitt, C. E. Limited Regeneration of Adult Salivary Glands after Severe Injury Involves Cellular Plasticity. *Cell Rep* **24**, 1464-1470 e1463, doi:10.1016/j.celrep.2018.07.016 (2018).

3 Chacon-Martinez, C. A., Koester, J. & Wickstrom, S. A. Signaling in the stem cell niche: regulating cell fate, function and plasticity. *Development* **145**, doi:10.1242/dev.165399 (2018).

4 Wei, C., Larsen, M., Hoffman, M. P. & Yamada, K. M. Self-organization and branching morphogenesis of primary salivary epithelial cells. *Tissue Eng* **13**, 721-735, doi:10.1089/ten.2006.0123 (2007).

5 Lukonin, I., Serra, D., Challet Meylan, L., Volkmann, K., Baaten, J., Zhao, R. *et al.* Phenotypic landscape of intestinal organoid regeneration. *Nature* **586**, 275-280, doi:10.1038/s41586-020-2776-9 (2020).

6 Serra, D., Mayr, U., Boni, A., Lukonin, I., Rempfler, M., Challet Meylan, L. *et al.* Self-organization and symmetry breaking in intestinal organoid development. *Nature* **569**, 66-72, doi:10.1038/s41586-019-1146-y (2019).

7 Sasai, Y. Cytosystems dynamics in self-organization of tissue architecture. *Nature* **493**, 318-326, doi:10.1038/nature11859 (2013).

8 Ito, K., Carracedo, A., Weiss, D., Arai, F., Ala, U., Avigan, D. E. *et al.* A PML-PPAR-delta pathway for fatty acid oxidation regulates hematopoietic stem cell maintenance. *Nat Med* **18**, 1350-1358, doi:10.1038/nm.2882 (2012).

9 Ly, C. H., Lynch, G. S. & Ryall, J. G. A Metabolic Roadmap for Somatic Stem Cell Fate. *Cell Metab* **31**, 1052-1067, doi:10.1016/j.cmet.2020.04.022 (2020).

10 Totaro, A., Zhuang, Q., Panciera, T., Battilana, G., Azzolin, L., Brumana, G. *et al.* Cell phenotypic plasticity requires autophagic flux driven by YAP/TAZ mechanotransduction. *Proc Natl Acad Sci U S A* **116**, 17848-17857, doi:10.1073/pnas.1908228116 (2019).

11 Lei, M., Schumacher, L. J., Lai, Y. C., Juan, W. T., Yeh, C. Y., Wu, P. *et al.* Self-organization process in newborn skin organoid formation inspires strategy to restore hair regeneration of adult cells. *Proc Natl Acad Sci U S A* **114**, E7101-E7110, doi:10.1073/pnas.1700475114 (2017).

12 Mayr, U., Serra, D. & Liberali, P. Exploring single cells in space and time during tissue development, homeostasis and regeneration. *Development* **146**, doi:10.1242/dev.176727 (2019).

13 Moor, A. E., Harnik, Y., Ben-Moshe, S., Massasa, E. E., Rozenberg, M., Eilam, R. *et al.* Spatial Reconstruction of Single Enterocytes Uncovers Broad Zonation along the Intestinal Villus Axis. *Cell* **175**, 1156-1167 e1115, doi:10.1016/j.cell.2018.08.063 (2018).

14 Medaglia, C., Giladi, A., Stoler-Barak, L., De Giovanni, M., Salame, T. M., Biram, A. *et al.* Spatial reconstruction of immune niches by combining photoactivatable reporters and scRNA-seq. *Science* **358**, 1622-1626, doi:10.1126/science.aao4277 (2017).

15 Gut, G., Herrmann, M. D. & Pelkmans, L. Multiplexed protein maps link subcellular organization to cellular states. *Science* **361**, doi:10.1126/science.aar7042 (2018).

16 Zhu, Q., Shah, S., Dries, R., Cai, L. & Yuan, G. C. Identification of spatially associated subpopulations by combining scRNAseq and sequential fluorescence in situ hybridization data. *Nat Biotechnol*, doi:10.1038/nbt.4260 (2018).
17 Takebe, T., Sekine, K., Enomura, M., Koike, H., Kimura, M., Ogaeri, T. *et al.* Vascularized and functional human liver from an iPSC-derived organ bud transplant. *Nature* **499**, 481-484, doi:10.1038/nature12271 (2013).
18 Yuki, K., Cheng, N., Nakano, M. & Kuo, C. J. Organoid Models of Tumor Immunology. *Trends Immunol* **41**, 652-664, doi:10.1016/j.it.2020.06.010 (2020).
19 Tsai, W. L., Huang, T. L., Liao, K. C., Chuang, H. C., Lin, Y. T., Lee, T. F. *et al.* Impact of late toxicities on quality of life for survivors of nasopharyngeal carcinoma. *BMC Cancer* **14**, 856, doi:10.1186/1471-2407-14-856 (2014).
20 Rocchi, C. & Emmerson, E. Mouth-Watering Results: Clinical Need, Current Approaches, and Future Directions for Salivary Gland Regeneration. *Trends Mol Med* **26**, 649-669, doi:10.1016/j.molmed.2020.03.009 (2020).
21 Pringle, S., Maimets, M., van der Zwaag, M., Stokman, M. A., van Gosliga, D., Zwart, E. *et al.* Human Salivary Gland Stem Cells Functionally Restore Radiation Damaged Salivary Glands. *Stem Cells* **34**, 640-652, doi:10.1002/stem.2278 (2016).
22 Pellegrini, G., Rama, P., Matuska, S., Lambiase, A., Bonini, S., Pocobelli, A. *et al.* Biological parameters determining the clinical outcome of autologous cultures of limbal stem cells. *Regen Med* **8**, 553-567, doi:10.2217/rme.13.43 (2013).
23 Marmary, Y., Adar, R., Gaska, S., Wygoda, A., Maly, A., Cohen, J. *et al.* Radiation-Induced Loss of Salivary Gland Function Is Driven by Cellular Senescence and Prevented by IL6 Modulation. *Cancer Res* **76**, 1170-1180, doi:10.1158/0008-5472.CAN-15-1671 (2016).
24 Wang, X., Bootsma, H., Terpstra, J., Vissink, A., van der Vegt, B., Spijkervet, F. K. L. *et al.* Progenitor cell niche senescence reflects pathology of the parotid salivary gland in primary Sjogren's syndrome. *Rheumatology (Oxford)* **59**, 3003-3013, doi:10.1093/rheumatology/keaa012 (2020).
25 Peng, X., Wu, Y., Brouwer, U., van Vliet, T., Wang, B., Demaria, M. *et al.* Cellular senescence contributes to radiation-induced hyposalivation by affecting the stem/progenitor cell niche. *Cell Death Dis* **11**, 854, doi:10.1038/s41419-020-03074-9 (2020).
26 Ferreira-Gonzalez, S., Lu, W. Y., Raven, A., Dwyer, B., Man, T. Y., O'Duibhir, E. *et al.* Paracrine cellular senescence exacerbates biliary injury and impairs regeneration. *Nat Commun* **9**, 1020, doi:10.1038/s41467-018-03299-5 (2018).
27 Xu, M., Pirtskhalava, T., Farr, J. N., Weigand, B. M., Palmer, A. K., Weivoda, M. M. *et al.* Senolytics improve physical function and increase lifespan in old age. *Nat Med* **24**, 1246-1256, doi:10.1038/s41591-018-0092-9 (2018).
28 Chang, J., Wang, Y., Shao, L., Laberge, R. M., Demaria, M., Campisi, J. *et al.* Clearance of senescent cells by ABT263 rejuvenates aged hematopoietic stem cells in mice. *Nat Med* **22**, 78-83, doi:10.1038/nm.4010 (2016).
29 Maimets, M., Rocchi, C., Bron, R., Pringle, S., Kuipers, J., Giepmans, B. N. *et al.* Long-Term In Vitro Expansion of Salivary Gland Stem Cells Driven by Wnt Signals. *Stem Cell Reports* **6**, 150-162, doi:10.1016/j.stemcr.2015.11.009 (2016).
30 de Vries, I. J., Lesterhuis, W. J., Barentsz, J. O., Verdijk, P., van Krieken, J. H., Boerman, O. C. *et al.* Magnetic resonance tracking of dendritic cells in melanoma

patients for monitoring of cellular therapy. *Nat Biotechnol* **23**, 1407-1413, doi:10.1038/nbt1154 (2005).
31 Hartman, R. E., Nathan, N. H., Ghosh, N., Pernia, C. D., Law, J., Nuryyev, R. *et al.* A Biomarker for Predicting Responsiveness to Stem Cell Therapy Based on Mechanism-of-Action: Evidence from Cerebral Injury. *Cell Rep* **31**, 107622, doi:10.1016/j.celrep.2020.107622 (2020).
32 Klein Nulent, T. J. W., Valstar, M. H., de Keizer, B., Willems, S. M., Smit, L. A., Al-Mamgani, A. *et al.* Physiologic distribution of PSMA-ligand in salivary glands and seromucous glands of the head and neck on PET/CT. *Oral Surg Oral Med Oral Pathol Oral Radiol* **125**, 478-486, doi:10.1016/j.oooo.2018.01.011 (2018).
33 Valstar, M. H., de Bakker, B. S., Steenbakkers, R., de Jong, K. H., Smit, L. A., Klein Nulent, T. J. W. *et al.* The tubarial salivary glands: A potential new organ at risk for radiotherapy. *Radiother Oncol*, doi:10.1016/j.radonc.2020.09.034 (2020).
34 Gronhoj, C., Jensen, D. H., Glovinski, P. V., Jensen, S. B., Bardow, A., Oliveri, R. S. *et al.* First-in-man mesenchymal stem cells for radiation-induced xerostomia (MESRIX): study protocol for a randomized controlled trial. *Trials* **18**, 108, doi:10.1186/s13063-017-1856-0 (2017).
35 Gronhoj, C., Jensen, D. H., Vester-Glowinski, P., Jensen, S. B., Bardow, A., Oliveri, R. S. *et al.* Safety and Efficacy of Mesenchymal Stem Cells for Radiation-Induced Xerostomia: A Randomized, Placebo-Controlled Phase 1/2 Trial (MESRIX). *Int J Radiat Oncol Biol Phys* **101**, 581-592, doi:10.1016/j.ijrobp.2018.02.034 (2018).