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Salivary Gland Development: A Template for Regeneration

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Abstract

The mammalian salivary gland develops as a highly branched structure designed to produce and secrete saliva. This review will focus on research on mouse submandibular gland development and the translation of this basic research towards therapy for patients suffering from salivary hypofunction. Here we review the most recent literature that has enabled a better understanding of the mechanisms of salivary gland development. Additionally, we discuss approaches proposed to restore salivary function using gene and cell-based therapy. Increasing our understanding of the developmental mechanisms involved during development is critical to design effective therapies for regeneration and repair of damaged glands.

Keywords

Salivary gland development; submandibular gland; branching morphogenesis; stem cells; progenitor cells; regeneration; parasympathetic innervation

Introduction

The salivary system of mice and humans contains three major pairs of glands; the parotid, submandibular (SMG) and sublingual glands, which together secrete 90% of the saliva in the oral cavity. Additionally there are numerous (600–1000) minor salivary glands in the submucosa throughout the oral cavity. The reader is referred to recent extensive reviews on salivary glands [1–3]. The major function of salivary glands is to produce saliva, which aids in lubrication, digestion of food, taste, immunity and oral homeostasis. The acinar cells produce either serous or mucous secretion, which contains water, salts and proteins, while the ductal cells modify the secretion, primarily by reabsorbing the salt. The stellate myoepithelial cells, which surround the acini and intercalated ducts, are innervated and are proposed to facilitate secretion by contraction, although this has not been directly demonstrated. There are three types of ducts based on their morphology and histological appearance; intercalated, striated and granular. Saliva flows from the acinar units through

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the ductal system into the oral cavity. Readers are referred to reviews on the physiology of salivary secretion [4–6].

1.0 Mechanisms of Development

1.1 Developmental origin

There is some controversy within the literature about the developmental origin of the epithelium of the major salivary glands i.e. are they ectodermal or endodermal in origin? While it is apparent that all 3 pairs of major glands are primarily derived from the oral epithelium, the issue is which part of the oral epithelium they arise from and where this is in comparison to the junction of the oral ectoderm with the foregut endoderm. During development this border is marked by the oropharangeal membrane that separates the stomodeum from the cavity of the primordial pharynx [7], but the exact position of this line as compared to sites of gland initiation is unclear. The use of genetic lineage tracing using lineage-specific Cre drivers has helped clarify the lineage of some cell types within the glands. The mesenchyme and nerves in the gland are neural crest in origin as shown by lineage tracing with Wnt1-cre [8]. However, there are conflicting reports of the embryonic origin of the epithelium. In text books, it has been suggested that the parotid is ectodermal, whereas the SMG and sublingual are endodermal [9]. An endoderm origin was proposed to be supported by data showing that adult salivary gland progenitors can differentiate into pancreatic β -cells and hepatocytes when transplanted into hepatectomized liver [10]. However, while it is clear that salivary gland progenitors can differentiate into these cell types in the appropriate extracellular microenvironment, i.e. when transplanted into the liver, it is not proof that in vivo the salivary epithelium is derived from the endoderm. Recent genetic lineage tracing experiments using the Sox17-2A-iCre/R26R mouse, which marks endodermal cells, showed that the epithelia of all three major salivary glands are not of endoderm origin, suggesting an ectodermal lineage [11]. In addition, animal models and human mutations that cause ectodermal dysplasias, developmental syndromes that specifically affect ectodermal organs, suggest that the major salivary glands arise from common multipotent precursors residing in the embryonic ectoderm. Hypohidrotic ectodermal dysplasia patients (HED) have abnormal salivary glands and similar phenotypes are observed in mouse models *Tabby* (*Eda^{Ta}*) and *downless* (*Edar^{dl}*) [12, 13]. Lineage tracing studies need to be performed with a specific ectodermal Cre to positively confirm the origin of the salivary gland epithelium.

1.2 Salivary Gland Initiation

Reciprocal interactions among the epithelium, and neural crest-derived mesenchyme, nerves, and blood vessels regulate the early events of SMG development (Figure 1). It is not known what signals cause the migrating neural crest cells to form a mesenchymal condensation at the appropriate location beside the oral epithelium. The mesenchyme provides instructive signals, resulting in the thickening of the oral epithelium to form a placode at embryonic day 11 of development. Knockout mice for *Fgf10*, *Fgfr2b*, *Pitx1* and *p63* lack salivary glands, emphasizing that these genes are critical for salivary gland initiation and patterning. In organs such as the liver and pancreas the endothelial cells provide critical cues for organogenesis [14], however the role of endothelial cells in salivary

gland initiation has not been investigated. By E12, the salivary placode invaginates into the mesenchyme, which begins to condense. The epithelial bud grows into the mesenchyme forming a primary bud on a stalk. The neural crest-derived neuronal precursors coalesce to form the parasympathetic submandibular ganglion (PSG), wrapping around the epithelial stalk that will become the major secretory duct. The signals that initiate this interaction have not been defined.

1.3 Branching morphogenesis

The major glands form by the developmental process of branching morphogenesis, which involves coordinated cell proliferation, clefting, differentiation, migration, apoptosis and reciprocal interactions between the epithelial, mesenchymal, neuronal and endothelial cells [15]. At E13 as the endbud enlarges, clefts in the epithelium delineate the first 3–5 buds, which correspond to major lobules of the gland, and in parallel, axons from the PSG extend along the epithelium to envelop the endbuds. By E14 the gland is highly branched and functional differentiation begins at E15 and continues to birth [1, 16]. In the next sections we review specific mechanisms involved in branching morphogenesis.

1.3.1 Clefting—Cleft formation is a stochastic and dynamic process that occurs as a result of two separate events; cleft initiation and progression. Basement membrane (BM) dynamics are a possible driving force for cleft formation. Fibronectin is a putative cleft initiation molecule [17] and its accumulation rapidly induces *Btdb7* (BTB (POZ) domain containing 7), which in turn induces expression of *Snail2* and suppresses E-cadherin levels [18]. This results in a loss of the columnar cell organization in the outer layer of the epithelial cells at the base of the forming cleft, and formation of intercellular gaps for cleft progression. Other extracellular matrix (ECM) proteins in the BM accumulate at the cleft sites including the laminin chains $\alpha 1$ and $\alpha 5$ [19], perlecan and heparanase, an endoglycosidase enzyme that cleaves heparan sulfate (HS) chains [20] (Figure 1). SMGs from laminin $\alpha 5$ null mice show a delay in branching morphogenesis with delayed cleft formation. In addition, expression of glycogen synthase kinase 3 beta (GSK3 β), an enzyme that phosphorylates β -catenin and targets it for degradation, is decreased in cells at the base of the clefts. Loss of GSK3 β by either pharmacological inhibition or reduced transcription promotes cleft formation [21].

Cytoskeletal dynamics are critical for clefting. Ultrastructural analysis of clefts revealed that a cytoplasmic shelf with a core of microfilaments occurs in cells at the base of the cleft [22]. The shelf may be a matrix attachment point to drive cleft elongation via cytoskeleton attachment and inhibition of the actin cytoskeleton polymerization inhibits clefts formation. However, a recent study has showed that cleft initiation and progression are physically and biochemically distinct [23]. It was proposed that a mechanochemical checkpoint involving the Rho-associated coiled-coil containing kinase (ROCK) regulates the transition of initiated clefts, which is proliferation independent, to a stabilized state competent to undergo cleft progression. The localized assembly of fibronectin results in epithelial proliferation and cleft progression. In contrast, inhibition of ROCK I or non-muscle myosin II activity prevents clefts at the initiation stage. Interestingly, ROCK also controls tissue organization by coordinating cell polarity via PAR-1b protein. PAR-1b is a regulator of BM deposition and

its activity is controlled by ROCK to maintain its localization in the outer epithelial cells [24].

1.3.2 Proliferation—The process of clefting is coordinated with cell proliferation during branching morphogenesis as the size of the epithelium increases. In the developing SMG, rapid proliferation is mainly localized at the peripheral endbuds, suggesting that they contain the proliferating progenitors (Figure 2). Fibroblast growth factor (FGF) signaling is essential for proliferation and survival of the salivary gland progenitors as *Fgfr2b*^{-/-} and *Fgf10*^{-/-} mice have no salivary glands, although, an epithelial bud forms but degenerates by E12.5 [1]. Exogenous FGF10 or FGF7 both bind Fgfr2b and increase SMG epithelial proliferation (Figure 2) but FGF7 induces budding whereas FGF10 induces duct elongation [25]. These differences are due to the binding affinities of the FGFs to HS as well as the endocytic recycling of the FGFR. FGF10 binds HS, which increases the affinity of FGF10 for its receptor FGFR2b to form an FGF10-FGFR2b-HS ternary signaling complex resulting in increased proliferation [26]. FGF10 also increases endocytic recycling of FGFR2b, which correlates with higher mitogenic activity, whereas FGF7 increases receptor ubiquitination and degradation [27].

Platelet-derived growth factor (PDGF) signaling also modulates FGF signaling. FGFs 1, 3, 7 and 10, which are produced by the mesenchyme, function downstream of PDGF signaling. Exogenous PDGF induces FGF expression and enhances epithelial proliferation, whereas loss of PDGF via siRNA-knockdown inhibits FGF expression [28]. In addition, the SMG branching defect caused by inhibition of PDGF can be rescued by exogenous FGF7 and FGF10, consistent with FGF being downstream of PDGF signaling.

The epidermal growth factors and their receptors are important for SMG proliferation. The SMGs of the EGFR-null mice have reduced proliferation, branching and maturation of the epithelium [29]. Also function-blocking antibodies to neuregulin 1 decrease ex vivo SMG branching, while exogenous Nrg1 increases branching [30]. Furthermore, acetylcholine (ACh)/muscarinic (M) receptor 1 signaling increases EGFR protein expression in the SMG, and HB-EGF increases proliferation of Keratin 5 (K5) progenitors in an EGFR-dependent manner [31].

Wnt signaling, involving the secreted Wnt ligands that signal through transmembrane Frizzled receptors, has many biological functions including proliferation, differentiation, organogenesis and cell migration [32]. Wnt signaling is highly dynamic during SMG development. During early stages it is localized in the mesenchyme but after ~ E14.5, it localizes to the ductal epithelium [33, 34]. A reduction in Wnt signaling with chemical inhibitors or conditional deletion of β -catenin in the SMG mesenchyme reduced epithelial branching [34]. Alternatively, forced activation of Wnt/ β -catenin in the epithelium by inhibiting GSK3 β also arrests branching, although proliferation was not affected [33]. Wnt signaling in the endbuds is repressed by FGF signals through induction of the Wnt antagonist sFRP. This repression maintains the endbuds in an undifferentiated state while the ductal structures continue to differentiate [33]. Others have shown that postnatal Wnt activity is detected in the intercalated ducts of SMGs, the putative stem cell compartment

[35]. Forced activation of Wnt/ β -catenin signaling specifically in the adult K5+ progenitors promoted ductal proliferation and progenitor expansion.

1.3.3 Cell movements, Cell-Cell and Cell-Matrix adhesions—Timelapse analyses of fluorescently-labeled epithelia have demonstrated a high degree of epithelial motility by individual or clusters of cells during early stages of SMG development [36]. Cell migration is dynamic and both outer columnar cells and the central core of polymorphic cells move randomly although the outer cells migrated more [22]. Similarly, tracking studies using a combination of photo-conversion of KikGR (Kikume green-red) show that cell motility is highest in cells that are in contact with the BM. This motility is integrin- and myosin-II-dependent but not E-cadherin-dependent. In contrast the motility of cells within the endbud was restrained by E-cadherin [37]. Thus, region-specific differences in cell-matrix interactions and cell motility within the epithelial endbuds, contributes to different processes during branching morphogenesis.

Cadherins are cell-cell receptors and during SMG development, two cell populations exist with distinct E-cadherin junctional organization and developmental outcome. The outer peripheral cells, which have well-organized junctions and express a neonatal acinar differentiation marker, are committed to the acinar lineage as early as E13.5 stage of development. In contrast the cells in the inner buds that have less-defined junctions express duct-specific markers such as K7 and form ductal structures [38]. Although, once ductal lumens are formed, E-cadherin junctions stabilize the ductal structures. Interestingly, inhibition of E-cadherin function only affects the inner bud cell organization and causes cell death indicating that these E-cadherin junctions provide a survival signal to the maturing duct cells. In addition, dilated lumens form in the mouse SMG in the absence of p120 catenin, which is a stabilizing partner of E-cadherin [39].

Integrins are heterodimeric receptors that bind ECM proteins in the BM. The SMGs of integrin *Itga3*^{-/-} embryos have defective apical-basal cell polarity and altered expression patterns of E-cadherin, α 5 integrin and fibronectin [40]. However, a more severe SMG phenotype occurs in the *Itga3*^{-/-}: *Itga6*^{-/-} double-knockout mice where a delay in epithelial branching and disorganization of the epithelial cells occurs [19]. The laminin α 5 knockout mice have a similar SMG phenotype as *Itga3*^{-/-}: *Itga6*^{-/-} null mice. Further studies using siRNA knockdown of *Lama5* show a decrease in branching, MAPK phosphorylation and *FGFR* gene expression. Addition of exogenous FGF10 restores branching in the *Lama5*-siRNA-treated SMGs and in turn FGFR-siRNA decreases *Lama5* suggesting that a reciprocal regulation of laminin and FGF signaling occurs. Together, these studies illustrate the dynamic role of ECM receptors and FGF signaling during SMG development.

1.3.4 ECM proteolysis during branching morphogenesis—Remodeling of the ECM and cell surface by matrix metalloproteinases (MMPs) generates bioactive cleavage products and releases growth factors stored in the BM [41]. However, most single MMP mouse knockouts have subtle phenotypes, likely due to compensation or overlapping functions. Mice lacking the membrane-type MMP, MT1-MMP (*Mmp14*^{-/-}), have decreased SMG branching morphogenesis [42]. Knockdown of MT2-MMP (MMP15) in ex vivo SMG culture decreases morphogenesis, epithelial proliferation, and the proteolytic release of NC1

domains from collagen IV, which increases the intracellular collagen IV [43]. Recombinant collagen IV NC1 domains rescue branching by increasing epithelial cell proliferation and MMP15 expression via β 1-integrin signaling. This in turn results in phosphorylation of AKT and downstream gene expression of *MT-MMPs*, *Hbegf* and FGF-related genes such as *Fgf1*, *Fgfr1b* and *Fgfr2b*. Furthermore, HBEGF increases the release of collagen IV NC1 domain, which rescues MMP15-siRNA treated SMGs and upregulates its own gene expression and that of *Mmp15*. This study highlights how protease activity affects various interconnected ECM and FGF signaling pathways during development.

1.3.5 NoncodingRNA regulation—MicroRNAs (miRNAs) are small, non-coding RNAs that target multiple RNAs to regulate gene expression at post-transcriptional level. Branching morphogenesis can be regulated by miR-21, a mesenchymal miRNA that downregulates two target genes *Reck* and *Pdcd4*. MiR-21 is upregulated by Egf and loss of miR-21 decreases epithelial branching [44]. miR-21 enhances branching via ECM degradation by MMPs that are activated due to inhibition of *Reck* and *Pdcd4*.

The miR-200c family is also highly expressed in epithelial endbuds and influence epithelial proliferation. Surprisingly, mir-200c targets very-low density lipoprotein receptor (Vldlr) function by decreasing expression of *Vldlr* and its ligand reelin, which affects downstream FGFR-dependent genes and proliferation [45]. miR-200c in the SMG also targets *Zeb1* and *Hs3st1*, which regulate E-cadherin and HS function, respectively. It is clear that further research on noncoding RNAs is required as they are likely to regulate other signaling pathways involved in development.

1.3.6 Post-translational regulation: Glycosylation—The carbohydrate structures of glycoproteins mediate diverse cellular and developmental processes. Many studies (reviewed previously in [16]) have focused on the function of glycosaminoglycans (GAGs) and their degradation during proliferation during branching morphogenesis. The activities of heparan sulfate proteoglycans (HSPGs) result from the sulfation patterns on their HS side chains, which can bind and activate growth factors or act as reservoirs in the ECM. An endoglycosidase, heparanase, releases FGF10 from perlecan HS in the BM to increase MAPK signaling, epithelial clefting, and lateral branching, which increases branching morphogenesis [20]. In addition, specific HS structures modulate FGF10-mediated morphogenesis by influencing proliferation, duct elongation, endbud expansion and differentiation [26]. Furthermore, modulation of FGF gradient within the ECM alters the cellular responses during SMG branching morphogenesis. Differences in HS binding between FGF7 and FGF10 underlie formation of different gradients that dictate distinct activities during branching, where FGF7 has low HS affinity and thus diffuses freely whereas FGF10 has high affinity for HS and only diffuses locally [46]. Together these studies highlight the importance of HS sulfation during SMG development.

Modification of E-cadherin by *N*-glycosylation has been found to influence SMG branching morphogenesis. Highly *N*-glycosylated E-cadherin was found in transient, unstable cell-cell junctions during early morphogenesis. Whereas, hypo-*N*-glycosylated E-cadherin was present in stable cell-cell junctions in cytodifferentiated SMGs [47]. Studies using MDCK cells shows that E-cadherin ectodomains modified with *N*-glycans impact the composition

and stability of the E-cadherin scaffold. Removal of complex-*N*-glycans from the ectodomains promotes the association of E-cadherin with the actin cytoskeleton [48]. Whereas hypoglycosylation of E-cadherin in these cells using siRNA to DPAGT1, a rate-limiting enzyme that initiates the synthesis of the oligosaccharide precursor for protein *N*-glycosylation, promotes tight junction assembly [48]. Thus *N*-glycosylation of E-cadherin is an important regulator of cadherin function.

Recently, *O*-glycosylation due to the enzyme *O*-glycosyltransferase 1 (ppGalNacT1) was shown to control the secretion of BM components such as collagen type IV and laminin during early SMG development [49]. The ppGalNacT1 is the most abundantly expressed isoform in the SMG, and mice deficient in this enzyme have a delay in early SMG branching due to intracellular accumulation of BM proteins, which results in endoplasmic reticulum stress, decreased FGF gene expression and cell proliferation [49]. In addition, these defects are dependent on interactions between the ECM and β 1-integrin signaling. Thus *O*-glycosylation influences the composition of the ECM, which influences a range of cellular responses. Taken together these studies highlight the importance of carbohydrate structures during SMG development and highlight the variety of cellular mechanisms that are influenced by the different types of glycosylation.

1.3.7 Innervation

1.3.7.1 Parasympathetic and sympathetic: Salivary glands are richly innervated by both parasympathetic and sympathetic nerves. The parasympathetic nerves release acetylcholine, which activates the muscarinic receptors to stimulate fluid secretion (Figure 1). The sympathetic nerves control salivation through the activation of α - and β -adrenoreceptors, which stimulate fluid-rich and protein-rich secretion, respectively [5, 50]. Recent research has focused on the instructive role of the developing PSG on SMG development. Ex vivo recombination experiments of epithelium and mesenchyme with or without the PSG show that in the absence of the PSG, expression of epithelial progenitor markers *Krt5*, *Krt15*, and aquaporin 3 (*Aqp3*) are reduced. In addition the number of K5+ cells in the epithelium decreases. Thus, proliferation and differentiation of K5+ epithelial progenitors into K19 cells is dependent on acetylcholine signaling, via the muscarinic M1 receptor and EGFR signaling [31]. Thus parasympathetic innervation maintains the epithelial progenitor population in an undifferentiated state during salivary organogenesis.

Not surprisingly, neurotrophic factors that control PSG function, such as neurturin (NRTN), also influence SMG development. NRTN binds its receptor GFRA2 and signals via RET, a tyrosine kinase coreceptor, and Src-kinase. Mice lacking *Nrtn*, *Gfra2* or *Ret* have smaller parasympathetic ganglia and display defects in salivary gland epithelial function as a result of decreased innervation (reviewed in [2]). Ex vivo cultures of isolated SMG PSG explants show that NRTN increases neurite outgrowth and reduces neuronal apoptosis. In addition, blocking antibodies to NRTN added to ex vivo SMG cultures show reduced branching morphogenesis [51].

In contrast the role of the sympathetic nerves has less effect on SMG development, although they may affect gland function. The most compelling evidence has come from studies of the noncanonical Wnt, Wnt5a. The specific reduction of Wnt5a in the neural crest using Wnt1-

Cre or in the sympathetic nerves using tyrosine hydroxylase (TH)-Cre results in incomplete sympathetic innervation of the SMG. However, no defects were observed in the development of the SMG or in overall tissue patterning, proliferation, migration and differentiation of the neuronal progenitors [52]. Furthermore, using compartmentalized neuronal cultures the Ror receptor tyrosine kinases were shown to be required in sympathetic axons to mediate Wnt5a-dependent axon branching. Further studies are required to determine whether the reduction in sympathetic innervation affects parasympathetic innervation or secretory function in the gland.

1.3.7.2 Axonal guidance cues: Since the innervation of SMGs is important for gland development and function, identifying the mechanisms of axonal guidance in SMGs is important. Semaphorins, a family of secreted and transmembrane axon guidance regulators, influence the development of various organs including the SMG. Semaphorin signaling affects SMG cleft formation where addition of exogenous SEMA3A or SEMA3C increases clefting and branching morphogenesis without affecting proliferation, whereas inhibition of the semaphorin receptor, neuropilin 1 inhibits cleft formation [53]. It was also shown that vascular endothelial growth factor (VEGF), which also binds neuropilin 1, did not affect SMG branching, suggesting the effects were semaphorin-dependent. However, this study did not directly investigate innervation in the gland, and the effects of semaphorins on PSG axons remains to be determined.

Sympathetic axons extend into the SMG along the vascular system. Endothelins are vascular-derived axonal guidance cues for sympathetic neurons. Mice lacking the endothelin 3 or the endothelin receptor type A have reduced sympathetic innervation of salivary glands from the superior cervical ganglion [54]. Endothelins also affect adult SMG function as their local release modulates the autonomic regulation of SMG secretion in rats [55]. These data highlight that endothelial-derived axonal guidance factors control sympathetic innervation of the SMG. The reader is directed to a recent review on the role of nerves in salivary glands [2].

1.3.8 Progenitor cells—It is evident that multiple progenitor populations exist both in the embryonic and adult salivary glands. Many nuclear, cytoplasmic and cell surface markers have been used to characterize salivary progenitors.

Progenitors expressing Kit have the capacity to regenerate damaged SMGs in a mouse model [56]. In both human and mouse adult SMGs, Kit⁺ cells are localized in the intercalated and excretory ducts. In the developing embryonic SMG, Kit is localized in the peripheral epithelial endbud cells and Kit signaling in concert with FGFR2b signaling maintains and expands the Kit⁺Keratin 14 (K14)⁺ distal progenitors [57]. In addition, epithelial Kit⁺K14⁺ cells direct ductal morphogenesis by communicating with the surrounding neuronal niche and proximal K5⁺ epithelial progenitors. This occurs because Kit⁺ cells produce NRTN, which promotes parasympathetic nerve survival and axon extension, which in turn maintains the K5⁺ progenitors and in concert with EGFR signaling promotes their ductal differentiation. Genetic lineage tracing has shown that K5⁺ cells are a progenitor population in the SMG, and K5 expressing cells are mainly localized in the ducts. As discussed in the previous section, the PSG is critical in the maintenance of these K5⁺

progenitors during development [31]. Interestingly, recent genetic lineage tracing experiments have shown that K14+ cells give rise to various cell types in the epithelial compartment. These include acini, myoepithelial cells and ducts, as well as K5-expressing cells. This indicates that K14+ cells are a multipotent epithelial progenitor population in the SMG [57](Figure 3).

Sox2 is important for maintenance of pluripotent stem cells and is required for the formation of several tissues during development. Sox2+ cells are putative stem/progenitor cells in the adult sublingual gland. Long-term lineage tracing experiments using Sox2-tamoxifen inducible Cre/R26-lox-STOP-lox-EYFP adult mice showed EYFP+ cells in the acini and ducts [58]. In addition, fetal Sox2+ progenitors give rise to adult Sox2+ cells in the SMG. Sox2 is expressed during embryonic development within the K5 population, where ~17% of the K5+ cells express Sox2 [59]. However, additional experiments are needed to determine whether Sox2+ cells in the adult SMG are stem cells.

Adult progenitors expressing the Ascl3 transcription factor are in the ducts of mouse salivary glands [60]. Lineage tracing experiments showed that they generated a subset of the adult ductal and acinar cell descendants [61–63]. Genetic ablation of the Ascl3-expressing cells showed that gland development occurred, the K5+ basal cells were present although the gland was smaller, suggesting that adult salivary glands harbors more than one population of progenitor cells and they can compensate for the loss of Ascl3+ progenitor cells. Ascl3+ salispheres generate multiple salivary cell types in culture over time but were not K5+, suggesting that K5 may be a separate population.

Salivary gland progenitors expressing $\alpha 6$ integrin (CD49f) and intracellular laminin were isolated following duct ligation, and differentiate into hepatic, pancreatic or salivary gland-like cells [10, 64]. Similar cells were isolated using heat stress-conditioned rats, where the number of $\alpha 6\beta 1$ -expressing cells increased ~5-fold, and their proliferation and clonal capability increased [65].

2.0 Translation towards therapy

2.1 Clinical need and proposed therapeutic approaches to restore salivary function

Head and neck cancer (HNC) is the fifth most common cancer and radiation therapy is the most common treatment. Therefore, salivary glands are often exposed to radiation and due to their exquisite radiosensitivity, irreversible hyposalivation is common (60–90%). Hyposalivation exacerbates dental caries and induces periodontal disease, causes mastication, swallowing and speech difficulties and affects taste, which impair the quality of life of patients. Understanding salivary gland development may provide a template for gland regeneration as well as tissue engineering approaches to build an artificial gland. Several strategies have been proposed; here we will review gene therapy, cell-based therapies and tissue engineering approaches to develop an artificial gland.

2.1.1 Repair using gene therapy—Gene therapy involves transfer of a gene into cells to treat a disease or correct a cellular dysfunction. A Phase 1 gene therapy clinical trial in patients suffering from radiation-induced salivary hypofunction has recently shown

promising results [66]. This trial involved transfer of the Aquaporin 1 (*Aqp1*) gene via retroductal cannulation of the parotid glands. In addition, human *KGF* (FGF7) gene therapy using a hybrid serotype 5 adenovirus vector in murine SMGs prevents radiation-induced salivary hypofunction [67]. An increase in acinar cell proliferation, number of endothelial cells and saliva flow was observed.

The Wnt/ β -catenin pathway has also been implicated in the control of stem/progenitors in the SMG. Wnt/ β -catenin signaling is activated after ligation-deligation of the main excretory duct, and its forced activation in the basal epithelia expands stem/progenitor cells [35]. Interestingly, damage as a result of radiation does not activate this signaling pathway. However, concurrent transient activation of Wnt/ β -catenin pathway in male mice prevents both acute and chronic hyposalivation by inhibiting apoptosis and preserving the stem/progenitor pool [68]. Further work is required to define specific targets that could be used either for gene therapy or as druggable targets.

The neurotrophic factor, neurturin (NRTN) could also be used in gene therapy to protect the neurons from damage due to acinar apoptosis and loss of the endogenous source of NRTN. Irradiation causes epithelial apoptosis within 1 day, and 3 days later a reduction in parasympathetic innervation due to subsequent neuronal apoptosis [51]. Addition of exogenous NRTN after radiation of fetal SMGs reduces neuronal apoptosis and restores parasympathetic function, which in turn promotes regeneration of the epithelium. Similarly, in human SMGs irradiation reduces parasympathetic innervation [51]. It remains to be determined whether gene therapy with NRTN will protect SMGs from radiation.

2.1.2 Gene activation/silencing—Activation of genes that improve regeneration following radiation is also being studied. Treatment of mice with Alda-89, a selective aldehyde dehydrogenase 3 (ALDH3) activator, enriches for Kit⁺/CD90⁺ progenitors and increases proliferation of salispheres [69]. These SMG progenitors express higher levels of *Aldh3* than non-progenitor cells. Alda-89 infusion may increase saliva production after radiation although optimization of drug dose and treatment duration is required.

Gene silencing approaches may include miRNA or naked RNA treatment that selectively target a single gene or pathway. A retroductal injection of siRNA-coated nanoparticles into mouse SMGs was an effective method to confer radioprotection. siRNAs targeting a proapoptotic *Pkc δ* gene administered prior to radiation prevented apoptosis and improved saliva secretion in irradiated animals [70].

2.1.3 Cell-based therapy—Cell therapy could involve isolating autologous progenitors from a patient biopsy before radiation, expanding and cryopreserving these cells during radiation, and then implanting them into the irradiated gland [56]. Alternatively, a gland bioengineered in vitro may be implanted into the salivary gland space to restore gland function. A recent major advance in the field showed a bioengineered gland made from fetal epithelium and mesenchyme can be transplanted into an adult mouse to form a new functional gland in the adult microenvironment [71]. This bioengineered gland contained a variety of fetal cells, including progenitors of epithelial, mesenchymal, endothelial and neuronal cells. Importantly, the gland reconnected with the existing ductal system and was

functional in terms of saliva secretion, protection of the oral cavity from bacteria, and restoration of normal swallowing. The goal now will be to use induced pluripotent stem cells or adult salivary progenitors to form a bioengineered rudiment that grows into a functional gland in the adult microenvironment.

In vitro spheroid culture of adult salivary gland cells has been used to identify adult progenitors for cell therapy. This salisphere culture enriches for progenitors expressing Kit, Sca-1, and Mushashi-1 [56]. Intraglandular transplantation of 300 Kit⁺ cells isolated from salispheres into irradiated recipient mouse SMGs restored the gland morphology and partly restored function. Furthermore, in serial transplantation experiments, only 100 Kit⁺ cells were required in a secondary transplant [56]. Similarly, salisphere-derived cells that express Kit with CD24 and CD49f also improve saliva production [72, 73]. After transplantation there was an increase in ductal cells and stem cells, normalization of vasculature and reduced fibrosis [73].

There is also the potential to use bone marrow-derived stem cells to regenerate SMGs [74] or even a bioactive lysate of these cells [75], although currently the mechanisms of regeneration are not well understood. In addition, intraglandular transplantation of bone marrow-mesenchymal stem cells improves saliva production, reduces apoptosis and increases microvessel density in irradiated mice. Transdifferentiation into acinar cells following transplantation was observed [76].

Recently, a personal stem cell bank was developed where salivary gland integrin $\alpha 6 \beta 1$ ⁺ cells were cryopreserved for up to 3 years without affecting their genetic or functional stability [77]. In addition, methods to enrich sufficient numbers of adult salivary stem cells for therapy are needed. Interestingly, salivary progenitors can be induced in culture to express pancreatic markers, therefore, they may be a potential source of cells for gland hypofunction and diabetes [10, 78].

2.1.5 Tissue engineering approaches—Tissue engineering of salivary glands requires cells that retain salivary biomarkers and a biocompatible scaffold that recreates the microenvironment of the gland. One approach is to create an artificial gland by seeding cells on 3D scaffold to mimic the in vivo gland microenvironment. Hyaluronic acid (HA) hydrogels can be seeded with primary human salivary gland cells that form spheroid structures, proliferate to form larger acini-like structures and can be maintained long-term in vitro. The structures signal in response to neurotransmitters and continue to secrete amylase when implanted in vivo into rats [79, 80].

Another scaffold is polylactic-glycolic acid (PLGA), which supports the attachment, proliferation and survival of salivary epithelial cells [81]. Furthermore, nanofiber PLGA scaffolds support branching of fetal SMGs and self-organization of dissociated primary gland cells into branched gland-like structures [82]. Lithographically micropatterning curved “craters” that mimic the physical structure of the BM increased the surface area and allowed apicobasal polarization and acinar differentiation [83]. Together, these studies provide a promising outlook for tissue engineering to regenerate salivary glands.

Conclusion

Salivary gland development involves the interaction of multiple cell types including epithelial, mesenchymal, endothelial and neuronal cells. This review is not exhaustive and we deliberately reviewed only recent literature on gland development and regeneration. However, there is still much to learn. For example, the role of the vasculature during development remains to be elucidated. Lineage tracing with an ectodermal-specific Cre is needed to confirm the ectodermal origin of the salivary glands. Little is known about the lineage relationships and the mechanisms that regulate the differentiation of salivary gland stem/progenitors cells. A deeper understanding of these populations will undoubtedly inform the cellular, genetic and bioengineering approaches to repair or regenerate salivary glands.

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Highlights

- Salivary glands develop by branching morphogenesis
- Development involves interaction among epithelial, mesenchymal, neuronal and endothelial cells
- The developing nervous system instructs epithelial progenitor cells
- There is dynamic interaction among multiple epithelial progenitor cells
- Regeneration strategies involve gene- and cell-therapy and tissue bioengineering

Figure 1.

Reciprocal interactions among the epithelium (Ecadherin staining, red), nerves (Tubb3 staining green), blood vessels (Pecam staining green) and basement membrane (Perlecan staining green) regulate branching morphogenesis during submandibular (SMG) and sublingual gland (SLG) development. The brightfield image shows and E13 SMG and SLG cultured overnight, the mesenchyme (Mes) and parasympathetic ganglia (PSG) are also visible. The fluorescent images are projections of multiple confocal sections, Scale bar 100 μ M.

Figure 2.

Proliferation of the progenitor cells occurs in the epithelial endbuds and increases the size of the glands. Cell proliferation in BrdU (red) labeling of an E13 SMG or SMG epithelial explants grown in the presence of FGFs. The epithelia are cultured in an extracellular matrix that contains heparan sulfate, which restricts FGF10 diffusion through the matrix. FGF10 treatment results in cell proliferation only at the tips of the endbuds, whereas FGF7 diffuses through the matrix and induces proliferation throughout the explant. The fluorescent images are projections of multiple confocal sections. The epithelium in the E13 SMG is stained with Ecadherin (green) and the nuclei in all images with DAPI (blue). Scale bar; 100 μ M.

Figure 3.

K14-lineage tracing in the post-natal day 1 SMG. By crossing K14Cre to Rosa^{mTmG} mice, K14+ cells and their progeny are visualized with membrane-bound GFP (mGFP, green). Cell types that are not derived from the K14-lineage express Tomato (mTm, blue). Single confocal sections (2 μ m) show K14+ cells (green) are progenitors of both epithelial acinar (a) and ductal (d) compartments of the SMG. Sections were also stained with an antibody to K5 (red), which shows the K5 cells are in the K14 lineage. Staining with an antibody to K14 (red) shows the endogenous K14+ cells. Refer to reference [57] for more detailed information.