Regeneration of irradiated salivary glands by stem cell therapy
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General Discussion and Perspectives

I haven’t failed, I’ve just found 10,000 things that don’t work.
ADULT STEM CELL THERAPY TO REGENERATE IRRADIATED GLANDS

During radiotherapy, coirradiation of salivary glands of head and neck cancer patients may lead to long-term hyposalivation, resulting in a secondary health problem, namely the dry mouth syndrome (i.e. xerostomia). Hyposalivation can partly be prevented by reducing the dose delivered to the tissue (using e.g. IMRT), transfer of the gland outside the radiation field, by radical scavengers like Amifostine® or by enhancing the compensatory response of unirradiated or radiation-surviving cells (pilocarpine). If unsuccessful, one can opt for symptom reduction using saliva-substitutes or sialogogues. All these, however, may be insufficient, and still many patients continue to suffer from xerostomia (Chapter 1). Therefore, scientists have been encouraged to develop new regenerative medicine strategies. While several tissue engineering concepts as culturing salivary gland tissues in vitro and post-irradiation gene therapy are currently being explored, in this thesis, the potential of stem cell therapy is investigated.

First, we showed that bone marrow-derived (stem) cells (BMCs), when mobilized to the blood stream, are able to contribute to the regeneration of irradiated salivary glands (Chapter 2&3). Next to this, we showed that post-irradiation adult tissue-specific stem cell transplantation has the potential to completely and profoundly induce long-term salivary gland regeneration (Chapter 4). In line with this, in vivo expansion of the number of stem/progenitor cells prior to and after irradiation, using ∆N23-KGF, pronouncedly restored salivary gland function (Chapter 6).

In this chapter, the experimental data described in this thesis will be discussed, and placed in the context of our current understanding of stem cell therapy.

1. BONE MARROW STEM CELL THERAPY

BMCs have been reported to engraft in a variety of organs (reviewed in Krause et al. 344), and promote functional regeneration of damaged organs such as liver 46, kidney 75, heart 151,160,163,166, and salivary glands (Chapter 2&3). However, the mechanism on how these BMCs contribute to organ repair is still an enigma. Several suggestions have been put forward, including trans-differentiation of BMCs into non-hematopoietic epithelial cells, fusion of BMCs with tissue cells thereby adopting the phenotype of the tissue, or via secretion of diffusible molecules (e.g. cytokines) which stimulate tissue repair.

In our study, mobilized BMCs clearly engrafted in irradiated mouse submandibular glands, which resulted in a partial restoration of tissue integrity and function. Since only very low percentages (< 1%) of acinar cells were BM-derived (Chapter 2&3), trans-differentiation of BMCs to salivary gland cells 345-346, gland cell fusion with e.g. macrophages 347-350 or mobilization and engraftment of rare salivary gland tissue-specific stem/progenitor cells which may reside in the bone marrow, 251,252 is at best very rare. Even considering the underestimation of the number of BMC-derived acinar cells due to transgene silencing 353 and false-negative Y-chromosome hybridizations, these low percentages are unlikely to be sufficient for the improvement of the gland after irradiation and mobilization. Therefore, we hypothesized that radiation-surviving salivary gland stem/progenitor cells are the main cell types responsible for tissue regeneration. The secretion of micro-environmental factors by engrafted BMCs 354,355 may stimulate these cells to proliferate/differentiate in order to repair the tissue. This stimulus is probably not caused by the cytokines that were administered to mobilize BMCs, since salivary gland duct and acinar cells lack G-CSF receptors, and addition of Flt-3L and SCF did not further increase repair. In contrast, improved vasculature could (partly) be responsible for the direct stimulation of endothelial cells, which do express G-CSF receptors and Flt-3. This, however, does not seem to add much to the post-irradiation improvement of submandibular gland function (Chapter 3).

Strikingly, the beneficial effect of BMC mobilization is radiation dose dependent (Fig. 1). The failure of repair at high dose rates can be explained by the lack of radiation-surviving stem cells in the gland, which would be in agreement with our hypothesis. At low doses (10 Gy), enough stem/progenitor cells survive so that extra stimulation seems not essential. Interestingly, a similar explanation was put forward for the protective effects observed by prophylactic pilocarpine treatment in exactly the same dose range 356 and after post-irradiation KGF treatment (Chapter 6). This not only indicates that the regenerative effect can be mimicked by exogenously applied pharmaceuticals, but also marks the limitation of the treatment.

It is not yet clear which BMCs may be responsible for such a paracrine effect. Two months after mobilization, we did observe many mesenchymal CD31+ cells (e.g. fibroblasts, myoepithelium cells), which are known in embryonic salivary glands to induce branching and acinar cell budding by secreting growth factors like FGF-7 and FGF-10 357. Mesenchymal stem cells (MSCs) have also been reported to reconstitute damaged tissues such as cartilage, bone, heart muscle, and tendon (reviewed in Bobis et al. 358), and are able to differentiate into liver, lung and gut cells (reviewed in Jiang et al. 354).
To directly test their potential we transplanted cultured mesenchymal stem cells (reviewed in Delorme et al. 359), and evaluated their potential to regenerate irradiated salivary glands. At 30 days post-irradiation, intra-glandular injection of 100,000 or 500,000 in vitro selected mesenchymal stem cells did not induce protection of the gland from malfunctioning (Kok T. et al., unpublished data). Instead, an enhanced fibrotic response was observed at the site of injection (Fig. 2, arrow & insert), emphasizing that one should be careful with the injection of certain cell types into an organ.

Next to this, intra-glandular transplantation of irradiated submandibular glands with in vitro FGF-1/2 enriched hematopoietic stem cells 360 also failed to induce regeneration (Kok T. et al., unpublished data). Even constitutive injections for two or three days of 2-10 million freshly isolated whole BM cells, either intra-glandular or intravenously injected, failed to induce repair (Kok T. et al., unpublished data). Negative results obtained from these experiments do not necessarily imply that the used cells are improper for gland repair. Assuming that cytokines secreted by the damaged organ (e.g. Stromal Derived Factor-1 361) attract circulating blood cells, the time of circulation of intravenously injected cells may be too short to yield substantial homing, or the cells might be less attracted compared to mobilized cells. Furthermore, results from the direct gland injection of MSC/hematopoietic cells indicate that next to appropriate cell populations and adequate cell numbers also the site of homing might be important for gland repair.

As mobilized BMC population consist of a variety of cells, including MSCs 178, endothelial and hematopoietic progenitor/stem cells 362, and mature bone marrow cells (e.g. myeloid cells, T-cells, granulocytes) 363, it might also be that cell types other than those we selected or a specific combination of cells is necessary to induce gland repair.

So far, cytokine-induced BMC therapy could be useful as a novel therapeutic strategy for irradiated salivary gland repair, but only after a limited radiation dose range when significant high numbers of gland stem/progenitor cells remain in the tissue.
Additionally, the lack of stem cell-related protein expression such as Sca-1, c-Kit, Notch-1, p63 and Musashi-1 in intercalated duct cells, and the high levels of CD29 and Musashi-2 in these cells, (Chapter 5), all indicate that intercalated ducts contain only progenitor cells whereas the more primitive stem cells reside in excretory ducts. If so, excretory duct cells should first differentiate into striated duct cells, then into intercalated cells, and finally into acinar cells. Since 4 days ΔN23-KGF (FGF-7) treatment increases the number of stem cells in the mouse submandibular gland (Chapter 6), such a transition should be visible when the different compartments are followed in time. Therefore, quantitative analysis of the area occupied by the different ductal compartments was performed. This revealed that excretory (ED) and intercalated duct (ID) areas were relatively increased at the expense of striated ducts (SD) (Fig. 3, day 4) immediately after the last treatment. Six days later, the ID area decreased, due to an expansion in acinar cells (Chapter 6). Within the following twenty days, the ID areas expanded again at the expense of SD areas, while the ED was unaffected. Gradually, all ductal areas returned to normal, reducing the ED ducts in favor of SD ducts and ID ducts in favor of acinar cells. Although these results do not prove our hypothesis that stem cells reside in excretory ducts, they certainly imply that excretory duct cells can convert into acinar cells via a striated and intercalated duct transition.

![Image](image_url)

**FIGURE 3. INFLUENCES OF KGF ADMINISTRATION ON DIFFERENT CELL COMPARTMENTS OF THE MOUSE SUBMANDIBULAR GLAND.** C75BL/6 mice were treated for 4 days with ΔN23-KGF for four constitutive days (starting at day -3), and salivary glands were morphologically evaluated at different time-points (day 4, 10, 30, 60, and 90). The mean area of the different ducts: intercalated (ID), striated (SD), and excretory duct (ED) were evaluated in mean ± SEM.

### 2.2 ISOLATION OF SALIVARY GLAND STEM/PROGENITOR CELLS

To successfully rescue lethally irradiated mammals from fatal hematopoietic failure, the isolation and transplantation of the bone marrow mononuclear cell fraction is sufficient. Solid tissue stem cell therapy appears to require a population enriched for stem cells to achieve clinical acceptable success rates (Chapter 4). Our salisphere culture system shows that more mature cells can relatively easy be removed, which automatically leads to enrichment for stem/progenitor cells. Nevertheless, additional cell type specific cell purification seems necessary, and can be used to obtain the most primitive stem cells from this heterogeneous salisphere population. Based on our (serial) transplantation studies (Chapter 4), high membrane expression of c-Kit was shown to be a reliable marker for the selection of stem cells. Still, the selected c-Kit+ population is heterogeneous, containing both progenitor and stem cells. Further enrichment is necessary to define the exact stem cells and to potentially increase transplantation successes. Immuno-histochemically, we proposed a broad spectrum of stem-cell related markers such as CD24, Msi-1 and Sca-1 (Chapter 5). Some of these (CD24, CD29, Sca-1) could easily be used for cell sorting and subsequent experimental transplantation purposes. So far, determination of cell types in our salispheres indicate that the majority of cells are Sca-1 and CD24 positive (81-90%) (Fig. 4). Similarly, 91% of these cells were CD29+ (β1-integrin) positive. Although acinar cells are also slightly positive for CD29, the brightest population resides in the ducts. These results, together with immuno-histochemical data (Chapter 5), support the notion that early salispheres (≤ D3) are enriched for duct cells. Although immuno-histochemistry suggested CK7 as an ideal duct cell marker, the expression of CK7 on salisphere-derived cells is rather low and only represents the brightest ~25% duct cell population. In contrast, CK14 expression is brightly expressed (~10%), but its use for duct cell determination requires additional markers to eliminate myoepithelial cells (Chapter 5). On the other hand, early salispheres contain ~12% CD133+ cells, that was shown to be a stem cell marker for prostate and neuron stem/progenitor cells. Although we have not determined the precise location of CD133+ cells, it might be a useful as an additional marker for stem/progenitor cell selection.

We also addressed the absence/presence of potentially therapeutically or experimentally interesting cell markers on the c-Kit+ cell population of our salispheres. Double staining with CD29, CD24, or Sca-1 (Fig. 4B) revealed that only a small population of c-Kit+ cells (0.3%) express these markers. Future research will be required to investigate the potential of these different cell populations to form salivary gland cell lineages and to rescue salivary glands from irradiation damage.
CHAPTER 7

Other studies claim to have isolated salivary gland stem cells using FACS sorting for \( \alpha_6\beta_1 \)-integrin cells \(^{144,145} \), CD49f\(^{+}\)Thy-1\(^{+}\) fractions (in humans \(^{147} \), rats \(^{145} \), and swines \(^{146} \)), or Sca-1\(^{+}\)c-Kit\(^{+}\) cells (mice \(^{143} \)). Although it cannot be ruled out that these markers are able to select for salivary gland stem cells, so far none of them has been shown to be able to \textit{in vivo} regenerate damaged salivary glands.

**Figure 4. Cell Surface Expression of Cells in Cultured Salispheres.** (A) FACS analysis of single cells obtained from salispheres cultured for 2 days, revealed the absence/presence of Sca-1, CD24, CD29, CD133, CK 7, and CK 14 proteins (\%, mean ± STDEV). (B) Double labelling for c-Kit and CD29, CD24, or Sca-1 visualized the co-expression of c-Kit cells for any of these epitopes. Gray box represent 0.3% c-Kit\(^{+}\) cells.

Next to stem cell selection based on expression of specific cell surface markers, enrichment based on Hoechst 33342 dye exclusion appears promising. Indeed, we have been able to select a salivary gland SP (Side Population), representing about 1% of the freshly isolated cell population (Fig. 5). This is comparable to what has been observed in other organs (reviewed in Challen \textit{et al.} \(^{104} \)). Determination of stem cell-related markers on SP cells, their putative presence in \textit{in vitro} selected c-Kit\(^{+}\) cell population, and their ability to form salivary gland cell types \textit{in vitro} and \textit{in vivo} will reveal their potential use in stem cell therapy.

To date, salivary gland stem/progenitor cell populations can be harvested via salisphere cultures. Subsequent sorting, using cell surface markers and/or Hoechst 33342 phenotype, may reveal the identity to the true stem cell. To achieve this, however, these cells need to be tested in additional \textit{in vitro} self-renewal (e.g. serial sphere formation) and cell lineage (cobble-stone like) assays that still have to be developed.

2.3 Differentiation and Self-Renewal \textit{In Vitro}

An \textit{in vitro} self-renewal assay (i.e. serial clonal formation from one single cell) has not yet been established for salivary gland stem cells. Although Kishi T. \textit{et al.} \(^{141} \) recently described a colony forming assay (\textit{Chapter 1}), many issues, as described in Chapter 1, need to be addressed before their assay can turn into a reliable method.

Alternatively, potential salivary gland stem cells can be studied in a 3D matrix environment for their capability to generate a gland-like structure (i.e. both duct and acinar cells) (\textit{Chapter 4}). In addition, this matrix may also be used to observe influences on stem/progenitor cell behavior caused by changing both matrix and/or medium composition.

**Figure 5. Side Population in the Normal Mouse Submandibular Gland.** (A) Staining for Hoechst 33342, with or without Verapamil (B), revealed an exclusive population of cells excluding the dye Hoechst 33342 (see box). This population (~1% of all cells) is defined as the Side population fraction.
In our experiments whole salispheres were placed into culture, but, ultimately, if possible, one single cell (type) will have to be seeded and evaluated.

So far, we have not been able to achieve stem cell self-renewal in our in vitro salisphere cultures to increase stem cell numbers. On the contrary, in time, the defined medium containing N_2, insulin, EGF and FGF-2 seemed to induce cell differentiation towards acinar cells. Morphologically, differentiation started after day 3 in culture (Chapter 4). Therefore, stem/progenitor cells were collected at that particular day for transplantation purposes. However, preliminary results indicate that this specific medium already influences the c-Kit\(^+\) cell fraction during the first three days of culturing. Therefore, transplantation of freshly isolated (D0) c-Kit\(^+\) cells might be more desirable in the future. Moreover, it is clear that media growth factor composition, other than described above, may enrich more/less for putative stem cells. We have strong evidence for the involvement of FGF’s (Fibroblast Growth Factors) in the regeneration of irradiated salivary glands since FGF-7 (KGF) administration in vivo induced a clear enhancement in salisphere formation (Chapter 6). It is therefore conceivable that other combinations of growth factors might enrich stem/progenitor cells in vitro, and/or enhance post-irradiation regeneration after stem cell therapy.

2.4 Transplantation of Salivary Gland Stem Cells

From our experiments, stem cell therapy to treat hyposalivation after radiotherapy seems promising and it should be relatively straightforward to translate this approach to clinical applications. Especially, the feasibility to isolate c-Kit\(^+\) stem cells from human salivary glands would launch its potential clinical use. The treatment of head and neck cancer patients follows a strict time-schedule, leaving a period for stem cell culture and selection when collected from biopsies of submandibular and/or parotid glands before the treatment. Similar to bone marrow transplantsations, salivary gland stem cells might be transplanted in the recipient organ following cryo-preservation for as long as needed after the conditioning therapy. Still, issues concerning cell regrowth after cryopreservation and other safety aspects related to the clinical therapy need to be investigated, as well as the future possibility of allogenic transplantation.

Intravenously injected day 3 salispheres or salisphere-derived cells did not yield any engraftment in irradiated glands (unpublished data). Direct intra-glandular injection as performed in the mouse model does not seem to be a preferable method for patients as it may not provide an optimal distribution in larger organs.

Alternatively, retrograde intra-ductal injection can be a clinical applicable way of administration in humans. Indeed, intra-ductal cell cannulation, as has been used in different animal models in the past for the delivery of adenoviral-mediated gene therapy in vivo (reviewed in Baum et al. \(366\)), indicated its potential. Upon successful intraductal injection (Fig. 6A), preliminary experiments documented a broad spectrum of salisphere-derived cells (35,000-520,000 cells per gland) 24hrs post-transplantation (Fig. 6B, arrows). This method may enhance regeneration as it is not associated with needle-induced injuries and reduces the need for migration. Despite the current differences in success rate in intra-ductal (3/11, 27\%) (Fig. 6C) and intra-glandular (42\%, 5/12 or 69\%, 9/13 for c-Kit injection) transplantations in mice, transfer of this procedure to larger species may prove to be beneficial.

2.5 Future perspectives

Understanding complex stem cell-mediated activity in normal and irradiated salivary glands requires knowledge of the anatomical organization of the stem/progenitor cell hierarchy and inter/intra-cellular signaling pathways influencing stem/progenitor cell behavior. Therefore, future research will have to be aimed at isolating more specific stem/progenitor single cells from both rodent and human glands. Subsequent in vitro 3D (differentiation) and 2D (clonogenic) assays, and in vivo transplantation procedures should clarify the distinction between more committed progenitor cells and primitive stem cells.

Secondly, it appears that the success in ameliorating radiation-induced hyposalivation, evoked by either pilocarpine, ΔN23-KGF or BMC treatment, is based on a similar principle. All treatments require a significant extent of (radiation-surviving) functional gland stem/progenitor cells that are, subsequently, stimulated to proliferate and/or differentiate into acinar cells. If (additional) stimulation of stem cells after transplantation into irradiated glands is required, we need more comprehensive understanding on the relation stem cell behavior and its environment.

Next to this, the radiation sensitivity of salivary gland stem cells, the minimal stem cell number needed to regenerate a salivary gland, the potential to which the gland stem cell can be used to generate other epithelial tissues, and the possibility to in vitro expand stem cells will (or should) be investigated in the near future. Therefore, it has been an exciting four years, but even more excitement lays ahead of us, which will reveal many features of putative salivary gland stem cells.
FIGURE 6. INTRA-DUCTAL INJECTION OF SINGLE CELL SOLUTION OF CULTURED SALISPHERES.
(A) Successful injection of cells into the duct system of the submandibular gland can be verified by the co-injected Indian ink, which is clearly absent in the sublingual glands. (B) Intra-ductal injection evokes a broad spectrum of cells around the ducts into the gland as noticeable by the pattern of Indian ink. (C) Three out of twelve transplanted mice produced higher saliva flow rate productions (○) compared to untreated irradiated mice (●).