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INVITED MEDICAL REVIEW

Salivary gland progenitor cell biology provides a rationale for therapeutic salivary gland regeneration

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An irreversible loss of salivary gland function often occurs in humans after removal of salivary tumors, after therapeutic radiation of head and neck tumors, as a result of Sjögren's syndrome and in genetic syndromes affecting gland development. The permanent loss of gland function impairs the oral health of these patients and broadly affects their quality of life. The regeneration of functional salivary gland tissue is thus an important therapeutic goal for the field of regenerative medicine and will likely involve stem/progenitor cell biology and/or tissue engineering approaches. Recent reports demonstrate how both innervation of the salivary gland epithelium and certain growth factors influence progenitor cell growth during mouse salivary gland development. These advances in our understanding suggest that developmental mechanisms of mouse salivary gland development may provide a paradigm for postnatal regeneration of both mice and human salivary glands. Herein, we will discuss the developmental mechanisms that influence progenitor cell biology and the implications for salivary gland regeneration.

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Salivary glands are composed of multiple cell types including epithelial, myoepithelial, mesenchymal, neuronal and endothelial cells. Complex interactions among these cell types are essential for normal physiologic function and maintenance of the glands. The salivary glands form during embryogenesis when the oral epithelium interacts with the mesenchyme, epithelial stem/progenitor cells are specified and a salivary gland placode forms. The stem/progenitor cells of the salivary epithelium then undergo a variety of processes such as maintenance, proliferation, lineage commitment and differentiation to form a variety of specialized salivary cell types. There are a number of reviews on salivary development in the mouse and the reader is referred to these for more information (Patel *et al*, 2006; Tucker, 2007). Stem cells are in general considered to be more 'primitive' and are the precursors of progenitor cells, which are more lineage-committed, have less capacity to self-renew and may be organ-specific. Research on the use of stem cells for regenerative medicine has focused on identifying organ-specific stem/progenitor cells. For example, it has been demonstrated that a single tissue-specific stem cell has the capacity to form the entire epithelial compartment of a mammary gland (Stingl *et al*, 2006) or gastric units (Barker *et al*, 2010). However, recent reports challenge the view that organ stem cells are a uniform pool and demonstrate that lineage-biased subtypes already exist within the stem cell population (Challen *et al*, 2010). In the salivary research field, a single stem cell has not been identified that gives rise to all epithelial cell types within the gland. It is also not known whether a number of different lineage-biased stem cell populations or subtypes exist and how these differ from progenitor cells. As such, in this review we will refer to the primitive cell populations that form the salivary epithelium as 'progenitor cells'. Based on our current work, we propose that complex interactions occur between parenchymal cell types and the epithelial progenitor cells, which influence the growth and development of the gland.

Characterizing the epithelial progenitor cell pool is critical for future therapies

There have been few studies that use genetic lineage tracing experiments in mice to identify progenitor cells in developing salivary glands. In one such study, an Ascl3$^+$ progenitor population was identified in the ductal compartment of the submandibular gland (SMG) and it gave rise to both ductal and acinar cells (Bullard *et al*, 2008). Importantly, not all SMG cells were derived from the Ascl3 cells, but only a subpopulation of acinar and ductal cells. The Ascl3$^+$ cell was therefore considered to be a progenitor cell.

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Table 1 Proteins and genes used to study progenitor cells showing their cellular location and some of their functions

Protein	*Gene*	*Localization*	*Function*
Keratin 5 or K5	*Krt5*	Cytoplasm	Intermediate filament protein, basal progenitor cell marker
Keratin 14 or K14	*Krt14*	Cytoplasm	Intermediate filament protein, basal progenitor cell marker
Keratin 19 or K19	*Krt19*	Cytoplasm	Intermediate filament protein, duct cell marker
c-Kit	*Kit*	Cell surface	Involved in progenitor cell maintenance in other organs
CD133, prominin1	*Prom1*	Cell surface	Progenitor cell marker in other systems
CD24	*CD24*	Cell surface	Progenitor cell marker in other systems
CD49f, α6 integrin	*Itga6*	Cell surface	Cell adhesion to extracellular laminin, in hemidesmosomes
Sca1	*Ly6a*	Cell surface	Lymphocyte antigen, progenitor cell marker
ΔNp^{63}	*Trp63*	Nucleus	Transcription factor, progenitor cell marker
Oct3/4	*Pou5f1*	Nucleus	Transcription factor regulating embryonic stem cell self-renewal
Nanog	*Nanog*	Nucleus	Transcription factor regulating embryonic stem cell self-renewal
Sox2	*Sox2*	Nucleus	Transcription factor regulating embryonic stem cell self-renewal
Sox9	*Sox9*	Nucleus	Transcription factor regulating stem cell maintenance in other organs
Sox10	*Sox10*	Nucleus	Transcription factor regulating stem cell maintenance in other organs
Klf4	*Klf4*	Nucleus	Transcription factor regulating stem cell maintenance in other organs
c-Myc	*Myc*	Nucleus	Transcription factor regulating embryonic stem cell self-renewal
Etv4, Pea3	*Etv4*	Nucleus	Transcription factor regulating progenitor cell maintenance in other organs
Etv5, Erm	*Etv5*	Nucleus	Transcription factor regulating progenitor cell maintenance in other organs
Ascl3	*Asl3*	Nucleus	Transcripton factor expressed in an SMG ductal progenitor
Aquaporin3	*Aqp3*	Cell surface	Water channel expressed in progenitor cells

In our own laboratory, we recently reported that removal or loss of function of the parasympathetic ganglion during early salivary gland development resulted in a reduction in keratin 5-expression (K5) (Knox *et al*, 2010). K5 is a cytokeratin that forms intermediate filaments, which are part of the cells cytoskeleton (Table 1, which lists genes and proteins often used to study stem and progenitor cells). K5 is also used as a marker of trachea and lung airway epithelial progenitor cells (Rock *et al*, 2009). We therefore analyzed the progeny of K5 positive ($K5^+$) cells via genetic lineage tracing in the developing SMG. Although the SMG epithelium at embryonic day 13 (E13) is only comprised of 9.7% $K5^+$ cells, their progeny were widespread in the ductal and acinar compartments of the salivary glands at birth (Knox *et al*, 2010). The $K5^+$ cells are thus considered a progenitor population in salivary glands (Figure 1). In addition, they are present in the duct compartment of the adult gland, which has long been postulated to contain progenitor cells [reviewed in Coppes and Stokman (2010)]. Thus, $K5^+$ cells may be useful for future cell therapies. However, to isolate them from tissue cell surface markers will need to be identified and their ability to form different cell types requires further investigation.

At present, the only progenitor cell population that has been used to regenerate salivary glands after irradiation was isolated based on the cell surface marker c-Kit. Remarkably, postirradiation stem cell treatment with $c\text{-}Kit^+$ adult salivary gland stem cells restored radiation-induced dysfunction (Lombaert *et al*, 2008a). Both acinar and ductal cells were generated and the fact that long-term restoration was feasible indicated the high self-renewal potency of the $c\text{-}Kit^+$ cells. In these experiments ~100 $c\text{-}Kit^+$ cells were used, although theoretically, a single tissue-specific stem cell could be used if it were identified within this cell pool. The relationship between the Ascl3 cells, c-Kit expressing cells and $K5^+$ progenitor cells remains to be determined.

Other groups have used cell surface markers such as Sca-1, CD133, CD24 and CD49f to enrich the epithelial progenitor cells in the salivary glands but a combination of definitive cell markers for salivary progenitor cells remain to be determined (David *et al*, 2008). Ultimately, we may need to identify cell surface markers to define not only the progenitor but also differentiation markers to define specific lineages. The identification of growth factors and conditions that influence progenitor cell proliferation and differentiation along a specific cell lineage, i.e. ductal, acinar, myoepithelial cell is also required.

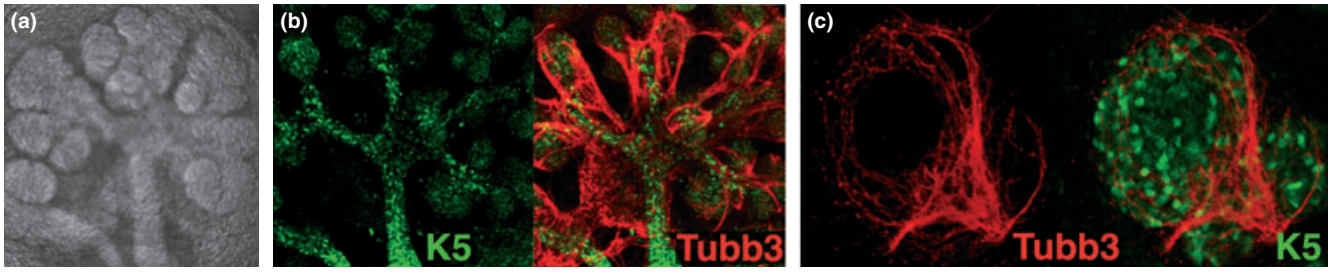

Figure 1 Localization of nerves and K5 progenitor cells in a mouse submandibular gland (SMG). (**a**) Bright field image of an E13 SMG. (**b**) Whole-mount staining for K5 (green) and neuronal tubulin (Tubb3, red) highlights the localization of K5 cells in the SMG ducts and end buds with the nerves surrounding both ductal and end bud epithelia. (**c**) Higher magnification image of a single end bud shows the nerves (red) wrapping around the end bud, which contains $K5^+$ cells (green). Images were taken with a confocal laser-scanning microscope and are a single projection of a stack of images

Human diseases and *ex vivo* models provide insight on growth factor control of epithelial progenitors

Two rare genetic syndromes in humans shed light on a growth factor/receptor signaling pathway that is essential for progenitor cells to initiate and form the salivary glands [reviewed in Patel *et al* (2006)]. Aplasia of lacrimal and salivary glands (ALSG: OMIM 602115) and lacrimo-auriculo-dento-digital syndrome (LADD: OMIM 149730), caused by mutations resulting in haploinsufficiency of fibroblast growth factor 10 (FGF10) or its receptor FGFR2b, have severe defects in the survival and function of salivary progenitor cells. In mice that have both copies of Fgf10 or Fgfr2b deleted (*Fgf10−/−* and *Fgfr2b−/−*), the embryos do not develop salivary glands. Therefore, salivary progenitor cells require Fgf10/Fgfr2b signaling to survive and initiate a salivary gland.

On the other hand, heterozygous mice with one copy of *Fgf10* or *Fgfr2b* are viable and fertile. However, they have hypoplastic salivary and lacrimal glands, which demonstrate that progenitor cells are particularly sensitive to levels of Fgf10/Fgfr2b signaling. It has also been demonstrated *in vivo* that Fgf7, another Fgfr2b ligand, has an effect on salivary gland progenitor cells (Lombaert *et al*, 2008b). Fgf7 injections before and following gland irradiation enhanced the number of progenitor cells. As a consequence, a higher number of progenitor cells remained after radiation forming more saliva-producing cells, which prevented radiation-induced hyposalivation.

Another human genetic syndrome with salivary developmental defects is hypohidrotic ectodermal dysplasia (HED:OMIM 305100 and 224900). This syndrome is due to mutations in ectodysplasin-A1 or its receptor Edar or a downstream signalling component Edar-rad. The patients have salivary hypofunction as well as hair, tooth and sweat gland developmental defects (Mikkola, 2009). Mutations in the same genes in mice phenocopy human HED patients and the mice have smaller salivary glands due to problems with development (Jaskoll *et al*, 2003). However, little is known about the role of Edar signaling in terms of progenitor cell biology in the salivary gland and this will be an important question to investigate.

The developing mouse SMG is an ideal model to investigate the effects of growth factors on progenitor cells during organogenesis (Figure 1). It enables us to study specific interactions among different cell types during development, providing a framework for understanding how we might maintain, expand and direct the specification of salivary progenitor cells to regenerate salivary glands in the adult. We analyzed the expression of genes in the mouse SMG that have been used as markers in other systems to define embryonic stem cell maintenance (*Oct3/4*, *Nanog*, *Sox2*, *Klf4*, *Myc*), stem/progenitor cell maintenance and differentiation in other organ systems (*Etv4*, *Etv5*, *Sox9*, *Sox10, Kit*) and basal progenitor cells in other epithelia (*Krt5*, *Krt14*, *Trp63*) (Table 1). The preliminary analysis highlighted that the developing gland already harbors multiple organ specific progenitors (Lombaert and Hoffman, 2010). We also used isolated SMG epithelium, cultured with specific growth factors to begin to identify if certain growth factors influenced the gene expression of specific markers. Our analysis suggested that a major specification of epithelial cells occurs at embryonic day 13 (E13), which defines the primary duct from the end bud epithelium. The E13 duct develops into Wharton's duct, connecting the adult gland to the oral cavity and the end bud forms the salivary gland. The end bud responds to increased Fgf10 signaling which influences the expression of progenitor cell markers. Fgf10 decreases *Sox2* and *Klf4* expression in isolated epithelia within 2 h, which suggests that a specific cell lineage decision and/or differentiation occurs in the end bud. The upregulation of other transcription factors (*Myc*, *Etv4*, *Etv5* and *Sox9*) suggests that Fgf10 promotes both end bud progenitor cell maintenance and differentiation. Despite obvious caveats to this simple approach, these experiments increase our understanding of how growth factors produced by the parenchymal cell types, such as the neuronal, endothelial and mesenchymal cells, impact epithelial progenitor cell development.

Nerves control salivary gland function and epithelial stem/progenitors

Nerves have been known to control salivary function for more than 100 years when Pavlov conditioned dogs to salivate at the sound of a bell (Pavlov, 1906). Both parasympathetic and sympathetic branches of the autonomic nervous system innervate the adult SMG and there is an abundant literature on salivary gland innervation and function which will not be reviewed here [see Proctor and Carpenter (2007)]. It has been known for some time that obstruction of a salivary duct results in degeneration of the salivary gland, which regenerates when the ductal blockage is removed. In addition, parasympathetic innervation is also critical for gland regeneration. Experimental models of salivary recovery after ductal ligation combined with parasympathetic denervation do not regenerate (Proctor and Carpenter, 2007). An obvious implication of these data is that the parasympathetic innervation must control epithelial progenitor cell function in the adult gland, either directly or indirectly.

The developing parasympathetic ganglion (PSG) maintains keratin 5^{+} progenitor cells during SMG development in a muscarinic receptor- and EGFR-dependent manner

Salivary gland development has been reviewed in detail and the reader is referred to these for more information (Patel *et al*, 2006; Tucker, 2007). Of importance for this review is that the parasympathetic ganglion (PSG), which is derived from the neural crest, condenses around the SMG at E12. One day later, axons from the PSG start to extend along the epithelium and remain in close contact with the epithelia (Figure 1). Using a simple tissue recombination approach, the impact of the PSG on epithelial progenitors and development was elucidated (Knox *et al*, 2010). Removing the PSG signif-

icantly reduced end bud formation but it was the PSG function, not just the physical presence of the nerves that was important for this to occur. Addition of chemical inhibitors that perturbed acetylcholine (Ach) signaling or knockdown of the epithelial Ach muscarinic receptors, also reduced progenitor cell markers, including K5, K15 and aquaporin 3 (Aqp3). We also demonstrated that carbachol (CCh), an ACh analog, increases gland morphogenesis by increasing $K5^+$ progenitor cell proliferation. Differentiation of the $K5^+$ cells in the salivary gland was similar to that described for prostate progenitor differentiation, with an increase in keratin 19 (K19) as ductal differentiation occurs. For example, as $K5^+$ ($K5^+K19^-$) cells differentiate, they co-express K19 ($K5^+K19^+$) and as differentiation proceeds, cells retain K19 but not K5 ($K5^-K19^+$). We observed that CCh signaling maintains the $K5^+$ and $K5^+K19^+$ progenitor cell population in an EGFR-dependent manner and that HBEGF/EGFR alone increases proliferation of the $K19^+$ cell. To determine whether $K5^+$ cells have subtypes with differences in their potency as progenitors, i.e. their progenitor capacity, we recently measured the expression of transcription factors regulating progenitor self-renewal. We observed that Sox2, which is essential in embryonic stem cell self-renewal, is differentially expressed within the K5 population (Figure 2). Sox2 is expressed by a subpopulation of $K5^+$ cells (17 ± 3%) and it is highly abundant in the committed $K5^+K19^+$ and $K5^-K19^+$ duct cells. This finding suggests that their potential to self-renew, driven by Sox2, is still present even as they differentiate along the duct lineage.

Future directions

Salivary gland hypofunction has a severe impact on the oral health of patients with Sjögren's syndrome ($1–2 \times 10^6$ cases in USA) and post-therapeutic radiation for the treatment of head and neck cancer (~30–40 000 new cases USA/year) affects more than 500 000 patients worldwide/year. The prospect of re-engineering salivary glands by repaired, redesigned, replaced or regenerated was proposed 10 years ago (Baum, 2000) and there has been a clear progress toward gene therapy (Baum *et al*, 2009), stem cell therapy (Feng *et al*, 2009) and tissue engineering (Aframian and Palmon, 2008). There have been recent reviews which the reader is referred to about the effects of radiation on salivary glands and potential therapies (Grundmann *et al*, 2009) and an extensive review on the use of stem cells to repair radiation damage (Coppes and Stokman, 2010). Exposure of salivary glands to irradiation results in permanent xerostomia (dry mouth) due to the destruction of the salivary gland progenitor cells. A possible therapeutic scenario is that prior to irradiation a head and neck cancer patient would have their salivary glands biopsied. The progenitor cells in the biopsy could be isolated, expanded in culture and cryogenically stored until the irradiation treatment is complete. After the irradiation treatment, the progenitor cells could be injected into the patients' salivary gland (Lombaert *et al*, 2008a). Unfortunately, there are at present no proven ways to either expand the progenitor cell population in culture without differentiation, along different cell lineages or to drive a cell fate decision pathway. Cell lines have been developed as a tool to define the conditions required for proper organ formation *in vitro* (Coppes and Stokman, 2010). It may also be needed to inhibit the differentiation or lineage commitment when expanding progenitors in culture. Alternatively, it may be desirable to drive a cell fate decision along a particular lineage pathway. This decision will also be critical for the use of induced pluripotent stem (iPS) cell therapy, which will require understanding

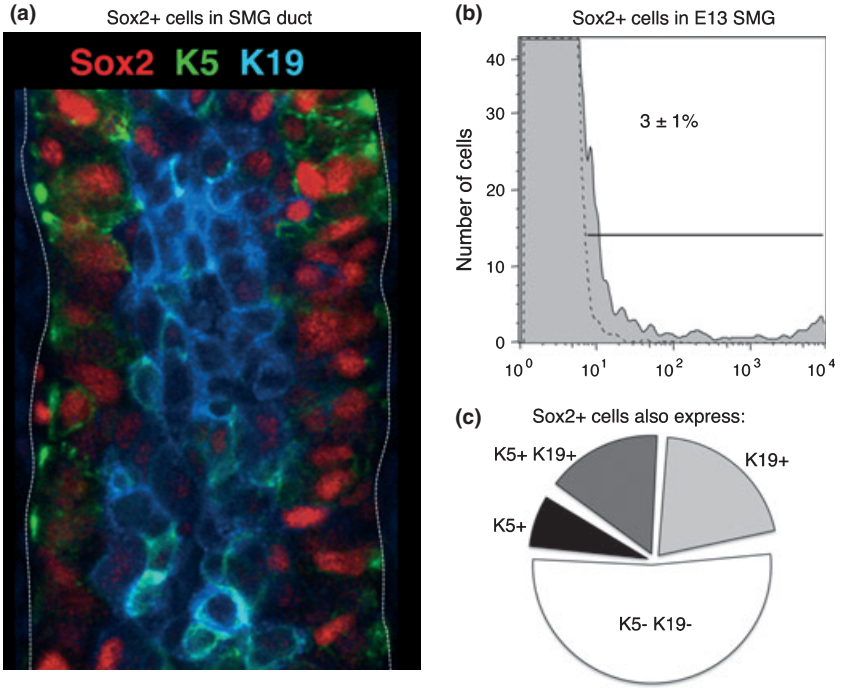

Figure 2 The K5 population expresses Sox2, a transcription factor involved in stem cell self-renewal. (**a**) Whole mount staining of Sox2, K5 and K19 in an E13 submandibular gland (SMG) duct demonstrates the expression of Sox2+ (red) in $K5^+$ (green), $K19^+$ (blue) and $K5^+K19^+$ (cyan) cells. (**b**) Histogram showing the percentage of Sox2-expressing cells in an E13 SMG (3 ± 1%). Data were obtained via Fluorescent Activated Cell Sorting (FACS) analysis by staining single SMG cells with an isotype antibody (depicted as the area defined by the dotted line) to offset the background against cells stained with anti-Sox2 antibody (Santa Cruz, polyclonal goat, gray area). Positive cells are represented by the horizontal line. (**c**) A diagram showing the percentage of the Sox2+ cell population that are also $K5^+$ (7 ± 2%), $K5^+K19^+$ (19 ± 5%), $K19^+$ (23 ± 6%) and $K5^-K19^-$ (51 ± 11%). Single SMG cells were stained for Sox2, K5 and K19 and evaluated by FACS analysis

how to drive iPS cells along a salivary gland lineage. In this scenario other cell types from the patient could be re-programed toward a primitive stem cell and then provided with appropriate conditions to form salivary gland tissue. However, we do not currently know how to drive the iPS cell to form a salivary-specific progenitor cell. Our recent results suggest that $K5^+$ progenitor cells from a patient biopsy might be maintained and expanded with an acetylcholine analog and HBEGF. Given that K5 is a cytoskeletal protein; work to identify cell surface markers on the $K5^+$ progenitor cells in the salivary gland ducts is currently being performed. Salivary glands from mice that have a fluorescent-tagged K5 protein (K5-venus) are being used to identify unique cell surface markers that would enable the cells to be isolated by FACS. Being able to maintain and expand a progenitor cell in culture is an important step in this process. Importantly, it remains to be determined whether an identical progenitor population exists in the human salivary gland. Recent work suggests that human salivary glands have a similar 'putative' c-Kit-expressing stem cell population as rodents, which is capable of *in vitro* differentiation and self-renewal (Feng *et al*, 2009). There is also evidence that stimulation of muscarinic receptors before or after irradiation treatment has a beneficial effect of salivary gland function after irradiation (Coppes *et al*, 2001). Therefore, the role of the PSG function in $K5^+$ progenitor cell maintenance suggests that muscarinic-EGFR-dependent mechanisms should be considered in regenerative therapies.

In conclusion, regeneration or repair of salivary glands requires understanding of the spatial and temporal interactions of the various cell types within the gland as it develops. Here, we have highlighted the critical role of growth factor signaling and the parasympathetic nervous system in the regulation of epithelial progenitor cell development in the SMG. These signaling systems will need to be incorporated into current models of salivary bioengineering as well as regenerative therapies for a successful outcome. A number of significant questions remain to be answered, including: How does radiation therapy affect neuronal function and/or the regulation of both the neuronal and epithelial progenitor cells? Do the recent advances using mouse model systems reflect the biology and/or pathology of human salivary glands? Answers to these questions as well as understanding of how progenitor cells are directed along a series of cell fate decisions to form a functional salivary gland, are all critical to understand organogenesis . Ultimately, this information will provide a template for future regenerative therapy in patients with an irreversible loss of salivary gland function.

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