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The contribution of specific cell subpopulations to submandibular salivary gland branching morphogenesis

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Abstract

Branching morphogenesis is the developmental program responsible for generating a large surface to volume ratio in many secretory and absorptive organs. To accomplish branching morphogenesis, spatiotemporal regulation of specific cell subpopulations is required. Here, we review recent studies that define the contributions of distinct cell subpopulations to specific cellular processes during branching morphogenesis in the mammalian submandibular salivary gland, including the initiation of the gland, the coordination of cleft formation, and the contribution of stem/progenitor cells to morphogenesis. In conclusion, we provide an overview of technological advances that have opened opportunities to further probe the contributions of specific cell subpopulations and to define the integration of events required for branching morphogenesis.

Keywords

submandibular salivary gland; branching morphogenesis; development; basement membrane; progenitor cell

Introduction

The developmental process by which the arborized structure of branched organs develops has fascinated biologists for decades. As with other branched organs, the major salivary glands (submandibular, sublingual, and parotid) all undergo a complex process known as branching morphogenesis during embryonic development to yield adult organs having a large surface area to volume ratio. While there are some commonalities in the genes and pathways that drive branching morphogenesis, each organ is unique; thus, it is worthwhile to

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study each organ. In this review, we focus on development of the mouse submandibular salivary gland (SMG), which has historically been the most studied of the salivary glands since these paired embryonic organs can be cultured *ex vivo*, mimicking *in vivo* morphogenesis [1-4] and cellular differentiation [5-7]. For a comprehensive overview, the reader is referred to several recent reviews on salivary gland development [8-13]. Here, we examine several specific questions regarding salivary gland branching morphogenesis towards which recent studies have provided significant insights. We discuss recent insights into the initiation of the gland, the coordination of cleft formation, and the contribution of stem/progenitor cells to branching morphogenesis. We conclude by highlighting the importance of defining and manipulating specific cell subsets within the epithelium and the mesenchyme, and with an overview of resources and emerging technologies that will facilitate the elucidation of molecular mechanisms driving branching morphogenesis.

How is the submandibular salivary gland initiated?

The submandibular salivary placode initiates from the oral epithelium at embryonic day 11.0 (E11.0) and invaginates into the surrounding mesenchyme by E12, which subsequently condenses around the epithelium (Figure 1). Recent Sox17 lineage tracing studies of oral endoderm indicate that all of the major salivary glands of the mouse are not endodermal but instead must be ectodermal in origin [14] (reviewed in [12]), and Wnt1-Cre lineage tracing demonstrated that the mesenchyme is derived from neural crest [15]. Early tissue recombination studies in which epithelium and mesenchyme derived from various sources and developmental time-points were recombined indicated that the mesenchyme has an instructive role in epithelial patterning but that the epithelium retains some autonomy in the control of its own cytodifferentiation, as reviewed in [8]. The epithelium is instructive up until E12.5 and is required to induce fibroblast growth factor 10 (Fgf10) production by the mesenchyme [16], which is then required for subsequent salivary gland development [3, 17]. Since the mesenchyme becomes instructive at E11.5 [16, 18], as distinct cell subpopulations arise, some of these cells may have a function in gland initiation. Acetylcholine-positive neurons that are not neural crest-derived, but rather are derived from nerve-associated Sox10-positive Schwann cell precursors, condense to form the parasympathetic ganglion (PSG) [19]. An endothelial plexus of unconfirmed origin is also present at gland initiation. Since endothelial cells are critical to the initiation of the liver [20], pancreas [21], lung [22, 23], and testis [24, 25] in a non-perfusional manner, the endothelial cell plexus may also be required for salivary gland initiation. The mechanisms by which the epithelium and mesenchyme mediate instructive signaling and the requirements for mesenchymal cell subpopulations in salivary gland initiation merit further investigation.

How is cleft formation coordinated?

Following initiation of the primary epithelial bud at E12, small invaginations of the basement membrane known as clefts form in the surface of this solid epithelial cell mass to start the process of branching morphogenesis. The mechanisms through which clefts initiate remain unclear but mesenchymally-induced activation of autocrine EGF signaling in the epithelium appears to promote cleft initiation [26], and lysophosphatidic acid (LPA) can synergize with EGF [27] (Figure 2). Semaphorin 3A/3C acting through the neuropilin

receptor 1 (Nrp1) [28] may also be involved. Deposition of fibronectin (FN) in clefts is required [29] but may not be the initial symmetry-breaking signal. Ectopic cleft initiation can occur with inhibition of Rho-associated protein kinase (ROCK) or non-muscle myosin (NMM) II function [30], suggesting that low contractility facilitates cleft initiation and that effective morphogenesis requires a contractility-dependent cleft stabilization step. How the placement of cleft initiations is determined remains unknown. Since computational modeling predicts that an optimal level of epithelial cell contractility is required to generate a progressing cleft [31], stabilization of clefts may be the critical event for effective branching morphogenesis.

Cleft progression occurs with the replacement of cell-cell adhesions by cell-matrix adhesions as basement membrane is assembled adjacent to the outer polarized epithelial cell layer in this very narrow structure [32]. Polarized deposition of basement membrane by these cells is maintained by ROCK1 in a Microtubule affinity-regulating kinase 2 (MARK2)/Partitioning-defective 1b (Par1b)-dependent manner [33]. Cleft progression requires FN assembly, which is regulated by ROCK1 in a NMM II-dependent manner to activate integrin $\beta 1$, and FAK, which is required to recruit focal adhesion proteins at the basal surface of the polarized outer epithelial cell population [30, 34]. This pathway may generate a feed-forward signal to propagate clefts [30]. Myosin light chain phosphatase 1 (MYPT1) can balance regulatory myosin light chain (rMLC) or microtubule deacetylase (HDAC6), to control contractility and microtubule acetylation, respectively, which are required for integrin $\alpha 5 \beta 1$ function and FN assembly [35]. Additionally, LIM kinase (LIMK)-mediated control of the actin and microtubule cytoskeletons facilitates FN assembly [36]. Mechanical signals applied by the mesenchyme also likely influence the progress of branching since a low environmental compliance is required to facilitate branching [5, 37]. The assembled epithelial basement membrane translocates from the tip of the endbud into progressing clefts where it accumulates adjacent to the nascent ducts, coincident with epithelial expansion [38, 39]. FN subsequently induces the expression of BTB domain containing 7 (Btbd7) in cells localized near the cleft region, promoting Btbd7-mediated E-cadherin inhibition and Snail expression for a transient, local epithelial-to-mesenchymal transition (EMT) [40]. Association of Taz with E-cadherin and α -catenin is important in downstream ductal morphogenesis [41]. How the EMT is reversed to facilitate the transition of terminating clefts into nascent ducts and how feedback controls generally limit branching and promote subsequent cytodifferentiation is currently unclear.

As clefts progress, the end buds must expand outward [30]. Interestingly, computational modeling predicts that epithelial contractility and proliferation are almost equally important to cleft progression [31]. While mesenchymally produced FGFs stimulate epithelial proliferation via FGF receptor 2b (FGFR2b) [18], mesenchymally-produced Wnt stimulates production of Eda that acts on EdaR in the epithelial cell to activate NF- κ B and Shh to promote branching [42] (Figure 3). Additional mechanisms are required to constrain bud outgrowth. Perforational matrix networks are specifically observed at the tip of the epithelial bud of many branching organs, and new data indicates that the basement membrane regulates the extent of cell outgrowth [38]. Treatment with broad-spectrum protease inhibitors and contractility inhibitors blocked the perforation and dynamic remodeling of salivary gland basement membrane (Figure 3). This study suggests that rapid endbud

expansion is due to a higher flexibility of basement membrane at the tips of endbuds than near ducts and clefts. Bioactive matrix fragments released during proteolysis subsequently stimulate bud outgrowth since membrane-type 2 metalloproteinase (MT2-MMP/MMP14)-mediated release of the collagen IV NC1 domain promotes integrin $\beta 1$ -mediated epithelial proliferation of the epithelium [43]. As the end buds expand, the epithelial cells undergo dynamic movements, and the basement membrane appears to regulate this movement. Time-lapse imaging and tracing of individual cell tracks in a fluorescent reporter mouse during SMG branching morphogenesis, and quantification of region-specific cell velocities revealed that the motility of the outer cells in the SMG endbud is higher than that of cells in the inner bud or in ducts [44], consistent with other work [39, 40]. The most migratory cell population is regulated by contact with the basement membrane via NMM II and integrins [44]. Progressive rearward translocation of the basement membrane results in accumulation of stabilized basement membrane at the base of clefts, which may stabilize emerging ducts. Building on these findings, a better understanding is needed of how the state of the basement membrane impacts specific subtypes of cells to coordinate branching morphogenesis and how mesenchymal signaling impacts the process.

How do epithelial stem cell populations contribute to branching morphogenesis?

Recent studies have contributed significantly to our understanding of where the progenitor populations are in the salivary gland and how they contribute to branching morphogenesis. While many studies have examined progenitor cell populations in postnatal glands, [45-48], three primary progenitor markers have been followed in the embryonic SMG: Achaete-Scute family BHLH transcription factor 3 (Ascl-3)/Sgn1 [49-51], cytokeratin 5 (K5) [7, 52], and kit [47, 52-54] (Figure 4). K14⁺ cells were traced but were found not to label a multipotent progenitor cell population [55]. Lineage tracing indicates that K5⁺ cells in the ducts of early developing glands can generate multiple epithelial cell types [7]. Ascl-3⁺ and K5⁺ progenitor cell populations are apparently distinct [51], whereas K5⁺ and kit⁺ cell populations are independent in early development, localizing in developing/mature ducts and endbuds/proacinar cells, respectively, these markers partially overlap in the same ductal cell populations later in development [52, 54].

Recent work has linked expansion of progenitor cell populations to morphogenesis and has furthered our understanding of the evolution of ducts and acini. Populations of kit+K14+ distal progenitors and kit+K5+ proximal progenitors have been defined [53]. Expansion of K5⁺ cells to grow the ducts by tubulogenesis is regulated by PSG innervation [7]. Nerve outgrowth from the PSG is regulated by Delta-like protein 1(DLK1)-mediated inhibition of NOTCH signaling [56]. PSG-derived vasoactive intestinal peptide (VIP) induces K19⁺ cell proliferation and expansion of these cells into developing ducts, eventually constructing a contiguous lumen via a cyclic AMP/protein kinase A (cAMP/PKA)-dependent manner [57]. miR-200 decreases *Krt5* gene expression in a Fgfr-dependent manner [58], possibly contributing to timing of end bud differentiation. Embryonic kit⁺ cells can be stimulated to proliferate by heparin sulfate modification in a Fgf10/Fgfr2b-dependent manner [59] and Fgfr2b signaling positively regulates kit ligand (kitl) expression within the epithelium [53].

kitl is also produced by the mesenchyme. Convergence of kit and Fgfr2b signaling within the epithelium is required for expansion of kit+K14+ progenitors, as loss of kit signaling reduced both the kit+K14+ and kit+K5+ progenitor cell populations. Interestingly, there is feedback between progenitor populations; kit signaling in the kit+K14+ distal progenitors is required for functional innervation and maintenance of the proximal K5+ progenitors by kit-stimulated production of neurotrophic factors by these distal progenitors [53] (Figure 4). Recent data reveal that FGF via repression of wnt signaling regulates the balance between progenitor maintenance and ductalization and timing of lumenization in the interior epithelial cell population [60]. Further work is needed to understand the lineage of each adult cell type. Additionally, an elucidation of the cellular and molecular mechanisms through which progenitor cell types are regulated to coordinate elaboration of structure and extent of differentiation with control of final organ size will be important for our understanding of organogenesis and also for future regenerative therapies.

Application of emerging technologies to define the roles for cell subpopulations in the control of branching morphogenesis

Interestingly, virtually all of the mechanisms controlling branching morphogenesis described herein involve significant interactions between the epithelium and mesenchyme, from gland initiation to intercompartmental paracrine growth factor signaling to innervation control of epithelial progenitor expansion. Recent studies are revealing a complex cross-talk between the molecular effectors. For example, mesenchymal FGFs and LPA cooperatively promote SMG epithelial branching via FGF-driven autocrine EGF signaling [26] (Figure 3). Upstream of FGFs, platelet-derived growth factors (PDGFs) have been implicated in the regulation of FGF signaling in salivary gland branching, and PDGF AA and BB regulate FGF expression by the neural crest-derived mesenchyme [61]. Similarly, neurturin (NRTN) produced by the epithelial endbuds promotes innervation and stimulates VIP expression by the PSG, which then regulates duct formation [57]. Further, a study demonstrating the dependence of SMG branching morphogenesis on the CXC chemokine receptor 7 (CXCR7) expressed on endothelial cells implies a requirement for developing vasculature in SMG development [62] (Figure 3), as is known in other organs [20-25]. Clearly, future studies in which specific cell subsets can be specifically manipulated are required to define the contributions of mesenchymal cell subpopulations to branching morphogenesis and determine the molecular mechanisms by which cell subsets control gland development. Specific epithelial cell subset manipulation is also needed, such as specific targeting of the basement membrane adherent outer cells and the Btbd7 expressing cleft cells, to better characterize the molecular effectors by which these cell subsets contribute to development and eventual tissue homeostasis.

Several tools and technologies are emerging to facilitate identification of cell subsets and to facilitate their manipulation and molecular characterization. To identify coordinated spatio-temporal gene expression, a map of temporal and spatial gene expression in the SMG and sublingual gland is publicly available [63] (<https://sgmap.nidcr.nih.gov/>), as is the developmental SMG and SLG expression pattern of a subset of proteins [52] (<http://sgdatlas.albany.edu/>) and a mouse embryo transcriptome analysis [64] (<http://>

www.eurexpress.org/). Future work using inducible tissue-specific loss-of-function and gain-of-function in vivo models, enabled by the application of CRISPR-Cas-based technology for genetic engineering [65–67], may facilitate such studies. For manipulation of gene expression in organ cultures, adenovirus [68], adeno-associated virus [69], and lentivirus [35] offer advantages, including the ability to deliver short hairpin RNAs. With the dynamic nature of branching morphogenesis, live imaging will continue to be a critical tool. Future application of intravital imaging [70] and live super-resolution imaging [71] promise to bring new insights into all cellular processes.

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Figure 1. Involvement of multiple cell subpopulations in salivary gland initiation

At E11 the epithelial placode initiates from the oral epithelium and protrudes into the surrounding mesenchyme. Salivary mesenchyme is composed of subpopulations derived from distinct origins, including *Wnt1*+/*PDGFR*+ cranial neural crest cells, *Sox10*+ Schwann cell-derived parasympathetic ganglion (PSG) precursors, and *PECAM*+ endothelial cell (EC) precursors of unknown origin. The epithelium has an instructive role to promote mesenchymal *Fgf10* expression, which subsequently induces epithelial morphogenesis (black half arrows). Possible contributions of the PSG and EC precursors to initiation are not known (red half arrows). By E12, the epithelium forms the primary bud and stalk as the mesenchyme condenses around it. The PSG surrounds the epithelial stalk and a primitive capillary system is established in the mesenchyme.

E12

Cleft Formation

Figure 2. Cooperational signaling in cleft formation to initiate branching morphogenesis

Cleft formation occurs through initiation, stabilization, and progression processes, ultimately maturing clefts into intralobular ducts. Cleft initiation steps are unclear but EGF signaling can stimulate clefts and transient low epithelial contractility facilitates clefts. Coordinated mesenchymal signals, such as FGF and LPA may stimulate cleft initiation. Computational modeling predicts that moderate levels of epithelial contractility stabilize clefts. LIMK-mediated actin stability may facilitate actomyosin contractility. ROCK1/NMMII-mediated actomyosin contraction resulting from LIMK-modulated actin stability stimulate activation of integrin $\beta 1$ complexes incorporating phosphorylated FAK to promote basal FN assembly. MT acetylation and MT stability also promote FN assembly via integrin $\alpha 5 \beta 1$. FN promotes Btbd7 expression in epithelial cells adjacent to the basal clefts, establishing a transient, local epithelial-to-mesenchymal transition (EMT). FN, together with various mesenchymal factors, stimulate epithelial proliferation to accomplish endbud outgrowth simultaneously with cleft progression, as detailed in Fig. 3.

Figure 3. Coordination of multiple processes in bud outgrowth during branching morphogenesis Distinct mesenchymal cell populations interact with the epithelium, including β III tubulin (Tubb3⁺) PSG and CXCR7⁺ primitive capillary EC. Epithelial NTRN production stimulates axonal growth and VIP production by the PSG, which enables ductal tubulogenesis (half arrows) while EC cells have unknown functions (red half arrows). Peripheral basement membrane (BM) remodeling triggered by proteases and facilitated by MYPT1-mediated actin and microtubule balance promotes epithelial outgrowth by proliferation. The BM dynamics make microenvironments that regulate compliance and control cell motility. Collagen IV NC1 fragments liberated by MT2-MMP stimulate epithelial proliferation. Other growth factors and signaling pathways assist branching morphogenesis. FGF signaling, downstream of PDGF signaling, acts cooperatively with LPA to induce EGF-driven epithelial branching morphogenesis and negatively regulate Wnt signaling for duct lumen formation. The outer epithelial cells adjacent to the BM are non-responsive to this FGF signal (red). Mesenchymal Wnt expression can regulate Eda signaling for epithelial cell growth and branching.

Figure 4. Epithelial stem/progenitor cell populations and their potential in developing salivary glands

Three epithelial stem/progenitor cell populations have been identified that can self-renew and differentiate into distinct epithelial subpopulations. $Ascl3^+$ cells can differentiate into acinar and ductal cells. $K5^+$ cells can differentiate into $K5^+/K19^+$ cells, $K19^+$ cells, and $K7^+$ ductal cells and other epithelial cell types. kit^+ cells differentiate into $kit^+/K5^+$ proximal and $kit^+/K14^+$ distal progenitor cells. Interestingly, $kit^+/K14^+$ cell-driven neurotrophic factors such as neurtin (NTRN) regulate $kit^+/K5^+$ cell maintenance through innervation.