Potential of salivary gland stem cells in regenerative medicine
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CHAPTER 3

LONG-TERM IN VITRO EXPANSION OF SALIVARY GLAND STEM CELLS DRIVEN BY WNT SIGNALS

Maimets, M., Rocchi, C., Bron, R., Pringle, S., Kuipers, J., Giepmans, BNG., Vries, RGJ., Clevers, H., de Haan, G., van Os, R., Coppes, RP.

INTRODUCTION

Tissue homeostasis and regeneration are maintained by resident stem cells that have the ability to self-renew and to generate all differentiated lineages that characterize a particular tissue. Self-renewal of stem cells should be achieved by asymmetric cell division to maintain sufficient numbers of stem cells and to allow ample production of mature, functional tissue specific cells. The balance between self-renewal and differentiation is stringently regulated by cell-intrinsic transcriptional programs and extracellular signals originating from a specialized microenvironment – the stem cell niche (Morrison and Spradling, 2008). Strict cell-extrinsic control is crucial to avoid the continuous self-renewal of stem cells and their possible progression into cancerous cells (Clarke and Fuller, 2006). An important feature of the stem cell niche model is the limited availability of self-renewing factors due to their local release and short signaling distance (Clevers et al., 2014). Understanding the nature of these factors and their effect on adult stem cells has been hindered due to the low abundance of stem cells and the limited number of functional assays.

The salivary gland is a useful model for studying adult stem cell maintenance due to the easy accessibility and its extensive regenerative capacity (Ball, 1974; Denny et al., 1997; Denny et al., 1993; Ihrler et al., 2002; Osailan et al., 2006). Salivary glands are complex secretory organs which are composed of saliva-producing acinar cells, myoepithelial cells which facilitate the saliva expulsion and ductal cells through which saliva is secreted into the oral cavity (Pringle et al., 2013). Intermingled with ductal cells reside salivary gland stem cells (SGSCs), which express c-Kit, CD49f, CD133, CD24, CD29 cell surface markers (Hisatomi et al., 2004; Lombaert et al., 2008a; Nanduri et al., 2011). Upon transplantation, SGSCs attenuate radiation-induced hyposalivation (Lombaert et al., 2008a; Nanduri et al., 2011) and improve tissue homeostasis necessary for long-term maintenance of the adult tissue (Nanduri et al., 2013). Although, recently we (Nanduri et al., 2014) and others (Xiao et al., 2014) have successfully purified SGSCs able to self-renew and differentiate in vitro and in vivo, the molecular cues underlying the maintenance of SGSCs and the existence of a specialized stem cell niche are still enigmatic.

The canonical Wnt/\(\beta\)-catenin signaling has been shown to play a crucial role in the maintenance of multiple types of adult stem/progenitor cells (Clevers and Nusse, 2012). The Wnt target gene Lgr5 has been identified as a marker of resident stem cells in the small intestine and colon (Barker et al., 2007), hair follicle (Jaks et al., 2008), stomach (Barker et al., 2010), kidney (Barker et al., 2012) and liver (Huch et al., 2013b). In adult salivary glands, Wnt/\(\beta\)-catenin signaling is weak, but is significantly activated during functional regeneration (Hai et al., 2010). Furthermore, concurrent transient activation of Wnt/\(\beta\)-catenin signaling ameliorates irradiation-induced salivary gland dysfunction (Hai et al., 2010).

SUMMARY

Adult stem cells are the ultimate source for replenishment of salivary gland (SG) tissue. Self-renewal ability of stem cells is dependent on extrinsic niche signals that have not been unraveled for the SG. The ductal compartment in SG has been identified as the location harboring stem cells. Here we report that rare SG ductal EpCAM^+ cells express nuclear \(\beta\)-catenin indicating active Wnt-signaling. In cell culture experiments, EpCAM^{high} cells respond potently to Wnt signals stimulating self-renewal and long-term expansion of SG organoids, containing all differentiated salivary gland cell types. Conversely, Wnt inhibition ablated long-term organoid cultures. Finally, transplantation of cells pre-treated with Wnt agonists into submandibular glands of irradiated mice successfully and robustly restored saliva secretion and increased the number of functional acini in vivo. Collectively, these results identify Wnt signaling as a key driver of adult SG stem cells, allowing extensive in vitro expansion, enabling restoration of SG function upon transplantation.

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et al., 2012). Whether Wnt proteins directly control normal salivary gland stem cell maintenance is still not known. In this study, we used a combination of cell culture and in vivo transplantation experiments to show that Wnt proteins serve as important self-renewing factors for SGSCs.

RESULTS

EpCAM$^+$ cells in salivary gland ducts co-express β-catenin

In the salivary gland, stem cells have been suggested to reside within the ductal compartment (Denny and Denny, 1999; Man et al., 2001). Therefore, a universal marker for ductal cells of adult submandibular gland would allow identification and enrichment of a population containing stem cells. EpCAM (Epithelial cell adhesion molecule) is present on most epithelial cells and has been used as a marker for self-renewing compartments in liver (Dan et al., 2006; Huch et al., 2015) and pancreas (Huch et al., 2013a). To assess the presence of EpCAM in the salivary gland, we stained whole gland sections using immunofluorescence. The expression of EpCAM was detected throughout the whole epithelia of the salivary gland (Figure S1A). However, we encountered most abundant and enhanced expression of EpCAM in the ductal compartment, marking excretory, striated and intercalated ducts (Figure 1A) and low EpCAM expression in acinar cells, which comprise most of the mouse submandibular gland. No background staining was detected in salivary gland sections treated without primary antibody (Figure S1B and D). Interestingly, transcription of EpCAM is activated upon Wnt/β-catenin signaling in other tissues (Yamashita et al., 2007). Therefore, we attempted to determine sites of Wnt-signaling in the salivary gland using β-catenin as a general reporter (Peifer et al., 1994). Indeed, highest β-catenin expression was observed to be confined to ductal cells (Figure 1B) of the salivary gland while the acinar cells showed low levels of β-catenin. Again, no background staining was detected without primary antibody (Figure S1C and D). Moreover, most cells positive for β-catenin were found to co-express EpCAM (Figure 1C and D). To quantify this, co-localization analysis was performed and Pearson correlation coefficient (PCC) for sub-region of interests (ROIs) were calculated. This revealed the strongest correlation of EpCAM and β-catenin occurring in excretory ducts (Figure 1E, ROI 2) (PCC=0.121644), which were previously suggested to contain the stem cells (Lombaert et al., 2008a; Pringle et al., 2013). Interestingly, striated ducts displayed more exclusive EpCAM$^+$ cells (Figure 1E, ROI 1) (PCC=0.032378) while intercalated ducts revealed more exclusive β-catenin expression (Figure 1E, ROI 3) (PCC=0.001784). Importantly, scanning of excretory ducts revealed rare basal cells with nuclear β-catenin expression (Figure 1F, arrows) suggesting an occurrence of active Wnt signaling in these cells. The existence of these rare cells agrees with the low level of cell turnover of the salivary glands (Aure et al., 2015).
Figure 1 EpCAM expressing ductal cells have the potential to be Wnt activated. (A) Immunofluorescence staining of EpCAM in striated, intercalated and excretory ducts. (B-D) β-catenin (red) co-localizes with EpCAM (green) in salivary gland ducts. (E) Scattogram of colocalization of EpCAM and β-catenin. Different regions of interests (ROIs) show which pixels (arrows) are included in the analysis. For each pixel in the fluorescent image, the two intensities (green, red) are used as coordinates in the scattergram. Images analyzed with ImageJ “Colocalization_Finder” plugin (Christophe Laumonerie; 2006/08/29: Version 1.2). (F) Nuclear localization of β-catenin in rare basal cells (arrow) of excretory ducts. Top: β-catenin; center: DAPI; bottom: overlay. Scale bars 20 μm (A-E) and 5 μm (F).

Recently, Wnt target genes Lgr5 (Barker et al., 2007) and Lgr6 (Snippert et al., 2010) have been identified as markers of stem cells in the intestine/colon and skin respectively. We utilized the LGR5-EGFP (Barker et al., 2007) and LGR6-EGFP (Snippert et al., 2010) knock-in alleles to determine the expression of LGR5 and LGR6 in the salivary gland. Both receptors are essentially undetectable in the tissue under steady-state conditions (data not shown). This is in line with recent findings in other slow turnover tissues such as liver (Huch et al., 2013b) and pancreas (Huch et al., 2013a) where under homeostatic conditions no expression of LGR5 was detected. These data indicate that EpCAM+
removing cell clumps, dead cells and debris, we depleted CD45 + and TER119 + hematopoietic and CD31+ endothelial cells (Figure 2A). Subsequently, salivary gland cells were subdivided into three distinct cell populations: EpCAM high, EpCAM med and EpCAM neg using Fluorescence Activated Cell Sorting (FACS) (Figure 2A). Purified cells were embedded into Matrigel containing enriched medium supplemented with Epidermal Growth Factor (EGF), Fibroblast Growth Factor 2 (FGF2), insulin and Y-27632 (Nanduri et al., 2014), to which Wnt3A and Rspo1 were added (WRY medium). Under these conditions, 0.9±0.2% of single cells from the EpCAM high population generated spheres within 24-48 hours (Figure 2B). In contrast, single cells from EpCAM med and EpCAM neg populations were unable to form spheres (<0.05%, Figure 2B). Moreover, only 0.05±0.01% of live, non-marker selected cells and 0.2±0.1% non-sorted cells were capable of generating spheres in WRY medium, further indicating that high EpCAM expression pronouncedly enriches for cells with in vitro self-renewing capabilities (Figure S2A). Similarly to gastric organoid units (Barker et al., 2010) supplementing the cultures with either Wnt3a or Rspo1 alone lead to the formation of a lower number of spheres (Figure S2B). We did not observe any cell growth in cultures not supplemented with Wnt proteins (Figure S2B) presumably due to the requirement of Wnt-signaling for the initiation of sphere-growth under these conditions.

Interestingly, within 9 days of culture 10.8±1.8% of the spheres formed differentiated organoid-like structures, which we termed miniglands (Figure 2C and S2C). Miniglands underwent extensive budding events during this time frame, and were up to 4-6 times bigger than co-cultured salispheres (89.2±1.8%), reaching up to 1 mm in diameter (Figure 2D and S2D) by day 15. Toluidine blue staining of miniglands revealed a lobular structure with evenly distributed lumina (Figure 2E). Moreover, miniglands consisted of differentiated CK18+ ductal (Figure 3A,C and S3A), Aqp5 + acinar (Figure 3B,C) and SMA-ɲ myoepithelial cells forming the outer layer of a lobe (Figure 3D and Movie S1), as shown by immunostaining, indicative of retention of differentiation potential of EpCAM high salivary gland stem cells in the presence of Wnt agonists. No background staining was detected in any of the samples treated without primary antibody even with enhancing the lasers of confocal microscope to excessive levels (Figure S3B). Interestingly, ultrastructural analysis of entire miniglands at high resolution with large scale electron microscopy (Sokol et al., 2015) further indicated representation of both, serous acinar (Figure 3E, arrows) and mucous acinar cells (Figure 3E, arrowheads) as recognized by characteristically electron dense and electron pale secretory vesicles, respectively (Movie S2). The basement membrane was lined with myoepithelial cells (Figure 3E, asterisk) distinguished by their elongated shapes. We also observed an abundance of CK5+ cells (Figure 3F), which is considered to be a progenitor cell population in embryonic salivary glands (Knox et al., 2010). Taken together, culturing single EpCAM high stem cell in vitro in Wnt-inducing conditions gives cells in salivary gland excretory ducts co-express β-catenin and therefore could potentially be activated by Wnt-signaling, albeit not through LGR5/6 receptors.

**Figure 2** Single EpCAM high cells generate spheres and miniglands. (A) Representative fluorescence-activated cell sorting (FACS) gating strategy for the analysis of ductal cells in the salivary gland. Left panel shows the exclusion of lineage marker-expressing cells. Right panel depicts the distribution of EpCAM high, EpCAM med and EpCAM neg cells in dissociated adult mouse salivary gland. FSC, forward scatter. (B) Sphere forming efficiency of EpCAM high, EpCAM med, and EpCAM neg populations (** *p* < 0.005). Data are expressed as the mean of ± SEM of three independent experiments. (C) Differential interference contrast image of a growing minigland until 9 days of culture. (D) Representative example of a salisphere and a minigland originating from single EpCAM high in 9-day-old culture. (E) Toluidine blue staining shows uniform lumen formation throughout minigland (arrows). Scale bars 100 μm (C,D) and 10 μm (E)

EpCAM high cells give rise to secondary salivary gland spheres and miniglands

Based on our observations in vivo, we next asked whether Wnt proteins could directly influence EpCAM+ salivary gland ductal cells in vitro by promoting their sphere-initiating ability. Therefore, salivary glands from adult healthy mice 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 2A). Subsequently, salivary gland cells were subdivided into three distinct cell populations: EpCAM\textsuperscript{high}, EpCAM\textsuperscript{med} and EpCAM\textsuperscript{neg} using Fluorescence Activated Cell Sorting (FACS) (Figure 2A). Purified cells were embedded into Matrigel containing enriched medium supplemented with Epidermal Growth Factor (EGF), Fibroblast Growth Factor 2 (FGF2), insulin and Y-27632 (Nanduri et al., 2014), to which Wnt3A and Rspo1 were added (WRY medium). Under these conditions, 0.9±0.2% of single cells from the EpCAM\textsuperscript{high} population generated spheres within 24-48 hours (Figure 2B). In contrast, single cells from EpCAM\textsuperscript{med} and EpCAM\textsuperscript{neg} populations were unable to form spheres (<0.05%, Figure 2B). Moreover, only 0.05±0.01% of live, non-marker selected cells and 0.2±0.1% non-sorted cells were capable of generating spheres in WRY medium, further indicating that high EpCAM expression pronouncedly enriches for cells with \textit{in vitro} self-renewing capabilities (Figure S2A). Similarly to gastric organoid units (Barker et al., 2010) supplementing the cultures with either Wnt3a or Rspo1 alone lead to the formation of a lower number of spheres (Figure S2B). We did not observe any cell growth in cultures not supplemented with Wnt proteins (Figure S2B) presumably due to the requirement of Wnt-signaling for the initiation of sphere-growth under these conditions.

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Single salivary gland stem cell expansion is Wnt-driven. Recently, we have shown pronounced expansion of SGSCs in vitro in the presence of Rho-kinase inhibitor Y-27632 (Nanduri et al., 2014), without Wnt agonists. However, this expansion was initiated from liquid cultures enriched for primary sphere forming cells and might involve a paracrine effect of Wnt-stimulation. To test the requirement of the Wnt pathway in this format, salivary glands from adult healthy mice were isolated and digested into dispersed cells, which formed primary salispheres within 3 days (Figure 4A). Next, single cells derived from dissociated primary spheres were embedded in Matrigel supplemented with expansion medium (EM) (Nanduri et al., 2014) or EM containing a panel of Wnt antagonists: IWR-1-endo (Figure 4D), which stabilizes Axin proteins -catenin destruction complex (Chen et al., 2009), IWP-2 (Figure 4E), which inactivates Porcn, a protein known to be essential for the production of Wnt ligands (Chen et al., 2009) and SFRP-1 (Figure 4F), which directly binds to Wnt proteins (Finch et al., 1997). Indeed, chemical inhibition of the Wnt pathway by IWR-1 endo compound completely suppressed growth of spheres while treatment of IWP-2 and sFRP1 dramatically reduced the growth of spheres (Figure 4G-H). DMSO treated cells (Figure 4C and G-H) did not have a significant effect on the population doublings or sphere forming efficiency. These data suggest that Wnt-signaling is essential for the initiation of sphere-growth, not only when isolating SGSCs directly from tissue (Figure 2A) but also in cultures enriched for salivary gland stem cells (Lombaert et al., 2008a; Nanduri et al., 2014). We next reasoned, that if Wnt inhibition leads to a lack of proliferation in salispheres, Wnt activation may lead to an increased proliferation and salisphere forming potential, and as such expansion of the SGSC pool. We tested this by seeding single cells derived from primary salispheres in Matrigel supplemented with WRY medium (Figure 4I). As expected, the presence of Wnt proteins had an enhanced effect on cell proliferation (population doubling 6.5±0.2) (Figure 4N). Furthermore, we observed a significant effect on sphere forming efficiency (15.7±1.1%) (Figure 4O) when compared to EM conditions (population doubling 3.2±0.2; sphere forming efficiency 12.0±1.0%). Disruption of Wnt-pathway by IWR-1-endo (Figure 4K), IWP-2 (Figure 4L) or sFRP1 (Figure 4M) in WRY conditions lead to a reduced cell growth (3.5±0.1; 0.6±0.2; 1.6±0.4 population doublings respectively) (Figure 4N) and weakened sphere forming efficiency (6.3±1.1%; 2.0±0.6; 3.3±1.2 respectively) (Figure 4O), indicative of incomplete inhibition of Wnt-signalling by Wnt antagonists in the presence of Wnt3a and R-spondin1. DMSO exposure (Figure 4J) did not alter cell growth or sphere formation (population doubling 5.9±0.3; sphere forming efficiency 14.3±1.9) (Figure 4N-O) compared to untreated condition. To test the self-renewal capacity of cells cultured in WRY conditions, every consecutive week, organoids were enzymatically digested into single cells and plated with the density of 10,000 cells per well (Figure 4A). The cultures maintained exponential growth for more than 8 months with rise to 3-dimensional structures which a) consist of all salivary gland cell lineages and b) contain large numbers of CK5+ putative progenitors.

Figure 3 Single-cell derived miniglands acquire fate of salivary gland cells. (A-D), Confocal images (z-stack projection) for salivary gland specific markers (A) CK18 (red, ductal cells), (B) Aqp5 (green, acinar cells), (C) Overlay of CK18 (red) and Aqp5 (green), (D) α-SMA (purple, myoepithelial cells), Counterstain, Hoechst 33342 (blue). (E) Electron microscopy demonstrates serous (arrows) and mucous (arrowheads) acinar cells and myoepithelial cells (asterisk). Data accessible: www.nanotomy.org , salivary gland organoid. Lu, lumen. (F) Confocal images (z-stack projection) for CK5 (red, embryonic SG progenitor cells). Counterstain, Hoechst 33342 (blue). Scale bars 30 μm (A-D,F upper panel), 10 μm (A-D,F lower panel) and 10 μm, 5 μm and 2 μm (E) left, center and right panel respectively.
Single salivary gland stem cell expansion is Wnt-driven

Recently, we have shown pronounced expansion of SGSCs in vitro in the presence of Rho-kinase inhibitor Y-27632 (Nanduri et al., 2014), without Wnt agonists. However, this expansion was initiated from liquid cultures enriched for primary sphere forming cells and might involve a paracrine effect of Wnt-stimulation. To test the requirement of the Wnt pathway in this format, salivary glands from adult healthy mice were isolated and digested into dispersed cells, which formed primary salispheres within 3 days (Figure 4A). Next, single cells derived from dissociated primary spheres were embedded in Matrigel supplemented with expansion medium (EM) (Figure 4B) (Nanduri et al., 2014) or EM containing a panel of Wnt antagonists: IWR-1-endo (Figure 4D), which stabilizes Axin proteins within the β-catenin destruction complex (Chen et al., 2009), IWP-2 (Figure 4E), which inactivates Porcn, a protein known to be essential for the production of Wnt ligands (Chen et al., 2009) and SFRP-1 (Figure 4F), which directly binds to Wnt proteins (Finch et al., 1997). Indeed, chemical inhibition of the Wnt pathway by IWR-1 endo compound completely suppressed growth of spheres while treatment of IWP-2 and sFRP1 dramatically reduced the growth of spheres (Figure 4G-H). DMSO treated cells (Figure 4C and G-H) did not have a significant effect on the population doublings or sphere forming efficiency. These data suggest that Wnt-signaling is essential for the initiation of sphere-growth, not only when isolating SGSCs directly from tissue (Figure 2A) but also in cultures enriched for salivary gland stem cells (Lombaert et al., 2008a; Nanduri et al., 2014). We next reasoned, that if Wnt inhibition leads to a lack of proliferation in salispheres, Wnt activation may lead to an increased proliferation and salisphere forming potential, and as such expansion of the SGSC pool. We tested this by seeding single cells derived from primary salispheres in Matrigel supplemented with WRY medium (Figure 4I). As expected, the presence of Wnt proteins had an enhanced effect on cell proliferation (population doubling 6.5±0.2) (Figure 4N). Furthermore, we observed a significant effect on sphere forming efficiency (15.7±1.1%) (Figure 4O) when compared to EM conditions (population doubling 3.2±0.2; sphere forming efficiency 12.0±1.0%). Disruption of Wnt-pathway by IWR-1-endo (Figure 4K), IWP-2 (Figure 4L) or sFRP1 (Figure 4M) in WRY conditions lead to a reduced cell growth (3.5±0.1; 0.6±0.2; 1.6±0.4 population doublings respectively) (Figure 4N) and weakened sphere forming efficiency (6.3±1.1%; 2.0±0.6; 3.3±1.2 respectively) (Figure 4O), indicative of incomplete inhibition of Wnt-signalling by Wnt antagonists in the presence of Wnt3a and R-spondin1. DMSO exposure (Figure 4J) did not alter cell growth or sphere formation (population doubling 5.9±0.3; sphere forming efficiency 14.3±1.9) (Figure 4N-O) compared to untreated condition. To test the self-renewal capacity of cells cultured in WRY conditions, every consecutive week, organoids were enzymatically digested into single cells and plated with the density of 10.000 cells per well (Figure 4A). The cultures maintained exponential growth for more than 8 months with

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We then asked whether the original organoids derived from EpCAM high cells (Figure 2) contain cells capable for self-renewal and therefore give rise to long-term SGSC cultures. To this end, salivary glands from adult healthy mice 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 S4A). As before, the cells were divided into three cell populations EpCAMhigh, EpCAMmed and EpCAMneg using FACS (Figure S4B) and embedded into Matrigel containing previously defined WRY-medium. During 10 days of culture, EpCAM high cells generated organoids (Figure S4C, arrows) as shown previously. As expected, single cells from EpCAM med and EpCAM neg populations were unable to form organoids (<0.05%) (Figure S4D-E). Next, organoids intiated from single EpCAMhigh cells were dissociated and re-plated into Matrigel supplemented with WRY-medium. Within the period of 3 passages (3 weeks) these cultures displayed exponential growth (Figure S4F) similar to cultures initiated from liquid cultures (Figure 4P). Additionally, as cells derived from EpCAMhigh population were passaged, an increase in the ability to form spheres was observed (Figure S4G), indicative of enrichment in stem/progenitor cells when cultured under Wnt-inducing conditions. This shows that EpCAM high cell population discerned from freshly isolated salivary glands in contrast to EpCAMmed and EpCAMneg populations contain cells with self-renewal potential.

In order to assess the suitability of expanded SGSCs for in vivo reconstitution experiments we beforehand tested their tumorigenicity and differentiation potential. First, when transplanting 8 x 10^5 passage 13 cells subcutaneously into immunocompromised mice, no tumor formation was detected after 1 year in any of the mice analyzed (n=5) (Figure S5A). Secondly, when embedding passage 18 spheres into our previously published differentiation assay containing Collagen Type IV and Basement Membrane Matrigel (50%:50%) (Nanduri et al., 2014) the growth of lobular organoids was observed within 14 days (Figure S5B) suggesting a normal differentiation potential when growing salivary gland cells for multiple passages with WR medium. Therefore, we conclude that passaging SGSCs in Wnt-inducing conditions allows massive expansion of salivary gland stem cell pool.

Transplantation of Wnt-induced cells unprecedentedly rescue irradiation-damaged salivary glands

Based on our in vitro observations showing that only EpCAMhigh cells give rise to secondary structures indicating the presence of stem cells, we finally tested the potential of these cells to rescue radiation-induced hyposalivation in vivo. For this purpose ACTB-DsRed (DsRed) mice were used as donor to enable tracing the donor cells after transplantation. First, sphere cultures from DsRed mice were initiated and cultured for 1 or 7 passages in Basement Membrane Matrigel supplemented with WR media (Figure 5A). Subsequently, cultures were collected, trypsinized into single-cell suspension and

**Figure 4** *In vitro* expansion of salivary gland stem cells, (A) Scheme showing isolation method of SGSCs and establishment of long-term salivary gland organoid culture. (B-F) DIC images of salivary gland organoid cultures grown in enriched medium (EM) (B) (Nanduri et al., 2014) in combination with DMSO (C) or Wnt antagonists IWR-1-endo (D), IWP-2 (E), sFRP1 (F). Scale bars 100 μm. (G-H), Population kinetics of salisphere-derived salivary gland organoid cultures during inhibition of Wnt-pathway grown in EM) representing population doubling (G) and sphere forming capability (H). (I-M) DIC images of salivary gland organoid cultures grown in WRY medium (I) in combination with DMSO (J) or Wnt antagonists IWR-1-endo (K), IWP-2 (L), sFRP1 (M). Scale bars 100 μm. (N-O), Population kinetics of salisphere-derived salivary gland organoid cultures during inhibition of Wnt-pathway grown in WRY medium representing population doubling (N) and sphere forming capability (O). (P) Population dynamics plot of salisphere self-renewal culture (formula in materials and methods). MM, minimal culturing media. WRY, minimal media supplemented with Wnt3a, R-spondin1 and y-27632. Data are expressed as the mean of ± SEM of three independent experiments (panels G,H,N-P). Scale bars 100 μm.

...cell doubling times essentially unchanged during the culturing period (Figure 4P, WRY). Furthermore, when compared to our previously published enriched medium (Figure 4P, EM) (Nanduri et al., 2014) cells grown in the presence of Wnt agonists displayed an enhanced capacity for stem cell expansion.
We then asked whether the original organoids derived from EpCAM\textsuperscript{high} cells (Figure 2) contain cells capable for self-renewal and therefore give rise to long-term SGSC cultures. To this end, salivary glands from adult healthy mice 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 S4A). As before, the cells were divided into three cell populations EpCAM\textsuperscript{high}, EpCAM\textsuperscript{med} and EpCAM\textsuperscript{neg} using FACS (Figure S4B) and embedded into Matrigel containing previously defined WRY-medium. During 10 days of culture, EpCAM\textsuperscript{high} cells generated organoids (Figure S4C, arrows) as shown previously. As expected, single cells from EpCAM\textsuperscript{med} and EpCAM\textsuperscript{neg} populations were unable to form organoids (<0.05%) (Figure S4D-E). Next, organoids initiated from single EpCAM\textsuperscript{high} cells were dissociated and re-plated into Matrigel supplemented with WRY-medium. Within the period of 3 passages (3 weeks) these cultures displayed exponential growth (Figure S4F) similar to cultures initiated from liquid cultures (Figure 4P). Additionally, as cells derived from EpCAM\textsuperscript{high} population were passaged, an increase in the ability to form spheres was observed (Figure S4G), indicative of enrichment in stem/progenitor cells when cultured under Wnt-inducing conditions. This shows that EpCAM\textsuperscript{high} cell population discerned from freshly isolated salivary glands in contrast to EpCAM\textsuperscript{med} and EpCAM\textsuperscript{neg} populations contain cells with self-renewal potential.

In order to assess the suitability of expanded SGSCs for in vivo reconstitution experiments we beforehand tested their tumorigenicity and differentiation potential. First, when transplanting 8 x 10\textsuperscript{e5} passage 13 cells subcutaneously into immunocompromised mice, no tumor formation was detected after 1 year in any of the mice analyzed (n=5) (Figure S5A). Secondly, when embedding passage 18 spheres into our previously published differentiation assay containing Collagen Type IV and Basement Membrane Matrigel (50\%:50\%) (Nanduri et al., 2014) the growth of lobular organoids was observed within 14 days (Figure S5B) suggesting a normal differentiation potential when growing salivary gland cells for multiple passages with WR medium. Therefore, we conclude that passaging SGSCs in Wnt-inducing conditions allows massive expansion of salivary gland stem cell pool.

Transplantation of Wnt-induced cells unprecedentedly rescue irradiation-damaged salivary glands

Based on our in vitro observations showing that only EpCAM\textsuperscript{high} cells give rise to secondary structures indicating the presence of stem cells, we finally tested the potential of these cells to rescue radiation-induced hyposalivation in vivo. For this purpose ACTB-DsRed (DsRed) mice were used as donor to enable tracing the donor cells after transplantation. First, sphere cultures from DsRed mice were initiated and cultured for 1 or 7 passages in Basement Membrane Matrigel supplemented with WR media (Figure 5A). Subsequently, cultures were collected, trypsinized into single-cell suspension and
after irradiation, in control animals (irradiated and non-transplanted), saliva production dropped to 17±3% of pre-irradiation values (Figure 5B-D). In contrast, saliva flow of mice transplanted with 100 or 10,000 passage 1 Wnt-induced cells increased significantly to 53±8% and 62±9%, respectively (Figure 5B and D). Furthermore, the transplantation was even more successful in mice transplanted with 100, 1000 or 10,000 passage 7 Wnt-induced cells, reaching levels of 63±8%, 67±3% and 79±6% of pre-irradiation saliva flow, respectively (Figure 5C and D). This indicates that our culturing conditions are optimized for the enrichment in salivary gland stem cell pool with in vivo reconstitution ability.

Figure 5 Transplantation of cultured Wnt-induced cells improves function of irradiated salivary gland tissue. (A) Scheme representing the transplantation protocol. (B) Transplants of 10,000 (blue), 100 (cyan) of passage 1 Wnt-induced cells in time course analysis of relative saliva production in comparison to irradiated control animals (black). Statistical analysis is shown in comparison to irradiated control group (*** p<0.001, ** p<0.01 at relevant time point). Data are expressed as the mean of ± SEM, n=8 animals per time point. (C) Transplants of 10,000 (blue), 1000 (purple), 100 (cyan) of passage 7 Wnt-induced cells in time course analysis of relative saliva production in comparison to irradiated control animals (black). Statistical analysis is shown in comparison to irradiated control group (*** p<0.001, ** p<0.01 at relevant time point). Data are expressed as the mean of ± SEM, n=8 animals per time point. (D) Relative saliva production at 120 days after irradiation in animals transplanted with 100, 10,000 passage 1 or 100, 1000, 10,000 passage 7 Wnt-induced cells per animal. Each data point represents a recipient animal. Note the uniform response of animals transplanted with passage 7 Wnt-induced cells.

transplanted intraglandularly into C57BL/6 recipient mice, which were previously locally irradiated with 15 Gy to the head and neck region (Lombaert et al., 2008a). Both glands in each mouse received equal cell numbers, so that a total cell number of 100, 1000 or 10,000 were transplanted per recipient mouse. The functionality of the transplanted glands was determined by pilocarpine stimulated saliva flow rate as described previously (Lombaert et al., 2008a). As expected, 120 days
after irradiation, in control animals (irradiated and non-transplanted), saliva production dropped to 17±3% of pre-irradiation values (Figure 5B-D). In contrast, saliva flow of mice transplanted with 100 or 10,000 passage 1 Wnt-induced cells increased significantly to 53±8% and 62±9%, respectively (Figure 5B and D). Furthermore, the transplantation was even more successful in mice transplanted with 100, 1000 or 10,000 passage 7 Wnt-induced cells, reaching levels of 63±8%, 67±3% and 79±6% of pre-irradiation saliva flow, respectively (Figure 5C and D). This indicates that our culturing conditions are optimized for the enrichment in salivary gland stem cell pool with *in vivo* reconstitution ability.

**Figure 6** Donor-derived cells regenerate destroyed salivary gland tissue. (A-E), H&E stainings of SG tissue: irradiated and transplanted with 100 (A), 1000 (B) or 10,000 (C) Wnt-induced cells, irradiated control (D) untreated control (E) showing presence of acini (asterisk). (F) Immunohistochemical staining for DsRed reveals incorporation of transplanted Wnt-induced cells into donor tissue and formation of ducts (arrows) and acini (arrowheads). Scale bars 100 μm (zoom-out panel) and 20 μm (zoom-in panel).
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Recovery of the glandular tissue was further demonstrated by improvement of general morphology (Figure 6A-C) and the re-appearance of functional acinar tissue (Figure 6A-C, asterisk). The histological improvement was observed in all transplanted mice when compared to irradiated controls (Figure 6D), returning to levels close to non-irradiated controls determined by strong increase in acini (Figure 6E). We also analyzed the engraftment of Wnt-induced cells by DsRed staining on serial sections of the entire salivary gland. We found DsRed⁺ ducts (Figure 6F, arrows) and acini (Figure 6F, arrowheads) incorporated in the tissue, indicating that they were derived from donor cells. No specific staining was detected in C57BL/6 salivary gland sections treated with DsRed antibody (Figure S5A) while most of the cells were positively stained in salivary gland sections of DsRed mice (Figure S5B). We did not observe any sign of tumor growth or dysplastic change in any of the transplanted areas indicating the non-transformed origin of cultured cells. Taken together, these data reveal that culturing SGSCs in Wnt-inducing conditions broadly expands SGSCs with enhanced potential to restore functionality in destroyed submandibular glands. However, with these experiments we cannot sufficiently conclude that Wnt proteins are responsible for restoring the function of irradiated salivary glands in vivo.

DISCUSSION

Wnt/β-catenin signaling is involved in many biological processes, including proliferation, differentiation, organogenesis and cell migration (Clevers and Nusse, 2012). It has been shown previously that combinations of Wnt- and R-spondin proteins support long term cultures of small intestine (Sato et al., 2009), stomach (Barker et al., 2010), liver (Huch et al., 2013b) and pancreas (Huch et al., 2013a). Until now, the role of Wnt signaling on key properties of salivary gland stem cell regulation has remained elusive. In this study, we provide evidence that Wnt proteins are required for salivary gland stem cell self-renewal and robustly promote their long-term expansion in culture. Our study further strengthens the role of Wnt-signaling as a universal self-renewal pathway (Lim et al., 2013; Reya et al., 2003; Zeng and Nusse, 2010).

A lack of stem cell assays in the adult salivary gland field has hindered studies aimed to assess functional properties and/or regenerative potential of putative stem cell populations. Our previous attempts for studying SGSC biology were based on in vitro cultures, already enriched for stem/progenitor cells (Lombaert et al., 2008a; Nanduri et al., 2014). Here we report the development of an optimized culture system for generating salivary gland organoids from primary adult submandibular glands in vitro, based on activation of Wnt-signaling. Although in addition to the common transmembrane expression of EpCAM we also detect an unexpected basal localization of
the protein in excretory duct cells of the salivary gland, FACS selection for single EpCAM$^{\text{high}}$ cells and subsequent culturing in the presence of Wnt-activating proteins efficiently generate three-dimensional salivary gland organoids that closely resemble primary salivary gland tissue.

Recently, Xiao and colleagues (Xiao et al., 2014) successfully isolated and cultured Lin$^-$/CD24$^c$/Kit$^+$Sca1$^+$ salivary gland stem cells as spheres lacking phenotypical hallmark of differentiation -branching morphogenesis. Contrary to this, the organoids presented here underwent series of budding events, until reaching sizes up to 1 mm (supplementary figure 2C). Combined with orthotopic transplantation (Ogawa et al., 2013), these findings may open up novel routes to organ replacement regenerative therapy. Using technique reported here we are able to expand salivary gland stem cells, derived from a single animal, to clinically relevant numbers without the use of specific stem cell markers. Previously reported methods have been required to start expansion cultures with cells derived from multiple animals, and used cell surface markers that are mouse specific (Xiao et al., 2014), rendering the expansion protocol less clinically relevant.

Achieving control of cell fate determination in adult tissues is one of the key goals of regenerative medicine. Given the central role of Wnt-mediated cellular responses in stem cell self-renewal we focused our attention on activation as well as inhibition of Wnt-signaling. By using a panel of chemical inhibitors of Wnt pathway we effectively show that Wnt signaling is required for the maintenance of salivary gland stem cell cultures. Although the ability of IWR and IWP compounds to selectively inhibit the Wnt pathway has been characterized elsewhere (Chen et al., 2009), it is still possible that chemical inhibition causes off target effects. Therefore, in the future it would be of interest to use conditional $\beta$-catenin loss-of-function mouse model (Huelsken et al., 2001) or CRISPR-Cpf1 genome editing system (Zetsche et al., 2015) for the ablation of Wnt-pathway.

Ultimately, this study provides proof of principle that SGSCs cultured under Wnt-inducing conditions can be used for stem-cell therapy to irradiation-damaged epithelium and possibly other cases of salivary gland dysfunction. Remarkably, the transplanted cells adhere and engraft into damaged tissue and contribute to the normal homeostasis of the salivary gland. We report an unprecedented improvement of saliva flow recovery over previously reported methods (Lombaert et al., 2008b; Nanduri et al., 2014; Nanduri et al., 2011; Xiao et al., 2014), which could have been achieved by transplantation of heterogeneous pool of cells containing stem, progenitor and differentiated cells present in these cultures. Although translation to the human situation is needed, the current study implies that in vitro expansion and transplantation of long-term cultured SGSCs may be a promising option for patients with severe salivary gland hypofunction.
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In conclusion, we provide clear evidence that Wnt-signals are necessary for SGSC maintenance in vitro. However, this does not exclude the relevance of other pathways in these processes. For example, platelet-derived growth factor receptor signaling, in concert with FGF signaling, has been shown to be essential for proliferation and survival of ex vivo cultures of embryonic salivary gland progenitors (Steinberg et al., 2005; Yamamoto et al., 2008). Furthermore, epidermal growth factors and their receptors are important for embryonic SMG proliferation (Haara et al., 2009; Knox et al., 2010). However, none of these pathways have been exploited to study adult stem cell maintenance in vitro to the extent as the Wnt-signaling pathway presented here. We believe that the efficient in vitro system reported here will be of vital use for validating and implementing further studies on adult SGSC biology and for the discovery of novel pathways involved in the salivary gland maintenance and regeneration.

EXPERIMENTAL PROCEDURES

Mice

8–12 week old female C57BL/6 mice were purchased from Harlan. NOD.Cg-PrkdcScidIl2rgTm1WjlSzJ, B6.Cg-Tg(ACTB-DsRed*MST)1Nagy/J and B6.129P2-Lgr5tm1(cre/ESR1)Cle/J animals were bred in Central Animal Facility of University Medical Centre Groningen. LGR6-EGFP (Snippert et al., 2010) were bred in Hubrecht Institute, University Medical Centre Utrecht. The mice were maintained under conventional conditions and fed ad libitum with food pellets (RMH-B, Hope Farms B.V.) and water. All experiments were approved by the Ethical Committee on animal testing of the University of Groningen.

Immunostaining

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: EpCAM (1:100; (Schnell et al., 2013)), β-catenin (1:100; Transduction laboratories, 610154), DsRed (1:100; BioVision, #3984-100). 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). Haematoxylin and Eosin staining was performed according to standard protocols. Visualization for bright field microscopy was
accomplished by addition of specific secondary biotin carrying antibodies (Dako), an avidin–biotin–horse radish peroxidase complex (ELITE ABC Kit, Vector Laboratories) and the diaminobenzidine (DAB) chromogen. Nuclear staining was performed with hematoxylin.

**Cell sorting and single cell salivary gland sphere culture**

Salivary glands were harvested from healthy adult mice, mechanically disrupted by gentleMACS Dissociator (Miltenyi) followed by enzymatic digestion with collagenase type II (0.63 mg/ml; Gibco), hyaluronidase (0.5 mg/ml; Sigma-Aldrich) and CaCl$_2$ (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), TER-119-PE/Cy7 (Biolegend, 116222) and EpCAM-APC antibody (eBiosciences, 17-5791-80) for 15’ on room temperature. After washing thoroughly cells were suspended in a solution containing propidium iodide (PI; 1mg/ml; Sigma-Aldrich), MgSO$_4$ (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. Sorted cells were embedded in Basement Membrane Matrigel (BD Biosciences) and seeded in 12-well. Cells were cultured in EM (Nanduri et al., 2014) or in WRY medium (DMEM:F12 containing Pen/Strep antibiotics (1X; Invitrogen), Glutamax (1X; Invitrogen), N2 (1X; Gibco), EGF (20 ng/ml; Sigma-Aldrich), FGF2 (20 ng/ml; Sigma-Aldrich), insulin (10 μg/ml; Sigma-Aldrich), dexamethasone (1 μM; Sigma-Aldrich), Y-27632 (10 μM; Sigma-Aldrich) 10% R-spondin1 conditioned media (provided by C. Kuo) and 50% Wnt3a conditioned media).

Primary salspheres were cultured as published previously (Lombaert et al., 2008a). Shortly, cell suspensions were prepared first by mechanical disruption with gentleMACS (Milteny) followed by enzymatic digestion with collagenase type II (0.63 mg/ml; Gibco), hyaluronidase (0.5 mg/ml; Sigma-Aldrich) and CaCl$_2$ (6.25 mM; Sigma-Aldrich). After washing thoroughly cell suspensions were re-suspended in DMEM:F12 medium containing 1X Pen/Strep antibiotics (Invitrogen), Glutamax (1X; Invitrogen), EGF (20 ng/ml; Sigma-Aldrich), FGF-2 (20 ng/ml; Sigma-Aldrich), N2 (1X; Gibco), insulin (10 μg/ml; Sigma-Aldrich) and dexamethasone (1 μM; Sigma-Aldrich), at a density of 400,000 cells per well of a 12-well plate.

**Self-renewal assay**

3-day salsphere cultures were dispersed to single cell suspensions using 0.05 % trypsin-EDTA (Invitrogen), enumerated and concentration adjusted to 0.4 x 10$^6$ cells/ml. 25 μl of cell suspension
was combined on ice with 50 μl of BM Matrigel and deposited in the center of 12-well tissue culture plates. After solidifying the gels for 20 minutes at 37 °C, gels were covered in minimal medium (MM), expansion medium (EM) (Nanduri et al., 2014), WR medium, WRY medium or combinations of EM or WRY medium containing Wnt antagonists IWR-1-endo (20 μM, Cayman Chemical), IWP-2 (1 μM, Merck Millipore) or sFRP1 (20 ng/ml, Peprotech). 7-10 days after seeding, Matrigel was dissolved by incubation with Dispase (1 mg/ml; Sigma) for 1 hour at 37°C. Spheres released from the gels were processed to a single cell suspension using 0.05 % trypsin EDTA, cell number and sphere number noted, and encapsulation in Matrigel repeated. This cycle was repeated up to 25 times (25 passages). Cell numbers seeded at the start of each passage and harvested at the end were used to calculate the number of population doublings that had occurred, using the following formula, where pd = population doublings and ln = natural log.

\[ pd = \frac{\ln (\text{harvested cells} / \text{seeded cells})}{\ln 2} \]

Whole-mount immunostaining

Matrigel in 10-day salisphere culture was dissolved by incubation with Dispase for 1 hour at 37°C. Released spheres and miniglands were FA fixed for 12 hours at 4°C, washed thoroughly and labeled for the following markers: Aqp5 (1,5 μg/ml; Alomone Labs, #AQP-005), CK18 (1:100; Abcam, ab668), α-SMA (1:100; Sigma-Aldrich, A2547) and CK5 (1:100; Covance, PRB-160P). 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 counterstaining was performed with Hoechst 33258 (Sigma-Aldrich).

Electron microscopy (TEM)

Large scale electron microscopic analysis was carried out essentially as described before (Sokol et al., 2015). 10-day salisphere culture in Matrigel was fixed in 2% glutaraldehyde in 0.1 M sodium cacodylate buffer for 24 h at 4 °C. After fixation in 1% osmiumtetroxide/1.5% potassiumferrocyanide (2 hr at 4 degree), salispheres were dehydrated using ethanol and embedded in EPON epoxy resin. 60nm sections were cut and contrasted using 2% uranylacetate in methanol followed by Reynolds leadcitrate. Images were taken with a Zeiss Supra55 in STEM mode at 29 KV using an external scan generator (Fibics) yielding mosaics of large area scans at 2 nm pixel resolution. These large scale TIF images were stitched and converted to html files using VE Viewer (Fibics). These html files can be opened using the following link: www.nanotomy.org, Salivary gland organoid. Annotations were done on the original TIFF files using Photoshop.
Irradiation and regeneration assay

The irradiation and regeneration assay employed here was described earlier (Lombaert et al., 2008a). Shortly, salivary glands of female C57BL/6 mice were irradiated with a single dose of 15 Gy (Precision X-ray Inc.). 4 weeks after irradiation, mice were anesthetized and SMG was exposed by small incision. As a source of donor cells passage 1 or passage 7 Wnt-induced or not induced salisphere cultures were dissociated into single cell solution, 100, 1000 or 10.000 cells suspended in equal volumes of α-MEM (Gibco) and injected into both submandibular glands of irradiated mice intra-glandular. Saliva was collected for 15 minutes at 30, 60, 90 and 120 days post irradiation.

Image analysis

Immunofluorescence images from tissue sections and images of cultivated cells were acquired with Leica TCS Sp8 confocal microscope. Images of time-lapse experiment were acquired with an Olympus IMT-2 inverted microscope. Immunohistochemical images of tissue sections were acquired with Leica 6000 series microscope or with Tissuegnostics TissueFAXS high throughput fluorescence microscope. Colocalization of 2 proteins were quantified by ImageJ “Colocalization_Finder” plugin (Christophe Laummonerie 2006/08/29: Version 1.2). Immunofluorescence images were reconstructed and analyzed using Imaris (Bitplane) software.

Data analysis

All values are represented as mean ± standard error of the mean (SEM). A 2-way analysis of variance (ANOVA) and Bonferroni post-hoc test with alpha values of 0.05 were applied to the time course analysis of saliva flow. n numbers for tested groups are stated in figure legend. All calculations were performed using GraphPad Prism (GraphPad software) software.

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Author contributions
M.M. designed and performed experiments, analyzed data, and wrote the manuscript. C.R. performed and co-analyzed cell culture experiments. R.B. performed and analyzed immunostaining experiments. J.K. generated electron microscopy data. S.P.; B.N.G.G. commented on and edited the manuscript. R.G.J.V.; H.C. and G.d.H. provided helpful discussions and edited the manuscript. R.v.O. and R.P.C. designed experiments, supervised the project and wrote the manuscript.

Conflict of interests
H.C. is an inventor of several patents involving the organoid culture system. The remaining authors declare no competing interests.

SUPPLEMENTAL INFORMATION

Figure S1. EpCAM expression in whole salivary gland section. (A) Immunostaining showing extensive EpCAM expression throughout the ductal compartment of salivary gland. (B-D) Control immunostaining without primary antibody for EpCAM (B), β-catenin (C) and overlay (D). AF – autofluorescence. Scale bars 500 μm (A) and 20 μm (B-D).
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Figure S2. Single EpCAM\textsuperscript{high} cells generate spheres and miniglands. (A) Sphere forming efficiency of EpCAM\textsuperscript{high}, EpCAM\textsuperscript{med}, and EpCAM\textsuperscript{neg} populations compared to live sorted and non-sorted cells. Data are expressed as the mean of ± SEM of three independent experiments. (B) Single EpCAM\textsuperscript{high} cells were seeded and supplemented with enriched media (EM) (Nanduri, 2014); EM+R-spondin-1 (EMR); EM+Wnt3a (EMW) and EM+R-spondin-1+Wnt3a (EMWR). Results are shown as mean of ±SEM of 2 independent experiments. (C) Representative image of single EpCAM\textsuperscript{high} –derived in vitro culture showing a mixture of spheres (upper right) and miniglands (lower right). (D) Single EpCAM\textsuperscript{high} cell gives rise to a minigland reaching 1 mm in diameter. Scale bars 100 μm.

Figure S1. EpCAM expression in whole salivary gland section. (A) Immunostaining showing extensive EpCAM expression throughout the ductal compartment of salivary gland. (B-D) Control immunostaining without primary antibody for EpCAM, α-catenin and overlay. AF – autofluorescence. Scale bars 500 μm (A) and 20 μm (B-D).
Figure S3. Cellular composition of miniglands. (A) Confocal images (z-stack projection) for ductal marker CK18 (red) depicting duct and lumen (Lu) formation within a minigland. (B-E) Control immunostaining without primary antibodies for A488 (B), A594 (C), DAPI (D) and overlay (E). Scale bars 10 μm (A) and 30 μm (B-E).

Figure S4. Expansion of EpCAM<sup>high</sup> cells isolated from salivary gland tissue. (A-B) Representative FACS gating strategy for the analysis of EpCAM expressing cells in the salivary gland. (A) Exclusion of lineage marker-expressing cells. (B) Distribution of EpCAM<sup>high</sup>, EpCAM<sup>med</sup> and EpCAM<sup>neg</sup> cells in dissociated adult mouse salivary gland. FSC, forward scatter. (C-E) Sphere forming efficiency of (C) EpCAM<sup>high</sup>, (D) EpCAM<sup>med</sup> and (E) EpCAM<sup>neg</sup> populations. (F) Population dynamics plot of the self-renewal culture of EpCAM<sup>high</sup> cells. (G) Sphere formation kinetics of cultures initiated from EpCAM<sup>high</sup> cells during serial passaging.
Figure S5. Tumorigenicity and differentiation potential of expanded SGSCs. (A) Transplantation of 800,000 passage 13 Wnt-induced cells subcutaneously on the side of NSG mice do not show any sign of tumour formation after 1 year follow-up. (B) Embedding passage 18 Wnt-induced spheres in matrigel/collagen (Differentiation assay, materials and methods) gives rise to terminally differentiated organoids after 15 days. Scale bar 100 μm.

Figure S6. Controls for dsRed staining in transplanted salivary glands. (A) Immunohistochemical staining showing no specific expression of DsRed in the salivary gland of a C57BL/6 mouse. (B) Immunohistochemical staining showing extensive DsRed expression throughout the salivary gland of a DsRed mouse. Scale bars 50 μm.
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