CHAPTER 4

IMPLICATIONS OF AN ACTIVELY CYCLING STEM CELL POPULATION WITHIN SALIVARY GLAND

Maimets, M., Bron, R., van Os, R., Coppes, RP.

Manuscript in preparation
SUMMARY

The maintenance of a stem cell population as a reservoir of undifferentiated cells is required for salivary gland (SG) development, homeostasis and regeneration. Despite the recent advancements in partial purification of mouse salivary gland stem cells (SGSC), there is currently no methodology that adequately isolates pure SGSCs. Here, we employ a cell-cycle state independent histone H2B-GFP pulse-and-chase system to characterize the putative SGSC population based on slow turnover. By combining tissue clarity technique with large-scale confocal imaging we provide volumetric data showing that H2B-GFP+ label retaining cells (LRCs) pulsed during embryonic development and chased until adulthood reside in excretory and striated ducts and not among acinar cells. Phenotypical profiling of LRCs reveals that LRCs co-localize with K8+ ductal luminal or Vimentin+ mesenchymal but not CK5+ or CK14+ putative progenitor cells. In culture, LRCs fail to generate organoids while a proportion of non-LRCs cells display differentiation and long-term expansion potential. Collectively, our data suggest that post-natally an active population of SGSC seems responsible for tissue remodeling and maintenance.

CHAPTER 4

INTRODUCTION

Adult stem cells are undifferentiated and long-lived cells, which have a remarkable capacity to replenish themselves through self-renewal and to give rise to either one (unipotent) or more (multipotent) downstream differentiated cell lineages. At present, there is a strong interest in the use of adult stem cells as a source of cellular therapy for developing therapeutic strategies to radiation treatment, immune diseases and even aging. Given their importance to body tissues, stem cells are called upon intermittently, leaving the majority of tissue regeneration to transiently dividing but committed progeny. Therefore, stem cells often remain quiescent for extended periods of time and are only called into action briefly upon initiation of homeostasis or injury (Fuchs, 2009). Quiescence of stem cells is critical to ensure lifelong tissue maintenance and to protect the stem cell pool from premature exhaustion under conditions of various stresses. In some cases, such as the blood (Foudi et al., 2009), skin epidermis (Clayton et al., 2007) or intestine (Barker et al., 2007), stem cells experience daily turnover as part of their normal differentiation process. In others, such as skeletal muscle (Collins et al., 2005) or brain (Doe et al., 1999) stem cells undergo extremely low or no division during normal homeostasis but can respond efficiently to stimuli or injury. Nevertheless, to date the population kinetics of salivary gland stem cells (SGSCs) during tissue turnover or following damage remains an enigma. Currently, a large body of work suggests and supports the presence of stem cell populations within salivary gland (SG). Ligation and subsequent de-ligation experiments of the major excretory duct have demonstrated SG’s extensive regeneration capacity (Cotroneo et al., 2010; Cotroneo et al., 2008; Osailan et al., 2006; Takahashi et al., 2004a; Takahashi et al., 2004b). Moreover, several antigens commonly identified in stem/progenitor cells in many organs such as c-Kit, Sca-1, CD133, CD24, CD29 and CD49f have been shown to be present in submandibular glands (Hisatomi et al., 2004; Lombaert et al., 2008; Nanduri et al., 2011). LinCD24+c-Kit+Sca1+(Xiao et al., 2014) and EpCAMhigh (Maimets et al., 2016) cells isolated directly from submandibular glands of adult mice and grown in 3-dimensional setting under defined conditions can be expanded into clinically relevant numbers of SGSCs in vitro. Furthermore, in transplantation experiments these SGSCs adhere and engraft into the donor tissue thereby salvaging irradiation-damaged epithelium (Maimets et al., 2016; Nanduri et al., 2014). However, knowledge about the specific localization and cellular phenotype of SGSCs within the salivary gland still remains scarce. Previously, using label retention as a surrogate marker for quiescence, label retaining cells (LRCs) have been found being distributed throughout the parenchyma of the SG (Chibly et al., 2014; Kimoto et al., 2008; Kwak and Ghazizadeh, 2015). However, information about the self-renewal and...
INTRODUCTION

Adult stem cells are undifferentiated and long-lived cells, which have a remarkable capacity to replenish themselves through self-renewal and to give rise to either one (unipotent) or more (multipotent) downstream differentiated cell lineages. At present, there is a strong interest in the use of adult stem cells as a source of cellular therapy for developing therapeutic strategies to radiation treatment, immune diseases and even aging. Given their importance to body tissues, stem cells are called upon intermittently, leaving the majority of tissue regeneration to transiently dividing but committed progeny. Therefore, stem cells often remain quiescent for extended periods of time and are only called into action briefly upon initiation of homeostasis or injury (Fuchs, 2009). Quiescence of stem cells is critical to ensure lifelong tissue maintenance and to protect the stem cell pool from premature exhaustion under conditions of various stresses. In some cases, such as the blood (Foudi et al., 2009), skin epidermis (Clayton et al., 2007) or intestine (Barker et al., 2007), stem cells experience daily turnover as part of their normal differentiation process. In others, such as skeletal muscle (Collins et al., 2005) or brain (Doetsch et al., 1999) stem cells undergo extremely low or no division during normal homeostasis but can respond efficiently to stimuli or injury. Nevertheless, to date the population kinetics of salivary gland stem cells (SGSCs) during tissue turnover or following damage remains an enigma.

Currently, a large body of work suggests and supports the presence of stem cell populations within salivary gland (SG). Ligation and subsequent de-ligation experiments of the major excretory duct have demonstrated SG's extensive regeneration capacity (Cotroneo et al., 2010; Cotroneo et al., 2008; Osailan et al., 2006; Takahashi et al., 2004a; Takahashi et al., 2004b). Moreover, several antigens commonly identified in stem/progenitor cells in many organs such as c-Kit, Sca-1, CD133, CD24, CD29 and CD49f have been shown to be present in submandibular glands (Hisatomi et al., 2004; Lombaert et al., 2008; Nanduri et al., 2011). Lin$^-CD24^-c$-Kit$^-$Sca1$^-$ (Xiao et al., 2014) and EpCAM$^{\text{high}}$ (Maimets et al., 2016) cells isolated directly from submandibular glands of adult mice and grown in 3-dimensional setting under defined conditions can be expanded into clinically relevant numbers of SGSCs in vitro. Furthermore, in transplantation experiments these SGSCs adhere and engraft into the donor tissue thereby salvaging irradiation-damaged epithelium (Maimets et al., 2016; Nanduri et al., 2014). However, knowledge about the specific localization and cellular phenotype of SGSCs within the salivary gland still remains scarce.

Previously, using label retention as a surrogate marker for quiescence, label retaining cells (LRCs) have been found being distributed throughout the parenchyma of the SG (Chibly et al., 2014; Kimoto et al., 2008; Kwak and Ghazizadeh, 2015). However, information about the self-renewal and
differentiation properties of LRCs collected from pulse-chase studies has been controversial. In one study, mice were labeled with EdU at postnatal day 10 and chased over a period of 8 weeks (Chibly et al., 2014). Subsequent immunohistochemical and in situ hybridization assays at the end of chase demonstrated co-localization between EdU+ and K5, K14 as well as kit mRNA. Furthermore, in sphere formation assays EdU+ cells showed proliferative potential since they co-localize with the proliferation marker Ki67 throughout sphere formation. These data indicate that some LRCs have stem cell characteristics. Yet, nucleotide exchange labelling studies rely upon at least one cell division. This is not the case for doxycycline inducible histone 2B-GFP labelling system. In a study conducted by Kwak and colleagues using the TetO-H2B-GFP reporter, mice were pulsed until 2 weeks after birth and chased for 12 weeks (Kwak and Ghazizadeh, 2015). Immunofluorescence revealed that subsequent to the chase GFP labeled cells were predominantly localized to the striated ducts and to the luminal cells in the excretory ducts. Moreover, in colony formation assays GFP+ cells failed to form colonies while substantial amount of colonies formed from the GFP- cells. These data suggests that cells with regenerative capabilities undergo at least one cell division while cells that do not divide at all may not include stem cells. Furthermore, since the in vitro assays used in both of these studies prohibited the assessment of the long-term self-renewal potential of LRCs, it is feasible that the conditions used were suboptimal for the ex vivo growth of stem cells. Additionally, considering that the bulk of stem cell activity occurs during embryonic development, analyzing LRCs labeled in embryonic development could yield to characterization of purified stem cells (Tumbar et al., 2004).

Therefore, in the present study we aimed to acquire new insights into the stem cell localization and population dynamics within the SG. For this, we employed an embryonic pulse and chase assay utilizing the ubiquitous tetracycline-regulated histone H2BGFP system (Foudi et al., 2009) allowing monitoring of cell division history. Furthermore, to directly estimate the potential of LRCs performance as SGSCs we challenged different LRC populations in vitro in organoid formation and self-renewal assays (Maimets et al., 2016).

RESULTS

Pulse-chase experiments reveal slowly cycling cells positioned in excretory and striated ducts

To enrich for SGSC population, we assessed the feasibility of using label retention to select for slowly cycling cells, given that a slower division rate respective to the more differentiated cells within a tissue is a universal characteristic of adult stem cells. In order to visualize and trace the fate of infrequently dividing LRCs in adult SG we used a mouse strain that allows ubiquitous, doxycycline-
inducible expression of an H2B-GFP fusion protein (Foudi et al., 2009). As shown in other tissues (Tumbar et al., 2004), we reason that the majority of stem cell activity occurs during embryonic development of the SG, rendering the postnatal tissue remodeling a task for the progenitor and more differentiated cells. Therefore, H2B-GFP label was induced in pregnant female mice by doxycycline administration from E18 to birth (Figure 1A, pulse) after which loss of fluorescence in the SG was monitored (Figure 1A, chase). Salivary gland is a complex organ with an inherent three-

---

Figure 1. Model for marking slowly cycling in salivary gland and monitoring their fate. (A) Experimental strategy. (B) 3D confocal reconstruction of a whole-mounted 1-day old salivary gland. Expression of H2B-GFP fusion protein is detected in ductal (arrows) and acinar compartments (arrowheads). Scale bars 300 µm (whole-mount) and 30 µm (enlargement). (C) Immunohistochemical staining for GFP shows expression of H2B-GFP fusion protein in ductal (arrows), acinar (arrowheads) and myoepithelial (asterisk) cells. Scale bars 100 µm (upper panel) and 50 µm (lower panel) (D) 4% formaldehyde fixed salivary glands with milky appearance. (E) PACT-processed salivary glands with transparent appearance. (Figure continues)
CHAPTER 4

dimensional (3D) ductal organization extending in many directions. This renders the localization of specific cells within a niche impossible on thin sections. Thus, to explore the presence of H2B-GFP label in the SG immediately after the pulse we developed a 3D confocal imaging strategy allowing in situ visualization of expansive areas of submandibular and sublingual gland tissue architecture (Figure 1B, Movie S1). Ductal (Figure 1B, arrows) and acinar (Figure 1B, arrowheads) compartments could be observed at high cellular resolution, spanning up to 2.5 mm of tissue. In agreement with 3D images, immunostaining using anti-GFP antibody on paraffin sections confirmed labeling of ductal (Figure 1C, arrows) and acinar (Figure 1C, arrowheads, Supplementary figure 1) as well as myoepithelial (Figure 1C, asterisk) cells, readily distinguished by their respective shape and position within the gland. No background staining was detected without primary antibody (data not shown). Consequently, these data show that the Col1a1-H2BGFP genetic mouse model is a powerful tool for studying the fate of slowly cycling salivary gland cells.

Figure 1 continues. (F) Whole-mount 3D confocal images of 40-day old salivary gland showing the position of LRCs surrounding the excretory duct (Panel 1) and in striated ducts (Panel 2). Scale bars 700 μm (whole-mount) and 20 μm (Panel 1 and 2). (G) 60-day old salivary glands showing no visible GFP+ cells. Scale bar 700 μm. Nuclei counterstained with DRAQ5. SMG, submandibular gland; SLG, sublingual gland.
Next, we analyzed SG-s for cells retaining H2B-GFP label over time. Postnatal SG is a transparent organ that allows optical access for recording physiologically relevant information. Conversely, adult salivary gland has an intrinsic milky appearance (Figure 1D) that creates an imminent light scatter, rendering imaging deep into a tissue volume problematic. Therefore, to enable whole-organ imaging we took advantage of the recently developed PACT reagents (Yang et al., 2014) for tissue clearing technique to successfully prepare translucent SG-s (Figure 1E). 40 days of chase dramatically reduced the number of cells expressing GFP. Notably, LRCs carrying H2B-GFP label were observed primarily in ductal (Figure 1F, Movie S2) and not in acinar compartments as distinguished by their respective shape and position in the gland. Specifically, we encountered GFP+ cells in excretory ducts (Figure 1F, panel 1) and striated ducts (Figure 1F, panel 2). Considered together, these data suggests that within the salivary gland cells with the slowest turnover reside in excretory and striated ducts, locations that were previously suggested to contain stem cells (Lombaert et al., 2008; Maimets et al., 2016; van Luijk et al., 2015).

**Phenotypical profile of LRCs is not reminiscent to SG stem cells**

After 60 days of tracing we were not able to detect LRC-s by large-scale microscopy, hypothetically due to the small number of LRCs residing within the SG (Figure 1G). Additionally, since at this point the glands are fully developed, 60 days was considered as an optimal chase period for characterization and isolation of a small pool of putative SGSCs. In order to provide molecular characterization of LRCs we next turned to conventional sectioning and immunostaining of the tissue by using panel of markers expressed in SG. Keratin 5 (K5) and Keratin 14 (K14) have been associated with salivary gland progenitors during development (Knox et al., 2013; Knox et al., 2010; Lombaert et al., 2013). Therefore, we aimed to determine whether salivary gland LRCs expressed these markers in vivo. Immunofluorescence staining on tissue sections from 60-day traced mice revealed LRCs scattered throughout the parenchyma of SG with an increased abundance in ductal compartments (Figure 2). K14 expression was found in the basal layer of excretory ducts (Figure 2A-B) where it, unexpectedly, did not co-localize with ductal LRCs (Figure 2B, arrows). Similarly, localization of K5 expressing cells was confined to basal cells in the excretory ducts (Figure 2C-D) where again no overlap with LRCs was found (Figure 2D, arrows). Conversely, antibody staining of SG sections with ductal luminal marker Keratin 8 (K8) (Stingl et al., 2001) (Figure 2E-F) using immunofluorescence revealed co-localization of LRCs and K8 in the luminal cells of excretory ducts (Figure 2F, arrows). Next, in order to characterize the remainder of LRCs residing outside of the ductal compartment we co-immunostained SG sections for the mesenchymal marker Vimentin.
(Eriksson et al., 2009) (Figure 2G-H). Indeed, co-expression with an abundant number of LRCs was observed (Figure 2H, arrows) indicative of a slower turnover of salivary gland mesenchymal cells compared to the glandular epithelium. These data suggests that slowly cycling cells during postnatal development of the salivary gland acquire the fate of 1) K8+ ductal luminal or 2) Vimentin+ mesenchymal but not CK5+ or CK14+ putative progenitor cells.

Figure 2. LRCs co-localize with K8+ ductal luminal or Vimentin+ mesenchymal cells. (A-D) Double immunofluorescence for (A-B) GFP and CK14 and (C-D) GFP and CK5 show no overlap between LRCs (arrows) and CK14+ and CK5+ (arrowheads) cells in the ductal compartment. (E-H) Double immunofluorescence for (E-F) GFP and CK8 and (G-H) GFP and Vimentin reveal co-localization (asterisk) of LRCs and ductal luminal cells (CK8+) and LRCs and mesenchymal cells (Vimentin+). Nuclei counterstained with DAPI. Scale bars 50 µm (left panel) and 20 µm (right panel).
Indeed, co-expression with an abundant number of LRCs was observed (Figure 2H, arrows) indicative of a slower turnover of salivary gland mesenchymal cells compared to the glandular epithelium. These data suggests that slowly cycling cells during postnatal development of the salivary gland acquire the fate of 1) K8+ ductal luminal or 2) Vimentin+ mesenchymal but not CK5+ or CK14+ putative progenitor cells.

Figure 2. LRCs co-localize with K8+ ductal luminal or Vimentin+ mesenchymal cells. (A-D) Double immunofluorescence for (A-B) GFP and CK14 and (C-D) GFP and CK5 show no overlap between LRCs (arrows) and CK14+ and CK5+ (arrowheads) cells in the ductal compartment. (E-H) Double immunofluorescence for (E-F) GFP and CK8 and (G-H) GFP and Vimentin reveal co-localization (asterisk) of LRCs and ductal luminal cells (CK8+) and LRCs and mesenchymal cells (Vimentin+). Nuclei counterstained with DAPI. Scale bars 50 µm (left panel) and 20 µm (right panel).

Figure 3. Regenerative potential of LRCs. (A) Experimental strategy. (B) Representative FACS gating strategy for the analysis of LRCs in the salivary gland. Left panel shows the exclusion of lineage marker-expressing cells. Right panel depicts the distribution of GFP\textsuperscript{high}, GFP\textsuperscript{med}, and GFP\textsuperscript{neg} cells in dissociated adult mouse salivary gland. FSC, forward scatter. (C) Fractions of of GFP\textsuperscript{high}, GFP\textsuperscript{med} and GFP\textsuperscript{neg} cells in 60-day old adult mouse salivary gland. (D-F) DIC images showing the outgrowth (arrows) of (D) GFP\textsuperscript{high}, (E) GFP\textsuperscript{med} and (F) GFP\textsuperscript{neg} cell populations after 12 days in culture. (G) Organoid formation efficiency of sorted GFP\textsuperscript{high}, GFP\textsuperscript{med} and GFP\textsuperscript{neg} cells. (H) Population dynamics plot of GFP\textsuperscript{high}, GFP\textsuperscript{med} and GFP\textsuperscript{neg} cells during serial passaging. (I) Organoid formation kinetics of GFP\textsuperscript{high}, GFP\textsuperscript{med} and GFP\textsuperscript{neg} cells during serial passaging.
CHAPTER 4

Label retaining cells do not promote growth of salivary gland organoids in vitro

We next sought to assess the regenerative potential of LRCs as a functional property of stem cells. For this, we focused on the growth of explanted cells into budding organoids, as this represents a well-defined experimental system reminiscent of in vivo regeneration (Maimets et al., 2016). As before, H2B-GFP label was induced in pregnant female mice from E18 to P0 (Figure 3A) and chased for 60 days. Subsequently, the salivary glands were isolated and digested into single-cell suspension. After removing cell clumps, dead cells and debris, we depleted CD45+ and TER119+ hematopoietic and CD31+ endothelial cells (Figure 3B, left panel). Next, salivary gland cells were subdivided into three cell populations, GFP$^{\text{high}}$, GFP$^{\text{med}}$ and GFP$^{\text{neg}}$ (Figure 3B, right panel) using fluorescence-activated cell sorting (FACS). The observed proportion of the cells in GFP$^{\text{high}}$ population was 1.8±0.2%, in GFP$^{\text{med}}$ 13.4±1.1%, and in GFP$^{\text{neg}}$ 84.9±1.1% (Figure 3C). Purified cells were embedded in Matrigel and supplemented with our previously defined salivary gland organoid culture conditions containing Wnt3a, R-spondin 1, epidermal growth factor (EGF), fibroblast growth factor 2 (FGF2), insulin, dexamethasone, N2 and Y27632 (WRY medium) (Maimets et al., 2016). Coinciding with what we had observed previously, during 12 days of culture GFP$^{\text{high}}$ (Figure 3D,G) and GFP$^{\text{med}}$ (Figure 3E,G) populations were effectively unable to initiate cultures (<0.06%) while GFP$^{\text{neg}}$ cells successfully generated organoids (Figure 3F,G) (0.47±0.1 organoid forming efficiency). Next, organoids derived from single GFP$^{\text{neg}}$ cells were dissociated and replated into Matrigel supplemented with WRY medium. Within the period of three passages (3 weeks) these cultures displayed exponential growth (Figure 3H) similarly to what we published previously (Maimets et al., 2016). In addition, as cells derived from GFP$^{\text{neg}}$ population were passaged, an increase in the ability to form spheres was observed (Figure 3I). Taken together, functional ex vivo regeneration analysis of slowly cycling cells in the salivary gland indicates that LRCs do not include cells with an in vitro high proliferative potential and therefore may not represent quiescent stem cells.

DISCUSSION

Here we provide evidence that during postnatal development quiescent cells within the submandibular gland do not display characteristics of tissue specific stem cells (SCs). Accurate identification of SCs in vivo remains one of the biggest hurdles to overcome in understanding SC biology. A common property of SCs is their quiescence in terms of the cell cycle (Cheung and Rando, 2013). Dormancy of SCs is believed to provide selective survival advantage, also under unfavorable conditions and to protect SCs from stress (Horsley et al., 2008; Kobilak et al., 2007). Our experiments were designed to employ a cell-state independent histone H2BGFP pulse-chase system
to determine the dynamics of stem/progenitor cells in the mouse submandibular gland and to characterize the putative quiescent stem cell population that may exist within. Despite efficient H2BGFP labeling of salivary gland parenchyma following embryonic pulse period, we found no evidence of a slowly dividing stem cell population ex vivo subsequent to animals reaching adulthood. However, in the future it would be of interest to study characteristics of LRCs by varying the timeframe of the pulsing strategy as it is feasible that due to changes in hormonal regulation glandular SCs could be activated during puberty (Van Keymeulen et al., 2011). As shown previously in mammary gland (dos Santos et al., 2013), it might therefore be feasible to combine label retention with previously identified salivary gland SC markers (Lombaert et al., 2008; Nanduri et al., 2014) which may lead to further purification of SGSCs.

Recently, two pulse-chase studies have been conducted in salivary glands with the goal to discern quiescent stem cells, if they existed. While in one study (Chibly et al., 2014) LRCs marked by EdU identified a cell population displaying putative salivary progenitor phenotype, in another (Kwak and Ghazizadeh, 2015) LRCs targeted with a histone H2BGFP model mapped to the more differentiated ductal compartments. The discrepancies between these studies can be explained by the EdU pulse-chase dependency on cellular proliferation, making it plausible that LRCs analyzed by Chibly and colleagues did not represent completely dormant cells. Furthermore, as in the case with hematopoietic stem cells it might be possible that the use of nucleotide analogues can also have an effect on stem cell activity (Kiel et al., 2007). In line with the results of Kwak and Ghazizadeh, our results show that postnatal quiescent cells acquire the fate of ductal luminal or mesenchymal cells and not putative progenitor cells. Previous studies have relied on 2D colony forming techniques for determining stem cell capabilities of LRC populations (Chibly et al., 2014; Kwak and Ghazizadeh, 2015). To strengthen our findings, we show in a 3D salivary gland organoid model that indeed, cells displaying any regeneration activity reside exclusively in the non-LRC population. Contrary to 2D colonies that focus on estimating the proliferative capacity of cells and therefore disregarding the fate of the progeny, organoids shown here underwent a series of budding events which based on our previous work (Maimets et al., 2016) is indicative of the retention of differentiation potential.

In conclusion, our H2BGFP pulse-chase experiments suggest the presence of an active stem/progenitor cell population within the salivary gland. For clinical application, in the future it would be of importance to characterize this population and define their role and potential during SG regeneration.
EXPERIMENTAL PROCEDURES

Mice

B6;129S4-Gt(ROSA)26Sortm1(rtTA*M2)IaeCol1a1tm7(tetO-HIST1H2BJ/GFP)Iae/J were purchased from the Jackson Laboratory. Animals were bred as homozygotes in the Central Animal Facility of University Medical Centre Groningen. The mice were maintained under conventional conditions and fed ad libitum with food pellets (RMH-B, Hope Farms B.V.) and water. For transgene expression, doxycycline (Sigma D9891, 2 mg/ml, supplemented with sucrose at 10 mg/ml) was administered to the pregnant female mother in drinking water or with doxycycline-impregnated food pellets (SDS Tecnilab, 625 ppm RM1) during embryonic development. All experiments were approved by the Ethical Committee on animal testing of the University of Groningen.

Two-photon microscopy

Postnatal H2BGFP (P1) salivary glands were harvested and the epithelium was separated by dispase (Gibco, 1mg/ml) treatment for 15 min at 37 °C. Defatted salivary glands were mounted in PBS and processed for imaging. Imaging was performed by using Zeiss 7 MP multiphoton system (Zeiss). For two-photon microscopy of green (GFP) fluorophore the laser (Coherent) was tuned to ~970 nm (940–970nm). A 20x dipping objective (Zeiss W Plan A 20x, 1.0 NA) was used for these experiments. 3D reconstruction was performed using Imaris (Bitplane) software.

Whole-organ confocal microscopy

Adult H2BGFP pulsed and chased (P40 and P60) mouse salivary glands were harvested, bisected longitudinally and processed for tissue clearing as reported elsewhere (Yang et al., 2014). Shortly, salivary glands were fixed in 4% formaldehyde solution overnight. Subsequently, fixed tissue sections were incubated at 4 °C overnight in AP40 solution (4% acrylamide in PBS) supplemented with 0.25% photo initiator VA-044 (Wako Chemicals), degassed with nitrogen for 1-5 minutes and then incubated for 2-3 hr at 37 °C to initiate tissue-hydrogel hybridization after which tissue-hydrogel matrices were transferred to 8% SDS in 0.1 M PBS (pH7.5) and incubated for 5 days. Prior to counterstaining with DRAQ5 (eBioscience) matrices were washed with PBS over the course of 1 day followed by mounting in RIMS imaging media containing 40g of Histodenz (Sigma) in 30 ml of 0.02M PB with 0.1% tween-20

Immunostaining

Adult H2BGFP pulsed and chased (P60) mouse salivary glands were 4% formaldehyde fixed (24 hours, RT) and processed for paraffin embedding. Following dehydration, the tissue was embedded in paraffin and sliced into 5 μ, boiled for 8 min in pre-heated 10 mM citric acid retrieval buffer (pH 6.0) containing 0.05 % Tween20, washed thoroughly prior to primary antibody exposure and labeled for the following markers: GFP (1:100, Chemicon, MAB3580), CK14 (1:400, Abcam, ab175549), CK5 (1:100, Covance, PRB-160-P), CK8 (1:50, Hybridoma bank, TROMA-I) Vimentin (1:50, Santa Cruz, sc-7557). For fluorescence microscopy Alexa Fluor 488 goat anti-rabbit (Life technologies, A11008) or Alexa Fluor 594 donkey anti-mouse (Life technologies, A21203) conjugates at 1:300 dilution were used as secondary antibodies. Nuclear staining was performed with DAPI (Sigma-Aldrich). Images were acquired with Leica DM6000 B microscope using LAS AF software.

Cell sorting and single cell salivary gland organoid culture

Salivary glands were harvested from adult pulsed and chased H2BGFP mice, mechanically disrupted by gentleMACS Dissociator (Milteny) followed by enzymatic digestion with collagenase type II (0.63 mg/ml; Gibco), hyaluronidase (0.5 mg/ml; Sigma-Aldrich) and CaCl2 (6.25 mM; Sigma-Aldrich). After filtering through 100 μm cell strainer the suspension was dissociated using 0.05% trypsin-EDTA (Gibco) following filtering through 35 μm strainer. Cell pellets were incubated with anti-mouse CD31-PE (eBioscience, 12-0311-82), CD45-PE (Biolegend, 103106) and TER-119-PE/Cy7 (Biolegend, 116222) antibodies for 15' on room temperature. After washing thoroughly cells were suspended in a solution containing propidium iodide (PI; 1mg/ml; Sigma-Aldrich), MgSO4 (10 mM; Sigma-Aldrich) and DNase I (50 μg/ml; Sigma-Aldrich). Pulse-width gating excluded cell doublets while dead cells were excluded by gating on PI negative cells. Positive gating was based on the comparison of non-stained and single antibody-stained samples in the case of CD31, CD45 and Ter-119 and on the comparison of not-induced and induced H2BGFP mice in the case of GFP. Sorted cells were
EXPERIMENTAL PROCEDURES

Mice

B6;129S4-Gt(ROSA)26Sortm1(rtTA*M2)JaeCol1a1tm7(tetO-HIST1H2BJ/GFP)Jae/J were purchased from the Jackson Laboratory. Animals were bred as homozygotes in the Central Animal Facility of University Medical Centre Groningen. The mice were maintained under conventional conditions and fed ad libitum with food pellets (RMH-B, Hope Farms B.V.) and water. For transgene expression, doxycycline (Sigma D9891, 2 mg/ml, supplemented with sucrose at 10 mg/ml) was administered to the pregnant female mother in drinking water or with doxycycline-impregnated food pellets (SDS Tecnilab, 625 ppm RM1) during embryonic development. All experiments were approved by the Ethical Committee on animal testing of the University of Groningen.

Two-photon microscopy

Postnatal H2BGFP (P1) salivary glands were harvested and the epithelium was separated by dispase (Gibco, 1mg/ml) treatment for 15 min at 37 °C. Defatted salivary glands were mounted in PBS and processed for imaging. Imaging was performed using the Zeiss 7 MP multiphoton system (Zeiss). For two-photon microscopy of green (GFP) fluorophore the laser (Coherent) was tuned to ~970 nm (940–970nm). A 20x dipping objective (Zeiss W Plan A 20x, 1.0 NA) was used for these experiments. 3D reconstruction was performed using Imaris (Bitplane) software.

Whole-organ confocal microscopy

Adult H2BGFP pulsed and chased (P40 and P60) mouse salivary glands were harvested, bisected longitudinally and processed for tissue clearing as reported elsewhere (Yang et al., 2014). Shortly, salivary glands were fixed in 4% formaldehyde solution overnight. Subsequently, fixed tissue sections were incubated at 4 °C overnight in AP40 solution (4% acrylamide in PBS) supplemented with 0.25% photo initiator VA-044 (Wako Chemicals), degassed with nitrogen for 1-5 minutes and then incubated for 2-3 hr at 37 °C to initiate tissue-hydrogel hybridization after which tissue-hydrogel matrices were transferred to 8% SDS in 0.1 M PBS (pH7.5) and incubated for 5 days. Prior to counterstaining with DRAQ5 (eBioscience) matrices were washed with PBS over the course of 1 day followed by mounting in RIMS imaging media containing 40g of Histodenz (Sigma) in 30 ml of 0.02M PB with 0.1% tween-20 and 0.01% sodium azide (pH7.5). Confocal microscopy was carried out on a LSM780 system (Zeiss) using Zen software. PlnApo 20x, 0.8 NA DICII lens was used with 488 and 633 laser lines. Image stitching was performed using Zen (Zeiss) software and 3D reconstruction was achieved using Imaris (Bitplane) software.

Immunostaining

Adult H2BGFP pulsed and chased (P60) mouse salivary glands were 4% formaldehyde fixed (24 hours, RT) and processed for paraffin embedding. Following dehydration, the tissue was embedded in paraffin and sliced into 5 μm sections. The sections were dewaxed, boiled for 8 min in pre-heated 10 mM citric acid retrieval buffer (pH 6.0) containing 0.05 % Tween20, washed thoroughly prior to primary antibody exposure and labeled for the following markers: GFP (1:100, Chemicon, MAB3580), CK14 (1:400, Abcam, ab175549), CK5 (1:100, Covance, PRB-160-P), CK8 (1:50, Hybridoma bank, TROMA-I) Vimentin (1:50, Santa Cruz, sc-7557). For fluorescence microscopy Alexa Fluor 488 goat anti-rabbit (Life technologies, A11008) or Alexa Fluor 594 donkey anti-mouse (Life technologies, A21203) conjugates at 1:300 dilution were used as secondary antibodies. Nuclear staining was performed with DAPI (Sigma-Aldrich). Images were acquired with Leica DM6000 B microscope using LAS AF software.

Cell sorting and single cell salivary gland organoid culture

Salivary glands were harvested from adult pulsed and chased H2BGFP mice, mechanically disrupted by gentleMACS Dissociator (Milteny) followed by enzymatic digestion with collagenase type II (0,63 mg/ml; Gibco), hyaluronidase (0,5 mg/ml; Sigma-Aldrich) and CaCl₂ (6,25 mM; Sigma-Aldrich). After filtering through 100 μm cell strainer the suspension was dissociated using 0,05% trypsin-EDTA (Gibco) following filtering through 35 μm strainer. Cell pellets were incubated with anti-mouse CD31-PE (eBioscience, 12-0311-82), CD45-PE (Biolegend, 103106) and TER-119-PE/Cy7 (Biolegend, 116222) antibodies for 15’ on room temperature. After washing thoroughly cells were suspended in a solution containing propidium iodide (PI; 1mg/ml; Sigma-Aldrich), MgSO₄ (10 mM; Sigma-Aldrich) and DNase I (50 μg/ml; Sigma-Aldrich). Pulse-width gating excluded cell doublets while dead cells were excluded by gating on PI negative cells. Positive gating was based on the comparison of non-stained and single antibody-stained samples in the case of CD31, CD45 and Ter-119 and on the comparison of not-induced and induced H2BGFP mice in the case of GFP. Sorted cells were
CHAPTER 4

embedded in Basement Membrane Matrigel (BD Biosciences) and seeded in 12-well plates. Cells were cultured in WRY medium (Maimets et al., 2016).

**Self-renewal assay**

Self-renewal assay was performed as published previously (Maimets et al., 2016). Shortly, 12-day organoid cultures were dispersed to single cell suspension using 0.05% trypsin-EDTA (Gibco). 25 μl of suspension containing 10,000 cells was combined with 50 μl of BM Matrigel and deposited in the center of 12-well tissue culture plates and covered in WRY medium. 7 days after seeding Matrigel was dissolved by incubation with dispase (1 mg/ml; Sigma) for 30 minutes at 37°C. Organoids released from the gels were again processed to a single cell suspension, cell number and sphere number noted, and encapsulation in Matrigel repeated. This cycle was repeated up to 3 times (3 passages).

**Data analysis**

All values are represented as mean ± standard error of the mean (SEM) (**p < 0.01). Student’s t-test was used for testing statistical significance in cell sorting and cell culture experiments. Numbers for tested groups throughout all experiments equal to or a larger than 3. All calculations were performed using GraphPad Prism (GraphPad software) software.

**AUTHOR CONTRIBUTIONS**

M.M. designed and performed experiments, analyzed data, and wrote the manuscript. R.B. performed and co-analyzed immunostaining experiments. R.v.O. and R.P.C. designed experiments, supervised the project and wrote the manuscript.

**ACKNOWLEDGEMENTS**

This work was supported by grants from The Netherlands Organization for Health Research and Development (ZonMW-Grant nr. 11.600.1023), the Netherlands Institute for Regenerative Medicine (NIRM, Grant No. FE0908) and the Dutch Cancer Society (RUG2013-5792). Part of the work has been performed at the UMCG Microscopy and Imaging Center (UMIC), which is sponsored by NWO grant
ACKNOWLEDGEMENTS

performed and co-analyzed immunostaining experiments. R.v.O. and R.P.C. designed experiments, M.M. designed and performed experiments, analyzed data, and wrote the manuscript. R.B. was dissolved by incubation with dispase (1 mg/ml; Sigma) for 30 minutes.

suspension containing 10 000 cells was combined with 50

performed at the UMCG Microscopy and Imaging Center (UMIC), which is sponsored by NWO grant (NIRM, Grant No. FE0908) and the Dutch Cancer Society (RUG2013-5792). Part of the work has been supported by grants from The Netherlands Organization for Health Research and Development (ZonMW-Grant nr. 11.600.1023), the Netherlands Institute for Regenerative Medicine (289-301).

CHAPTER 4

(175-010-2009-023). We thank K. Sjollema for expert assistance in confocal microscopy and G. Mesander and H. Moes for expert cell sorting assistance.

Conflict of interests

All authors declare no competing interests.

REFERENCES


