Salivary gland stem cells

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Chapter

An in vitro screen for putative salivary gland stem cell populations

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ABSTRACT

Stem cell therapy provides an option to rescue patients from life-long salivary gland impairment, which leads to hyposalivation and xerostomia. We have previously shown that murine primary salisphere-derived c-Kit⁺, CD133⁺, CD24⁺, and CD49f⁺ cells all can rescue hyposalivation albeit with different potencies. Therefore, our aim here is to identify the most potent stem cell in the salivary gland by assessing self-renewal and differentiation into mature salivary gland cell types of various stem cell candidates.

To test self-renewal, primary salisphere-derived single cells were FACS sorted based on expression of the cell surface markers such as c-Kit, CD133, CD24, CD49f, and were seeded as single cells in a 3D matrix to allow secondary-sphere formation. Spheres were serially passaged to test the proliferative capacity of each population. To assess their differentiation capacity, single cell-derived secondary spheres were subjected to salivary gland organoid inducing conditions. Our results revealed that primary salisphere cells that are c-Kit⁺, CD133⁺, CD24⁺, and CD49f⁺ can be self-renew for up to >7 passages. The highest sphere-forming potential was found for CD24⁺ (5.95±1.12%) and CD49f⁺ (10.35±1.36%) cells at the end of passage-1.

Among the unfractionated cells, 18.9 ± 13.0% of the cells were able to form organoids. This is higher than the organoid forming ability of purified populations, which may indicate that unfractionated cells contain more committed/progenitor cells. Collectively, these results imply that CD24⁺ and CD49f⁺ cells showed relatively high self-renewal and low differentiation ability, suggesting an enrichment of stem/progenitor cells. Therefore, CD24⁺ and CD49f⁺ cells are best candidates to be studied further to fully understand their stem cell potential.
1. INTRODUCTION

Hyposalivation may lead to xerostomia with symptoms such as dry mouth, dry nasal passages, sore throat, loss of oral hygiene, dental caries, oral candidiasis, loss of taste, and difficulties with swallowing and speaking. These symptoms drastically reduce the patient’s quality of life and no sufficient treatments are currently available. Therefore, autologous adult stem cell transplantation could be an option to mitigate the suffering of these patients.

The ability to self-renew, differentiate and regenerate damaged tissues are the properties that distinguish stem cells from more committed cells of a tissue and are the driving force of life-long tissue homeostasis. In the search for the salivary gland stem cell, in vitro self-renewal and differentiation assays for salivary gland stem cell populations can be very instrumental. In other tissues such as blood\(^1\), intestine\(^2\), and liver\(^3\), in vitro assays have been reported that monitor long-term growth (self-renewal) and differentiation of adult stem cells. The report by Barker et al. 2010\(^2\) showed for the first time that in murine intestine, single Lgr5\(^+\) cells are able to differentiate into organoids that can be maintained (i.e. they self-renew) under Wnt-pathway stimulated conditions and suggest that these cells represent a genuine stem cell population. Other in vitro stem cell studies have been reported for liver (from Lgr5\(^+\) cells)\(^3\) and brain (from unfractionated cells, cerebral organoids)\(^4\). In addition to characterizing stem cells, these in vitro self-renewal and differentiation assays have been used to study tissue regeneration and to model disease conditions\(^4\). Although actual in vivo proof of function of these stem cells is rare, the short-term in vitro studies provide preliminary evidence to support their role as potential candidate stem cell populations.

In the salivary gland, mesenchymal and epithelial cells\(^5\), have been shown to exhibit signs of differentiation in vitro by the expression of duct markers, but no morphological development or gene expression analysis confirming differentiation were performed. These studies did not provide concrete evidence of differentiation as reported in other tissues such as intestinal organoids\(^2\). Therefore, in this study we aimed to develop single cell based self-renewal and differentiation assays in vitro, to select putative stem cell populations that can be further tested to functional rescue salivary glands in vivo.
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2. MATERIALS AND METHODS

2.1. Animals

8-12 week old female C57BL/6 mice were purchased from Harlan (The Netherlands). Transgenic mice C57BL/6-Tg(CAG-EGFP)1Os/J (GFP), B6.Cg-Tg(CAG-DsRed*MS-T)1Nagy/J (DsRed) both containing beta actin promoter (Jackson laboratories) were bred in house and used as source of DsRed and GFP salivary glands. The mice were maintained under conventional conditions and fed ad libitum with food pellets (RMH-B, Hope farms B.V., Woerden, The Netherlands) and water. The Ethical Committee on animal testing of the University of Groningen approved all experiments.

2.2. Primary sphere culture

Primary salispheres were cultured from mouse salivary glands in DMEM:F12 medium with growth factors (referred to as minimal medium, MM from here on) as described in Chapter 2, this thesis. To make single cells, these spheres were dissociated using 0.05% trypsin-EDTA (Gibco, Invitrogen). To perform flow-cytometry, single cells were incubated with fluorescently labelled antibodies for c-Kit/CD117 (Biolegend 105808), CD133 (eBioscience 12-1331-82), CD49f (Biolegend 313613), CD24 (Biolegend 101820) or CD29 (BD555005) at 4°C for 20-30 min followed by a washing step with PBS/0.2% bovine serum albumin. Finally, stained cells were suspended in Propidium Iodide (PI, 1 µg/ml) and were either analyzed on a FACS LSR-II Flow Cytometer (BD) or sorted using a Moflow Astrios (BD). All gates were set based on isotype controls and single stain controls. Propidium Iodide negative cells were used as “unfractionated” cells in our experiments.

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

Single unselected cells (live, unstained cells) and those positive for stem cell markers were sorted. 10000 cells were plated in 75 µl gel/well (50 µl matrigel (BD356235) + 25µl cells in MM) in a 12-well plate and were solidified for 10-15min at 37°C. After solidification 1ml of MM or enriched medium, EM (MM + Rho-inhibitor, Y-27632) was added gently on top of the gels and incubated for 5-7 days in a 37°C incubator. Spheres appeared (in 4-7days) were counted per well and percentage of sphere-forming cells per group was calculated. To test long-term self-renewal ability, these secondary spheres are passaged every 5-7 days. First, medium
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on top of gel is gently removed and 1 ml of Dispase (1 mg/ml) was added directly to the gels to dissociate the matrigel and incubated for 1 hour at 37°C. This was followed by a washing step with PBS/0.2% bovine serum albumin and centrifuged 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 is repeated at the end of every passage.

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

To test differentiation/organoid-formation ability of spheres, at the end of any/some passages in self-renewal, medium on top of gels is removed and the matrigel is pipetted with P1000 to release spheres from the gel (No dispase and No trypsin is used in this step) and were collected and pelleted by centrifugation. Pelleted spheres are suspended into 10X DMEM (1/10th of total collagen) with 1M NaOH and collagen (BD354236) (40 collagen:60 matrigel). Later matrigel (BD354230) (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 minimal medium (MM) with 10% Fetal Calf Serum (FCS) was added on top of each gel and incubated at 37°C. Spheres in each well were counted and observed every 2-3 days for the development of organoids, that were pictured and quantified.

### 2.5. Immunohistochemistry

Mice were exsanguinated under isoflurane anesthesia and the submandibular glands were carefully dissected. In order to perform immuno-histochemical staining extirpated mouse submandibular 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: Cytokeratin 7 (Millipore MAB3226), Aquaporin 5 (Alomone, AQP5), Cytokeratin 18 (Abcam ab668)

Visualization for bright field microscopy was accomplished by adding specific secondary biotin carrying antibodies (DAKO cytotation) an avidin-biotin-horse radish peroxidase complex and the diaminobenzidine (DAB) chromogen. Nuclear counterstaining was performed with hematoxylin. Control sections without primary antibodies did not show positive immuno- staining. For confocal imaging, organoids were scooped out from the differentiation gel and digested with collagenase I (5min at 37°C) followed by washing with PBS 1% bovine serum
albumin and centrifugation. Pelleted organoids were fixed with 4% formaldehyde and stained with primary antibodies overnight in 0.05% triton at 4°C. For confocal imaging fluorescently labeled secondary antibodies were used to visualize the staining and imaged with either Leica SP8 or Zeiss 780 microscope.

2.6. \textit{in vivo} model of salivary gland regeneration

To enable reconstitution of endogenous salivary gland cells, recipient mice were locally irradiated. Irradiation of salivary glands was done with a single dose of 15 Gy (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 animals. Saliva flow rate was determined every 30 days post-irradiation. Pilocarpine injection (2.5 mg/kg, s.c.) was given to animal and placed in a restraining device. Saliva was collected for 15 min and the quantity was determined gravimetrically, assuming density of 1g/ml for saliva. 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 \textit{in vivo} regeneration is the most rigorous test for stem cell function.

To test for regenerative capacity of various cell populations, primary-salisphere derived cells were sorted for c-Kit$^+$/CD24$^+$/CD29$^+$ and c-Kit$^-$/CD24$^+$/CD29$^+$, and dissociated cells from Passage-2 and Passage-13 spheres were suspended in α-mem with 2% FCS so that 1400 - 5000 cells/5µl could be transplanted into each gland of a recipient mouse. Thirty days post-irradiation mice with irradiated salivary glands were anaesthetized under isoflurane and a small incision is made in the neck region to visualize the submandibular glands. 5 µl of cell suspension was injected intra-glandular into each gland with a micro syringe and needles (Hamilton 7803-02) and the skin was sutured. Animals were administered with analgesic with in 24 hours of surgery.

2.7. Microarray expression profiling and analysis

RNA was extracted from all the samples used in the array using Qiagen RNAeasy micro kit. Highly pure total RNA (300 ng/sample) was used for expression profiling on Illumina WG-6 v2.0 expression bead chip kit. The data was normalized using the R version 3.0.1 neqc function of the BioConductor version 2.12 library limma 3.16.5 by control background correction, quantile normalization and log2 transformation and batch effects between arrays. Differential expression analysis was performed using eBayes function of the BioConductor library limma and an adjustment method BH (Benjamini Hochberg) was used with a pvalue of 0.05. Gene
ontology analysis was performed using Amigo labs (http://amigo.geneontology.org/cgi-bin/amigo/term_enrichment?session_id=). Heat maps were made by R library heatmap.plus 1.3 and RcolorBrewe 1.0-5.

3. RESULTS

3.1. Single cell self-renewal in vitro

To assess self-renewal and differentiation potential of salivary gland stem/progenitor cells, in vitro single cell based assays were developed. First, to test self-renewal, dispersed murine salivary gland cells were cultured into floating primary spheres (Fig. 1A) containing stem/progenitor cells, as published previously. PI negative primary sphere derived single cells were FACS sorted cells and seeded into matrigel-based matrix (10,000 cells/well), supplemented with

Figure 1: Single cell self-renewal in vitro. (A) Diagrammatic representation of primary-sphere culture from murine SG after enzymatic digestion of a gland, followed by steps for secondary-sphere-formation using trypsin, self-renewal by continuous re-plating into matrigel based matrix. (B) Graph showing the percentage of sphere-formation (y-axis) of FACS sorted single unfractionated cells under minimal and enriched (with Rho-inhibitor) culture medium conditions (t-test ***p<0.001). (C) Pictures showing individual wells with DsRed and GFP primary-sphere derived single cells (Scale bars 50µm). Picture of well showing predominantly (99.2%) single cell derived spheres from murine DsRed and GFP SG cells individually and co-seeded in culture.
quantification of secondary spheres within 5-7 days showed that 0.44 ± 0.03% of the single cells formed secondary spheres (Fig. 1B, MM). To minimize dissociation-induced stress, the Rho-inhibitor Y-27632, known to protect against dissociation-induced cell stress, was added in enriched medium (EM). This enhanced the initial secondary sphere-formation percentage of unfractionated cells almost 3-fold to 1.15 ± 0.08% (Fig. 1B, EM). To establish that the secondary spheres were indeed single cell-derived, unfractionated single cells sorted individually from DsRed and eGFP transgenic mice were mixed and co-seeded. More than 99% of all secondary spheres were from cells of only a single color (Fig. 1C), indicating that the spheres were not formed by clumping of cells, but rather due to the outgrowth from single cells.

To test the long-term self-renewal of the unfractionated cells, secondary spheres were dissociated and the resulting single cells were re-seeded (Fig. 1A, Self-renewal). Similarly, tertiary, quaternary etc. spheres could consistently be established, indicating extensive in vitro self-renewal. Serial replating of these secondary sphere cells showed that they could self-renew for more than 20 passages (Fig. 2A). Surprisingly, during early passages the percentage of spheres formed increased and further stabilized between 10 and 40%, indicative of enrichment for sphere-forming stem/progenitor cells. During passing of unfractionated
cells, a striking $1,442 \pm 529$ fold (passage-7), $13,889 \pm 5450$ fold (passage-16) and $53,147 \pm 937$ fold (passage-28) increased number of cells were generated from 20,000 cells (Fig. 2B). Thus, a pronounced expansion of putative SG stem cells can be obtained with *in vitro* cultures.

### 3.2. Single cell derived-organoids *in vitro*

To test their differentiation potential, single cell-derived secondary spheres from unfractionated cells were removed from the 3D matrix with only matrigel and seeded into another 3D matrix with matrigel and collagen (40:60), supplemented with minimal medium containing 10% fetal calf serum (Fig 3A). Careful analysis of this culture for multiple consecutive days showed that within 1-2 weeks after inducing differentiation, the single cell-derived secondary

**Figure.3:** Single cell derived-organoids *in vitro*. (A) Diagrammatic representation of secondary-sphere-formation and steps following differentiation assay in matrigel/collagen. (B) Pictures showing gradual growth of two organoids during different days in culture, indicating the growth and diversity of the organoids. Scale bars 50µm. Images showing lobular organoids (C) and ductal organoids (D) in culture and with confocal imaging after staining for actin (with phalloidin) and nuclei (DAPI). Scale bars 50µm.
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spheres underwent major morphological changes into organoids resembling salivary glands (Fig.3B). Based on the morphology of the organoids, they could be categorized into two types.

1) Lobular organoids that are almost exclusively spherical, with compact cellular arrangement resembling salivary gland acini (Fig.3C).

2) Ductal organoids that contain long-tubular extensions connected to form 3-dimensional structures, resembling the duct cells of the salivary gland (Fig.3D).

In order to confirm the existence of differentiated salivary gland cell types, these organoids were embedded in paraffin, sectioned and stained for salivary gland signature markers.

HE staining on organoids showed the general morphology and compact/organized arrangement of cells in organoids (Fig.4A). Some of the structures resembled acini or ducts that can be found in the normal salivary gland. Furthermore, immunostaining with antibodies specific for ductal cell markers showed that organoids contained cells that expressed ductal cell markers like Cytokeratins 18 and 7 (Fig.4A, Cytokeratin), indicating duct cell differentiation. The acinar cell marker Aquaporin-5 was also detectable in some, but not all, organoids (Fig.4B, AQP5).

3.3. Gene expression analysis of in vitro derived organoids

To further provide insight into their differentiation state, a genome-wide gene expression

Figure.4: Single cell derived-organoids in vitro. (A) Pictures of organoids in culture and stained with HE (for general morphology), Cytokeratins (CK18 and CK7), duct cell markers; and (B) Aquaporin5 (AQP5). Inset showing higher magnification of the positive (brown) staining. Scale bars 50µm.
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A total of 302 probes were differentially expressed between organoids and primary spheres (Fig.5A) with a threshold p<0.0001. Among these, 102 probes were up regulated (>logFC 2.0) in organoids (Fig.5B) compared to primary spheres. These probes were tested for genre ontology classifiers. This analysis revealed significant enrichment in biological processes including gland morphogenesis (GO:0022612), gland development (GO:0048732) and many others listed in Table 1. At the individual gene level these 102 genes include, calcium-channel related proteins (CPNE8), secretin (SCT) (role in pancreatic duct secretion)\textsuperscript{10}, cranio-facial development related RBMS3 gene\textsuperscript{11}, vitamin-K-dependent protein (PROS1), vesicle associated membrane protein-VAMP4\textsuperscript{12}, STRA6\textsuperscript{13} known to be expressed in salivary gland, salivary gland branching morphogenesis related (TGM2, NTN4), embryonic salivary gland end bud differentiation marker (FGF1)\textsuperscript{14}. Collectively, these findings show that single-cell-derived spheres that are capable of secondary sphere-formation in vitro can form differentiated salivary gland lineages.
CD49f expressing cells had the highest self-renewal potential in this assay.

To further test the long-term self-renewing potential of these purified cells, secondary spheres were enzymatically dissociated into single cells and re-plated with EM to allow tertiary and quaternary sphere formation. The results show that secondary spheres, derived from c-Kit, CD133, CD24 or CD49f expressing cells, were able to maintain at least 7 passages (Fig.6C) indicating their self-renewing potential. No apparent differences between the various purified cell populations were detected.

To test their differentiation potential, single cell-derived passage-5 spheres from multiple populations were subjected to differentiation. Results showed that passage-5 derived spheres from CD133+, CD24+ and CD49f+ cells all were able to differentiate into organoids (Fig. 6D) albeit with different potencies. Quantification of differentiation revealed that 18.86 ± 13.01% of unfractionated, 12.27 ± 6.21% of CD133+, 10.81 ± 9.83% of CD24+ and 3.14 ± 4.06% of

### Table 1: Gene ontology results. Table showing the results of gene ontology analysis performed on the 102 genes that are highly (>logFc 2.0) expressed in organoids compared to primary spheres.

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3.4. *in vitro* self-renewal and differentiation of stem cell marker expressing cells

Next, we tested whether enrichment for stem cells from the salisphere-derived population using specific cell surface markers would improve *in vitro* self-renewal and differentiation potential. Primary-sphere-derived single cells expressing c-Kit, CD133, CD24 or CD49f were FACS-sorted and plated into matrigel (Fig.1A) to allow secondary-sphere-formation. Results showed that 1.175 ± 0.29% of unfractionated, 2.12 ± 0.47% of c-Kit+, 1.54 ± 0.46% of CD133+, 5.95 ± 1.12% of CD24+ and 10.35 ± 1.36% of CD49f+ cells formed secondary spheres (Fig.6A and 6B).
CD49f⁺ cells formed organoids (Fig.6E).

These results show that unfractionated cells showed higher organoid-forming ability, CD49f⁺ cells showed higher sphere-forming cells, whereas CD24⁺ cells showed both self-renewal and differentiation properties.

3.5. Presence and functional activity of CD24⁺/CD29⁺ cells in the salivary gland

Studies of other glandular tissues, such as the mammary gland, have identified Lin⁻ CD29hi/CD24⁺ expressing cells as potential stem cells¹⁵. Hence, we tested for the presence of CD24⁺ and CD29⁻ cells in murine salivary gland tissue using immunohistochemistry.
Our results showed that both CD24⁺ and CD29⁺ cells were exclusively located in the larger excretory ducts (ED) and striated ducts (SD), while the acinar cells (AC) were completely devoid of these cells (Fig. 7A). Also, CD24⁺/CD29⁺ cells are enriched in the primary spheres obtained from culturing salivary glands (Fig. 7B). These cells can self-renew for more than 7 passages in the newly developed self-renewal assay (data not shown). Next, to test the in vivo regeneration potential of primary sphere-derived CD24⁺/CD29⁺ cells, these cells were transplanted into the irradiated salivary glands of mice. Transplanted CD24⁺/CD29⁺ cells were able to ameliorate radiation-induced hyposalivation to 63.30±15.89% of pre-irradiation saliva levels (Fig. 7C, data from Chapter 2 8). However, recipient mice showed variable responses after transplantation. This could be due to the existence of sub-populations within the CD24⁺/CD29⁺
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cell fraction that may differ in regenerative potential (see chapter 5 for further discussions on this issue).

Our previous studies (Chapter 2 and Lombaert et al.20087) showed that primary sphere-derived c-Kit⁺ cells are able to regenerate radiation-damaged glands. Flow cytometric analysis of murine primary spheres showed an overlap of c-Kit⁺ cells with CD24⁺/CD29⁺ positive cells (Fig.7B, FACS plot). Therefore, we tested whether further selection for c-Kit⁺ or c-Kit⁻ cells within CD24⁺/CD29⁺ population might identify an even more potent population. However, the results showed that transplantation of primary sphere-derived c-Kit⁻/CD24⁺/CD29⁺ and c-Kit⁺/CD24⁺/CD29⁺ did not show significant differences in recovery of irradiated salivary glands. This showed that enrichment of c-kit⁺ cells within the CD24⁺/CD29⁺ does not further increase stem cell potential (Fig. 7C).

4. DISCUSSION

This study demonstrates the development of single cell-based in vitro self-renewal and differentiation assays that are able to identify potential stem cell candidates within the murine salivary gland. Although previous reports have suggested the existence of self-renewing and differentiating stem cells in the salivary gland5, 7, the exact nature of these cells was not well established. Here, we show for the first time in salivary gland that primary sphere-derived single cells can self-renew for more than 25 passages. In addition, we show that these single cell-derived spheres were able to differentiate into salivary gland organoids in vitro.

These in vitro assays are similar to those developed in the intestine2 and liver3, however, without the extensive Wnt signaling used in these studies. Such assays open windows for applications, like understanding the role of genes involved in either salivary gland physiology or disease pathogenesis and potentially for testing of therapeutics.

We previously showed (Chapter 2, Fig.4 and Nanduri et al 20118) that primary-sphere derived c-Kit⁺, CD133⁺, CD24⁺ or CD49f⁺ salivary gland cells can rescue hyposalivation to some extent. However, each cell population does so with different potencies. Here we observed that CD49f⁺ cells possess higher self-renewing potential, indicative of their stemness. Although all the tested candidates were able to form organoids in the differentiation assays, unfractionated cells showed highest and CD49f⁺ cells showed lowest organoid-forming efficiencies, respectively. In addition, we know from in vivo studies Chapter 2 (Fig.4, Nanduri et al 20118) that CD49f⁺ cells showed the lowest regeneration potential. Together, these data suggest that although CD49f⁺
cells contain sphere-forming cells, they are more difficult to differentiate. This does not make CD49f+ cells a promising stem cell population, as they would be predicted to lack of short-term regeneration in vivo.

Another promising cell surface molecule that appeared to predict cellular activity in vitro assays is CD24. Primary sphere-derived CD24+ cells showed both self-renewal and differentiation potential indicating it may indeed be useful as a potential stem cell marker for the salivary gland. Previously, we showed that transplantation of CD24+/CD29+ cells into irradiated salivary glands can rescue radiation-induced hyposalivation8. Hence, it is important to study CD24+ cells and assess the extent to which it overlaps with other putative markers of tissue stem cells. In the salivary gland, c-Kit was shown to be one of these markers7,8. As an extension of this, in this study we showed that separation of c-Kit+ or c-Kit− cells within murine primary sphere-derived CD24+/CD29+ cells does not label a more potent population. This excludes c-Kit as a useful additional marker and indicates the possible existence of subsets in CD24+/CD29+ that differ in their stem cell potential. Therefore, in future studies using the in vitro self-renewal and differentiation assays developed in this chapter we aim to identify the most potent stem cells among the murine primary sphere-derived CD24+/CD29+ cells (see chapter 5).

The assays developed here can be used in the future to validate the effect of either knock-down or overexpression of genes or factors predicted to regulate self-renewal or differentiation in salivary gland. In conclusion, the short-term in vitro assays developed in this study not only screen potential stem cell candidates of the salivary gland but also reduce the number of animals required for in vivo testing.

5. ACKNOWLEDGEMENTS

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