Potential of salivary gland stem cells in regenerative medicine

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CHAPTER 7

SUMMARY AND FUTURE PERSPECTIVES

SUMMARY

Head and neck cancer is the sixth most common cancer worldwide, with an estimated annual burden of 387,100 new cases in 2012 (Torre et al., 2015). The majority of these patients will be treated with radiotherapy alone or in combination with chemotherapy and/or surgery. Although radiotherapy treatment significantly improves the patient's chances of survival, it often coincides with side effects due to the unavoidable co-irradiation of normal tissues surrounding the tumor, including salivary glands. For these patients, severe hyposalivation is a very common irreversible side effect, resulting in alterations in speech and taste, difficulties with mastication and deglutition, and increased risk of developing oral infections and dental caries (Vissink et al., 2015). Even with the current state-of-the-art intensity-modulated radiotherapy still about 40% of the patients suffer from oral dryness, which severely hampers their quality of life. Considering there is currently no satisfactory treatment to reverse salivary gland damage, intense interest is now focused on bio-therapeutic approaches such as stem cell (SC) replacement via transplantation.

Radiation-induced hyposalivation is the result of the inadequacy of salivary gland stem cells (SGSCs) to produce a sufficient number of mature functional cells (van Luijk et al., 2015). Collection of SGSCs prior to radiotherapy and transplantation into the glands after treatment could offer a potential therapy in order to restore the secretory function of the recipient tissue. However, the amount of biopsy material that can safely be obtained from a patient prior to radiotherapy treatment is limited. Therefore, a precise characterization and controlled expansion of SGSCs is crucial for further extrapolation to clinic. Although a large body of work supports the presence of SC populations within salivary gland (Bullard et al., 2008; Lombaert et al., 2008; van Luijk et al., 2015; Xiao et al., 2014) information about the localization of the SGSC and molecular interactions with the surrounding environment is scarce. Therefore, answering fundamental questions in regards to SGSC biology is of great importance.

The work presented in this thesis is focused on uncovering the identity of SGSCs within their local niche and furthering our understanding on the molecular signals that govern their maintenance. This Chapter summarizes the main findings of the thesis and puts these studies into perspective.

In **Chapter 1**, as an introduction, an overview of the available data on somatic SCs in the context of exocrine glands was provided. Moreover, differences and similarities between sebaceous, sweat, prostate, mammary and salivary gland were highlighted. Subsequently, the potential clinical relevance of culturing and expanding glandular tissue specific SCs in a laboratory setting was considered. The use of pivotal techniques in identifying glandular SCs, tracing their progeny during development and tissue maintenance and profiling their unique characteristics was discussed.

Finally, the importance of the utilization of novel three-dimensional organoid culture systems was emphasized and the potential opportunities and threats that are associated with steamrolling these systems towards the clinic were considered.

In **Chapter 2**, the aim was to improve the characterization of salivary gland stem/progenitor cell population residing in the mouse salivary gland based on a panel of SC markers previously identified in other adult SCs. First, the expression of various SC-associated markers in homeostatic salivary gland was established and observed that cells displaying these markers reside in the ductal compartment of the gland, previously known for harboring SCs. Next, quantitative comparison of the fraction of cells expressing these SC-markers between SG tissue and an in vitro SGSC (salisphere) culture was performed. The results obtained broadly indicated that salispheres were enriched for cells with SC characteristics. Finally, the regenerative potential of SC-marker expressing cells was tested in an in vivo transplantation assay. Subsequently, we reported that multiple cell populations expressing various SC-markers were capable of recovering salivary gland function. However, the exact hierarchy of SGSC compartment, if existing, still required further research.

As suggested in Chapter 2 and indicated previously by others (Denny and Denny, 1999; Man et al., 2001), the ductal compartment of salivary gland contains SGSCs. Therefore, the molecular signals governing this niche with an emphasis of Wnt/β-catenin pathway were explored in **Chapter 3**. First, EpCAM was identified as a universal marker for ductal cells of the SG. Simultaneously, the basal expression of β-catenin, a general indicator of activation of the Wnt-pathway, in the SG was established. Importantly, rare cells within excretory ducts were observed displaying nuclear expression of β-catenin, a hallmark of active Wnt-signaling. This in mind, single $EpCAM^{+}$ cells from healthy adult mouse salivary glands were isolated using fluorescence-activated cell sorting (FACS). Subsequently, sorted cells were embedded in a three-dimensional (3D) setting using Matrigel as 3D basal extra-cellular matrix and induced with high Wnt signaling by combining Wnt3a ligand and Rspo1 in addition to our previously reported growth factors in the culture medium. In these conditions, $EpCAM^{high}$ cells responded potently to Wnt signals stimulating self-renewal and long-term expansion of SG organoids while retaining their ability to generate differentiated SG epithelial cells. Conversely, by using a panel of chemical inhibitors of Wnt pathway, the requirement of Wnt signaling for the maintenance of SGSC cultures was shown. Finally, by assessing the in vivo regeneration potential of SG organoid-derived cells in transplantation assay, the possible use of SGSCs cultured under Wnt-inducing conditions for stem cell therapy to irradiation-damaged epithelium and possibly other cases of salivary gland dysfunction was shown.

By developing optimized *in vitro* organoid system for expansion of SGSCs retrieved directly from tissue in Chapter 3, we opened up a novel avenue for testing candidate SGSCs based on their i) phenotypical or ii) functional characteristics. Consequently, in **Chapter 4** the identification of SGSCs based on a universal stem cell-associated functional property – quiescence, was attempted. A cell-state independent histone H2B-GFP pulse-chase system was employed in order to characterize the putative dormant SGSC population that may exist within. We hypothesized that the majority of stem cell activity occurs during the embryonic development of the SG rendering the postnatal tissue remodeling as a task for the progenitor and more differentiated cells. Therefore, H2B-GFP label was induced in pregnant mothers until the birth of the litter after which the loss of the label was monitored in time as the mice aged. Subsequently, when the mice reached adulthood, label retaining cells (LRCs) were detected primarily positioned in excretory and striated ducts and not among acinar cells. Interestingly, subsequent to phenotypical profiling of LRCs with known SG markers, co-localization of LRCs and $K8^+$ ductal luminal or $Vimentin^+$ mesenchymal but not $CK5^+$ or $CK14^+$ putative progenitor cells was observed. Next, by challenging these cells in the organoid formation assay the regenerative potential of LRCs was assessed. Coinciding with the previous observations, LRCs failed to generate organoids while a proportion of non-LRCs cells upheld differentiation and long-term expansion potential. Collectively, these data suggested that throughout postnatal development SGSCs are an active population of cells responsible for tissue re-modeling and maintenance.

In many elderly people salivary gland dysfunction with consequential dry-mouth syndrome (Xerostomia) is a major complaint, which commonly results in reduced oral health with, e.g., an increased chance on aspiration pneumonitis. However, whether aging-related decline in SG functionality can be accredited to a reduced responsiveness to proliferation stimuli of SGSCs remains enigmatic. Therefore, in **Chapter 5** previously developed techniques (Chapter 3 and (Nanduri et al., 2014) were applied in order to study phenotypical and functional properties of SGSCs isolated from young and old mice. First, an increased number of $CD24^{hi}/CD29^{hi}$ putative SGSCs residing in old mice was observed. However, when these SGSCs were isolated from aged salivary glands they exhibited a reduced functional potential when compared to young glands. Nevertheless, culturing the remainder of aged SGSCs *in vitro* in a previously described 3D setting unveiled a similar intrinsic expansion and *in vivo* regeneration potential to their young counterparts. Taken together, data gathered in this Chapter suggested that age-dependent decline in SG regenerative potential might be caused due to age-related changes in microenvironment, possibly reversible through exposure to extrinsic factors.

In **Chapter 6** the prospect of translating our previous findings from murine salivary glands into clinical application was addressed. Therefore, the existence and regenerative potential of human salivary gland stem cells (hSGSCs) isolated from healthy patient biopsies was studied. First, by utilizing

previously published conditions (Feng et al., 2009), we reported that human salispheres contain stem/progenitor cells capable of both self-renewal and multilineage differentiation *in vitro*. Next, the regenerative potential of salisphere-derived cells was investigated *in vivo* by challenging them in transplantation assay. The capability of proliferation, functional restoration and long-term engraftment after xenotransplantation into an irradiated environment of human salisphere-derived cells was demonstrated. Particularly, among salispheres a subset of c-Kit+ cells was defined that required dramatically lower cell numbers for functional recovery compared to their unfractionated counterparts. In line with what is described in Chapter 5 the frequency of c-Kit+ cells in primary cultures appeard to decrease with age suggesting that techniques for controlled expansion of hSGSCs would hold great therapeutic promise. Additionally, contributing to the noted functional recovery, the combination of human cell engraftment but also regenerative signals emanating from the surviving cells in the recipient SG was detected. This observation was further investigated by performing whole genome mRNA expression analysis on regenerating SG-s comparatively with irradiated controls. The expression analysis revealed enhancement of ECM protein expression and stimulation of several stem cell-associated signaling pathways. Specifically, in line with the observed dependence of SGSC on Wnt pathway (Chapter 3), this Chapter indicated the involvement of Wnt/β-catenin signaling in initiating functional recovery of salivary glands treated with human salisphere-derived cells.

FUTURE PERSPECTIVES

The work described in this thesis is focused on unraveling the identity of salivary gland stem cells (SGSCs) and the molecular signals governing them in their native niche. In addition, we studied the intrinsic regenerative capacity of SGSCs with the aim of future exploitation in clinical therapy. We will further discuss the progress achieved in these directions and speculate how the field may develop in the future.

Salivary gland stem cell identity

In recent years, the existence of SGSCs has been postulated from evidence that salivary gland demonstrates an extensive regeneration capacity subsequent to complete atrophy (Cotroneo et al., 2010; Cotroneo et al., 2008) and that salivary gland can be regenerated by transplantation of ductal cells (Lombaert et al., 2008). However, until now, bona fide SGSCs have not been functionally identified owing to the lack of unique markers and the absence of stem cell assays (investigated in

Chapter 2, 3 and 4). In Chapter 3 of this thesis, based on co-activation of Wnt/β-catenin signaling, conditions for culturing and expansion of SGSCs isolated directly from tissue were developed. In the future, the availability of this robust *in vitro* assay will allow the screening of putative SGSC populations for the differentiation and self-renewal capacity. Moreover, performing transcriptome profiling via RNA-sequencing (reviewed in (Wang et al., 2009)) on cells obtained from salivary gland organoid cultures can provide crucial information about genes responsible for the long-term self-renewal of SGSCs. However, it is likely that the fraction of true stem cells in organoid cultures is not very large. Therefore, an ideal approach for capturing rare cells amongst heterogeneous pool would be to profile the cell type composition of the whole culture. Over the last six years single cell mRNA-sequencing has been introduced as an unbiased method to discriminate cell types in healthy tissues (Jaitin et al., 2014; Zeisel et al., 2015), to study differentiation dynamics (Treutlein et al., 2014) or to discover rare cell types (Grun et al., 2015). Therefore, applying single cell sequencing technology on salivary gland organoid cultures could lead to the identification of true SGSCs and a possible discovery of other previously not know cell types within salivary gland. However, since any single-cell sequencing technique is based on amplification of minute amounts of material leading to substantial technical noise (Brennecke et al., 2013; Grun et al., 2014), data processing and analysis require extra care.

Another option for interrogating the potency and fate of putative stem cells in their native context is lineage tracing (reviewed in (Kretzschmar and Watt, 2012)) using genetic introduction of either tamoxifen-regulatable or a tetracycline-responsive version of *cre* gene into mice. In recent years, cell fate studies have provided essential information about contribution of various cell populations in the development, homeostasis and regeneration of multiple tissues including mammary gland (Rios et al., 2014; van Amerongen et al., 2012; Van Keymeulen et al., 2011; Wang et al., 2015), which as discussed in Chapter 1 has many similarities with salivary gland regarding tissue composition and development. Nonetheless, in postnatal salivary gland, lineage tracing studies for most of the putative stem cell populations are not yet available. Previously, in a study conducted by Bullard and colleagues, transcription factor Ascl3 was shown to mark progenitors that generate acinar and duct cells (Bullard et al., 2008). However this study was conducted in a mouse line where *cre* allele was constitutively active. Therefore, it is feasible that the acinar and ductal cells were labeled in a prenatal state. More recently, the same group conducted fate-mapping experiments using tamoxifen-inducible *cre* under the control of acinar-specific Mist1 locus (Aure et al., 2015). Within a period of 6 months no decrease in the number of labeled acinar cells was observed. Moreover, by crossing Mist1-cre line with *Rosa26*$^{Brainbow2.1}$ reporter strain, over time clusters of unicolored cells were detected. Together, these data demonstrate the proliferative activity and clonal expansion of

acinar cells in the adult salivary gland. However, the direct contribution of stem cells residing in the ducts on the development and regeneration of the salivary gland was not investigated. Therefore, in the future initiating lineage tracing experiments from luminal K8 (Van Keymeulen et al., 2011) and basal K5 (Van Keymeulen et al., 2011), K14 (Van Keymeulen et al., 2011) and possibly Procr (Wang et al., 2015) and Axin2 (van Amerongen et al., 2012) promoters would be essential in ascertaining the relative contributions of these lineages to development, homeostasis and regeneration.

Molecular signals governing the niche

Stem cell activity is often dictated by the microenvironment (the niche) so that stem cell output is precisely shaped to meet homeostatic needs or regenerative demands. In salivary gland, the existence of a specialized stem cell niche and the signals governing it has not been fully established. Previously, Wnt/β-catenin signaling pathway has been implicated in the control of stem/progenitor cells in the salivary gland. More specifically, Wnt signaling is activated after ligation and subsequent deligation of the main excretory duct and its forced activation in the basal epithelia expands stem/progenitor cells (Hai et al., 2010). Interestingly, as a result of radiation damage the activation of this pathway was not observed. However, in male mice transient activation of Wnt signaling during irradiation prevents both acute and chronic hyposalivation by inhibiting apoptosis and preserving the stem/progenitor pool (Hai et al., 2012). In Chapter 3 of this thesis, we provided evidence regarding the direct requirement of Wnt/β-catenin signaling in SGSC cultures. Still, *in* vivo manifestation of the effect of Wnt/β-catenin signaling as a regulator of SGSCs is until now missing. Therefore, in the future it would be of great interest to utilize mouse models carrying loss-of-function (Brault et al., 2001; Huelsken et al., 2001) or gain-of-function (Harada et al., 1999) mutations of β-catenin for further investigation of how changes in β-catenin expression affect the SGSC compartment in tissue development, homeostasis and repair. Furthermore, employing an epitope-tagged functional Wnt3a knock-in mouse model (Farin et al., 2016) recently developed by the Clevers laboratory could provide spatial answers to how and which cells create the stem cell niche of the salivary gland.

In addition to Wnt signaling, other core molecular pathways such as Hedgehog (Jaskoll et al., 2004), Notch (Garcia-Gallastegui et al., 2014) and FGF/FGFR (Lombaert et al., 2013) are essential for the organogenesis of the salivary gland and therefore also require thorough investigation in adulthood. Notch transduction pathway has been shown to play a role in growth and differentiation of adult salivary precursor cells and branching morphogenesis (Dang et al., 2009). Moreover, inhibition of Notch signaling via DLK1 leads to abrogated branching morphogenesis and innervation in embryonic salivary gland explant cultures (Garcia-Gallastegui et al., 2014). Currently, in our laboratory we are

investigating the direct involvement of Notch signaling to SGSCs (Serrano-Martinez et al., unpublished). Our preliminary data suggest that Notch receptors play a role in controlling salivary gland progenitor cell fate specification coinciding with observations from other tissues such as small intestine (Fre et al., 2005).

Previously, Xiao and colleagues successfully identified and isolated a population of $Lin^{-}CD24^{+}c\text{-}Kit^{+}Sca1^{+}$ stem cells in the submandibular glands of adult mice, which improved salivary gland condition and increased salivation when transplanted into irradiated animals (Xiao et al., 2014). Subsequent gene-expression analysis of this SGSC population revealed that a subpopulation of cells highly expressed glial cell line-derived neurotropic factor (GDNF). Importantly, mice given GDNF exhibited enhanced function and integrity of irradiated salivary glands without any apparent effects on head and neck tumor growth. These data suggest that administration of GDNF may serve as a pharmacological approach for improving salivary gland function. In a more recent study Kumar et al., successfully created a mouse model for hyper-regulation of endogenous GDNF protein which displayed two-fold elevated GDNF expression following introduction of a transcription stop signal in the 3'untranslated region (UTR) of the endogenous *Gdnf* gene (Kumar et al., 2015). Although the authors describe the occurring GDNF hypermorphism events on kidney branching morphogenesis, they do not study the salivary glands. As such, investigating the effects of elevated levels of GDNF on salivary gland development and regeneration could serve as a next step in taking GDNF as a potential (gene-) therapy for enhancing survival and function of SGSCs towards clinic.

Extrapolation to the clinic

Salivary gland stem cell therapy is expected to transition from a research promise to clinical reality in the upcoming years. For this, previously we have developed a method for culturing human salivary gland stem cells (hSGSCs) as spheres (salispheres) from submandibular glands (Feng et al., 2009). When placed in 3D matrix salispheres were able to develop organoids with differentiated salivary gland cell types present and displayed limited (up to 7 passages) self-renewal capability *in vitro*. In the work described in this thesis (Chapter 6) *in vivo* functionality, long-term engraftment and functional restoration of hSGSCs in immune-suppressed mouse xeno-transplantation model was shown. Indeed, transplanted salisphere-derived cells restored saliva production, gland weight and greatly improved intrinsic regenerative potential of irradiated salivary glands. Collectively, these data shows that salispheres cultured from human submandibular glands contain stem/progenitor cells capable of self-renewal, differentiation and rescue of saliva production. However, before the implantation of hSGSCs could occur, patients undergoing radiotherapy treatment would require a

time period of 15 weeks, including diagnosis, treatment and recovery. Thus, during this period hSGSCs will be either 1) cryopreserved until the patient is ready for transplantation or 2) cultured and expanded *in vitro* for a maximum cell yield. Previously, long-term cryopreservation of rat SG progenitors with little effect on cellular characteristics has been demonstrated (Neumann et al., 2012). Furthermore, in our laboratory isolation and cryopreservation of hSGSCs is already possible using current good manufacturing practice (cGMP)-approved reagents (unpublished data). However, with regards to *in vitro* hSGSC expansion there are still considerable challenges to overcome. Although, the studies conducted in mouse (Chapter 3) serve as a proof-of-principle and a beneficial starting point for human system, it is likely that as is the case in other organs (Bartfeld et al., 2015; Boj et al., 2015; Huch et al., 2015), cultures of long-term human SGSCs could require modification of growth media. Therefore, currently in our laboratory we are testing a panel of growth factors and inhibitors for organoid-forming efficiency, phenotype of the organoids and longevity of the hSGSC cultures (Rocchi et al., unpublished data). In addition, not all materials utilized in the mouse study (Chapter 3) are cGMP-compliant. The use of biologically based culture system such as conditioned medium as a source for Wnt3a and R-spondin1 proteins will not be permitted according to strict cGMP-guidelines due to concerns over xenobiotic contamination and batch-to-batch variability. This complexity within a culture system makes it more difficult to understand and control the cell/material interface, which are prerequisites to developing a scalable and reproducible hSGSC culture system. Therefore, the production of large quantities of recombinant human Wnt3a and R-spondin1 protein would be of use for the future development of hSGSC therapy. Furthermore, current methods for culturing SGSCs rely on the use of Matrigel, a proprietary basement membrane-enriched extracellular matrix gel enriched for laminin, collagen IV and entactin (Kleinman et al., 1982). As Matrigel is derived from Engelbreth-Holm-Swarm (EHS) mouse sarcoma cells it will provoke immune responses in other species. This feature disqualifies the use of Matrigel as a matrix for future translational therapies given stringent requirements instituted by the U.S. Food and Drug Administration (FDA) (Lee et al., 2010) and the European Medicines Agency (EMA). Therefore, further investigations on the use of artificial matrices such as Synthemax (Jin et al., 2012), StemAdhere (Nagaoka et al., 2010) or CellStart (Swistowski et al., 2009) as growth substrates can provide aid in translating hSGSC therapy to clinic.

Overall, the work presented in this thesis summarized and contributed to the current knowledge regarding salivary gland stem cell identity and the core molecular pathways crucial for salivary gland regeneration. In addition, in various contexts, we highlighted the potential of salivary gland stem cells in regenerative medicine. Thus, these data provides a promising start to design a therapy for salivary gland dysfunction, associated diseases or even ageing.

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