Stem Cell Research (2012) 8, 379–387

Available online at www.sciencedirect.com

SciVerse ScienceDirect

www.elsevier.com/locate/scr

Ascl3 marks adult progenitor cells of the mouse salivary gland

Anastasia Rugel-Stahl [1, 2], Marilyn E. Elliott [2], Catherine E. Ovitt*

Center for Oral Biology, Department of Biomedical Genetics, University of Rochester School of Medicine and Dentistry, Rochester, NY 14642

Received 15 September 2011; received in revised form 17 January 2012; accepted 23 January 2012
Available online 31 December 2012

Abstract The Ascl3 transcription factor marks a subset of salivary gland duct cells present in the three major salivary glands of the mouse. *In vivo*, these cells generate both duct and secretory acinar cell descendants. Here, we have analyzed whether Ascl3-expressing cells retain this multipotent lineage potential in adult glands. Cells isolated from mouse salivary glands were cultured *in vitro* as non-adherent spheres. Lineage tracing of the Ascl3-expressing cells within the spheres demonstrates that Ascl3+ cells isolated from adult glands remain multipotent, generating both duct and acinar cell types *in vitro*. Furthermore, we demonstrate that the progenitor cells characterized by Keratin 5 expression are an independent population from Ascl3+ progenitor cells. We conclude that the Ascl3+ cells are intermediate lineage-restricted progenitor cells of the adult salivary glands.

Introduction

Saliva produced by the three major pairs of salivary glands is critical for many facets of oral function and health. Radiation treatment of head and neck cancers, drug therapies, and autoimmune diseases, such as Sjögren's syndrome, all inflict damage on the salivary glands, causing a significant decrease in saliva production. Although there is currently no satisfactory treatment to reverse salivary gland damage, intense interest is now focused on bio-therapeutic approaches such as cell replacement, or the development of artificial salivary glands. A high priority for both approaches is the identification of suitable donor cells with the ability to repopulate damaged or artificial glands, in order to restore salivary function.

In a search for cells responsible for salivary gland maintenance and renewal, several candidate progenitor cell

Abbreviations: Ascl3, achaete scute-like homolog 3; Krt5, Keratin 5; Krt8, Keratin 8; Sabpa, salivary androgen binding protein, alpha subunit.

* Corresponding author at: Center for Oral Biology and Department of Biomedical Genetics, University of Rochester School of Medicine and Dentistry , Box 611, 601 Elmwood Avenue, Rochester, New York 14642. Fax: +1 585 276 0190.
E-mail address: Catherine_ovitt@urmc.rochester.edu (C.E. Ovitt).
[1] Present Address: Department of Microbiology and Immunology, University of Texas Health Science Center, San Antonio, Texas 78229.
[2] These authors contributed equally.

1873-5061/$ - see front matter © 2012 Elsevier B.V. All rights reserved.
doi:10.1016/j.scr.2012.01.002

populations have been identified in the mouse (Bullard et al., 2008; Hisatomi et al., 2004; Kim et al., 2008; Knox et al., 2010; Lombaert et al., 2008). For the progenitor populations expressing the markers cKit, $\alpha 6\beta 1$ integrin, Ascl3 or Keratin 5, there is direct evidence that these cells have either multi-lineage or regenerative potential within the salivary glands (Bullard et al., 2008; Hisatomi et al., 2004; Knox et al., 2010; Lombaert et al., 2008). It is not yet clear how these cell groups are related, but the prevailing hypothesis is that multiple progenitor cell types are involved in salivary gland development, maintenance and repair (Arany et al., 2011; Lombaert et al., 2011).

There are three identified members of the mammalian achaete scute homolog (*Ascl*) gene family. All encode transcription factors expressed by tissue-specific progenitor cells, and are implicated in cell fate determination and differentiation events (Battiste et al., 2007; van der Flier et al., 2009). Progenitor cells characterized by expression of *Ascl3* are present in all three major salivary glands of the mouse (Bullard et al., 2008). During prenatal development of the glands, only a small number of epithelial duct cells express the Ascl3 transcription factor (Yoshida et al., 2001). Lineage tracing showed that these Ascl3-expressing (Ascl3+) cells are progenitors of both duct and acinar cells in the mature glands (Bullard et al., 2008). These data suggested that Ascl3+ progenitor cells are involved in normal maintenance and renewal of the salivary glands. However, the lineage tracing was based on a constitutively active Cre recombinase. Thus, it was not clear if acinar and duct cells are generated from Ascl3+ progenitors only during gland development, or also in the adult gland. In order to directly test whether the Ascl3+ cells are progenitor cells in the adult gland, we have used an *in vitro* culture system to generate salivary gland spheres (Lombaert et al., 2008).

In vitro formation and culture of non-adherent spheres from single-cell suspensions has been reported for many tissues, including the salivary glands (Lombaert et al., 2008; Dontu et al., 2003; Reynolds and Weiss, 1996; Weiss et al., 1996). The cultivation of non-adherent spheres in serum-free media permits experimental characterization of cell proliferation and differentiation, and can be used to demonstrate the presence and viability of undifferentiated stem or progenitor cells within adult tissues (reviewed in (Pastrana et al., 2011)). Salivary gland spheres have been shown to include stem cells able to restore secretion and tissue regeneration in radiation-damaged salivary glands of mice (Lombaert et al., 2008). Using the sphere assay, we have analyzed the differentiation capacity of Ascl3+ cells isolated from adult salivary glands, and have investigated the relationship of Ascl3+ progenitors to the Keratin 5 progenitor cell population.

Results

Formation and characterization of salivary gland spheres

Spheres are formed after culturing dissociated cell suspensions from adult mouse salivary glands for two to three days in serum-free media (Lombaert et al., 2008). While a majority of the cells undergo cell death, we obtained approximately 3×10^3 spheres from 1.5×10^6 dissociated cells of an adult submandibular gland. Cell-mixing experiments were conducted to ascertain that sphere expansion is not due to cell aggregation. Spheres were generated using dissociated salivary gland cells from wild type C57Bl/6 and from CAG-EGFP mice. The latter express EGFP in a ubiquitous manner, driven by the chicken beta-actin promoter (Okabe et al., 1997). Cell suspensions of submandibular glands were separately prepared from same-sex animals, to rule out sexually dimorphic differences present in rodent salivary glands (Pinkstaff, 1998). Cells were mixed and plated in a 1:1 ratio of each genotype. At 3, 5 and 8 days after plating, the spheres were scored based on their composition: all wild type cells, all GFP+cells, or mixed wild type and GFP+(data not shown). The majority of the spheres (>80%; 884/1056 counted; $P=0.0001$) were exclusively either wild type or GFP+. We conclude that salivary gland spheres form predominantly from proliferation of component cells, and only minimally from random aggregation of cell clumps.

BrdU incorporation for 24 h has been used to show that cells within the spheres are undergoing proliferation (Lombaert et al., 2008). Because BrdU is retained in labeled cells through several divisions, we repeated this experiment, shortening the labeling time to 3 hours. In spheres treated at days 3, 5 and 7 of culture, cells located on the outer periphery of the spheres were rarely labeled with BrdU after the 3-hour labeling period. However, BrdU was incorporated into proliferating cells located at the center of the spheres (Fig. 1A and B). This is consistent with earlier reports (Lombaert et al., 2008; Banh et al., 2011) and suggests that proliferating cells are located within the spheres.

We examined the cellular composition of spheres formed from dissociated submandibular gland by immunostaining with lineage-specific markers for differentiated cell types. Mist1, a transcription factor, is expressed exclusively in differentiated serous acinar cells of the salivary glands (Yoshida et al., 2001; Pin et al., 2000). Antibody to Mist1 was used to stain submandibular gland spheres after 7 days of culture, and detects Mist1-positive cells localized at the peripheral surface of the submandibular gland spheres (Fig. 1C and D). Acinar cells of the salivary glands also express the water channel, aquaporin 5 (AQP5) (Matsuzaki et al., 1999). Antibody to AQP5 also labels cells at the periphery of the spheres (Fig. 1E and F), indicating that differentiated acinar cells are localized to the outside of the spheres. Antibody to Keratin 8 (Krt8), a cytoskeletal component of duct cells in the submandibular and parotid glands (Born et al., 1987; Draeger et al., 1991), also consistently labeled cells that were on the outer periphery of the spheres (Figures G and H). These data suggest that the SG spheres display distinct cellular organization, with proliferating cells inside and differentiated cells at the periphery.

Salivary gland spheres have been shown to contain undifferentiated stem or progenitor cells (Lombaert et al., 2008). We used RT-PCR to monitor the expression of several putative stem cell markers in spheres at days 0, 2, 3, 4 and 7 of culture. Prior to day 3, spheres are not distinguishable from cell aggregates, and RNA samples most likely include non-sphere cells. Primers to LE32, a 60S ribosomal protein, served as controls. The expression level of four genes known as stem or progenitor cell markers, cKit, Sca1

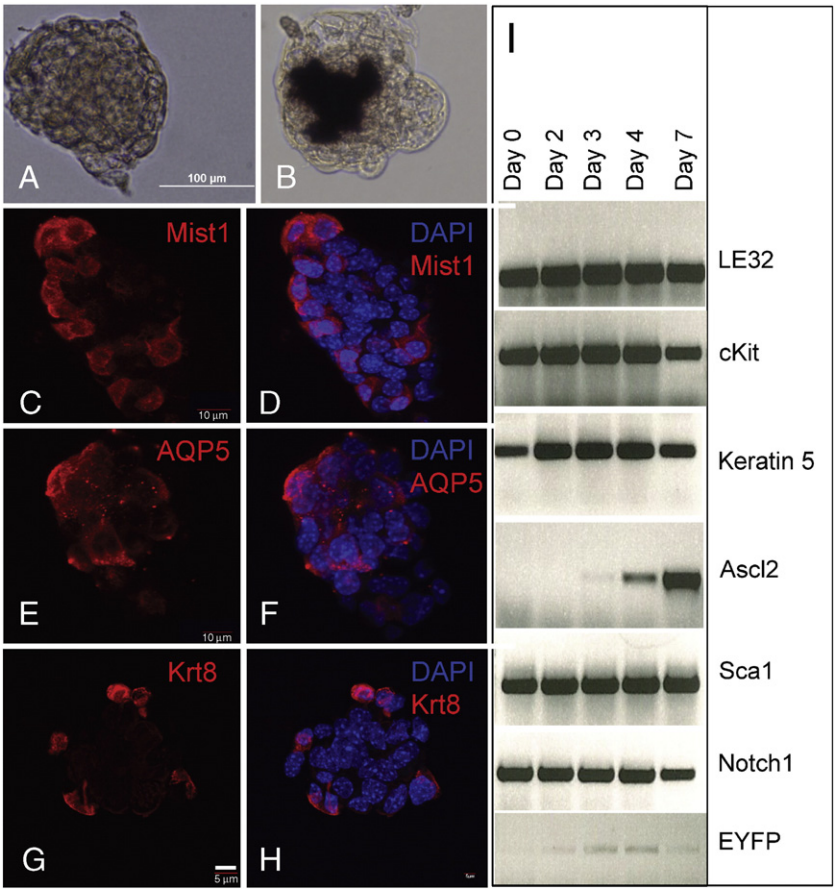

Figure 1 Salivary gland spheres include both differentiated and undifferentiated cells. (A, B) Submandibular spheres cultured for 5 days were labeled for 3 h with BrdU, fixed and stained with antibody to BrdU. (A) Control, no primary BrdU antibody. (B) Staining with antibody to BrdU reveals that proliferating cells are concentrated in the center of the sphere. Scale bar = 100 μm. (C, D) The acinar cell-specific transcription factor Mist1 antibody labels cells localized at the periphery of the spheres. (E, F) Antibody to acinar cell-specific aquaporin 5 also labels cells at the surface of the spheres. Scale bars = 10 μm. (G, H) Antibody to Keratin 8, a marker of differentiated duct cells, labels cells located only at the periphery of the spheres. Scale bar = 5 μm. (I) The expression of several stem or progenitor cell markers in spheres was analyzed by RT-PCR at days 0, 2, 3, 4 and 7 of culture. Primers to LE32 detect a 60S ribosomal protein and were used as a control for RNA quality. The expression of most stem/progenitor cell markers remains constant over time in culture. In contrast, the transcription factor Ascl2 is upregulated as the spheres mature. EYFP expression is increased over time in culture, reflecting the increase in labeled descendants of Ascl3+ progenitor cells.

(Lombaert et al., 2008), Keratin 5 (Krt5, (Knox et al., 2010)), and Notch1 (Dang et al., 2009), remained relatively constant within the spheres over time in culture (Fig. 1I). Unexpectedly, *Ascl2*, a member of the achaete scute-like gene family and a stem cell-associated factor in the intestine (van der Flier et al., 2009), is not expressed in such a constant manner. Instead, *Ascl2* is activated in spheres only after more than 3 days of *in vitro* sphere culture. *Ascl2* expression is found predominantly in the salivary gland duct cells of developing embryos and is significantly upregulated in adult glands (http://sgmap.nidcr.nih.gov/sgmap/sgexp.html; our unpublished observations). The increase in *Ascl2* expression within the spheres over time could reflect differentiation to duct cells, and suggests that Ascl2 is not a progenitor cell marker in the salivary gland. We also note an increase in the expression of enhanced yellow fluorescent protein (EYFP), a lineage reporter for descendants of Ascl3+ cells (see Fig. 3D and E).

Salivary gland spheres include Ascl3+ progenitor cells

To determine if Ascl3+ progenitor cells are included in the salivary gland spheres, the *Ascl3* $^{EGFP\text{-}Cre}$ mouse strain was used for sphere formation. In this strain, Ascl3+ cells express EGFP driven by the endogenous Ascl3 promoter (Bullard et al., 2008). At day 7 of culture, EGFP-positive cells were present in spheres derived from submandibular (Fig. 2A, arrowheads), sublingual, and parotid glands (not shown). The outer cells of the sphere are stained with antibody to the differentiated acinar cell marker salivary androgen-binding protein (Sabpa; also known as *Scgb1b*), a secretoglobin protein (Wickliffe et al., 2002). Ascl3 expression within the spheres was analyzed by RT-PCR to confirm that the EGFP expression reflects that of endogenous Ascl3, using RNA isolated from spheres at days 0, 2, 3, 4 and 7 of culture. At day 0, the RNA sample is derived from the total cell

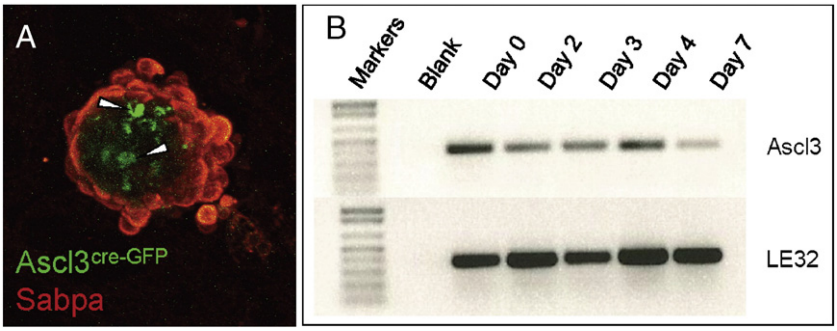

Figure 2 Ascl3-expressing progenitor cells are localized within salivary gland spheres. (A) Confocal slice image of a day 7 sphere stained with antibody to Sabpa (red). Ascl3-expressing cells are marked by EGFP expression (green) driven from the Ascl3 promoter, and are located only within the spheres (arrowheads). (B) Spheres were collected at days 0, 2, 3, 4 and 7 of culture, and RNA isolated. RT-PCR was performed using primers for Ascl3 and LE32, used as a control. Ascl3 expression is detected at all stages of sphere culture.

suspension and includes many differentiated cells. Primers were designed to detect Ascl3 or LE32, a ribosomal protein, used as an internal control. Expression of Ascl3 mRNA was clearly detectable in salivary gland spheres (Fig. 2B), as well as in secondary spheres after 7 days of culture (data not shown).

Ascl3+ cells from adult salivary glands retain progenitor capacity in vitro

By crossing the *Ascl3* $^{EGFP\text{-}Cre}$ mice with a Cre-activated reporter strain (*Rosa26*^{LacZ}), progeny derived from Ascl3+ cells can be detected and traced, even if the *Ascl3* gene expression is down regulated (Bullard et al., 2008). *Ascl3* $^{EGFP\text{-}Cre}$ /*Rosa26*^{LacZ} mice were used for the preparation of spheres from all three major salivary gland cell types. At day 4 of culture, the spheres were fixed and stained for β-galactosidase (LacZ) expression. The expression of LacZ is dependent on activation by Cre recombinase driven by the Ascl3 promoter, and indicates that the cell has either expressed Ascl3 or is descended from an Ascl3+ cell. Spheres formed from submandibular, sublingual and parotid salivary gland cell preparations all included LacZ+descendants (Fig. 3A–C).

The *Rosa26*^{YFP} reporter mouse was also crossed to *Ascl3* $^{EGFP\text{-}Cre}$ for lineage tracing. The EYFP reporter allowed us to maintain the spheres in culture and follow the generation of descendant cells over time. A small number of peripheral cells expressing EYFP were present in spheres at day 4 of culture (Fig. 3D), and this number increased over time, indicating that the Ascl3+ cells continue to generate progeny (Fig. 3E). Furthermore, the EYFP-positive progeny are

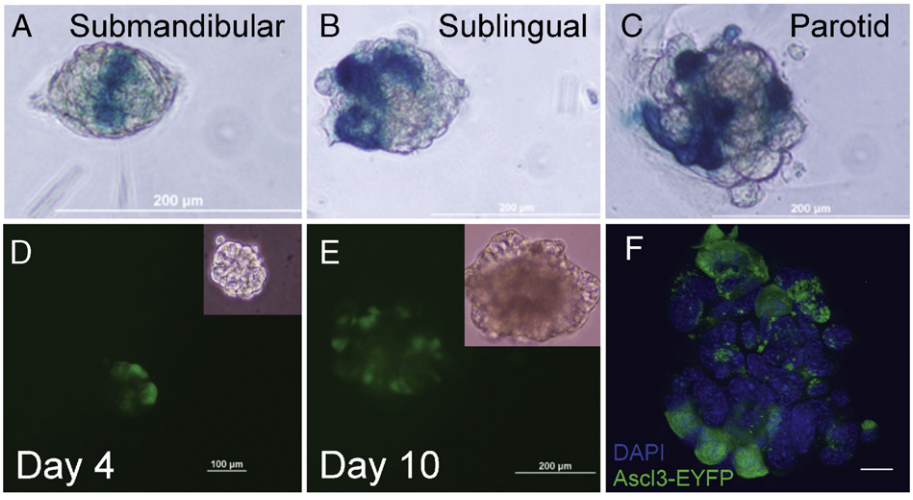

Figure 3 Ascl3-expressing cells from adult glands retain progenitor capacity *in vitro* within the spheres. (A-C) Day 4 spheres generated from single-cell suspensions of submandibular, sublingual or parotid glands isolated from Ascl3$^{EGFP\text{-}Cre}$/R26R^{LacZ} mice, and stained for beta-galactosidase (LacZ) activity. LacZ+labeled progeny (blue), derived from Ascl3-expressing progenitor cells, are found in spheres derived from (A) submandibular, (B) sublingual, and (C) parotid glands. Scale bars=200 μm. (D, E) Lineage tracing in spheres derived from the Ascl3$^{EGFP\text{-}Cre}$/R26R^{EYFP} reporter mouse strain, in which all descendants of Ascl3 progenitors are labeled by EYFP expression (green). An increased number of EYFP-labeled descendants is evident with increased time in culture, from day 4 (D) to day 10 (E). Insets show bright field image of each sphere. Scale bars are labeled. (F) Day 7 sphere from Ascl3$^{EGFP\text{-}Cre}$/R26R^{EYFP} reporter mouse, stained with DAPI to label nuclei. EYFP+descendants are located at the periphery of the spheres. Scale bar=20 μm.

generally located at the periphery of the cultured spheres (Fig. 3F). RT-PCR analysis confirmed an increase in EYFP expression (Fig. 1I). These results demonstrate that adult Ascl3+ progenitor cells retain the capacity to generate progeny *in vitro*.

In order to identify the cell types generated from adult Ascl3+ progenitors, spheres from *Ascl3* $^{EGFP\text{-}Cre}$; *Rosa26*^{EYFP} mice were analyzed with antibodies to cell type-specific markers. Antibody to Sabpa detects seromucous acinar cells (Wickliffe et al., 2002). Labeling of a day 4 sphere reveals significant co-localization of EYFP expression with Sabpa, indicating that adult Ascl3+ progenitor cells can generate seromucous acinar cells (Fig. 4A–C). In contrast, no co-localization was found in numerous spheres labeled with antibody to Mist1, a transcription factor expressed in serous acinar cells (Pin et al., 2000) (Fig. 4D–F). The absence of overlap between Ascl3+ descendants and Mist1-expressing cells suggests that Ascl3+ progenitors do not generate serous acinar cells.

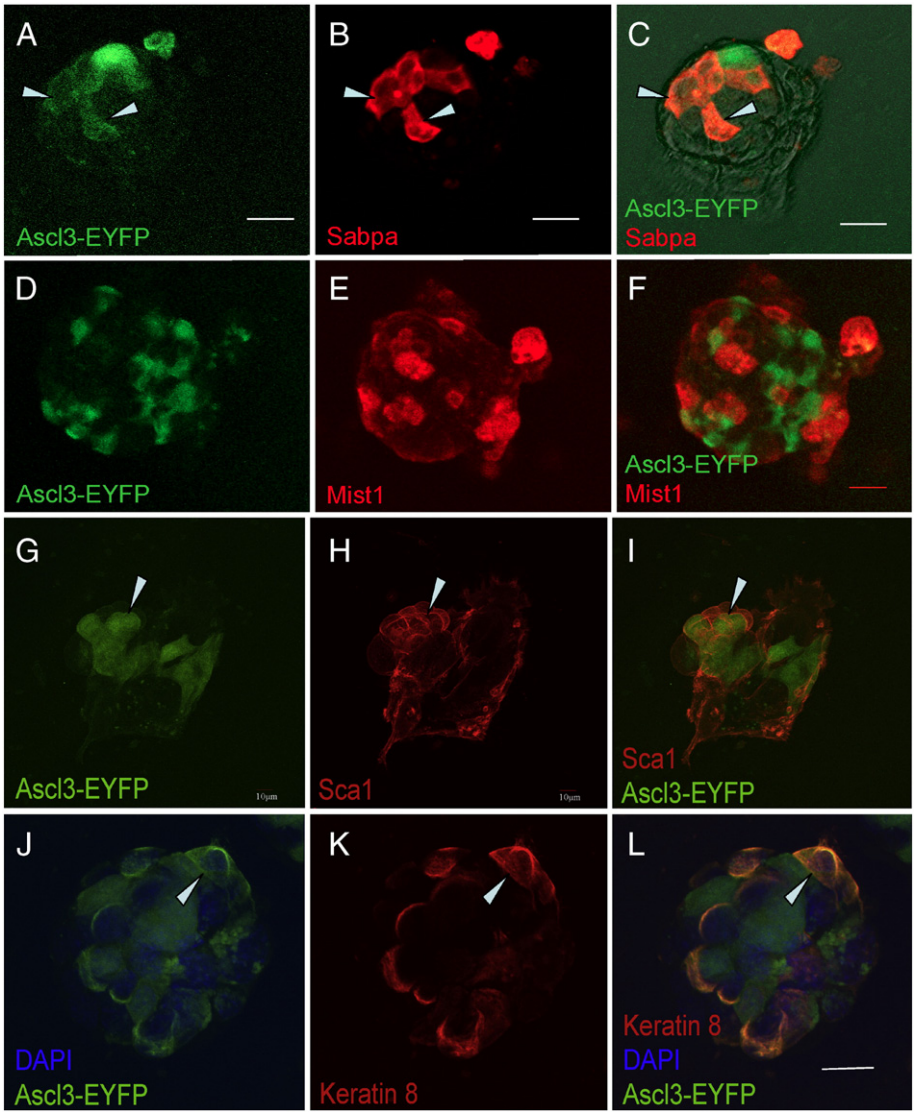

Figure 4 Ascl3+ progenitors isolated from adult glands can generate more than one cell type in cultured spheres. (A) EYFP-labeled descendants of Ascl3+ progenitor cells (green; arrowheads). (B) Antibody to Sabpa labels distinct cells in the spheres (red; arrowheads). (C) Merged image of (A) and (B). EYFP-labeled descendants of Ascl3+ progenitors co-localize with cells labeled by Sabpa antibody (arrowheads). (D) EYFP-labeled descendants of Ascl3+ progenitor cells (green). Scale bars = 50 μm. (E) Antibody to serous acinar cell-specific transcription factor, Mist1, labels cells in the spheres (red). (F) Merged image of (D) and (E) shows that Ascl3+ descendants expressing EYFP do not co-localize with cells labeled by Mist1 antibody. Scale bar = 20 μm. (G) EYFP-labeled descendants of Ascl3+ progenitor cells (green; arrowhead). (H) Antibody to Sca1 labels the membranes of cells located at the periphery of spheres (red; arrowhead). (I) Merged image of (G) and (H). EYFP + descendants of Ascl3+ progenitors co-localize with cells labeled by antibody to Sca1 at the surface of cultured spheres (arrowhead). Scale bars = 10 μm. (J) EYFP-labeled descendants of Ascl3+ progenitor cells (green; arrowhead). The sphere was stained with DAPI to label cell nuclei. (K) Antibody to Keratin 8, a duct cell-specific marker, labels cells located exclusively at the periphery of the spheres (red; arrowhead). (L) Merged image of (J) and (K). EYFP + descendants of Ascl3+ progenitor cells express the ductal cell marker Keratin 8 (arrowhead). Scale bar = 20 μm.

This result is consistent with earlier findings that few Ascl3+ progenitor cells can be lineage traced in the parotid gland, which is predominantly comprised of serous acinar cells (Bullard et al., 2008).

Additional co-localization experiments were performed to test whether other cell types are also derived from Ascl3+ progenitors. Although Sca1 has been suggested to delineate salivary gland progenitor cells (Hisatomi et al., 2004), it is broadly expressed in salivary gland duct cells (Lombaert et al., 2008). Sca1 antibody labels cells that are located at the periphery of spheres (Fig. 4H), and many of these are also EYFP-positive (Fig. 4G–I), leading us to conclude that the Sca1+/EYFP+cells are differentiated duct cells derived from Ascl3+ progenitors. Antibody to Keratin 8 also labels cells located exclusively at the periphery of the spheres (Fig. 4K). The Keratin 8+ duct cells are co-localized with EYFP-labeled Ascl3+ descendants (Fig. 4J–L). Taken together, these data demonstrate that adult Ascl3+ cells retain multilineage potential, and can act as adult progenitor cells, to generate both duct and acinar cell types.

Ascl3+ progenitor cells are not precursors of Krt5+ basal cell progenitors

The relationship between the populations of putative progenitor cells so far identified in the salivary glands (Bullard et al., 2008; Hisatomi et al., 2004; Kim et al., 2008; Knox et al., 2010; Lombaert et al., 2008; David et al., 2008; Kishi et al., 2006) is not yet clear. Ascl3+ progenitor cells are located in the ducts *in vivo*, as are the basal cell Krt5+ progenitors (Knox et al., 2010). We analyzed spheres by lineage tracing to determine whether the Krt5+ cells are derived from the Ascl3+ progenitor lineage. In spheres, YFP-labeled descendants of Ascl3+ progenitors do not co-localize with Krt5+ cells (Fig. 5A–C). In fact, the staining patterns demonstrate that Krt5+ and YFP+cells are clearly separate populations. Thus, Ascl3+ cells are not precursors of the Krt5+ progenitors. Lineage tracing of the Krt5+ cells will be required to determine whether they might be upstream precursors of Ascl3+ progenitor cells.

Discussion

Non-adherent spheres formed *in vitro* from dissociated salivary gland tissue have been shown to include progenitor and/or stem cells that contribute to the restoration of secretory function in damaged glands (Lombaert et al., 2008). Here we demonstrate that cells expressing the transcription factor Ascl3 are adult progenitor cells present in all three major salivary glands.

In the adult salivary gland, Ascl3+ cells are found exclusively in the ducts, from the smallest intercalated, to the striated, granular, and large excretory ducts, but represent less than 5% of total duct cells (Bullard et al., 2008). In developing glands, Ascl3+ cells are mitotically active (Arany et al., 2011), and lineage tracing has established that they are precursors of both duct and acinar cells (Bullard et al., 2008). To establish that Ascl3+ cells continue to act as progenitors in the adult gland, we have cultured spheres from adult gland tissue and demonstrated that they generate differentiated progeny over time in culture. Furthermore, the Ascl3+ cells isolated from adult glands retain their multilineage potential, generating both duct and acinar cell types *in vitro* within the spheres. Notably, no sphere was found in which all cells were generated from Ascl3+ progenitors. Furthermore, the co-localization experiments indicate that Ascl3+ cells are not capable of generating all cell types in the salivary gland, and there was no evidence of self-renewal within the spheres. Thus, the Ascl3+ cells are clearly not stem cells, but represent an intermediate lineage-restricted progenitor population of the adult glands.

In this report, co-localization studies using lineage tracing and immunohistochemistry revealed that Ascl3+ progenitors in the spheres are precursors of duct cells expressing the differentiation marker Keratin 8. In addition, some Ascl3+ descendants express the stem/progenitor cell marker Sca1. Although Sca1 was identified as a progenitor cell marker in the salivary gland, it is widely expressed in differentiated epithelia of several tissues (van de Rijn et al., 1989). We conclude that the Sca1+/EYFP+cells are likely differentiated duct cells derived from Ascl3+ progenitors. Co-localization was also found between Ascl3+ descendants and secretory cells expressing salivary androgen binding protein (Sabpa), a member of the secretoglobin protein family. Sabpa, for which at least six paralogues have been detected in the mouse genome (Laukaitis et al., 2005), is expressed by a subpopulation of acinar cells in the submandibular gland, which are presumed to be seromucous cells (Wickliffe et al., 2002). In contrast, we saw no co-localization of the Mist1 transcription factor with EYFP+progeny of Ascl3+ cells. In the salivary glands, Mist1 expression is found in serous-secreting acinar cells, but not in serous demilune or

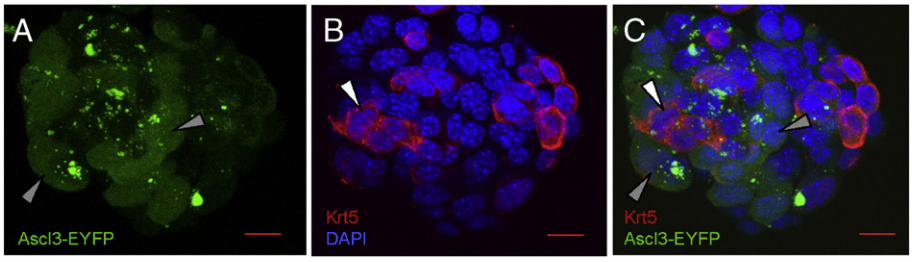

Figure 5 Ascl3+ progenitors are not precursors of ductal Krt5+ progenitor cells. (A) EYFP-labeled descendants of Ascl3+ progenitor cells (green; gray arrowheads). (B) Sphere stained with antibody to keratin 5 (red; white arrowhead) and DAPI to label nuclei (blue). (C) Merged image of (A) and (B). Cells positive for Keratin 5 antibody (red; white arrowhead) show no overlap with EYFP+descendants of Ascl3+ progenitor cells (gray arrowheads). Bright green punctate stain is non-specific background. Scale bars=20 μm.

mucous-secreting cells (Pin et al., 2000). We have previously demonstrated that Ascl3+ cells are progenitors of serous demilune cells in the sublingual glands (Bullard et al., 2008). Thus, it is clear that secretory acinar cells are a heterogeneous population, and may arise from separate progenitors. We propose that Ascl3+ progenitors generate ductal, mucous-secreting, and possibly, seromucous type cells, but that they are not precursors of serous acinar cells. Expression of a closely related transcription factor, *Ascl1*, was recently shown to define one of several competence-restricted progenitor populations in the retina (Brzezinski et al., 2011). In an analogous manner, Ascl3 expression may define a restricted set of progenitor cells in the salivary gland.

A number of progenitor cell populations have been identified in the salivary gland (Bullard et al., 2008; Hisatomi et al., 2004; Knox et al., 2010; Lombaert et al., 2008; David et al., 2008; Kishi et al., 2006). In addition to Ascl3, c-Kit and Keratin 5 also mark progenitor cells (Knox et al., 2010; Lombaert et al., 2008). To begin to establish the relationship between these cell populations, we analyzed Ascl3+ descendants in the spheres for Krt5 expression. We found no co-localization of EYFP-labeled progeny with cells expressing Krt5. Thus, Krt5+ cells are not lineally derived from Ascl3+ progenitors. We recently generated a targeted cell ablation model in the salivary glands in order to establish the role of the Ascl3+ progenitor cells. In the absence of Ascl3+ cells, the salivary glands appeared to develop normally, and were able to efficiently regenerate after ductal ligation (Arany et al., 2011). Consistent with the lineage tracing results reported here, the Krt5+ basal cells are still present in the Ascl3-cell ablation model (Arany et al., 2011). These results clearly indicate that adult salivary glands harbor more than a single population with progenitor cell capacity. Observations in other tissues suggest that rather than being redundant, these cell populations may contribute differently to maintenance and repair (Li and Clevers, 2010; Rawlins and Hogan, 2006; Rock and Hogan, 2011; Watt and Jensen, 2009).

We observed a distinct spatial organization of cell types within the spheres. Distinct cellular organization has also been reported in spheres derived from mammary gland (Dontu et al., 2003) and tracheal basal cells (Rock et al., 2009). In the salivary gland spheres, cells expressing differentiation-specific markers, including the acinar cell markers Mist1 and Sabpa, as well as the duct cell-specific marker Krt8, were located on the outer periphery. In contrast, proliferating cells incorporating BrdU were localized in the center of the spheres. Furthermore Ascl3+ cells were found within the spheres, while differentiated descendants marked by EYFP were predominantly found at the periphery. Absence of Ascl3 expression in peripheral cells is consistent with our earlier report that Ascl3 expression is down regulated in differentiated progeny (Bullard et al., 2008). However, in contrast to the internal localization of Ascl3+ progenitor cells, immunostaining demonstrated that Krt5+ progenitor cells are localized at the outside of the spheres. This was also observed of Sca1+ cells, another proposed marker of progenitor cells (Lombaert et al., 2008). Both Krt5+ and Sca1+ are expressed by ductal basal cells *in vivo*. Thus their external localization on the spheres may reflect a similarity in interaction of their cell membranes with neighboring cells. The biological relevance of cellular organization within the spheres is not yet clear, and further characterization of each progenitor type is required to resolve their individual roles.

The use of stem or progenitor cells for therapeutic treatment requires precise knowledge of their differentiation potential. Using lineage tracing to definitively establish the progenitor-progeny relationship, we have shown that Ascl3 + cells are multipotent lineage-restricted progenitors of the adult salivary glands. The characterization of such progenitor cells is a critical step in the process of developing cell replacement therapies.

Methods

Mice

Generation of the Ascl3 $^{EGFP\text{-}Cre}$ mouse strain has been previously described (Bullard et al., 2008). Expression of Ascl3 is detected through EGFP or Cre recombinase expression driven by the Ascl3 promoter. Lineage tracing was done using progeny from crosses of Ascl3 $^{EGFP\text{-}Cre/+}$ with the *ROSA26*^{LacZ} (Gt(ROSA)26Sortm1Sor; (Soriano, 1999)) or *ROSA26*^{EYFP} reporter mouse strains. The *CAG-GFP* (C57BL/6-Tg(CAG-EGFP) stock # 006567) strain was purchased from Jackson Laboratory. This transgenic line expresses EGFP, driven by the chicken beta-actin promoter, in nearly all tissues of the adult mouse, including the salivary glands (Okabe et al., 1997). Wild type C57BL/6 J mice were purchased from Jackson Laboratory. The University Committee on Animal Resources at the University of Rochester approved all procedures and protocols involving animals.

Generation and culture of salivary gland spheres

Single-cell suspensions were prepared from salivary glands isolated from either male or female mice, 2 weeks to 9 months of age as described (Lombaert et al., 2008). All three pairs of major salivary glands, submandibular, sublingual, and parotid, were dissociated individually, in HBSS containing CaCl2 and MgCl2, with 10 mg Collagenase type II, Hyaluronidase (100 mg/mL, Applichem) warmed to 37 °C. Cell suspensions were filtered thru 0.40 micron mesh, and plated in serum-free media [1:1 DMEM (Cellgro Mediatech), and Ham's F-12 media (Cellgro Mediatech), with PennStrep (1x, Gibco), Glutamax (1x, Gibco), EGF (20 ng/mL, Sigma-Aldrich), bFGF-2 (20 ng/mL, Invitrogen), N2 supplement (1x, Gibco), Insulin (10 μg/mL), Dexamethasone (1 μM, Sigma-Aldrich)] on non-coated plastic dishes at a concentration of 3×10^5 cells/ml. Cells were incubated at 37 °C in 5% CO_2. Media was changed every 48 hours by gently pelleting the spheres, and resuspending in fresh media. Spheres became evident between 2 and 3 days.

Secondary spheres were generated after 6 or 7 days in culture by rinsing gently pelleted spheres in 1xPBS for 10 min, followed by pelleting and resuspension in trypsin for 2 min. Spheres were then mechanically disrupted by rapid trituration, rinsed, and single cells were replated in sphere medium as above.

Cell mixing experiments

Single-cell suspensions of submandibular gland cells were prepared independently from wild type C57Bl/6 and from CAG-EGFP mice, as described above. All preparations were made from same sex animals, because of distinct sexually dimorphic differences in rodent salivary glands. Equal numbers of cells of each genotype were mixed in a 1:1 ratio, and the mixtures were incubated in serum-free media for 5 days. Spheres were then scored as non-fluorescent ($EGFP^-$), completely fluorescent ($EGFP^+$), or mixed, by direct visualization using a Nikon Eclipse TS100 microscope with Nikon intensilight C-HGFI illuminator. Three separate experiments were performed. Total number of spheres scored was 1315.

BrdU labeling

BrdU labeling of dividing cells was performed at days 3, 5 or 7 after culturing in serum-free media. 10 μM BrdU was added directly to the media for 3 hours, and incubation continued at 37 °C. Spheres were fixed in Carnoy's fixative, and immunostained with antibody to BrdU according to the manufacturer's instructions (Roche).

Staining for ß-galactosidase activity

Spheres were fixed in 4% PFA for 20 minutes at room temperature and then rinsed in detergent buffer [2 mM $MgCl_2$, 0.01% sodium deoxycholate, 0.02% IGEPAL (Sigma-Aldrich), in 0.1 M PO_4 buffer]. Staining for B-galactosidase activity was performed as previously described (Bullard et al., 2008). Spheres were photographed using an Olympus DX41 microscope and DSL camera, with DSL software.

Immunohistochemistry

Non-adherent spheres were gently pelleted, resuspended in 800 μl serum-free media (described above), plated onto 4-well Permanox™ plastic chamber slides (LabTek), and allowed to attach. Subsequent steps were performed on attached spheres in chamber slides. Spheres were fixed in 4% PFA for 20 minutes, washed in 1xPBS, followed by 1 mM glycine. For the Mist1 antibody stain only, permeabilization was performed with 0.5% TritonX-100 in PBS, followed by 1xPBS wash. Spheres were then incubated in 10% normal donkey serum in 1xPBS. Antibodies used for immunocytochemistry included: anti-rabbit Mist1 diluted 1:2000 (gift from Dr. Stephen Konieczny, Purdue University); anti-rabbit salivary antigen binding protein alpha subunit (Sabpa) diluted 1:100 (gift from Dr. Art Hand, University of Connecticut); anti-chicken Keratin 8 (Krt8) diluted 1:500 (Sigma-Aldrich); anti-rabbit Sca1 diluted 1:400 (R&D Systems); anti-rabbit Keratin 5 (Krt5) diluted 1:500 (Covance). Antibodies were incubated overnight. Secondary antibodies used were Cy3-labeled anti-rabbit, or anti-chicken diluted 1:500 (Jackson Immunoresearch). Following the last wash, 10 μg/mL DAPI (Invitrogen) in PBS was applied to tissue for 5 minutes at room temperature. The tissue was rinsed 3×5 min in PBS. The plastic chamber scaffold was removed, and the slides were mounted using Immu-mount (Thermo Scientific).

Imaging

Imaging was performed using an FV1000 Olympus laser scanning confocal microscope with sequential scanning to eliminate bleed-through. The intensity for each fluorophore was optimized using gray scale analysis to insure each fluorophore was imaged in the linear range. Post-processing and 3D analyses were performed using FV1000 post-processing capabilities. Photographs in Fig. 5 were made using the Bright Z feature of the FV1000 software.

RT-PCR expression analysis

Total RNA was isolated from aliquots of spheres taken at days 0, 2, 3, 4 and 7 after plating. RNA was isolated according to manufacturer's instructions, using the RNA-easy Qiagen mini-kit. DNAse treatment was included in the protocol. cDNA was synthesized from 1 μg of RNA using the iScript cDNA Synthesis Kit (BioRad) according to the manufacturer's instructions. Primers (Integrated DNA Technologies) used for RT-PCR were the following: Ascl3F: GGTGAAAGGAAACGATGGACACC; Ascl3R: TTCTCCAGGTAGTCCTCCGGA; LE32F: TTCATCAGGCAC-CAGTCAGACC; LE32R: ACACAAGCCATCTACTCATTTTCTTCG; ckitF: CGGGCCTACAATGGACAGCC; ckitR: CCCAAGCCATG-CAAACGGTGC; Krt5F: GCCTGGACCTGGACAGCATCATC; Krt5R: CTGGTACTCCCGCAGCAGCCTGG; Ascl2F: GCCCGTGAAGGTG-CAAACGTCCA; Ascl2R: CTCATCGGACGGCGGAGTAGCG; EYFP F: GCGAGGGCGAGGGCGATGCCAC; EYFP R: CAGCTCGTCCATGCC-GAGAGTGA; Sca1F: CTCTGAGGATGGACACTTCTCAC; Sca1R: GGTCTGCAGGAGGACTGAGCTCA; Notch1F: GCAAGAGGCTTGA-GATGCTCCC; Notch1R: AGCGATAGGAGCCGATCTCATTG. EYFPF: AAAGTCGCTCTGAGTTGTTAT; EYFPR: AAGACCGCGAA-GAGTTTGTC. RT-PCR conditions were: 94° 3 minutes, followed by 35 cycles of 94°, 30 s; 60°, 30 s; 72°, 30 s. RT-PCR products were separated on 1% agarose gels and photographed using an Alpha Imager (TM).

Acknowledgments

We are grateful to Linda Callahan for technical advice and skilled assistance with the confocal microscopy. We thank Michael Rogers, Laurie Koek and Yasna Jamarillo for excellent mouse husbandry and genotyping. Sabpa antibody was generously donated from Drs. Art Hand (Univ of Connecticut) and Lily Mirels (UC Berkeley). The antibody to Mist1 was a generous gift from Dr. Stephen Konieczny (Purdue University). We thank Dirk Bohmann for critical reading of the manuscript. This work was supported by grants R01 DE008921 and R01 DE018896 from NIDCR (CEO).

References

Arany, S., Catalan, M.A., Roztocil, E., Ovitt, C.E., 2011. Ascl3 knockout and cell ablation models reveal complexity of salivary gland maintenance and regeneration. Dev. Biol. 353, 186.

Banh, A., et al., 2011. A novel aldehyde dehydrogenase-3 activator leads to adult salivary stem cell enrichment in vivo. Clin. Cancer Res. 17, 7265.

Battiste, J., Helms, A.W., Kim, E.J., Savage, T.K., Lagace, D.C., Mandyam, C.D., Eisch, A.J., Miyoshi, G., Johnson, J.E., 2007. Ascl1 defines sequentially generated lineage-restricted neuronal

and oligodendrocyte precursor cells in the spinal cord. Development 134, 285.

Born, I.A., Schwechheimer, K., Maier, H., Otto, H.F., 1987. Cytokeratin expression in normal salivary glands and in cystadenolymphomas demonstrated by monoclonal antibodies against selective cytokeratin polypeptides. Virchows Arch. A Pathol. Anat. Histopathol. 411, 583.

Brzezinski, J.A.T., Kim, E.J., Johnson, J.E., Reh, T.A., 2011. Ascl1 expression defines a subpopulation of lineage-restricted progenitors in the mammalian retina. Development 138, 3519.

Bullard, T., Koek, L., Roztocil, E., Kingsley, P.D., Mirels, L., Ovitt, C.E., 2008. Ascl3 expression marks a progenitor population of both acinar and ductal cells in mouse salivary glands. Dev. Biol. 320, 72.

Dang, H., Lin, A.L., Zhang, B., Zhang, H.M., Katz, M.S., Yeh, C.K., 2009. Role for Notch signaling in salivary acinar cell growth and differentiation. Dev. Dyn. 238, 724.

David, R., Shai, E., Aframian, D.J., Palmon, A., 2008. Isolation and cultivation of integrin alpha(6)beta(1)-expressing salivary gland graft cells: a model for use with an artificial salivary gland. Tissue Eng. Part A 14, 331.

Dontu, G., Abdallah, W.M., Foley, J.M., Jackson, K.W., Clarke, M.F., Kawamura, M.J., Wicha, M.S., 2003. In vitro propagation and transcriptional profiling of human mammary stem/progenitor cells. Genes Dev. 17, 1253.

Draeger, A., Nathrath, W.B., Lane, E.B., Sundstrom, B.E., Stigbrand, T.I., 1991. Cytokeratins, smooth muscle actin and vimentin in human normal salivary gland and pleomorphic adenomas. Immunohistochemical studies with particular reference to myoepithelial and basal cells. APMIS 99, 405.

Hisatomi, Y., Okumura, K., Nakamura, K., Matsumoto, S., Satoh, A., Nagano, K., Yamamoto, T., Endo, F., 2004. Flow cytometric isolation of endodermal progenitors from mouse salivary gland differentiate into hepatic and pancreatic lineages. Hepatology 39, 667.

Kim, Y.J., Kwon, H.J., Shinozaki, N., Hashimoto, S., Shimono, M., Cho, S.W., Jung, H.S., 2008. Comparative analysis of ABCG2-expressing and label-retaining cells in mouse submandibular gland. Cell Tissue Res. 334, 47.

Kishi, T., Takao, T., Fujita, K., Taniguchi, H., 2006. Clonal proliferation of multipotent stem/progenitor cells in the neonatal and adult salivary glands. Biochem. Biophys. Res. Commun. 340, 544.

Knox, S.M., Lombaert, I.M., Reed, X., Vitale-Cross, L., Gutkind, J.S., Hoffman, M.P., 2010. Parasympathetic innervation maintains epithelial progenitor cells during salivary organogenesis. Science 329, 1645.

Laukaitis, C.M., Dlouhy, S.R., Emes, R.D., Ponting, C.P., Karn, R.C., 2005. Diverse spatial, temporal, and sexual expression of recently duplicated androgen-binding protein genes in Mus musculus. BMC Evol. Biol. 5, 40.

Li, L., Clevers, H., 2010. Coexistence of quiescent and active adult stem cells in mammals. Science 327, 542.

Lombaert, I.M., et al., 2008. Rescue of salivary gland function after stem cell transplantation in irradiated glands. PLoS One 3, e2063.

Lombaert, I., Knox, S., Hoffman, M., 2011. Salivary gland progenitor cell biology provides a rationale for therapeutic salivary gland regeneration. Oral Dis. 17, 445.

Matsuzaki, T., Suzuki, T., Koyama, H., Tanaka, S., Takata, K., 1999. Aquaporin-5 (AQP5), a water channel protein, in the rat salivary and lacrimal glands: immunolocalization and effect of secretory stimulation. Cell Tissue Res. 295, 513.

Okabe, M., Ikawa, M., Kominami, K., Nakanishi, T., Nishimune, Y., 1997. 'Green mice' as a source of ubiquitous green cells. FEBS Lett. 407, 313.

Pastrana, E., Silva-Vargas, V., Doetsch, F., 2011. Eyes wide open: a critical review of sphere-formation as an assay for stem cells. Cell Stem Cell 8, 486.

Pin, C.L., Bonvissuto, A.C., Konieczny, S.F., 2000. Mist1 expression is a common link among serous exocrine cells exhibiting regulated exocytosis. Anat. Rec. 259, 157.

Pinkstaff, C.A., 1998. Salivary gland sexual dimorphism: a brief review. Eur. J. Morphol. 36 (Suppl), 31.

Rawlins, E.L., Hogan, B.L., 2006. Epithelial stem cells of the lung: privileged few or opportunities for many? Development 133, 2455.

Reynolds, B.A., Weiss, S., 1996. Clonal and population analyses demonstrate that an EGF-responsive mammalian embryonic CNS precursor is a stem cell. Dev. Biol. 175, 1.

Rock, J.R., Hogan, B.L., 2011. Epithelial Progenitor Cells in Lung Development, Maintenance, Repair, and Disease. Annu. Rev. Cell Dev. Biol. 27, 493.

Rock, J.R., Onaitis, M.W., Rawlins, E.L., Lu, Y., Clark, C.P., Xue, Y., Randell, S.H., Hogan, B.L., 2009. Basal cells as stem cells of the mouse trachea and human airway epithelium. Proc. Natl. Acad. Sci. U. S. A. 106, 12771.

Soriano, P., 1999. Generalized lacZ expression with the ROSA26 Cre reporter strain. Nat. Genet. 21, 70.

van de Rijn, M., Heimfeld, S., Spangrude, G.J., Weissman, I.L., 1989. Mouse hematopoietic stem-cell antigen Sca-1 is a member of the Ly-6 antigen family. Proc. Natl. Acad. Sci. U. S. A. 86, 4634.

van der Flier, L.G., et al., 2009. Transcription factor achaete scute-like 2 controls intestinal stem cell fate. Cell 136, 903.

Watt, F.M., Jensen, K.B., 2009. Epidermal stem cell diversity and quiescence. EMBO Mol. Med. 1, 260.

Weiss, S., Dunne, C., Hewson, J., Wohl, C., Wheatley, M., Peterson, A.C., Reynolds, B.A., 1996. Multipotent CNS stem cells are present in the adult mammalian spinal cord and ventricular neuroaxis. J. Neurosci. 16, 7599.

Wickliffe, J.K., Lee, V.H., Smith, E., Tandler, B., Phillips, C.J., 2002. Gene expression, cell localization, and evolution of rodent submandibular gland androgen-binding protein. Eur. J. Morphol. 40, 257.

Yoshida, S., Ohbo, K., Takakura, A., Takebayashi, H., Okada, T., Abe, K., Nabeshima, Y., 2001. Sgn1, a basic helix-loop-helix transcription factor delineates the salivary gland duct cell lineage in mice. Dev. Biol. 240, 517.