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Epithelial Stem/Progenitor Cells in the Embryonic Mouse Submandibular Gland

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Abstract

Salivary gland organogenesis involves the specification, maintenance, lineage commitment, and differentiation of epithelial stem/progenitor cells. Identifying how stem/progenitor cells are directed along a series of cell fate decisions to form a functional salivary gland will be necessary for future stem cell regenerative therapy. The identification of stem/progenitor cells within the salivary gland has focused on their role in postnatal glands and little is known about them in embryonic glands. Here, we have reviewed the information available for other developing organ systems and used it to determine whether similar cell populations exist in the mouse submandibular gland. Additionally, using growth factors that influence salivary gland epithelial morphogenesis during development, we have taken a simple experimental approach asking whether any of these growth factors influence early developmental lineages within the salivary epithelium on a transcriptional level. These preliminary findings show that salivary epithelial stem/progenitor populations exist within the gland, and that growth factors that are reported to control epithelial morphogenesis may also impact cell fate decisions. Further investigation of the signaling networks that influence stem/progenitor cell behavior will allow us to hypothesize how we might induce autologous stem cells to regenerate damaged salivary tissue in a therapeutic context.

Introduction

Submandibular gland (SMG) organogenesis involves inductive interactions between mesenchymal and epithelial cells. Recent research on SMG development has focused on the cross-talk between mesenchymal and epithelial cells that control branching morphogenesis [1–6]. The SMG mesenchyme plays an instructive role and produces many growth factors that regulate epithelial morphogenesis by controlling processes such as proliferation, differentiation, migration, and cell death. During epithelial morphogenesis primitive stem/progenitor cells also undergo a series of cell fate decisions that give rise to more differentiated cell types while simultaneously maintaining a reservoir of stem/progenitor cells. Therefore, a major challenge in the field is to identify epithelial salivary gland stem/progenitor cells and determine which signaling pathways direct their cell fate decisions along different cell lineages. Here, we will provide background on SMG development that will offer insight into stem/progenitor cells, and then review what is known about adult salivary gland stem/progenitor cells, focusing on some of the known stem/progenitor cell markers identified in other developing organ systems. Since there are no reports identifying stem/progenitor cells in embryonic SMGs, we have included some preliminary data: microarray and qPCR analyses that begin to define what types of salivary stem/progenitor cells exist in the SMG and experiments investigating whether growth factors influence epithelial stem/progenitor cell fate.

By definition, a stem cell is capable of both unlimited self-renewal and differentiation into all mature gland cell types. As stem cells differentiate into more committed progenitor cells,

they lose their self-renewing capacity and become more restricted to one cell type lineage. These progenitor cells, also termed transit amplifying (TSA) cells, are highly proliferative and can give rise to multiple differentiated cell types. Alternatively, a stem cell might produce one daughter cell that immediately commits to a differentiated cell without the need for further cell division or for a TSA cell [7]. However, there is currently no clear distinction between stem cells and TSA progenitor cells in the SMG. Therefore, we have defined all primitive cells in this review as stem/progenitor cells. Nevertheless, in both cell types, differentiation occurs by either silencing or activating gene transcription. Genes involved in self-renewal will be highly expressed in the stem/progenitor cells and become undetectable in differentiated cells. Genes with low expression in the stem/progenitor cells may increase their expression in one or both matured daughter cells as they differentiate. These cell fate decisions are regulated in a spatiotemporal manner during gland development and are controlled by intrinsic signals and/or by the environment, which includes growth factors, cell-cell interactions, and the extracellular matrix.

As reviewed in other chapters of this book, the SMG initiates at approximately 11 days post-coitum (E11), when an ectoderm-derived oral epithelium interacts with the neural crest-derived mesenchyme, forming an epithelial placode. The epithelium invaginates into the mesenchyme by E12, where an end bud enlarges on a stalk of epithelium. At E13, clefts form on the enlarged end bud and branching morphogenesis begins, along with lumen formation (Fig. 1). At this stage two distinct epithelial cell types, the primary duct and the end buds, are morphologically observed during dissection under a light microscope and can be easily separated and analyzed. Major cell differentiation occurs at E15, followed by postnatal gland maturation [8–12]. Classic experiments using heterotypic tissue recombination have provided insight into the tissue patterning and the plasticity of the epithelial progenitor cells as they respond to different inductive mesenchymal signals [1,3]. The branching pattern is dependent on the source of the organ-specific mesenchyme so that lung, mammary, and pituitary epithelia develop a “salivary-like” branching morphology when combined with embryonic salivary mesenchyme [2,4–6]. However, SMG epithelial growth could only be induced by the addition of either urogenital or SMG mesenchyme. These data suggest that specific SMG mesenchyme-secreted growth factors are needed when evaluating SMG epithelial cell fate decisions. Importantly, the capacity of the mesenchyme to re-imprint the epithelial cell fate only occurs with early epithelia (< E16), suggesting that later stages of development have less stem/progenitor cells and more committed differentiated cells. Consequently, we have focused our studies on identifying stem/progenitor cell populations present early in development by evaluating E13 SMG epithelium.

Important information regarding genes that are critical for SMG epithelial stem/progenitor cell survival and maintenance comes from the analysis of genetically modified mice. (Reviewed in [13]). Studies of mice lacking fibroblast growth factors have been particularly instructive. Conditional loss of fibroblast growth factor 8 (FGF8) in the ectoderm-derived epithelium results in severe gland hypoplasia; only a rudimentary epithelial bud develops [14], and loss of its mesenchymal receptor FGFR2c produces a similar effect [15,16]. FGF8 signaling modulates both mesenchymal FGF10 and epithelial sonic hedgehog signaling, which explain the severe phenotype with the loss of epithelial FGF8. Salivary gland agenesis further occurs with the loss of either FGFR2b or its mesenchymal-derived ligand FGF10, although a single hypoplastic end bud does initially form [15,17]. Similar effects occur in p63 null mice, with p63 normally being expressed in the basal stem/progenitor layers of many ectodermal organs [18]. As such, FGF8 and FGF10 signaling, and p63, are critical for the survival and growth of epithelial stem/progenitor cells. In addition, epidermal growth factor receptor (EGFR)-null mice have SMG hypoplasia, but differentiation appears normal [19]. This suggests that the number of progenitor cells may be reduced, resulting in a

smaller gland. However, direct analyses of the stem/progenitor populations in these genetically modified mice have not been performed.

Adult salivary gland stem/progenitor cells

Interest in identifying progenitor cells in the adult gland has arisen from the potential therapeutic application of regenerating salivary tissue after therapeutic irradiation of head and neck tumors or for replacing damaged glands with bioengineered artificial tissue [20]. The adult gland is comprised of two major epithelial compartments; the ducts, which transport and modify saliva, and the acinar cells, which produce the saliva. These cells are surrounded by a stromal matrix containing contractile myoepithelial cells, (myo)fibroblasts, immune cells, endothelial cells, and neurons. Adult stem cells could potentially be found in many different niches within an organ, in a similar manner to skin stem cells, which reside in the interfollicular epidermis, hair follicles, sebaceous glands, and neural crest mesenchyme (reviewed in [21]). Therefore, multiple stem/progenitor cell types may reside within the SMG epithelium and mesenchyme and a variety of techniques have been used to identify these.

Lineage tracing with genetically marked cells is the most direct genetic method of determining the progenitor cell populations in the gland. *Ascl3* is a transcription factor localized in the duct cells of the salivary glands that increases in expression during postnatal development [22]. Recently, *Ascl3*-expressing SMG duct cells in mice were shown to form both duct and acinar cells by lineage tracing using a Cre-recombinase reporter system [23]. As such, a direct progenitor-progeny relationship was demonstrated between the multipotent *Ascl3*-expressing duct cells and the acinar cell compartment. Reversible duct ligation is another commonly used technique to identify stem/progenitor cells and is described in detail in chapter 7. Duct ligation causes atrophy of the gland with major acinar cell loss. Following the removal of the ligation, intercalated duct cells proliferate and reform acinar cells. This observation suggested intercalated duct cells were acinar progenitors [24,25], which was further supported by acinar differentiation in vitro of duct cells from human, macaque [26] and rat [27] SMG/parotid glands.

Another way to identify stem/progenitor cells is to localize label-retaining cells (LRCs) in adult glands. These cells are slowly dividing and retain DNA-binding dyes, such as BrdU, long after a transient exposure. LRCs have been identified in acinar cells, ducts, myoepithelium, and connective tissue cells [24,25,28,29], suggesting multiple progenitor cell-types exist in different cell compartments in the adult salivary glands. Fluorescent-activated cell sorting (FACS) is a common and direct technique to label and isolate stem/progenitor cells from adult tissue. The initial characterization of cell surface markers that identify stem/progenitor cells from fresh or post-duct-ligated glands showed they were *Sca1*⁺ and *cKit*⁺ [30,31], $\alpha 6 \beta 1$ -integrin⁺ [32] or *CD49f*⁺($\alpha 6$ -integrin) and *Thy1*⁺ [33,34]. Cells isolated from neonatal rat and adult rat/human glands, termed salivary gland stem cells (SGSC), trans-differentiated into either pancreatic or hepatic cells [30,31,34,35], and regenerated hepatectomized livers after transplantation. These SGSCs are similar to in vitro cultured pancreas stem cells [36], highlighting the plasticity of early organotypic stem cells, which suggests that similarities exist between different exocrine organs. SGSCs also express *c-Kit*, and the pluripotency markers *Nanog* and *Oct3/4*. However, this heterogeneous cell population has mesenchymal-like stem cells properties based on their cell surface *CD44*⁺, *CD90*⁺, and *CD105*⁺ expression. Other stem/progenitor cells that have been characterized by FACS are the side population (SP) cells, which have a unique ABC-transporter channel that excludes toxic dyes such as rhodamine or Hoechst-33342. SP cells have been identified in adult salivary glands and make up 1% of the cell population although their capacity to self-renew and differentiate has not been evaluated [37].

Many cell types mentioned in this section have been hypothesized to be salivary gland stem/progenitor cells but have not been tested for their capacity to regenerate damaged SMGs. However, epithelial c-Kit⁺ duct cells isolated by FACS from adult mouse SMGs have been shown to functionally and morphologically regenerate irradiated adult mouse SMGs [38]. Autologous SMG stem cell transplantation is a clinical approach to potentially treat head and neck cancer patients suffering from radiation-induced hyposalivation. However, this heterogeneous c-Kit⁺ cell population likely contains multiple cell types including self-renewing stem/progenitor cells with multipotent differentiation capacities.

In summary, there are numerous examples in the literature of postnatal salivary gland cell types that exhibit self-renewing, transdifferentiating, and multipotent capacities. However, it remains to be determined from which stem/progenitor cell type they originate during embryonic gland development, and what influences their cell fate decisions as they develop along different cell lineages.

Embryonic salivary gland stem/progenitor cells

To date, no lineage tracing studies identifying early embryonic SMG stem/progenitor cells have been reported and few molecular markers have been identified in the epithelial compartment of the embryonic gland. However, in other developing organ systems, specific transcription factors (TFs) have been used to identify stem/progenitor cell types from cells undergoing early cell fate decisions. These cell fate decisions are coordinated by TFs that regulate genes involved in self-renewal and differentiation. Additionally, stem/progenitor cells have also been characterized with cytoskeletal markers, particularly the intermediate filament keratins, to define their cellular differentiation state. Therefore, we analyzed whether TFs involved in stem cell self-renewal (Sox2, Klf4, Nanog, Oct3/4, cMyc, and the Etv family), progenitor differentiation (other Sox genes), and basal progenitor cell markers (cytokeratins) were expressed in the SMG.

We hypothesized that early epithelial cell fate decisions have occurred within the epithelium before branching morphogenesis begins (E13) and that TFs and cell markers involved in stem/progenitor cell self-renewal and early cell lineage specification will already be expressed in distinct epithelial cell compartments. As development and gland maturation proceeds and cells become committed to a particular cell lineage, the expression of genes involved in self-renewal and cell lineage specification would change. Therefore, the presence of these genes in the SMG was evaluated by genome-wide microarray analysis or qPCR at important time-points during development when major cell fate changes are likely occurring; at E13 as branching morphogenesis begins, at E15 when end bud differentiation starts, and in the fully functional adult gland (Fig. 1B). For genes implicated in stem cell self-renewal, embryonic stem cells were used as a positive control. Furthermore, we analyzed their expression within the two morphologically distinct E13 epithelial compartments; the initial duct and end buds (Fig. 1C). We also hypothesized that growth factors that regulate SMG development would also regulate epithelial cell fate decisions. Therefore, we added growth factors that are important for SMG development to isolated E13 epithelium and measured the early (2 hr) downstream transcriptional effects on the genes implicated in stem/progenitor cell self-renewal (Fig. 2). Importantly, this is by no means a comprehensive analysis of all the growth factors and genes involved, but provides an initial starting point for discussion.

1. The SMG expresses TFs involved in embryonic stem (ES) cell self-renewal

In 2006, a groundbreaking report showed that differentiated cells could be reprogrammed to become pluripotent by forced viral transduction of four TFs, Sox2, Klf4, cMyc, and Oct3/4 [39]. The cells were called induced pluripotent stem (iPS) cells, and exhibited

morphological properties of ES cells, suggesting that these TFs were major regulators of stem cell maintenance. Unexpectedly, *Nanog*, another TF thought to be involved in ES cell maintenance, was dispensable for iPS formation. Nonetheless, these TFs have become the hallmark of stem cell self-renewal, but whether they are present in stem/progenitor cells within the SMG is unknown.

Oct3/4 was the first TF to be identified as a master regulator of pluripotency in ES cells. Apart from ES cells, *Oct3/4* is present in adult stem cells such as bone marrow mesenchymal stem cells, prostate neuroendocrine cells [40], muscle-derived progenitors [41] and also in multiple cancers. Both *Oct3/4* and *Nanog* expression were reported in cultured adult SGCS cells [36]. However, in our analyses *Oct3/4* and *Nanog* expression were undetectable by qPCR at any stage of SMG development (Fig. 1B). We used cDNA from embryonic stem cells to confirm that the primers amplified *Oct3/4* and *Nanog* with our qPCR conditions.

Sox2 is a TF expressed in many tissues during embryonic development including the pancreas, retina, brain [42–44], neural crest, tongue, pituitary gland [45], the lungs, tongue, and esophagus [46–49]. Generation of *Sox2* null embryos demonstrated that it was required for epiblast and extra-embryonic ectoderm formation. Conditional deletion of *Sox2* in early tissue development results in abnormal differentiation of organs with characteristic loss of basal cells and differentiated lineages [50]. Consistent with *Sox2* being a stem/progenitor cell marker, our data shows that *Sox2* expression gradually decreases from E13 towards adulthood (Fig. 1B). At an E13 it is specifically localized to the epithelial ducts (Fig. 1C), suggesting that this region may be a source of stem/progenitor cells during gland development.

Klf4 acts as a tumor suppressor by inhibiting proliferation via p21-activation, but is also involved in cell differentiation of epithelium of the intestine, skin, lung, testis, and cornea. *Klf4* increases during SMG development (Fig. 1B), and our analysis at E13 reveals relatively more expression of *Klf4* in the duct, similar to *Sox2* (Fig. 1C). Interestingly, *Klf4* may regulate cytokeratin 19 (*Krt19*) expression [51], which we show to be expressed in the SMG ducts [52], and is known to be expressed by TSA cells in the pancreas [53].

cMyc is a basic helix-loop-helix TF involved in the maintenance of ES cells, in cell growth, differentiation, and proliferation. As a proto-oncogene, it promotes cell cycle progression of G1 into S phase and counteracts the anti-proliferative effect of *Klf4* [54]. In the developing SMG, a relative increase in *cMyc* expression is detected at E15 (Fig. 1B) when proliferation and gland expansion are rapidly occurring. Additionally, *cMyc* is expressed more in the E13 end buds than the duct (Fig. 1C), which is analogous to the *cMyc*⁺ proliferating tip-precursor cells in the pancreas [55] and our previous reports that highly proliferative cells are present in the SMG end buds [56]. Thus, *cMyc* expression may indicate proliferating progenitor cells within the end buds.

Taken together, these data suggest that two distinct progenitor cell populations exist in the E13 SMG; the duct containing the more “primitive” or pluripotent cell types compared to the end bud, i.e. higher relative expression of the TFs involved in ES cell maintenance, namely *Sox2* and *Klf4*. Whereas *cMyc* was more abundant in the end buds, consistent with the hypothesis that the end bud cells have gone through an early cell fate decision and are more proliferative. The observation that *Oct3/4* and *Nanog* were not detectable by E13, suggests that the SMG stem/progenitor cells present no longer have the same capacity to self-renew as embryonic stem cells.

2. ETS and Sox-related transcription factors are expressed in the epithelial end bud

ETS TFs are a large family of TFs associated with stem cell maintenance, cell proliferation, differentiation, and tumorigenesis [57,58], and some of them may also function as transcriptional repressors. The maintenance of mesenchymal stem cells [59] requires *Etv5* expression, and both *Etv4* and *Etv5* are expressed in proximal pancreatic progenitor cells that are regulated by mesenchyme-secreted FGF10 [60]. *Etv4* is maintained in the gland until E15 and decreases towards adulthood, whereas *Etv5* expression markedly decreases at E15 and is undetectable in the adult gland, suggesting they are only involved in early SMG development. In the E13 SMG, both TFs were highly expressed in the end bud compared to the duct. Similar to the pancreas, the SMG end bud is surrounded by an FGF10 producing mesenchyme [13]. Therefore, both *Etv5* and *Etv4* may be associated with the maintenance of putative end bud progenitor cells under the control of FGF10.

In addition to the embryonic stem cell-related Sox2; other Sox TFs also play a role in stem cell specification/maintenance in a variety of tissues; marking both early and late stem/progenitor cells. Sox9 is involved in the development of many organs including the pancreas, pituitary gland, kidney, intestine, and gonad [61–63]. Sox9 is not critical for the initial formation of organotypic stem cells, but it is required for their specification, maintenance, survival, and proliferation [64]. However, it is the level of Sox9 expression that determines its role in stem/progenitor cell regulation. For example, in intestinal epithelium Sox9 is abundant in post-mitotic Paneth progenitor cells but is expressed less in the highly proliferative crypt columnar stem cells. Increased levels of Sox9 repress cMyc and CyclinD1-regulated proliferation and induce Paneth cell specification [65]. This is in contrast to the crypt stem cells where low Sox9 levels maintain the stem cells as they proliferate. Thus, Sox9 can have different effects depending on its level of expression in different types of stem/progenitor cells within a tissue, and this also occurs in the pancreas [66,67], hair [68,69], heart valve [70], cartilage [64], and neural crest [71,72]. In the SMG, *Sox9* is expressed in the E13 epithelial end bud cells, suggesting it might exert a similar effect on the maintenance or early specification of the end bud cells as they proliferate.

Sox10 is also associated with stem/progenitor maintenance/specification. Human mutations in Sox10 cause the Waardenburg-Shah syndrome, characterized by hypopigmentation of the skin, heterochromia irides, deafness, and absence of enteric ganglia. Therefore, Sox10 is required for the development of multipotent neural crest cells and their derivatives, the Schwann cells, glia, and melanocytes. Sox10 can also have different effects depending on its level of expression in different stem/progenitor cells within a tissue. Before lineage segregation, low Sox10 expression increases neural crest progenitor cell survival, whereas later in development, higher Sox10 levels induce lineage determination of glial cells. During adulthood Sox10 remains in glial cells only, suggesting it might be required to prevent differentiation into other neuronal cell lineages [73].

In E13 SMG's, the epithelial end buds express *Sox10* (Fig. 1C). Sox10 expression was previously reported in E14.5 end bud cells and in several types of duct cells [74]. If Sox9 and Sox10 proteins have a similar function during SMG development as in neural crest and intestinal formation, our analyses suggest that SMG end bud progenitor cells have undergone some specification. This hypothesis is supported by the observation that *Sox9* and *Sox10* expression increases at E15, the time of onset of cell differentiation (Fig. 1B). In addition, in adult human SMGs, Sox10 was located in myoepithelial cells [75]. Taken together, these data suggest that Sox10 might be involved in later basal/myoepithelial cell specification and maintenance. However, it is not known if they are co-expressed in the same cells in the early SMG or what their function is.

3. SMG epithelia contain basal progenitor cells

An alternative approach to defining stem/progenitor lineages is by evaluating the cytoskeleton keratin profiles of cells. Keratins occur as heterodimer pairs of a type I and type II keratin, and are expressed in spatiotemporal and tissue-specific manners. Keratin mutations lead to disorders in several tissues such as pancreas, liver, and intestine. The keratin expression patterns have been used to characterize basal, intermediate, and luminal cell types in many epithelial tissues. The basal Krt5⁺ and Krt14⁺ cell layer contains the niche for multiple tissue stem/progenitor cells in all multilayered epithelia such as prostate, mammary glands and skin bulge stem cells [76]. Krt5/Krt14 expression is downregulated during differentiation, and there is a transition to other keratin types as the cells move into suprabasal layers. Depending on the tissue, the cells transition to express keratins such as Krt1/10 in the epidermis and Krt8/Krt18 in luminal prostate and mammary gland cells. In the oral epithelium and mammary duct cells, basal cells first lose Krt14, which is replaced by Krt15, Krt17 and Krt19 [77]. Krt15 and Krt17 are limited to basal intermediate cells, while cells moving from the basal into the luminal layer only maintain Krt19. Further terminal differentiation leads to loss of Krt19 and increased Krt8/Krt18 expression [53]. Surprisingly, keratin profiles of the early SMG have not been thoroughly characterized. Krt8 staining (Troma-1 antibody) was observed throughout the E13 SMG epithelium, although it appeared higher in the ducts than the end buds [78]. Also, Krt7 has been used as a luminal duct cell marker in the E13.5 SMG [79]. We observed Krt5 expression in basal duct cells of the E13 SMG [52] as well as a subpopulation of cells within the end bud region. The expression pattern of Krt5 and Krt14 is different, with *Krt5* expression decreasing in the adult SMG (Fig. 1B), whereas *Krt14* has increased expression at E15 and is similar in E13 and adult. Our array analysis also shows at E13 there is relatively more expression of *Krt5* in the duct (Fig. 1C) whereas *Krt14* is more broadly expressed and could label a basal cell population in both the duct and end bud area.

The p63 protein is an additional stem cell marker, and p63^{-/-} mice lack ectodermal tissues [80]. There are two p63 isoforms and a correct balance between TA and ΔNp63 is required for tissue development [81]. ΔNp63 is highly expressed by stem cells and is related to other basal epithelial cells [80]. It is present in basal prostate cells [82], limbal cells, myoepithelial cells, and its expression is also required for the maintenance and proliferation of thymus and basal epidermis stem cells [83]. Furthermore, loss of p63 prevented the full commitment of early endodermal cells to the prostate lineage. However, secretory cells are still formed, suggesting p63 is not required for the commitment to this specific cell lineage [84,85]. The p63^{-/-} mice do not develop SMG's, suggesting that basal progenitor cells are required for proper SMG development. In the developing SMG there is a relative increase in *p63* expression at E15 and it is still detected in the adult SMG. Our array analysis also shows that it is expressed in both the duct and the end buds of the E13 SMG, which might indicate their expression in basal cells of both compartments.

Influence of mesenchyme-produced growth factors on progenitor cell fate decisions

Our observation that epithelial cells from E13 SMG's express a variety of stem cell-related TFs and markers further indicates that the bud, duct, and basal cell niches harbor heterogeneous cell subpopulations. This compartmentalization also raises the possibility that different signaling pathways are needed to maintain/specify these cell populations. Removal of the SMG mesenchyme enables us to study the effect individual growth factors on epithelial cell fate. The culture of isolated SMG epithelium has been extensively used to study growth factor matrix interactions during epithelial morphogenesis. Here, we have included experimental data where we added exogenous growth factors individually to

isolated E13 epithelial rudiments cultured in 3D laminin-111 matrix for only 2 hours to measure early downstream changes in gene expression of stem/progenitor cell-related genes associated with cell fate decisions. A short incubation time was chosen to detect early transcriptional changes and to minimize downstream effects of epithelial cell growth as no change in morphogenesis was observed at 2 hours. The effect of growth factors was evaluated by qPCR and normalized to control epithelia cultured in serum-free medium alone (DMEM/Ham's F12 + Vitamin C + Transferrin). The concentrations of growth factors added were similar to those reported in the literature; i.e. HBEGF at 1ng/mL; TGFβ1 and TGFβ2 at 10ng/mL; FGF2, FGF8b, BMP2, BMP4 at 100ng/mL; FGF7 at 200ng/mL; and FGF10 at 400ng/mL. There are obvious caveats to this approach but it allows us to address whether a growth factor regulates gene expression of stem/progenitor cell markers present in the E13 epithelium.

1. Different ES-related maintenance genes are downregulated by FGFs

In our analysis, ES-related TF Sox2 was downregulated more than 2-fold by the addition of FGF7, FGF10, and BMP2 (Fig. 2). BMP4 also gave identical results to BMP2 (data not shown). *Klf4* expression was also down-regulated by FGF7 and FGF10, which are expressed around the end bud region in vivo, suggesting they promote end bud cell fate. The down-regulation of *Sox2* by FGF10 has been observed in other organs such as stomach [86], lung [47] and esophagus [49]. Sox2 may be important for the maintenance of SMG duct cells and its expression may be maintained by other growth factors, such as FGF2 and HBEGF.

2. FGF7 and FGF10 control bud-related maintenance/differentiation genes

The end bud-associated TFs *cMyc*, *Etv4*, *Etv5*, and *Sox9* are all upregulated by FGF10 and/or FGF7 (Fig. 2). The mesenchyme produces FGF10 and FGF7 in vivo and they induce SMG end bud cell proliferation, which is associated with activation of MAPK, ERKs, and cMyc. Since cMyc is highly expressed in the E13 end bud, and both FGF10 and FGF7 promote epithelial growth, cMyc may be both a proliferative marker and label self-renewing end bud progenitor cells. Accordingly, BMPs and TGFβs promote cell cycle exit and differentiation via cMyc [87,88] and BMP2 and BMP4 (not shown) also decrease cMyc expression in the E13 SMG epithelium. Although EGF increases proto-oncogenes like cMyc in mES cells [89], E13 SMG cells maintain their *cMyc* expression with HBEGF treatment. Interestingly, none of the tested growth factors influenced Sox10 expression, suggesting it is regulated further downstream (i.e. 2 hrs is too short) or directly controlled by other growth factors.

3. Basal cells are regulated by TGFβs

TGFβs have been proposed to maintain basal stem/progenitor cells in their niche [90]. In our experiments TGFβ1 (Fig. 2) and TGFβ2 (not shown) gave similar results, both increasing *Krt5* and *Krt14* gene expression (Fig. 2). Therefore, the TGFβ family may be important for maintaining the basal stem cell niche. On the other hand, BMP2 and BMP4 (not shown) also increased *Krt14* expression. Interestingly, *ΔNp63* is reported to regulate expression of both *Krt5* and *Krt14*, however no direct regulation of *ΔNp63* was observed with any growth factor within 2hrs. Since *ΔNp63* is a basal marker, interaction with the basement membrane, extracellular matrix, or cell adhesion receptors may influence its expression, rather than with a growth factor alone. Our data also suggests that TGFβ signaling and *ΔNp63* regulate the basal keratin expression by different mechanisms.

Conclusion

In this review, we demonstrate that TFs associated with epithelial stem/progenitor cells are present in the E13 SMG, and that, for most, their expression is downregulated towards adulthood. This suggests that several TFs associated with stem/progenitor cell maintenance are not needed in the adult gland, or that the stem/progenitor cells in the adult gland are different from those present in embryonic gland. We have further demonstrated that the E13 SMG epithelium end buds and duct contain different cell types based on their transcriptional profiles. The ductal compartment contains more primitive stem/progenitor cell types based on the expression of TFs related to embryonic stem cell self-renewal (Sox2 and Klf4) and higher levels of basal cell keratin (Krt5). Whereas the end buds contain stem/progenitor cell types that express TFs related to progenitor cell maintenance and specification (Etv4, Etv5, Sox9, Sox10). Furthermore, we show that different growth factors produced by the SMG mesenchyme in vivo, directly influence the cell fate of distinct cell populations by regulating the transcriptional level of TFs and cytokeratins located in the end bud region, duct area, and the basal cell compartment. Understanding the cell lineage of progenitor cells within the salivary glands will be important from the clinical perspective where progenitor cells of specific lineages may be more appropriate than pluripotent stem cells for clinical transplantations to regenerate irradiation-damaged salivary glands.

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Figure 1. Gene expression analysis of stem/progenitor cell-related markers in the developing SMG

(A) Branching morphogenesis of the mouse submandibular gland (SMG) begins at E13 as the end bud clefs. At E15, cell differentiation occurs, which is followed by post-natal cell maturation to form the adult gland. E13 glands were mechanically separated into a mesenchyme and epithelial compartment. The latter was further mechanically separated into an end bud and duct compartment. (B) Relative gene expression analysis of markers related to embryonic stem cell maintenance (*Oct3/4*, *Nanog*, *Sox2*, *Klf4*, *cMyc*), stem/progenitor cell differentiation/self-renewal (*Etv4*, *Etv5*, *Sox9*, *Sox10*), and basal stem/progenitor cells (*Krt5*, *Krt14*, *p63*) in E13, E15, and Adult (Ad) SMGs. The expression of *Oct3/4* and *Nanog* were not detected by qPCR. *Sox2*, *Etv4*, *Etv5* and *Krt5* decrease during development, while *Klf4* increases towards adulthood. Other genes such as *cMyc*, *Sox9*, *Sox10*, *Krt14* and $\Delta Np63$ are present throughout development with maximum expression at E15. (C) The relative gene expression was compared between the E13 epithelial end buds and ducts. Ducts express higher levels of *Sox2* and *Klf4* and *Krt5*. In contrast, *cMyc*, *Etv4*, *Etv5*, *Sox9*, and *Sox10* were detected in the end bud compartment. *Krt14* and $\Delta Np63$ were expressed in both the duct and end bud compartments. Agilent whole mouse genome microarray analysis was performed on triplicate pooled biological samples; gene expression was normalized to the level of expression at E13. A probe for *Oct3/4* is not present on the Agilent microarray and the expression of *Nanog* was low; therefore qPCR was used to detect their gene expression, which was normalized to the housekeeping gene *29S*. Error bars represent SEM.

Figure 2. Growth factors involved in SMG development regulate stem/progenitor cell-related gene expression in E13 SMG epithelium

Different growth factors produced by the SMG in vivo such as FGF2, FGF7, FGF8b, FGF10, BMP2/4, TGFβ1/2, and HBEGF were added to isolated SMG epithelium for 2 hrs. Both FGF10 and FGF7 downregulate *Sox2* and *Klf4*, which are mainly expressed in the duct. FGF7 and FGF10 also upregulate *cMyc*, *Etv5*, and *Sox9*, which are expressed in the end bud, and FGF10 specifically upregulates *Etv4*. BMP2 also downregulates *Sox2* and *cMyc* expression. Basal stem cell-related genes *Krt5* and *Krt14* are both upregulated by TGFβ1. BMP2 also increases *Krt14* expression. In contrast, *Sox10* and *ΔNp63* are not regulated by any of the growth factors. In addition, BMP4 (not shown) gave similar results as BMP2, and TGFβ2 (not shown) gave similar results to TGFβ1. Gene expression was normalized to the housekeeping gene *29S* and normalized to epithelia cultured in media alone for 2 hours. At least 5 epithelial rudiments were used for each condition and the results of 3 independent experiments were combined. Mean + SEM. Statistical analyses were performed using a one way ANOVA or t-test with *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.