Regeneration of irradiated salivary glands by stem cell therapy
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The Adult Mouse Submandibular Gland Stem/Progenitor Cell – its Localization and Fate after Post-irradiation Transplantation

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ABSTRACT

In the previous chapter, we have shown that transplantation of c-Kit+ cells, derived from cultured submandibular salivispheres, can rescue recipient glands from radiation-damage. The ability to self-renew, proliferate and differentiate into all major tissue cell types after (serial) transplantation demonstrated that the selected cell population contained stem cells. Salivary gland stem cell transplantation has the potential to be translated to a clinical application to treat patients at risks for life-long hyposalivation after radiotherapy. However, comprehensive characterization of the stem/progenitor cell population is a prerequisite to ultimately isolate and transplant the most optimal cell type. In this study, we investigated the localization of potential murine submandibular gland stem/progenitor cells and their fate in irradiated tissue after transplantation.

Cytoskeleton CK 7, CD24, and Sca-1 are all specifically expressed by duct cells, which are thought to contain the stem/progenitor cells of salivary glands. Within the ductal compartment, stem cell proteins expression such as c-Kit, Musashi-1, p63, and Notch-1 were restricted to a specific cell population in the excretory ducts. In irradiated tissue, immunostaining for virtually all these markers was negative. After cell transplantation into irradiated glands, tissues were again positive for these stem cell markers, indicating a recovery of the tissue from the radiation insult by virtue of the graft.

In conclusion, our results strengthen the notion that the salivary gland stem cells reside in the excretory ducts. Most salivary gland cell types, including excretory duct cells, are completely obliterated by irradiation leading to irreversible tissue damage. This can partly be restored by cell transplantation. Selection of cells based on the expression of a combination of proteins such as CD24, CD29, Sca-1 and c-Kit may allow further isolation of the most primitive submandibular gland stem/progenitor cell for future use in stem cell therapy.

INTRODUCTION

Radiation-induced sterilization of tissue stem cells impairs the replacement of differentiated cells, and consequently results in organ dysfunction. In patients treated for head and neck cancer, unavoidable co-irradiation of the salivary glands occurs, and this can lead to life-long hyposalivation. Consequent oral/dental damage and elicited xerostomia (=dry mouth syndrome) severely hamper the quality of life of these patients. Recent experimental studies show that post-irradiation stem cell treatment can rescue the salivary gland from radiation-induced dysfunction. Improved salivary gland function and morphology were obtained after cytokine-induced bone marrow (stem) cell (BMC) mobilization following irradiation. However, although BMCs did contribute to an improved vascularization, virtually no trans-differentiation into submandibular gland cells was observed, indicating that irradiation-surviving resident salivary gland stem cells were stimulated by the infiltrated BMCs. These studies indicate that post-irradiation stimulation of surviving stem cells indeed restores tissue function. Strikingly, an almost complete restoration of saliva flow rate and tissue morphology was obtained after transplantation of submandibular gland specific stem/progenitor cells. Serial transplantation of selected c-Kit+ cells isolated from cultured salispheres appeared to be highly successful. In contrast, the c-Kit negative progenitor population could only temporarily ameliorate radiation-induced hyposalivation. However, the exact nature of stem cells responsible for the tissue regeneration still needs to be determined.

Until recently, salivary gland stem cells were believed to reside in the intercalated duct compartment. In contrast, our cell transplantation study indicates that c-Kit+ cells are confined to the excretory duct compartment. Apparently, intercalated ducts contain progenitor cells responsible for acinar cell formation, while excretory ducts contain the actual stem cells, which provide long-term reconstitution of all cell types.

More profound characterization of salivary gland stem cells may improve their purification and will be important to identify and characterize their niche. This is relevant for potential multiplication of the tissue specific stem cells in vitro, and may be a prerequisite for clinical application. Identification of specific cell-surface markers and subsequent selection and transplantation of cells expressing these markers, have led to the prospective isolation of pluripotential mammary gland stem cells. Such stem cell-related markers include a variety of proteins, some of which are tissue specific. Potential salivary gland candidate genes include cytoskeleton proteins, such as (cyto)keratin 15 used for the identification of hair follicle stem cells.
In addition, the membrane-related CD29 (β1-integrin) protein has been correlated with murine mammary gland 101, skin 245, human liver 246, and rodent/human brain 247-249 stem/progenitor cells. Similarly, CD24 is used to isolate mammary gland 101,102 stem cells, but also for early B-cell progenitor cells 250, renal progenitor cells 251, prostate stem cells 252, and brain progenitor cells 253. A general stem/progenitor cell-related marker seems to be Sca-1, which is present on mouse hematopoietic stem cells 254-255, mammary gland 238, prostate 233, lung 257, dermis 230,258, liver 260, heart 118, and even skeletal muscle cells 261-262. Unfortunately, as there is no human homologue of Sca-1, this protein is of little clinical applicability. On the other hand, the receptor for Stem Cell Factor, c-Kit, is not as commonly expressed as Sca-1, and has so far only been found in a small group of tissues (pancreas 263, gut 264, mammary gland 265,266, and hematopoietic stem cells 267).

Other potential stem cell markers may be proteins involved in self-renewal, such as Notch-1 and Msi-1 (murine small intestine 268, hair 269, stomach 270, and mammary gland 271) or proteins necessary for developmental tissue structure such as p63, which is correlated with ocular surface cells 272, skin 273, mammary gland 274, lung 275 and prostate 276 stem cells.

The aim of this study is to determine the localization in the salivary gland of cells expressing putative stem cell markers and, subsequently, to determine how they respond to irradiation and cell transplantation. Irradiation will induce a loss of salivary gland stem cells 243 which can be visualized and quantified by assessing marker expressions. Cells expressing stem cell markers should re-appear after stem cell transplantation, enabling the determination of potential stem cells in the tissue. Our results provide information that can be used for selection and characterization of salivary gland stem cells, and reveal information on the stem cell niche.

RESULTS/DISCUSSION

THE GENERAL MORPHOLOGY OF THE MURINE SUBMandIBULAR GLAND

The salivary parenchyma has a well-known structure that comprises three major cell types 277. The largest part of the submandibular gland (±60%) consists of typically triangle shaped mucin-expressing (dark purple, PAS stained) saliva-producing acinar cells (AC) 185 (Fig. 1A,B, Control, insert). Spherically joined acinar cells called acini (Fig. 1A, Control, insert-dashed line) surround a small lumen that collects the primary saliva. Contractile myoepithelial cells (MY) assist duct cells (PAS-) (Fig. 1B, Control, insert-dashed line) to further modify and transport the saliva to the oral cavity 31. The submandibular gland ductal system is thought to contain the stem/progenitor cells 128. It consists of three different ductal cell types aligned as intercalated duct (ID) cells, striated duct (SD) cells or granular containing tubules (GCT, profoundly in males), and excretory duct cells (ED). Ninety days after irradiation, the tissue is virtually devoid of acinar cells and mainly striated duct cells remain 243 (Fig. 1A,B, IR). Transplantation of c-Kit bright cells obtained from 3 days old cultured salispheres can prevent the loss of acinar cells (Fig. 1A,B, IR-SCT) 220, albeit with a somewhat less well-organized tissue structure when compared to control situation. Apparently, a severe lack of cell replacement has resulted in the relative survival of striated duct cells and an almost complete loss of other cell types. Evaluating the expression of (stem) cell markers after irradiation and transplantation will provide important information on the localization of the tissue stem cells.

CYTOSKELETAL PROTEINS

Cytoskeletal cytookeratin (CK) filaments, which ensure cell shape, cell motion, and regulate cellular divisions, are differentially expressed in each tissue. This makes them potentially useful for fingerprinting of certain cell types. Therefore, CK expression patterns have been widely examined in many studies. Human 278,279, rabbit 280-282, dog 283 and rat 284-286 parotid/submandibular glands widely express Cks, although information on murine glands is scarce 287. For example, CK 8 was shown to be expressed on acinar and duct cells and not on GCT cells 287. We observed that this is not only true for CK 8 (Fig. 2A, Control), but also for CK 18 (Fig. 2B, Control). Moreover, the expression of both Cks was most pronounced in intercalated duct (ID) and excretory duct (ED) cells. Even more specific staining was observed with CK 19 (Fig. 3A, Control) and CK 7 (Fig. 3B, Control), which mark only the three different duct cell types, whereas CK 14 (Fig. 3C, Control) is restricted to ducts in the excretory and striated duct compartment and to myoepithelial cells.
Clearly, irradiation affects the number of cells expressing these cytokeratins (Fig. 2, IR and 3, IR). After cell transplantation, some highly CK expressing cells re-appeared locally in the duct compartment, suggesting duct reconstitution (Fig. 2A, B, IR-SCT, Fig. 3C, IR-SCT). Interestingly, also acinar cells with normal CK levels re-appeared (Fig. 2B, IR-SCT). Additionally, at the site of transplantation, clusters (CC) of CK expressing cells were observed (Fig. 2B, IR-SCT).

In summary, CKs are indeed clearly differentially expressed among duct and acinar cells. The strong reduction after irradiation reflects a strong reduction in acinar and ductal cells, but may also be an expression of loss of tissue integrity. Apparently, both parameters are (partly) re-established after transplantation. CK 7 and 19 are the most appropriate for selection of duct cells. Additional precision may be obtained with selection based on expression levels of CK 8, 18 (high/low) and CK 14. CKs, however, do not select for stem/progenitor cells, and can only be used as co-markers to localize the position of such a cell.
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FIGURE 3. CK 19, CK 7 AND CK 14 EXPRESSION IN THE MOUSE SUBMANDIBULAR GLAND.

Control: Filaments CK 19 (A), CK 7 (B) and 14 (C) are exclusively expressed by ductal cells (intercalated ducts (ID), striated ducts (SD), and excretory ducts (ED)) and completely absent on acini (AC). Still, CK 14 is also expressed by myoepithelial cells (MY). Irradiation (IR) dramatically affects the CK expression which was partly restored after IR-SCT. Scale bar = 50 μm, insets = 20 μm.

MEMBRANE CD (CLUSTER OF DIFFERENTIATION) PROTEINS

CD29 (β1 integrin receptor)

Beside cytoskeletal proteins, adhesion molecules may be used for cell type selection. By re-arranging the internal actin cytoskeleton, integrin receptors influence cellular activities like cell growth, adhesion, polarization and differentiation. High expression of one of these proteins, β1 integrin receptor (CD29), has been related to the regulation of stem cell maintenance and stem cell migration during differentiation, and has been found to be expressed on stem cells of mammary gland, skin, liver, and brain. In normal mouse submandibular glands, CD29/β1-integrin seems ubiquitously expressed, with intermediate expression on striated duct cells (SD) (Fig. 4A, Control) and especially high expression on interlobular excretory duct cells (ED). CD29 expression is strongly reduced after irradiation (Fig. 4A, IR). Although CD29 was still present on striated duct cells (SD), hardly any CD29high expressing excretory duct cells (ED) could be found. The importance of CD29 is further emphasized by a study on a human salivary gland cell line HSG. Once CD29 expression was lost, any further duct cell formation was blocked, resulting in immediate acinar cell-like differentiation and loss of migration. Assumingly, after irradiation this would result in the loss of acinar cells and tissue integrity.

After transplantation, normal CD29+ cells were found in regenerating glands, indicative of restored tissue integrity (Fig. 4A, IR-SCT). Moreover, many CD29high expressing cells in EDs were observed, indeed suggesting that the salivary gland stem cells express high levels of CD29.

These data show that environmental changes resulting from radiation damage have impact on extracellular matrix-cell protein interactions, and are indicative of the incapability to regenerate. Furthermore, it proposes CD29high excretory duct cells as potential stem cells.

CD24

Like CD29, CD24 is suggested to play a role in the proliferation/differentiation of progenitor cells. Possibly, CD24 is expressed by potential stem/progenitor cells in salivary glands, as has been shown for mammary gland stem cells, and prostate stem cells. In the submandibular gland, CD24 expression is restricted to duct cells and is highest on excretory duct cells (ED) (Fig. 4B, Control). From striated ducts (SD) to intercalated duct cells the expression seems to be progressively reduced. Strikingly, CD24 expression is severely affected after irradiation, leaving only few CD24 expressing cells (Fig. 4B, IR).
In IR-SCT glands, CD24<sup>high</sup> cells re-appear in the entire duct system (Fig. 4B, IR-SCT). These findings are in agreement with the expected loss of stem cells after irradiation and re-appearance after stem cell transplantation. It also indicates that CD24<sup>bright</sup> cells may well be potential stem cells of the submandibular gland, and may well be used as a selection marker for stem cells.

**FIGURE 4. EXPRESSION OF CD29 AND CD24 BY THE MOUSE SUBMANDIBULAR GLAND.** Control: (A) All gland cell types express CD29, albeit to variable levels. (B) CD24 is exclusively expressed in duct cells (intercalated ducts (ID), striated ducts (SD), and excretory ducts (ED)), and brightly CD29- and CD24-expressing cells seem to be present in the transplanted glands (IR-SCT) when compared to irradiated only glands (IR). Scale bar = 50 μm, insets = 20 μm.

**PROTEINS INVOLVED IN DEVELOPMENT**

**14-3-3 σ (STRATIFIN)**

The group of 14-3-3 proteins, which consist of 7 isoforms, interact with many molecules, exerting numerous crucial intracellular functions such as cell proliferation and differentiation and cellular trafficking. Interestingly, it has been demonstrated in skin that the isoform 14-3-3σ (stratifin) is expressed by cells that are about to undergo differentiation. Functional inactivation of stratifin leads to continued proliferation and immortalization. In submandibular glands, 14-3-3σ/stratifin was expressed in cells of all the duct types (Fig. 5A, Control). In striated (SD) and excretory ducts (ED), several high stratifin expressing cells were observed, probably indicating ongoing differentiation. Strikingly, hardly, if any, expression of stratifin was observed after irradiation (Fig. 5A, IR), indicative of a strongly reduced differentiation activity. As expected, after IR-SCT, the gland contains many stratifin expressing cells, which is in agreement with the high level of proliferation observed before both in the duct compartments as well in cell clusters (CC) at the injection site (Fig. 5A, IR-SCT). These data confirm the strong capability of duct cells to proliferate, differentiate, and initiate the regeneration process. Stratifin could be used as negative marker in combination with other markers to select for stem cells.

**ΔNp63**

The protein p63, the most ancient member of the p53 family, became interesting as a stem cell marker when it was shown that mice lacking this gene (p63<sup>-/-</sup>) die neonatally due to the absence of a large number of tissues, among which craniofacial organs. Although p63 expression is not limited to stem cells, it has been reported that these express the highest levels of p63. Its exact function is still not fully elucidated since the two isoforms, TAp63 and ΔNp63 seem to have opposing functions. A balance between both isoforms is required for normal development where in stem cells the ΔN isoform is highly expressed.

In mouse submandibular glands, ΔNp63 is expressed in myoepithelium cells (MY) and in certain cells in the excretory duct (ED) (Fig. 5B), similar to what has been observed in human salivary glands. After irradiation a dramatic reduction in ΔNp63 expressing cells is observed (Fig. 5B, IR). Strikingly, after cell transplantation, ΔNp63<sup>+</sup> cells were arranged either around or within duct segments, and at the site of injection as clustered cells (CC) in IR-SCT glands (Fig. 5B, IR-SCT). These observations indicate that ΔNp63 expressing cells may be involved in regeneration processes.
Although the precise function of p63 needs to be elucidated for the submandibular gland cells, it might be a putative marker for excretory duct stem cells, when myoepithelial cells can be excluded. This observation further indicates a particular role of ∆Np63 in the myoepithelial cells, which have been less well studied, compared to other epithelial cell populations. However, it was recently hypothesized that the myoepithelial cell population might have crucial input on the stem cells \(^3\text{00,301}\), and have its own progenitors \(^3\text{02}\).

**FIGURE 5. EXPRESSION OF 14-3-3σ (STRATIFIN) AND ΔNp63 BY THE MOUSE SUBMANDIBULAR GLAND.** Control: (A) 14-3-3σ (stratifin) expression is confined to intercalated ducts (ID), striated ducts (SD), and excretory ducts (ED). (B) ΔNp63 is uniquely expressed in excretory duct cells (ED) and myoepithelial cell types (MY). Both 14-3-3σ and ΔNp63 expression is affected by irradiation (IR) and restored after transplantation. (CC) clustered cells at the site of injection of transplantation. Scale bar = 50 μm, insets = 20 μm.

**STEM CELL SELF-RENEWAL/Maintenance**

**Sca-1**
Sca-1 (Stem cell antigen-1 or lymphocyte activation protein-6A (Ly-6A)) is one of the best-known stem cell markers \(^3\text{03}\), which has been shown to be expressed in a variety of mouse tissues \(^2\text{28,233,238,254-257}\). Its expression pattern in the adult submandibular gland has already been addressed in our previous study \(^2\text{22}\). In short, Sca-1 is exclusively expressed by striated duct (SD), excretory duct (ED), and endothelial cells (Fig. 6A). However, differences are found with males (Fig. 6B): the GCT (Granular Convoluted Tubules) ducts, mostly absent in females, lacked expression of the protein. Also, Sca-1 expression is greatly reduced after irradiation (Fig. 6C), and re-appears after IR-SCT, especially in excretory ducts (ED) (Fig. 6D) and in clustered cells (CC) at the transplantation site. The observation that excretory duct cells express Sca-1 more brightly than striated cells indicates that it can be used as a selection marker for stem cells.

**c-Kit**
The stem cell marker c-Kit is the receptor for Stem Cell Factor (SCF). Expression of c-Kit was found to be related to survival and renewal of the earliest multi-lineage hematopoietic progenitors \(^3\text{03}\) and other tissues like skin and testis \(^3\text{04}\). Cells expressing this marker are able to rescue the salivary gland form irradiation-damage \(^2\text{22}\). They are exclusively expressed by excretory duct cells (ED) (Fig. 6B, Control). c-Kit expression is very much reduced after irradiation (Fig. 6B, IR) and re-appears after IR-SCT (Fig. 6B, IR-SCT), c-Kit cells were again abundantly present in the excretory ducts and in clustered cell (CC) groups at the place of transplantation. This suggests that c-Kit cells contain the essential cells that are able to (re-)generate organ function and morphology. Although c-Kit mutant mice are viable, this model showed the importance of these cell types in other tissues for adequate sperm cell formation, skin pigmentation \(^3\text{05}\) and hematopoietic activity \(^3\text{06}\). Expression of the c-Kit marker may be a starting point for selection of salivary gland stem cells.

**Notch-1**
Stem cell fate is partly regulated by signaling pathways such as Wnt, Hedgehog, and Notch \(^3\text{07}\). Depending on the circumstances, Notch can be involved in inducing differentiation and maintenance of stem cells as it is involved in a variety of pathways regulating stem cell number, cell survival, and lineage decisions \(^3\text{08}\).
Most of the Notch genes, which are transmembrane receptors (Notch 1-4), have been found in organs such as skin \(^{309}\), brain \(^{249}\), mammary gland \(^{310}\), pancreas \(^{311}\), the hematopoietic system \(^{312}\), and intestine \(^{313}\).

In the submandibular gland, Notch-1 was found to be expressed in the cytoplasm of interlobular excretory duct cells (Fig. 7A, Control). This suggests that Notch-1 is only expressed in a small percentage of unique cells. Ninety days post-irradiation, only a single Notch-1+ surviving cell was observed (ED, arrow) out of all sections evaluated (Fig. 7A, IR). In IR-SCT glands, Notch-1 expressing cells were found in close proximity to excretory duct structures (Fig. 7, IR-SCT). These observations indicate that Notch-1 signaling plays a role during the regeneration of irradiated glands and be a selection marker.

Musashi-1

The RNA binding protein Musashi-1 (Msi-1) seems to function in cooperation with Msi-2 to activate Notch signaling, and as such seems to be involved in stem cell self-renewal. Although not much is known about Msi-1, it was originally associated with asymmetric division of neural progenitor cells, and seems to be down-regulated with differentiation into progeny \(^{314}\). In the submandibular gland, a high nuclear Msi-1 expression was correlated with specific interlobular excretory duct cells, and some striated duct cells (Fig. 7B, Control). After irradiation (IR), none of these cells could be observed, and only very infrequently Msi-1+ cells were noticed in a regenerated gland (IR-SCT). Nevertheless, if Msi-1 down-regulation is linked with differentiated progeny, this does strengthen the hypothesis that the majority (but not all) of the excretory duct and a minority of striated duct cells have some level of stem cell potential. Thus, potential Msi-1 could be used as a marker although its exact role in the regeneration process still needs to be determined.

Musashi-2

Musashi-2 also belongs to a conserved group of RNA binding proteins, exhibits high sequence similarity Msi-1, and is thought to play role in the proliferation and maintenance of tissue specific stem cells \(^{315}\). Although Msi-2 has been correlated with neural stem/progenitor cells \(^{316}\) and hair follicle stem cells \(^{309}\), its expression pattern in the gland does not seem to be as specific as Msi-1. High expression levels were observed in intercalated and excretory duct cells and in some large small striated duct shaped cells. Despite the reduction in Msi-2 expression in some duct cells, and especially the excretory ducts, after irradiation, it was not completely diminished. Although IR-SCT glands did show some Msi-2 expressing clustered cells at injection places, the protein seems to be not suitable for isolating gland stem/progenitor cells.

**FIGURE 6. EXPRESSION OF SCA-1 AND c-KIT.** Control: (A) Sca-1 expression in glands of female mouse is confined to excretory (ED), striated duct cells (SD) and endothelium. Male glands only show Sca-1 expression on excretory duct cells and endothelium. Granular convoluted tubules (GCT) lack Sca-1. Sca-1 expression is abrogated after irradiation (IR), while regenerated glands (IR-SCT) contained again Sca-1 expressing cells in both duct system (ED) as (CC) clustered cells at the site of injection of transplantation. (B) c-Kit is exclusively expressed by excretory duct cells (ED). Similar to Sca-1 is c-Kit expression diminished post-irradiation (IR), but bright cells could be noticed again after stem cell transplantation (IR-SCT). Scale bar = 50 μm, insets = 20 μm.
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CONCLUSION

Irradiation clearly affects many glandular protein immunostaining. Although the loss of almost the complete acinar, intercalated and excretory duct compartment would on its own already affect overall expression of the proteins, it is clear that the loss of cytoskeletal regulation, CD antigens and proteins involved in development and stem cell maintenance are severely affected. This is caused by, or the result from, loss of tissue integrity after irradiation. It also emphasizes the general lack of ability to regenerate the tissue although still some viable cells may be present. The remaining protein-expressing cells either lost their functional ability to restore the gland and/or their architectural position and connection with their environment to be functional.

In contrast, in regenerating IR-SCT glands, the re-appearance of cells expressing both membrane, CK, developmental and self-renewal-related proteins implies that the transplanted cells might either integrate/fuse into the remaining duct system or even initiate the formation of new ducts, restoring tissue integrity.

Our data indicate a possible location of stem cells in the excretory ducts and reveal potential markers to isolate and study the submandibular gland stem/progenitor cell. Isolation of enriched duct cells could be performed using combinations of CD29<sup><sup>high</sup></sup> or CD24 (Table 1). On top of this, Sca-1 and/or c-Kit selection seems to be promising for stem cell selection (Table 1). Next to this, p63, Msi-1 and/or Notch-1 expression could be used to verify the localization of excretory duct stem cells. Based on the morphological observations of these stem cell-related markers, this investigation opens new abilities in the search towards the salivary gland stem cell.

Further research need to be performed to characterize and test cell types expressing these proteins and include isolation, repopulation and regeneration studies, both in vitro and in vivo in irradiated recipients in order to reveal their potential.

FIGURE 7. NOTCH-1, MUSASHI-1 AND -2 EXPRESSION. Control: (A) The receptor Notch-1 is only present on few cells in the interlobular excretory duct system. (B) Musashi-1 is expressed in the nucleus of excretory (ED) and striated duct compartment (SD). (C) Musashi-2 is expressed by both acinar and gland duct cells, but a clear high expression pattern is noticed in intercalated (ID), excretory duct cells, and unique cells in the striated duct (arrows). Expression of all these proteins is clearly affected after irradiation (IR), while some bright expressing cells re-appeared after IR-SCT. (CC) Clustered cells at the site of injection of transplantation. Scale bar = 50 μm, insets and A and B = 20 μm.
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MATERIALS AND METHODS

ANIMALS

Female C57BL/6 mice, 8-12 weeks old, were purchased from Harlan (Horst, The Netherlands), kept under clean conventional conditions, and fed ad libitum with food pellets (RMH-B, Hope Farms B.V., Woerden, The Netherlands) and acidified tap water (pH 2.8). All experiments were approved by the Animal Ethical Committee on animal testing of the University of Groningen.

IRRADIATION OF SALIVARY GLANDS AND CELL TRANSPLANTATION

Salivary glands were locally irradiated with a single dose of 15 Gy of X-rays (Philips CMG 41 X, 200kV, 10mA, 5 Gy/minute). This radiation dose is known to induce sufficient damage without compromising the general health of the animals 185. Mice were protected from off-target radiation by a 3 mm lead shield. One group of mice received a submandibular gland cell transplant at 30 days post-irradiation, as described previously 220.

In short, cells from hyaluronidase and collagenase dissociated submandibular gland cells were transferred to defined DMEM/Ham's F12 medium containing EGF, FGF-2, N2, and insulin. After 3 days, ~8,000 salispheres per digested submandibular gland were formed. Subsequently, the spheres were trypsinized and purified c-Kit+ cells (100-1,000) were intra-glandularly injected in the irradiated submandibular glands of recipient mice.

Submandibular glands from mice, sacrificed 90 days after irradiation (IR) only or irradiation + stem cell transplant (IR-SCT), were used for morphological examination. After sacrificing the mice, submandibular glands were extirpated for further processes. At least three mice (6 glands) per group, and a minimal of three sections per gland were evaluated.

IMMUNOHISTOCHEMISTRY

The extirpated submandibular glands were incubated for 30 hours at 4°C in 4% buffered formaldehyde. Following dehydration, the tissue was embedded in paraffin. Five-micrometer sections were analyzed for the presence of various epitopes. The pre-treatment protocol and company name of each antibody used is depicted in Table 2. Visualization was further performed using biotinylated secondary antibodies (Dako, Carpinteria, CA), an avidin-biotin-horseradish peroxidase complex (Vector Elite Avidin-Biotin Complex kit) and DAB. Nuclei were stained with Hematoxylin. Periodic Acid Schiff's base staining, which detects mucins, was used to show acinar cells. Control sections without primary antibodies were all negative. Tissue sections of glands were analyzed using a bright field microscopy (Olympus CX40, Germany).

Table 2. Specific antibodies and conditions used.

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Table 1. Expression pattern of proteins in the mouse salivary gland.

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Sca-1 | c-Kit | Notch-1 | Msi-1 | Msi-2

MATERIALS AND METHODS

ANIMALS

Female C57BL/6 mice, 8-12 weeks old, were purchased from Harlan (Horst, The Netherlands), kept under clean conventional conditions, and fed ad libitum with food pellets (RMH-B, Hope Farms B.V., Woerden, The Netherlands) and acidified tap water (pH 2.8). All experiments were approved by the Animal Ethical Committee on animal testing of the University of Groningen.