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Salivary gland stem cells

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Chapter

7

Summary and future perspectives

1. SUMMARY

Salivary glands of head and neck cancer patients undergoing radiotherapy are often in the field of irradiation, which is often unavoidable. As a result these patients often suffer from symptoms related to salivary dysfunction. This radiation damage persists life-long and thus, impairs the function of salivary gland irreversibly. Currently no adequate treatments are available to restore radiation-damaged salivary glands. Stem cell therapy is one alternate to rescue these patients from hyposalivation. To achieve this, first, stem cells of salivary glands need to be identified and characterized. In this thesis, we studied the rodent salivary glands with the aim of identifying stem cells, develop assays to characterize them, and unravel their potential to rescue hyposalivation.

1.1. Identification of putative stem cells of salivary gland

In most tissues, stem cells are distinguished from differentiated cells by their quiescent nature, ability to self-renew, differentiate and localized in a specialized niche. Adult stem cells can be identified using several approaches, characteristic and unique for each specific tissue type. In a tissue with extensive proliferation like skin and bone marrow, non-proliferative/quiescent stem cells can be distinguished by their ability to retain DNA/membrane specific label, whereas this label is diluted upon cell division in proliferating cells¹. For the mouse intestine two different stem cells have been suggested, being Lgr5⁺ at the bottom of the crypt² and Bmi1⁺ cells³ at position four of the crypt. Later, Yan Kelley et al 2012³ confirmed using label-retaining strategy that Lgr5⁺ cells marks mitotically active, Wnt sensitive intestinal stem cells (ISC's) whereas Bmi1⁺ cells marks the quiescent stem cells that are insensitive to Wnt signals, with concomitant resistance to high-dose of radiation. In other tissues, like mammary gland and prostate gland, a cell surface marker based approach was used to identify candidate stem cells revealing that CD24, CD29 and/or CD49f positive cell population contain stem cells^{4, 5}.

In this thesis, a surface marker based approach was implemented for the identification of murine salivary gland stem cells. A previous study⁶ has shown that salivary gland stem/progenitor cells can be cultured into salispheres (primary spheres). This study suggested that salisphere derived c-Kit-expressing cells are potential candidate stem cells due to their ability to rescue hyposalivation to some extent.

In **Chapter 2** we tested whether some known glandular stem cell markers like c-Kit, CD24, CD133 and CD49f expressing cells possess salivary gland stem/progenitor characteristics.

In this chapter, it is described that culturing salivary gland cells into primary spheres enriches for cells expressing these stem cell markers. In order to identify the most potent stem cell, c-Kit⁺, CD24⁺/CD29⁺, CD133⁺ and CD49f⁺ putative stem cells needed to be tested for their stemness. However, the lack of *in vitro* or *in vivo* assays to test functionality of the putative stem cells delayed the progress in salivary gland stem cell identification in general. Therefore, as a first step, to test putative stem cell candidates we have optimized the *in vivo* model of radiation-induced hyposalivation, which had been developed earlier⁶. In this model, salivary glands of mice are irradiated using X-rays (15Gy) inducing hyposalivation to mimic the situation in patients. One month later, GFP-donor derived putative stem cell populations were transplanted via intra-glandular injection. Pre-irradiation and 1, 2 and 3 months post-transplantation saliva flow was measured as a functional read-out of transplantation, and was assumed to reflect tissue regeneration. In **Chapter 2**, to identify the regenerative potential of murine primary sphere-derived CD24⁺, CD133⁺, CD49f⁺ and CD24⁺/CD29⁺ cells, they were transplanted into the irradiated salivary glands of mice. Saliva measurement post-transplantation revealed that mice transplanted with 10,000 CD24⁺/CD29⁺ cells showed an improved salivary gland function compared to the irradiated non-transplanted mice and mice that were transplanted with 10,000 CD133⁺ and 134,000 CD49f⁺ cells. The fact that only 10,000 CD24⁺/CD29⁺ cells were able to resolve hyposalivation suggests the existence of potent stem cells in this population. Moreover, it is unlikely that CD133⁺ and CD49f⁺ cells contain stem cells in reasonable numbers, since transplantation of either similar number of CD133⁺ cells or higher numbers CD49f⁺ cells did not sufficiently recover hyposalivation.

1.2. Stem cell transplantation

The primary goal of stem cell transplantation is to restore the function of a damaged tissue by adding new differentiated/functional cells and/or cells that can produce sufficient numbers of functional cells over an extended period of time. Maintenance of epithelial cells in a healthy tissue is dependent on the neighboring endothelial cells, blood vessels, neuronal innervation, and supply of nutrients and growth factors necessary for communication between different cells. Radiation influences many of these, thereby creating a disturbance in the homeostasis and to functional deterioration of the organ itself⁷. Hence, during stem cell transplantation, it is important to evaluate the effect of stem cell transplantation on the recipient tissue's homeostasis. Therefore, in **Chapter 3** the effect of transplantation on salivary gland homeostasis was investigated.

Earlier reports showed that stem cell transplantation into irradiation-damaged salivary glands rescues functional acinar tissue⁶. First we confirmed that transplantation of either c-Kit⁺ cells, or subpopulations of c-kit⁺ cells can recover radiation-induced hyposalivation. Moreover, further analysis of the general morphology showed the formation of new donor-derived duct cells with the restoration of the number of stem cell marker expressing cells indicative of potential recovery of glandular homeostasis. Interestingly, post-transplantation murine salivary glands showed less fibrosis, less inflammation and a normalized vasculature all indicative of a more healthy tissue⁸. These results support the fact that stem cells recover tissue homeostasis necessary for the long-term functional recovery and maintenance of the damaged tissue. At this moment, it remains unclear whether the transplanted stem cells are solely responsible for this effect or that cytokine signals (released by stem cell or accessory cells) induce endogenous stimulation of homeostasis regulatory pathways. Preliminary data point to the latter.

So far we have shown that using an *in vivo* mouse model of radiation-induced hyposalivation, putative stem cell candidates can be tested for their regeneration potential (Chapter 2). Further transplanted tissues can be analyzed (Chapter 3) for the extent of recovery obtained. However, this procedure involves large numbers of animals and takes months. Also, it does not show extended self-renewal, necessary to determine the long-term regenerative potential of the transplanted tissue. Short-term *in vitro* assays that can screen a library of conditions would be instrumental to accelerate the identification of adult stem cells, assess self-renewal potential and induce expansion.

1.3. Methods to screen stem candidates *in vitro*

In the field of stem cell biology, the gold standard test for the identification of stem cells is to test for their regenerative/repair potential *in vivo*. However, as mentioned above this has certain constraints. Hence, in this thesis we also aimed to develop short-term *in vitro* assays that could provide quantitative information on a test population of cells, and would enable pre-screening of candidates before proceeding *in vivo*. This way one of the 3R's – reduction in number of animals, according to the ethics of animal experiments can be very well satisfied.

In **Chapter 4**, we demonstrated self-renewal and differentiation of putative stem cells using *in vitro* assays. First, we isolated single cells from primary spheres and seeded them into matrigel-based matrix. This allowed the assessment of single cell self-renewal potential measured as ability to form secondary spheres. Our results showed that CD24⁺ and CD49f⁺ cells

had a higher sphere-forming potential when compared to unfractionated, CD133⁺ and c-Kit⁺ cells. Next, we dissociated the secondary spheres into single cells and serial re-plated them to form tertiary, quaternary, etc. spheres. This procedure allows quantification of and selection for sphere-formation ability as a measure of self-renewal potential. Using this assay, cells expressing c-Kit⁺, CD24⁺, CD133⁺ and CD49f⁺ could be kept in self-renewing conditions for >5-passages. The CD24⁺ and CD49f⁺ cells showed higher-sphere forming potential. This suggests that the population of cells expressing CD24 and CD49f contain more cells capable of self-renewing stem/progenitor cells of salivary gland. Further testing of CD24⁺ and CD49f⁺ cells for differentiation potential might characterize these cells better.

To maintain/repair a damaged tissue, adult stem cells need to be able to differentiate into all cell types of the tissue of origin. Therefore, to investigate the differentiation potential of putative stem cells, we have seeded single cell-derived secondary spheres under differentiation-inducing conditions *in vitro* (Chapter 4). This method required substantial optimization, but finally we were able to obtain structures resembling salivary gland morphology. It was shown that spheres can undergo tremendous morphological changes from a spheroid shape to 3-dimensional structures, generally referred to as organoids (or mini-glands) that are made up of either long tubular extensions (ductal organoids) or compact lobule like structures (lobular organoids). Confocal imaging, immunohistochemical and gene-expression analysis on these organoids showed the existence of differentiated salivary gland cells from all lineages. Different cell populations were characterized for their potential to form organoids. Among the tested candidates, unfractionated cells and CD24⁺ cells showed relatively higher organoid-forming (or differentiation) ability. This suggests that unfractionated and CD24⁺ cells contain stem/progenitor cells that are capable of differentiating into salivary gland cell types, and may therefore be considered suitable candidates to test *in vivo* for their ability to can recover hyposalivation.

In Chapter 2, we showed that among stem cell candidates tested *in vivo*, c-Kit⁺ and CD133⁺ cells showed some functional recovery. CD24⁺ cells showed significant higher recovery when combined with CD29, whereas CD49f⁺ cells did not show any recovery. Using the same candidates from Chapter 2, here we tested whether the *in vitro* assays from Chapter 4 will identify similar differences between the candidate stem cells.

In vitro experiments revealed that CD49f⁺ cells although possessing some self-renewal, failed to show significant differentiation (Fig.6, Chapter 4), which is in agreement with *in vivo* study that these cells could not differentiate into acinar cells *in vivo* thereby not showing any

functional recovery (Fig.4, Chapter 2). Moreover, CD24⁺ cells were able to both self-renew and differentiate, characteristic of stem cells (Fig.6, Chapter 4). This is in line with the *in vivo* data indicating significant recovery when co-selected with another marker being CD29 (Fig.4, Chapter 2). These results suggest that, *in vitro* assays developed in this chapter can be used to measure the stem cell activity of individual cells from their ability to self-renew and differentiate. This striking outcome related *in vivo* results with the *in vitro* studies, thereby confirming that *in vitro* assays described in Chapter 4, may help us to better understand and characterize a particular population of putative salivary gland stem cells.

In conclusion, the assays described in this thesis not only provide a quick, reliable screening method for stem cell candidates, but also open wide applications (see future perspectives) for deeper understanding of salivary gland stem cells. Therefore, the development of these short-term *in vitro* assays being confirmable with *in vivo* regeneration assays can be considered breakthrough in the field salivary gland stem cell biology.

1.4. Expansion of adult stem/progenitor cells

Since CD24 was identified as a potential stem cell marker in Chapter 4, we utilized its expression in combination with CD29, in analogy with the mammary gland, to search for even more potent populations. Though CD24/CD29 positive cells seem to contain stem-like cells (Chapter 2), it is unlikely that all these cells are stem cells of the gland warranting enrichment. Therefore, in **Chapter 5**, using flow cytometry analysis we were able to identify four subsets within CD24/CD29 cell population namely CD24^{hi}/CD29^{lo}, CD24^{hi}/CD29^{hi}, CD24^{med}/CD29^{hi} and CD24^{lo}/CD29^{hi} cells. These subsets are not as easily distinguishable as in mammary gland in flow cytometric analysis. Therefore we used an arbitrary gating-strategy to isolate these cells as separate subsets.

Once isolated as single cells, the four subsets were tested using the *in vitro* assays described in Chapter 4, for their self-renewal and differentiation potential (Chapter 5). The results showed that CD24^{hi}/CD29^{hi} cells have a significantly higher secondary sphere-forming ability ($2.5 \pm 0.68\%$), and were able to self-renew for >5-passages, whereas CD24^{med}/CD29^{hi} cells could not be maintained for more than 4-passages. Interestingly, CD24^{med}/CD29^{hi} cells were able to form only ductal organoids, whereas CD24^{hi}/CD29^{hi} cells formed organoids containing both duct and acinar cells, indicating their multi-lineage differentiation potential. These results established CD24^{hi}/CD29^{hi} cells as the population containing the most potent stem cells in *in*

vitro self-renewal and differentiation.

The limited availability of patient-biopsy material, and the very low number of potential stem cells that can be obtained from it, puts a halt on the progress in the research on human salivary gland stem cells. Therefore, it could be of eminent importance to find strategies that can expand patient biopsy-derived cells to obtain sufficient numbers of cells of high quality for autologous stem cell transplantation for patient treatment and scientific research. However, expansion of functional stem cells is one of the major challenges in the field of adult stem cell biology. Hence, in this thesis we tested whether the CD24^{hi}/CD29^{hi} cells could be expanded and, most importantly, would remain functional after such *in vitro* expansion. After culturing of primary sphere-derived CD24^{hi}/CD29^{hi} cells for more than 7-passages a very substantial expansion was observed when compared to unselected cells (Chapter 5). More strikingly, when the expanded cells were transplanted into radiation-damaged salivary glands, they were able to rescue hyposalivation very consistently and to a significantly higher level than any cell type with the same cellular dose tested in this thesis. Mice transplanted with cells from late passages (passage-13) showed an increased and more homogeneous recovery of saliva production, than those transplanted with early passage cells (passage-2). This indicates that serial passaging of CD24^{hi}/CD29^{hi} cells not only increased sphere-forming cells but also enriched for functional stem cells able to reconstitute irradiated salivary glands.

In order to understand the signaling pathways involved in the enrichment of stem cells in culture, we performed genome wide gene expression analysis on these cells. Results suggested the potential role of Wnt and Hedgehog signaling pathways, which were known to be associated with regulation of stem cells in other adult tissues. However, individual genes involved in each pathway need to be tested to confirm their regulatory effect on stem cell enrichment during culturing (see future perspectives). Confirmation of the involvement of these pathways in the process of self-renewal and expansion could lead to approaches that manipulate these pathways and benefit to their clinical application.

1.5. Method of stem cell transplantation

We developed a rat animal model that enables us to investigate other important aspects of clinical transplantation. For example, to study the feasibility of stem cell transplantation in a larger animal and to develop a better, potentially more convenient delivery of stem cells to the patients. The advantage of this model is that we have access to both submandibular and parotid

salivary glands (like in humans) and can test the method of retrograde ductal injection of stem cells potentially a clinical preferred way to transplant stem cells in patients.

First, we needed to isolate stem/progenitor cells from the rat salivary glands. Although murine primary sphere medium can successfully be used to culture submandibular gland stem cells, it might not be suitable for culturing parotid glands of rats. So, in **Chapter 6** we optimized the culture medium and established culturing of salispheres from rat parotid and submandibular glands similar to those of mice. In addition, we showed that both parotid and submandibular gland primary spheres contain cells capable of self-renewal and differentiation. Further, we tested for the expression of stem cell markers in the rat salivary glands using immunohistochemistry and derived primary spheres from flow-sorted cells. Results showed that, similar to mice, CD24, CD133 and c-Kit markers are expressed in rat salivary gland duct cells and cells with these markers are enriched in cultured rat salispheres. This is in agreement with findings from mice that stem/progenitor marker expressing cells are indeed localized in the major ducts and can be isolated from salisphere cultures.

We know from murine *in vivo* studies that intra-glandular transplantation of stem cells can rescue hyposalivation. However, it does not result in uniform delivery of cells in the correct anatomical location in all situations. In patients, an intra-glandular method of transplantation might not be ideal due to the complexity of the gland and the necessary surgical procedure. However, parotid and submandibular gland ducts open directly into the oral cavity as small openings in the cheek and below the tongue. These are easily assessable through the mouth. Since the ducts are suggested to be the niche for the stem cells, we tested whether retrograde intra-ductal delivery of stem cells might be an efficient transplantation method.

30 days after local irradiation of the rat salivary glands, primary sphere-derived single cells (unselected or selected for c-Kit) were transplanted retrogradely into the submandibular and parotid glands. Pre and post-transplantation saliva measurements are currently under progress. Preliminary experiments showed some recovery with this retrograde method of injection of stem/progenitor cells, but not equivalent to the recovery observed with intra-glandular injected cells. Therefore, at present our findings do not support retrograde delivery of cells as a superior transplantation method to intra-glandular injection. Hence, alternate methods of transplantation, such as echo-guided duct injection might be necessary. This could be tested in larger animal model.

2. FUTURE PERSPECTIVES

The findings described in this thesis may be implemented extensively for broader research applications. First and foremost the *in vivo* salivary gland hyposalivation model can be implemented in understanding various aspects of salivary gland biology. Even with the most modern radiotherapy, there are side effects on normal or healthy tissues, which is unavoidable in some cases. Considering this, studies on the effect of radiation on normal tissues are still prerequisite. Our salivary gland models to study radiation-induced damage are of use to test the possibilities of stem cell transplantation to rescue hyposalivation. However, they may be further developed to understand mechanisms of radiation-induced normal tissue damage and regeneration. In our rat model of hyposalivation, long-term effects of radiation can be studied and closely monitored using immuno-histochemical analysis and saliva measurement as read out. As shown in earlier reports⁹ changes in the protein, electrolyte and enzyme composition of saliva post-irradiation can be extensively studied.

Self-renewal and differentiation are the major properties to be tested to characterize potent stem cells. The *in vitro* self-renewal and differentiation techniques described in this thesis are the first in the field of salivary gland stem cell biology. Implementing these assays, we characterized individual stem cell marker expressing cells based on their ability to self-renew and differentiate. The major advantage is that these methods can be extrapolated to both fundamental and translational studies.

For instance, the role of individual genes predicted to regulate the self-renewal and differentiation of salivary gland stem cells can be investigated using the newly standardized *in vitro* assays. Gene-expression analysis (Chapter 5) has identified up-regulation of KRT5, CCND2, SLUG, PORCN genes in long-term self-renewing CD24^{hi}/CD29^{hi} cells. However, to what extent these genes regulate self-renewal in these cells is not clear. To understand this, we could knockdown one of these genes in the primary-sphere cells and follow-up in the *in vitro* self-renewal assay. Any change in the growth kinetics of these cells, would reveal whether the silenced gene is promoting self-renewal. On the other hand, a relatively quicker method is to check for any reporter mice that have knockdown these genes like Slug^{-/-} mice¹⁰ and analyze the salivary glands in these mice for any phenotypic changes resulting from the gene knock down.

Differentiation of salivary gland stem cells *in vitro* has not been reported till date. Here, we established method to differentiate primary sphere derived single cells into the 3D organoids. Gene expression analysis (Chapter 5) identified genes regulating organ development, gland

morphogenesis indicating progress of differentiation in the developing organoids. In addition, organoids express aquaporin-5, cytokeratin 18, the signature markers of differentiated salivary gland cells. Organoid (mini-gland) formation from other tissues, like brain (mini brains) and intestine, are used to study diseases like microencephaly¹¹ and cystic fibrosis¹², respectively. Similarly the organoid-assay developed in this thesis might be used to study aging (25% of the elderly have xerostomia), salivary gland diseases like Sjögren's syndrome and screen drugs to evaluate response to chemotherapeutics for cancer and potentially other diseases.

In vivo testing of stem cells is the gold standard for testing of regeneration ability. In this thesis, we have shown that *in vitro* expanded CD24^{hi}/CD29^{hi} cells showed a very significant recovery of radiation-damaged salivary gland. Though the amount of saliva produced is the key evidence to support recovery, immunohistochemical understanding of the transplanted tissues provided a clear idea of recovery of tissue homeostasis. Using immunohistochemistry we have shown that GFP-donor derived cells contribute to the recovery of radiation-damage tissue. However, the GFP mouse model used in this study is not ideal, since the GFP positive salivary gland itself does not label all the cells, especially the differentiated acinar cells. Therefore, an alternate model like DsRed transgenic mice can be used as donors for more reproducible tracing and follow-up of transplanted cells. To compare the regeneration potential of different candidates, GFP-donor derived and Dsred-donor derived populations can be mixed and transplanted into same mouse and resultant lineages can be analyzed like in mammary gland¹³. This would reveal the regeneration competition between the transplanted populations and their dependency on each other if any *in vivo*. Another option would be barcoding of cells (transfection of donor cells with genetic barcode) similar to that used in hematopoietic stem cell studies¹⁴ that help to identify and quantify the contribution of donor cells to multiple lineages in the transplanted tissue.

In this thesis we showed that murine primary sphere-derived CD24^{hi}/CD29^{hi} population contain the stem cells of salivary gland able of extensive self-renewal and differentiation *in vitro* and capable of potent rescuing of radiation-induced hyposalivation. Since the ultimate goal of our group is to achieve stem cell therapy for xerostomia patients, it is important to investigate the possibilities of extrapolating the findings in mice to humans.

As described earlier, in view of the demand for expanding the patient biopsy material, the self-renewal method of expansion developed in this thesis need to be extrapolated to humans. Though the method of obtaining primary spheres is almost the same in humans and mice, it is important to note that human cells might be relatively more/less sensitive to treatments like

sorting, dissociation, genetic instability via culturing and so on¹⁵. From human salivary gland studies (Pringle *et al* in preparation) we know that unlike in mice human primary sphere-derived CD24 cells do not co-express CD29. So, there is need to find more candidate markers in human salivary gland that either co-express or not with CD24 and test for their potential to differentiate and self-renew. Therefore basic differences between species might be critical factors while extrapolating findings from mice to humans.

Despite of the difficulties involved, human salivary gland research has progressed quite far. First, human salivary gland stem/progenitor cells can be cultured into primary spheres, however, often with variation in the amount of spheres obtained from different patients. This could be due to the fact that the number of stem/progenitor cells varies depending on the age/sex/healthy status of the patient. Using flow cytometry and immunohistochemistry several known stem cell markers (c-Kit, CD24 and CD49f etc.) have been identified in salivary gland tissue and primary spheres (Pringle *et al* in preparation). Currently these marker-expressing cells are being tested for their stem like potential using *in vitro* and *in vivo* techniques. However, a larger screen for surface markers can be done on human salivary gland cells with the new surface marker antibody screening panels commercially available (BD Lyoplate™ 560747). This would generate a greater list of the candidates including markers that co-express, which can be tested *in vitro* in a shorter duration.

Similar to mice human salivary gland primary sphere cells can be self-renewed and differentiated *in vitro* but not yet as extended as in mice. Further optimization is in progress and may allow screening for the most potent stem cell candidates and expand potential stem cell numbers. In mice studies, in the differentiation assay (Chapter 4, organoid assay) along with EGF, N2, Insulin, fetal bovine serum is a major constituent. It is important to consider that human cells might respond different to these ingredients. Moreover, a surface marker based approach to identify stem cells often involves, isolation and subsequent sorting of cells followed by culturing, for instance in self-renewal assay which needs frequent dissociation of cells. It is important to focus that human salivary gland cells might be more sensitive to these procedures. Even though murine salivary gland cells are sensitive to an extent, addition of factors like Rho-inhibitor rescued them from dissociation induced stress (Chapter 4). Hence, as used in mice Rho-inhibitor or better its Good Manufacturing Practices (GMP)-certified alternative must be considered for the survival of human salivary gland cells in the *in vitro* assays. Despite of these challenges, development of the *in vitro* methods will accelerate the human salivary gland stem cell research.

In addition, these assays can be used in the clinic to determining the stemness quality of the patient-derived human salivary gland stem cells prior to the transplantation.

To test the potential human stem cell candidates *in vivo* candidate cells were transplanted into irradiated salivary glands of immune compromised mice and monitored for saliva production as a read out of transplantation success. However, tracing of the transplanted cells is a bigger challenge. Membrane labels (PKH26) and human nuclei stainings are available, but long-term follow-up is not possible due to dilution of label (PKH26) upon proliferation of cells. Immunostaining for human nuclei might not provide precise information especially when searching for very low number of transplanted cells and cannot be used for isolation (sorting) of transplanted cells for further studies. Therefore, prior to transplantation human salivary gland primary sphere cells may be expanded (by optimizing expansion method developed in mice) and transfected with stronger label (like GFP) enabling tracing after transplantation. This label is not lost due to proliferation of cells and can be used to isolate live cells thereby facilitates deeper analysis of transplanted cells.

In addition to this, transplanted cells need to be isolated, expanded in culture and serial-transplantations need to be performed to test the long-term *in vivo* regenerating potential. This has been shown feasible in mice salivary gland stem cell transplantation experiments⁶, however it is a bigger challenge to isolate human cells after transplantation from the potentially recovered irradiated salivary glands of immune-compromised mice. The huge background of mouse cells may compromise the selection of rare human cells. These however might now be eliminated using mouse-cell depletion kits (Miltenyi biotec 130-104-694), which are normally used to deplete mouse cells from xenograft tumors, potentially purifying the transplanted human cells from the recipient mouse cells.

The long-term goal of this project is to establish a clinically translatable stem cell therapy protocol for patients at risk to develop Xerostomia from radiotherapy for head and neck cancer. To achieve this, the first step is to obtain transplantable stem/progenitor cells from human salivary glands. Knowledge available from the current and related projects strongly supports the isolation of stem/progenitor cells from mouse and human salivary glands as primary spheres. We have shown that primary sphere derived stem cell marker selected cells either or not followed by expansion could rescue hyposalivation in our mouse model. For the clinic, the generation of human salivary gland primary spheres and their cryo-preservation, necessary to store for future use, need to be performed under GMP conditions, which is currently being developed. Part of

the GMP process includes the testing of the quality of the stem cells to be transplanted for every patient. In mice (this thesis) we showed different *in vitro* assays, which could identify the stem-like potential of the cells. Development of these assays for human system is ongoing, but preliminary data are encouraging (Pringle *et al* under preparation). For therapy, stem cells generated without cell sorting procedure are preferred, avoiding complicated GMP certified FACS procedures and use of GMP certified antibodies. Here, we have shown generation of mouse SG stem cells via expansion of selected (sorted) and unselected cells. For patients, without selection (no sorting) would be preferable, however recent studies showed CD34⁺ cells obtained via magnetic sorting under GMP conditions can be used for successful treatment of acute ischemic stroke¹⁶. Therefore, recent development of cell sorting under GMP conditions (Clini-MACS) could be valuable for future sorting of putative human salivary gland stem cells.

With the knowledge from this thesis and current research testing isolation, preservation and transplantation of human SG stem cells under GMP conditions, in very near future phase-I clinical trials for xerostomia can be expected. Hopefully this will result in a improvement of quality of life at first as autologous transplantation for patients at risk for suffering from radiation-induced hyposalivation and may be in the future as allogeneic transplantation for patients suffering from other salivary gland diseases caused by autoimmune disease such as Sjogren's syndrome and ageing.

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