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Chapter 6

Retrograde intra-ductal salivary gland stem cell transplantation

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ABSTRACT

Transplantation of salivary gland stem cells is an attractive option to treat reduced gland function. However, the optimal administration route needs to be determined. In mice, intra-glandular transplantation of primary salisphere derived c-Kit+, CD133+ and CD24+ cells was shown to induce recovery of radiation-damaged salivary glands. Often, transplanted cells were found to integrate into the ducts, at a location where stem cells normally reside in the salivary gland. However, the observed non-uniform functional response after transplantation may be due to improper homing of the cells due to the intra-glandular injection. Therefore, the aim of this study is to investigate whether retrograde intra-ductal injection of stem cells can be used as an alternative method of transplantation into radiation damaged-salivary glands. This required the use of larger animal model, for which we used rats.

Dispersed rat salivary gland cells were cultured into primary salispheres. Salisphere derived cells were evaluated for expression of stem cell markers using flow cytometry, and for their differentiation and self-renewal potential. Putative stem/progenitor cells were retrogradely injected into ductal orifices of irradiated (15Gy) rat submandibular and parotid salivary glands and saliva flow rates were compared with intra-glandular transplantation.

Stem cell markers such as c-Kit, CD29 and CD133 were expressed by cells of the ducts of rat salivary glands and in primary salispheres. Primary salisphere derived cells were able to form secondary spheres and were found to be able to self-renew for up to 4 passages. In addition, rat primary salispheres differentiated in vitro into organoids containing differentiated duct and acinar-like cells of salivary gland. Preliminary data show that retrograde injection of stem/progenitor cells did not improve post-irradiation salivary gland function when compared to intra-glandular injection. Therefore, retrograde intraductal injection of stem cells into the orifices of main salivary gland duct does not seem to improve transplantation success. Alternative methods need to be tested to optimize transplantation of salivary gland stem cells for patients.
1. INTRODUCTION

Hyposalivation, a severe reduction in saliva flow, may lead to xerostomia which is characterized by symptoms such as dry mouth, dry nasal passages, sore throat, loss of oral hygiene, dental caries, oral candidiasis, loss of taste, difficulties with swallowing and speaking. Collectively, these symptoms and lack of treatment options drastically reduce the patient’s quality of life. Stem cell therapy may be a future alternative to improve salivary gland (SG) function in these patients.

Recently, we showed that intra-glandular transplantation of murine primary-sphere derived c-Kit^+ , CD133^- and CD24^+ cells can functionally rescue irradiated salivary glands (Chapter 2, 1). However, an inhomogeneous response was observed, ranging from animals in which a complete functional recovery was apparent, to animals in which no response at all was seen. We speculate that this could be the result of poor homing (inappropriate deposition and lodging) of the transplanted cells to their niche, as it may be unavoidable using random intra-glandular injection of cells.

The anatomical location, or habitat, of stem cells within a tissue is referred to as the “niche”. The niche is composed of different types of cells, which provide signals for stem cells to respond under physiological conditions or when the tissue is challenged such as after damage to tissue. Hence, it is well accepted that the niche has a major influence on stem cell function. Therefore, delivery of stem cells targeted to the tissue specific niche may be a critical determinant for successful stem cell transplantation. Considering the future prospects of salivary gland stem cell transplantation, an efficient and reproducible method that can deliver stem cells to their niche, or allowing stem cells to easily migrate towards the niche, may be necessary.

The duct-ligation induced salivary gland damage model has been used to understand regeneration in SG. In this model, damage is inflicted by ligation of the main ducts, which leads to death and ultimately the disappearance of acinar cells, followed by a marked proliferation of residual ductal cells. Reopening of the ducts repopulates the lost acinar cells, a resultant of activation of stem/progenitor cells. Matsumoto et al 2007 3 showed that in a duct ligation damage model of SG, post-ligation proliferating (BrdU positive) cells were observed within the ducts of the SG. Moreover, they showed that these cells expressed known stem cell markers like CD29 and c-Kit. This suggests that stem/progenitor cells in the ducts proliferate and reconstitute the lost acini as a response to the damage. In agreement with this, the presence of CD133^+ , c-Kit^+ , CD24^- and CD49f^- cells in the intercalated (ID), striated (SD) and the larger excretory ducts
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(ED) of SG, indicate that indeed stem/progenitor cells reside in the major ducts. These results collectively support the notion that the ducts contain the SG stem cells niche. The success of transplantation is most likely dependent on the location of injected cells and/or the capacity of the cells to migrate to their niche. Therefore, transplantation of stem/progenitor cells exactly into the ducts (the prospective niche) might provide better engraftment and subsequent homing, and may result in a more successful outcome.

Retrograde injection is the method of injecting proteins, cells, drugs or genes against the flow of body fluids, for instance saliva flow. In this case, the flow of saliva into the excretory ducts should be temporarily suppressed to allow for adhesion to the ducts and migration and integration into the tissue. This can be achieved, for instance, by Atropine, which is a competitive antagonist for the muscarinic acetylcholine receptor types M3, which is involved in the secretion of saliva by acinar cells. Subsequently the solution of interest (either viruses, proteins, drugs, or cells) can be intra-ductally injected. If feasible, there will be no need to surgically open the skin to access the glands as is required for intra-glandular injection, and thus reducing post-operative stress and complications.

Therefore, the aim of the current study is to investigate the potential of retrograde ductal injection as a method of salivary gland stem cell transplantation.

The excretory duct of the parotid gland (Stenson’s duct) open in to the buccal mucosa (cheek of oral cavity) near the upper teeth, whereas the submandibular excretory duct (Warton’s duct) open underneath the tongue. Since the ductal openings are microscopically small, it is difficult to validate the method in mice, hence we performed this study in rats. However, no prior knowledge of stem/progenitor cells from rat SGs is available. Knowledge of the rat stem/progenitor cells is a prerequisite before proceeding to in vivo experiments. Therefore, in this study we first tried to identify and characterize stem cells in rat submandibular and parotid glands and further cultured these cells into salispheres allowing the selection of stem cells. Subsequently, retrograde injection was tested using salisphere cells injected into irradiated salivary glands of rats, using saliva production as a functional read out of transplantation.
2. MATERIALS AND METHODS

2.1. Animals

Wistar (WU) outbred rats were purchased from Harlan (The Netherlands) and Wistar Kyoto (WKY/NCr) rats from Charles River (USA), respectively. Animals were fed *ad libitum* with food pellets (RMH-B, Hope farms B.V., Woerden, The Netherlands) and water. All experiments were approved by the Ethical Committee on animal testing of the University of Groningen.

2.2. Primary sphere culture

Rats were sacrificed under anesthesia and submandibular and parotid glands were carefully dissected and suspended into HBSS (Invitrogen) with 1% BSA. The glands were mechanically and enzymatically digested with Collagenase (23 mg/ml) and Hyaluronidase (40 mg/ml) and Calcium chloride (50 mM) for 40 minutes at 37˚C incubation under gentle shaking conditions. The cell suspension was centrifuged at 400 g for 5 minutes and new HBSS 1% BSA buffer and enzymes were added followed by 40 minutes incubation at 37˚C. For submandibular gland cells, the resulting cell suspension was washed twice with HBSS 1% BSA and filtered through a 100 µm filter and suspended into DMEM:F12 with EGF (20 ng/ml), FGF-2 (20 ng/ml), N2 (10 µl/ml), Insulin (10 µg/ml) and Dexamethasone (1 µM). Parotid gland cells were suspended into Mammocult medium (Stem Cell technologies) with hydrocortisone (0.48 µg/ml) and heparin (0.0004%) mixed with proliferation medium (5 ml in 50 ml of Mammocult).

2.3. *in vitro* secondary sphere-formation and self-renewal assay

10,000 primary sphere-derived single unfractionated cells (live, unstained cells) were plated in 75 µl gel/well (50 µl matrigel + 25 µl cells in primary sphere medium) in a 12-well plate and were solidified for 10-15 min at 37˚C. After solidification, 1 ml of enriched medium, EM (primary sphere medium + Rho-inhibitor, Y-27632) was added gently on top of the gels and incubated for 5-7 days in a 37˚C incubator. Spheres that appeared (in 4-7 days) were counted per well and the percentage of sphere-forming cells per group was calculated. To test long-term self-renewal ability, these secondary spheres were passaged every 7-10 days. First, medium on top of gel was gently removed and 1 ml of Dispase (1 mg/ml) was added directly to the gels and incubated for 1 hour at 37˚C to dissociate the matrigel. This was followed by a washing step with PBS/0.2% bovine serum albumin and centrifugation at 400 G for 5 min. Pelleted spheres
were dissociated with 0.05% trypsin-EDTA and passed through 40 µm filter to filter out clumps to obtain single cells. Single cells obtained were counted and re-plated (10000 cells/well) for the next passage and this procedure was repeated at the end of every passage.

2.4. *in vitro* differentiation/organoid-formation assay

To test differentiation/organoid-formation ability of primary spheres, all spheres were collected after 3-4 days of culture and pelleted by centrifugation. Pelleted spheres were suspended into 10x DMEM (1/10th of total collagen) with collagen (40 collagen:60 matrigel) with 1M NaOH, necessary to solidify the gel. Later matrigel (at 4°C) was added and gently mixed for uniform distribution of spheres and plated immediately as 100 µl/well into a flat bottomed 96-well plate and allowed to solidify for 15-20 min at 37°C. After solidification, 150 µl of primary sphere medium with KGF, R-spondin or Fetal Calf Serum (FCS) was added on top of each gel and incubated at 37°C. Spheres in each well were quantified and imaged every 2-3 days for the development of organoids.

2.5. Flow cytometry

After 3-4 days of culture primary salispheres were evaluated. Spheres were centrifuged at 400 g for 5 minutes to pellet them, 1 ml trypsin was added to the pellet and incubated for 10-20 minutes, and the cell suspension was passed through 50 µM filters using a 5 ml syringe (with 25G needle) to obtain a single cell suspension. Subsequently, the cells were centrifuged at 400g for 5 minutes and single cells in the pellet were carefully resuspended into PBS/0.2%BSA, and stained with fluorescent-labeled antibodies like CD24, CD117. Stained cells were washed with PBS/0.2%BSA and measured on LSR-II (BD biosciences) for positive staining or sorted on Moflo® Astrios (Beckman Coulter) for living cells. Propidium Iodide (1 µg/ml) was used to detect dead cells.

Anti-CD117 (c-Kit) antibody was custom designed and developed with Eurogentec. Peptide sequence of the epitope of rat c-Kit molecule was determined (GEDYVKSDNQSNIR). The antibody was raised in guinea pig against this pre-designed peptide sequence of the rat c-Kit molecule. Thus produced antibody was tested on rat salivary gland tissue using immunohistochemical staining procedure.
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2.6. Immunohistochemistry

Rats were sacrificed under anesthesia and submandibular and parotid glands were carefully dissected without contamination from other organs and fat. In order to perform immunohistochemical staining, both the extirpated glands were incubated for 30 h at room temperature in 4% buffered formaldehyde. Following dehydration, the tissue was embedded in paraffin and sliced into 5 µm sections. The sections were dewaxed and labeled for the following markers: CD117/c-Kit, CD24 (Santa Cruz, sc7034), CD133 (abcam, ab19898), Cytokeratin (CK) 18 (abcam, ab668), PCNA (Dako Cytomation, MO879), Aquaporin5 (abcam, ab85905) and α-smooth muscle actin (SMA, Sigma A2547). Visualization for bright field microscopy was accomplished by adding specific secondary biotin carrying antibodies, an avidin-biotin-horse peroxidase complex (Vectastain ABC) and the diaminobenzidine (Impact DAB) chromogen. Nuclear counterstaining was performed with hematoxylin. Control sections without primary antibodies did not show positive immunostaining.

2.7. Irradiation and saliva collection

Submandibular and Parotid glands of rats (9-11 week old) were irradiated with a single dose of 15Gy (Precision X-ray Inc. – X-rad 320, 200kV, 20mA, 1.843Gy/min). This dose is known to induce hyposalivation without compromising the general health of the animals6. Pilocarpine injection (2.5 mg/kg, s.c.) was given to animals under anesthesia and saliva flow rate was determined every 30 days post-irradiation. Vacuum pump connected miniaturized Lashley cups were placed on the opening of duct (cheek-parotid gland; under tongue-submandibular) and saliva was collected for 30 min (for Parotid gland with second pilocarpine injection) and 15 min (for submandibular gland) and the quantity was determined gravimetrically 7, assuming density of 1 g/ml for saliva. Since the parotid gland ducts open into left and right cheek separately, saliva was collected separately from these two. Percentage flow rate of saliva of an animal is calculated by denoting the pre-irradiation saliva as 100%. The principle behind this functional assay is that the in vivo regeneration is the most rigorous test for stem cell function.

2.8. Transplantation

Primary sphere (salispheres) derived single cells from both submandibular and parotid gland were transplanted without further manipulation, or were sorted to select PI negative cells (referred to as “viable” from now on (50,000-70,000/gland), or were sorted based on c-Kit+
expression (5000-7000 cells/gland). Sorted cells were suspended into α-mem with 2% FCS with total volume of 200 µl/gland for retrograde and 25–50 µl/gland for intra-glandular transplantation. Thirty days post-irradiation two groups of animals were transplanted with retrograde and intra-glandular method of transplantation individually. Cell suspension was injected intra-glandular into each gland with a micro-syringe and needles (Hamilton 7803-02) and the skin was sutured. For retrograde injection 125-150 µl of cell suspension was injected into the duct openings of the parotid (in the cheek) or submandibular (under the tongue) gland. Animals were administered with analgesic immediately following surgery.

3. RESULTS

3.1. Isolation of rat salivary gland stem/progenitor cells using *in vitro* salisphere cultures

To isolate and culture stem/progenitor cells from the rat salivary glands we developed an *in vitro* floating sphere culture method. The method is schematically shown in Fig.1A.

**Figure.1:** (A) Diagrammatic representation of isolation of stem/progenitor cells from rat submandibular and parotid gland tissue. (B) Pictures of primary spheres from submandibular and parotid gland tissue in culture from 0 to 6 days in culture. (C) Quantification of primary spheres obtained per mg of submandibular (SUB) and parotid gland tissue (PAR) (D) anti-BrdU staining on primary spheres showing nuclei positive for BrdU.
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Exirpated parotid and submandibular glands were individually subjected to mechanical and enzymatic digestion using hyaluronidase and collagenase. Due to the high endogenous enzyme content of parotid gland, it was difficult to obtain sufficient viable cells. Therefore different concentration of enzymes, at various times of digestion, culture medium were tested and optimized for parotid gland (see methods). The resultant cell suspension was filtered to remove clumps and was suspended into DMEM:F12 with growth factors (submandibular gland) and Mammocult medium with growth factors (parotid gland) and incubated at 37°C. Three days after initiation of the culture, round spheroid structures were observed which closely resemble the primary spheres obtained from murine and human salivary gland cell cultures. In rats, both submandibular (Fig.1B, submandibular) and parotid (Fig.1B, parotid) gland could be cultured as salispheres.

Figure 2: (A) quantification of primary spheres obtained per mg of submandibular and parotid gland tissue (B) anti-BrdU staining on primary spheres showing nuclei positive for BrdU (green).

Quantification of these salispheres yielded 116 ± 16 spheres/mg tissue of submandibular gland and 122 ± 12 spheres/mg tissue of parotid gland (Fig.2A). BrdU labeling indicated the existence of proliferating cells in these spheres (Fig.2B). The morphology of these spheres seems similar to that of murine primary spheres.

3.2. Characterization of rat salivary gland stem cells

Next, rat salivary gland tissue and primary spheres were tested for the presence of stem/progenitor cells, based on expression of known stem cell markers. First, submandibular and parotid gland tissues were immunohistochemically stained for c-Kit, CD133 and CD24.

Since rat specific antibodies were not available, we have used anti-mouse antibodies for CD133 and CD24. For c-Kit, we have developed a custom-designed antibody specific for rat (see methods) for this study. Indeed, cells in the striated (SD) and excretory duct (ED) cells
of the submandibular gland tissue (Fig.3A, submandibular) stained positive for c-kit (CD117), CD133 and CD24 markers. Similar expression of c-Kit was observed predominantly in duct cells of parotid gland tissue (Fig.3A, parotid).

Regretfully, the serous enzymatic content of the parotid gland created substantial background staining, making it impossible to reliably detect the presence of CD133 and CD24. These results indicate the presence of putative epithelial stem/progenitor cells in the rat salivary gland ducts, which is in agreement with observations in mouse (Chapter 2, this thesis) and human tissue.

To evaluate the presence of putative stem cells in primary spheres obtained from rat salivary gland tissue was enzymatically dispersed to single cells, and stained with fluorescently labeled antibodies specific to c-Kit (CD117), CD133 and CD24. Flow cytometry showed that 2.9 ± 1.7% of primary sphere-derived cells from submandibular gland express c-Kit (CD117), 83.9 ± 10.4% express CD29, 1.0 ± 1.4% express CD133, and 0.6 ± 0.6% express CD24 (Fig.4A, submandibular), whereas 5.3 ± 5.1% of parotid gland spheres express c-Kit/CD117 and 74.8 ± 10.7% express CD29 (Fig.4A, parotid).

These results suggest the presence of putative stem cells in the epithelial ducts of both submandibular and parotid glands, as well as in cultured primary spheres.
3.3. Rat primary spheres can differentiate in vitro

The ability of stem/progenitor cells to differentiate into the tissue of origin is an indicator of their stemness. To test whether primary sphere cells have any differentiation potential, both submandibular and parotid gland primary spheres were placed into 3D matrix (collagen:matri-gel; 1:1) and supplemented with primary sphere culture medium containing factors that induce differentiation (see methods). Primary spheres from both submandibular and parotid glands underwent massive morphological changes in 4-25 days (Fig.5A) and developed long tubular extensions (similar to ducts of salivary gland). We refer to these structures as organoids.

To visualize the general morphology and assess the expression of salivary gland differentiation markers, organoids were embedded in paraffin, cut into 5 μm sections and stained with H&E and processed for immunohistochemistry (IHC). HE staining (Fig.5B) determined the cellular organization in the organoids and revealed lumen formation (arrows), indicating the presence of ducts. IHC showed cells expressing PCNA, indicating the proliferative nature of the organoids, cells expressing Cytokeratin18 (a duct cell marker) and cells expressing Aquaporin5 (an acinar cell marker) (Fig.5B).

These results show that primary spheres derived from the submandibular and parotid glands are able to form organoids that proliferate and contain differentiated duct and acinar-like cells of the salivary gland, indicating the presence of putative stem cells in the salispheres.
3.4. Rat primary spheres can self-renew in vitro

Stem cells have to be able to self-renew to maintain their progeny throughout the life time of an organism. As previously described for murine salivary gland (Chapter 4, this thesis), the ability of single cells derived from primary-sphere to grow into secondary spheres is a measure of the self-renewal potential of putative stem cells in vitro. Therefore, submandibular and parotid gland derived primary spheres were tested for their self-renewal potential.
Primary spheres from both parotid and submandibular glands were cultured for 3 (D3) and 6 days (D6) and were subsequently enzymatically dissociated into single cells, seeded into matrigel (10000 cells/well). To allow secondary sphere formation primary sphere culture medium is added that is enriched with Rho-inhibitor (Y-27632) known to protect cells from dissociation-induced apoptosis.

Seven to ten days after seeding, secondary spheres developed from single primary cells. Both submandibular and parotid gland derived day 3 (D3) primary sphere cells formed higher number of secondary spheres in comparison to day 6 primary sphere cells (Fig.6A, submandibular, parotid). This suggests that culturing of relatively older spheres (6 days) does not enhance sphere-formation, but rather reduces this ability presumably because of differentiation into more committed cell types under these conditions.

Further, to test their long-term self-renewal, secondary spheres were enzymatically dispersed into single cells and re-plated. This procedure was repeated for several passages. Rat submandibular and parotid sphere cells were shown to be able to self-renew for at least 4 passages (Fig.6B), albeit with declining efficiency. These results indicate that primary spheres from both submandibular and parotid glands contain cell capable of self-renewing to some extent. Highest secondary sphere forming potential was present in spheres that had been cultured for 3 days (D3). The in vitro culture conditions need to be optimized to facilitate prolonged self-renewal as established for murine salivary glands (Chapter 4).
3.5. **Retrograde method of salivary gland injection *in vivo***

The larger ducts (Stenson’s duct) of parotid gland open into the buccal mucosa, whereas excretory ducts from the submandibular glands (Wharton’s duct) are underneath the tongue (Fig 5A). To establish retrograde/intra-ductal injection as a method to transplant salivary gland stem cells, first the technique itself needed to be tested in our rat model. To visualize the technical success of injection, first, Indian ink in PBS was injected retrogradely into both parotid and submandibular duct openings (Fig.7A). Fig.7B shows that the injected ink was widely dispersed throughout the gland. To further visualize the distribution of injected fluid, indian ink injected gland were stained with HE. Indeed, these sections showed distribution of injected ink at various places (Fig.7C) along the path of ducts, indicative of successful retrograde injection. This method shows the delivery but not the potential migration of transplanted cells.

![Figure 7](image)

**Figure 7:** (A) Photomicrographs of oral cavity of rats showing parotid and submandibular gland ductal openings (arrow, circle). (B) Pictures of parotid gland of rat that received indian ink via retrograde/intra-ductal injection for a technical validation. (C) HE stained pictures of the same showing distribution of injected ink near/via ducts of the parotid gland.

3.6. **Functional activity of salivary glands after retrograde injection of stem/progenitor cells**

To test the ability of retrogradely injected stem cells to rescue hyposalivation, we have delivered primary sphere-derived single cells into rats with radiation-damaged (15Gy) salivary...
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glands, and compared that with direct intraglandular injections, as performed previously in mice. Viable male and/or female rat primary sphere-derived (50,000-70,000 PI negative cells/gland) and c-Kit\(^+\) (7,000 cells/gland) cells were sorted, labeled with cell membrane tracker dye (PKH26 –red) and injected into locally irradiated (15Gy, X-rays) rat salivary glands. Saliva flow and gland weight were measured 360 days after irradiation.

After 90 days intra-ductally transplanted tissues were sectioned to visualize the fate of transplanted cells. PKH26 positive cells were seen at various sites in the gland (Fig.8A, site of injection) in clusters, and as individual cells dispersed at various places, confirming that the injected cells were able to localize and proliferate into the gland.

![Figure 8](image)

**Figure 8:** (A) Photomicrographs of salivary glands of rats, showing cell tracker labeled (PKH positive, red) cells at the site of injection and their distribution over different places in the gland. Nuclei (DAPI) counter stained. Graphs showing weight of irradiated (B) parotid and (C), submandibular glands 360 days post-irradiation, mg of tissue on y-axis. Weight of irradiated non-transplanted parotid gland is 155±7 mg and submandibular gland is 97±1 mg.

After 360 days of retrograde transplantation, parotid glands showed some increase in gland weight when compared to the irradiated non-transplanted glands (Fig.8B, Parotid). However, submandibular glands did not show any improvement in gland weight, with retrograde
method of cell delivery (Fig. 8B, Submandibular).

As expected, a drop in parotid and submandibular saliva production was seen after irradiation. Retrograde transplantation with live (viable PI negative) or c-Kit⁺ cells induced a recovery in saliva production in the parotid gland when compared to intra-glandular transplanted animals (Fig 9A and B). Transplanted submandibular glands also showed some recovery but no significance difference between the two transplantation methods could be observed. These preliminary results indicate that some recovery can be induced by retrograde transplantation with live and c-kit⁺ cells.

4. DISCUSSION

In the current study retrograde/intra-ductal injection as an alternative to intra-glandular injection of salivary gland stem/progenitor cells was investigated. We found that, similar to mouse and human tissue, CD24⁺, CD133⁺ and c-Kit⁺ cells can be detected in the striated and excretory ducts of the rat salivary glands. We successfully established isolation and culture methods of rat salivary gland cells. Rat salispheres were found to contain cells that express CD24, CD133 and c-kit and are able to self-renew for 4 or more passages in vitro, and can differentiate into at least some major salivary gland cell types suggesting stem/progenitor potential.

When transplanted via retrograde injection into irradiated rat salivary glands, salisphere cells (with or without enrichment for cells expressing specific cell surface markers) induced an increase in parotid gland weight and some recovery of saliva flow from submandibular and parotid glands. However, in these preliminary experiments the extent of recovery for both methods was

![Figure 9: Graphs showing saliva (% of pre-irradiation, y-axis) from (D) parotid and (E) submandibular glands 360 days post-irradiation](image)
similar, rendering it difficult to suggest that retrograde injection of stem cells is more efficient than intra-glandular injection.

Intra-ductal injection of viral-mediated gene transfer and nano-particle mediated siRNA transfer has been shown to be successful in salivary gland. We hypothesized that intra-ductal injection of stem/progenitor cells should facilitate higher affinity of stem cells to localize in the larger ducts compared to intra-glandular injection. However, intra-ductal transplantation of stem cells was not more successful than intra-glandular injection. This could be due to flushing out/release of cells early after injection. Cells require adhesion to ductal cells and further migration and/or integration into the tissue. It is plausible that cells need more time for integration into the tissue and are removed from the gland by resumption of saliva flow before they can properly integrate. Moreover, irradiation of salivary glands might have an effect on the excretory ducts that hinders the homing of transplanted stem cells. It needs to be further investigated whether the homing of transplanted salivary gland cells can be optimized.

Therefore, alternative methods of transplantation, including echo-guided injection or retrograde endoscopy-guided injection, need to be tested for the transplantation of stem cells in patients. The small size of the oral cavity in rats makes it difficult to test these methods, therefore, this need to be tested in a relatively bigger animal model (such as rabbits or pig). Unfortunately, salivary glands stem cells have not been investigated in these animals and the lack of proper culture media and antibodies may delay such studies.

The findings from this study, such as isolation and culturing of rat primary spheres from both submandibular and parotid glands, in vitro self-renewal and differentiation of primary spheres and radiation-damaged model of rat salivary glands, will be instrumental in deciphering fundamental and translation studies in rat salivary gland biology.

5. REFERENCES


