

J. Harunaga, J.C. Hsu, and  
K.M. Yamada\*

Laboratory of Cell and Developmental Biology, National Institute of Dental and Craniofacial Research, National Institutes of Health, Bldg. 30, Room 426, 30 Convent Drive, MSC 4370, Bethesda, MD 20892-4370, USA; \*corresponding author, kenneth.yamada@nih.gov

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## ABSTRACT

Salivary glands form during embryonic development by a complex process that creates compact, highly organized secretory organs with functions essential for oral health. The architecture of these glands is generated by branching morphogenesis, revealed by recent research to involve unexpectedly dynamic cell motility and novel regulatory pathways. Numerous growth factors, extracellular matrix molecules, gene regulatory pathways, and mechanical forces contribute to salivary gland morphogenesis, but local gene regulation and morphological changes appear to play particularly notable roles. Here we review these recent advances and their potential application to salivary gland tissue engineering.

**KEY WORDS:** developmental biology, cell-matrix interactions, microscopy, gene expression, cell signaling, tissue engineering.

# Dynamics of Salivary Gland Morphogenesis

## INTRODUCTION

Salivary glands are created during embryonic development by complex and remarkably dynamic processes that yield the final, precisely organized architecture of normal adult glands. Efficient functioning of these salivary glands is essential to produce the approximately half-liter of saliva daily that maintains oral health. Salivary hypofunction and xerostomia can result from local surgery, therapeutic radiation for head and neck cancer, Sjögren's disease, and side-effects of certain medications. Hypofunction can result in severe dental caries and oral ulcers, as well as difficulty in swallowing and a substantial loss of quality of life (Napenas *et al.*, 2009; Vissink *et al.*, 2010).

Normal salivary gland function requires a large surface area of saliva-secreting epithelium packaged efficiently into a compact gland composed of densely packed, well-organized networks of acini and ducts. A key goal of restorative tissue engineering and/or regeneration of damaged or lost salivary glands will be to replace this large expanse of secretory epithelium, ideally packaged in a similarly compact structure. One approach may be to try to mimic nature by learning how embryos generate glands and using these mechanisms to generate gland replacements. As we review below, a recurring theme in recent studies of salivary gland development is the importance of dynamic local cell and tissue re-organization regulated by novel pathways controlling cellular and extracellular matrix interactions.

## BRANCHING MORPHOGENESIS

During embryonic salivary gland development, a simple epithelial bud is dramatically remodeled through repetitive branching (Figs. 1, 2) in a process termed 'branching morphogenesis'. This process generates a secretory organ containing many thousands of acini connected to secretory ducts to provide sufficient secretory epithelium (Patel *et al.*, 2006; Tucker, 2007). Branching morphogenesis also creates other branched organs, such as lungs, kidneys, and mammary and prostate glands (Lu and Werb, 2008; Affolter *et al.*, 2009; Andrew and Ewald, 2010; Costantini and Kopan, 2010; Morrisey and Hogan, 2010). Branching morphogenesis of salivary glands has been studied extensively, providing new insights into oral biology and morphogenesis of other crucial organs (Grobstein, 1953; Melnick and Jaskoll, 2000; Kadoya and Yamashina, 2005; Patel *et al.*, 2006; Tucker, 2007; Gresik *et al.*, 2009; Andrew and Ewald, 2010; Hsu and Yamada, 2010; Larsen *et al.*, 2010; Sequeira *et al.*, 2010).

Although some morphogenetic mechanisms are shared, others are likely to be organ- or process-specific, *e.g.*, the differences between tube and bud formation, and differing modes of branching or extension (Lu and Werb, 2008; Andrew and Ewald, 2010). For example, *Drosophila* salivary glands develop as relatively linear tubular structures, and major recent progress has identified

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key genetic regulatory pathways for their morphogenesis (Andrew and Ewald, 2010; Pirraglia and Myat, 2010). In contrast, mammalian salivary gland development involves more complex three-dimensional branching and tissue remodeling (Patel *et al.*, 2006; Tucker, 2007; Andrew and Ewald, 2010; Hsu and Yamada, 2010). Because of space limitations, we focus this review on research published during the past three years that has provided new insights into the dynamics and mechanisms of mammalian salivary gland development.

### Regulatory Gene and Growth Factor Regulation of Branching Morphogenesis

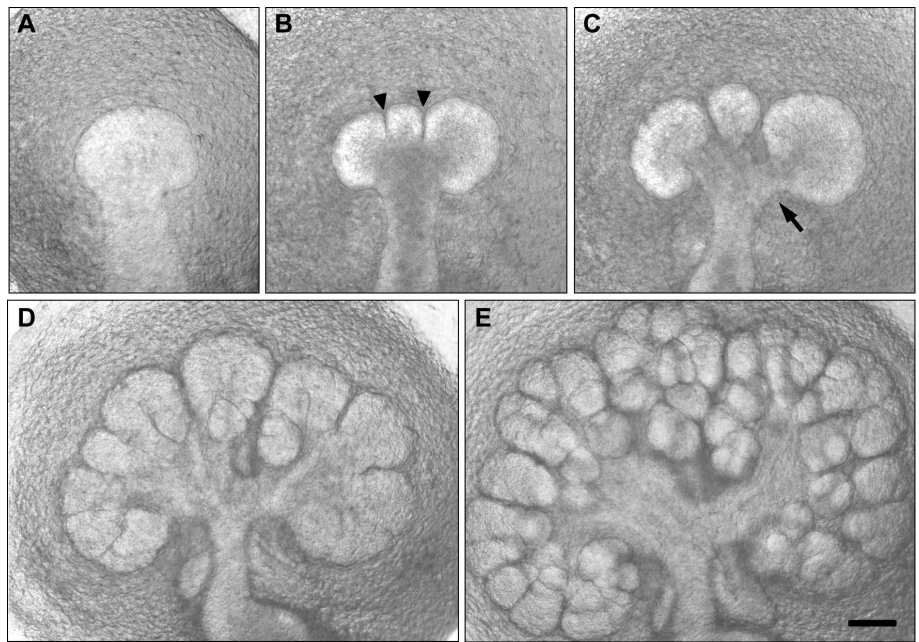
As expected for a complex developmental process, salivary gland branching requires several dozen individual genes and proteins (Fig. 3; Appendix Table), which include transcription factors, growth factors, receptors, and extracellular matrix molecules. In many cases, the precise roles of these molecules remain to be identified, but several new pathways regulating morphogenesis have been defined recently, as reviewed below.

### Salivary Gland Tissue Dynamics

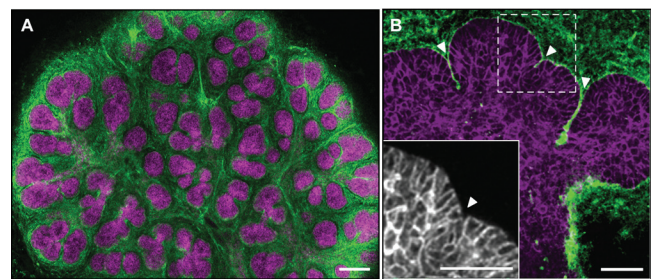
A major challenge facing embryonic organs undergoing branching morphogenesis is to generate organ architecture sufficiently rapidly and reliably during fetal development. For example, the branched submandibular gland structure shown in Fig. 2A took only three days to establish after initiation at mouse embryonic day 12, with continued branching over subsequent days. Consequently, it is not surprising that these tissues undergo rapid major remodeling that involves cycles of cleft formation and bud outgrowth to generate increasing numbers of buds. A recent surprise, however, was the discovery that the cells within these tissues are themselves involved in extensive migratory movements (Appendix Movie 1, discussed below).

### Cleft Formation

Cleft formation can be visualized in real time by time-lapse microscopy (Larsen *et al.*, 2006; Kadoya and Yamashina, 2010; Onodera *et al.*, 2010). One or more indentations appear on the outer surface of a bud, which then progress to form deepening clefts. The site of cleft formation does not appear to be precisely predetermined. Small clefts can form and regress, and sometimes two clefts can merge to form a single larger cleft (*e.g.*, see Larsen *et al.*, 2006) in a highly dynamic process. Because cleft

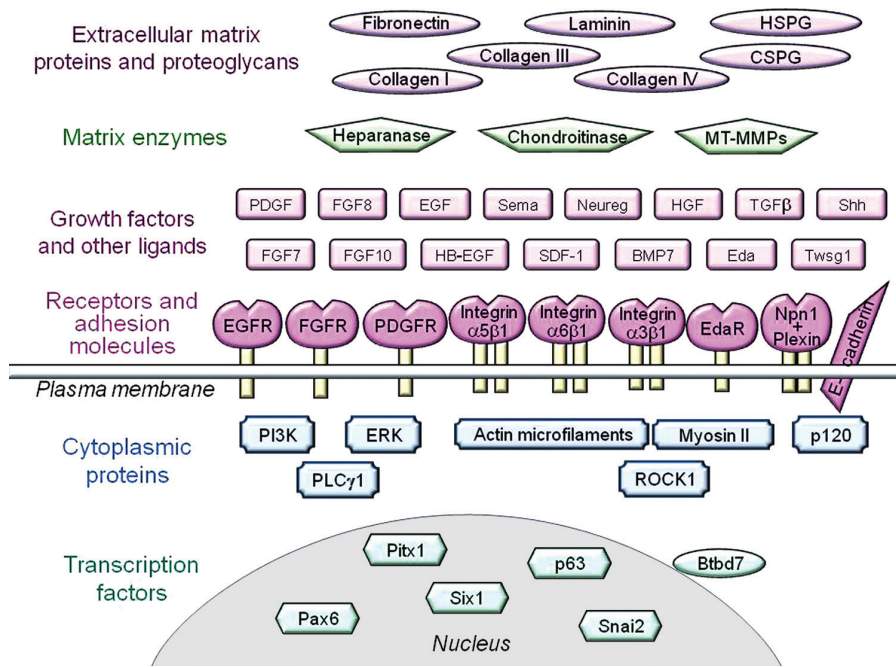


**Figure 1.** Salivary gland branching morphogenesis. Transmitted light microscopy of a living submandibular salivary gland dissected out of a mouse embryo after 12 days of gestation (panel A), placed into explant culture, and photographed after being cultured for an additional 12 hrs (B), 24 hrs (C), 48 hrs (D), and 72 hrs (E). The gland starts as a single bud, which is subdivided by clefts (arrowheads), which progressively deepen. The clefts eventually widen to define secondary ducts (arrow) connected to the main duct. This process of branching morphogenesis progresses rapidly from a single bud to a complex branched structure in 3 days, and it continues to branch as the embryo develops. Scale bar = 100  $\mu\text{m}$ .



**Figure 2.** Immunostaining of salivary glands. Mouse submandibular glands were fixed with paraformaldehyde and stained for the cell-to-cell adhesion molecule E-cadherin and the extracellular matrix protein fibronectin. (A) Low-magnification view of an entire salivary gland at embryonic day 14.5 showing E-cadherin (magenta) at epithelial cell junctions and the extracellular matrix protein fibronectin (green) primarily in the mesenchyme. (B) Higher-magnification view of an epithelial end bud from an embryonic day 13 salivary gland showing E-cadherin staining (magenta) and fibronectin (green) accumulating in clefts at different stages of progression (arrowheads). The inset (grayscale) shows a magnified region of the bud, highlighting the more-columnar cells by E-cadherin staining at the periphery of the bud attached to the basement membrane. Scale bars = 100  $\mu\text{m}$ .

formation is such a central feature of salivary gland branching morphogenesis, it is obviously important to identify the mechanisms of cleft formation and propagation. Additional mechanisms of bud extension and duct formation contribute to branching after clefting.



**Figure 3.** Schematic overview of proteins required for salivary gland branching morphogenesis. Multiple proteins are necessary during embryogenesis for successful branching morphogenesis, as documented in the Appendix Table. These proteins include multiple extracellular matrix proteins and proteoglycans (abbreviations: HSPG = heparan sulfate proteoglycan and CSPG = chondroitin sulfate proteoglycan); several matrix enzymes (MT-MMPs = membrane-type matrix metalloproteinases, e.g., MT2-MMP); many growth factors and ligands (Sema = soluble form of semaphorin, Neureg = neuregulin, Shh = sonic hedgehog, Eda = ectodysplasin-A, and Twsq1 = twisted gastrulation 1); and a variety of transmembrane receptors and adhesion molecules (EGFR = EGF receptor, FGFR = FGF receptor, PDGFR = PDGF receptor, EdaR = ectodysplasin-A receptor, and Npn1+Plexin = non-covalent complex of neuropilin-1 and plexin). Molecules routinely described by abbreviations, such as PDGF, are not defined here.

One approach to understanding cleft formation is to identify the key genes and proteins associated with, and especially required for, branching morphogenesis. Gene expression patterns have been determined with the use of microarrays or SAGE (serial analysis of gene expression) at early embryonic stages (Hoffman *et al.*, 2002; Sakai *et al.*, 2002). This approach has recently identified a daunting number of candidate genes for regulating and mediating branching morphogenesis. Conversely, genetic approaches have proven quite fruitful for identifying essential individual genes, as reviewed previously (Patel *et al.*, 2006; Melnick *et al.*, 2009; Larsen *et al.*, 2010). These two approaches have been combined with functional studies involving tests of candidate growth factors and matrix molecules by localization and function-inhibition studies, which have identified numerous genes and proteins needed for successful salivary gland branching morphogenesis (Appendix Table). These findings provide a solid foundation from which to discover how these molecules interact to form clefts, buds, and ducts. To use an engineering analogy, we currently have a large parts list, and we now need to learn better how the machine works.

Over the past three years, a half-dozen different approaches have provided new insight into the mechanisms of salivary branching. These approaches include: (i) visualizing the dynamic movements of cells and tissues by fluorescence confocal time-lapse microscopy, (ii) establishing roles of mechanical forces in

salivary development, (iii) identifying distinct subsets of cells in developing glands that play unique roles, (iv) determining the gene expression patterns of local subsets of cells involved in different aspects of morphogenesis, (v) identifying regulatory pathways and generating models to begin to explain how gene regulation can mediate branching morphogenesis, and (vi) taking preliminary steps toward reconstituting branching morphogenesis, with the ultimate goal of regenerating salivary gland function. In the following sections, we will review findings discovered using these complementary research approaches.

### Cell Motility during Morphogenesis

Epithelial cells in salivary glands undergoing branching morphogenesis display strikingly high levels of cell motility (Larsen *et al.*, 2006; Wei *et al.*, 2007), as determined by confocal time-lapse microscopy of fluorescently labeled cells in developing glands in organ culture. As shown in Appendix Movie 1, these cell movements generally appear random, although peripheral bud cells can undergo a characteristic circular motion in which they relinquish contact with the basement membrane, move away, and then circle back to the basement membrane; the biological significance of these characteristic motions is not yet clear. In contrast to bud epithelial cells, the cells in duct regions tend to have lower motility. Cell motions virtually cease by the time the mice are born (Larsen *et al.*, 2006). The signaling mechanisms that drive this motility and its function(s) remain to be identified, but this continual restless motion may provide plasticity to the embryonic organ to facilitate rapid remodeling.

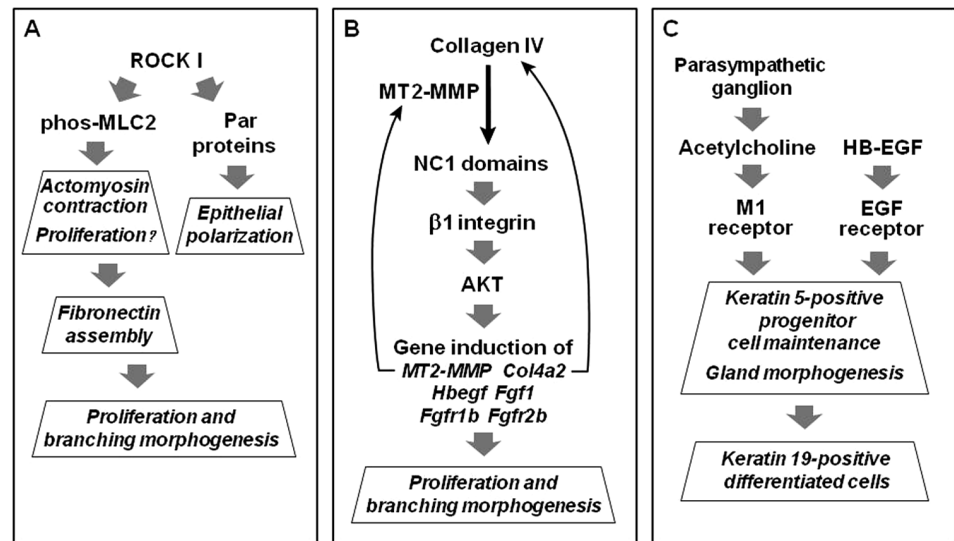
A more detailed examination of these cell motility patterns reveals that the cells at the base of deepening clefts can briefly separate from each other to form gaps. These transient gaps between cells can be visualized as either dark areas in glands in which GFP (enhanced green fluorescent protein) is expressed in the cytoplasm of all cells, or as bright red slits or gaps if the extracellular space is labeled with a fluorescent marker (Onodera *et al.*, 2010; Appendix Movie 2). The local opening of these tiny spaces for cleft progression appears stochastic, but the progression of the main cleft by this transient gap formation proceeds relatively steadily at  $\sim 5 \mu\text{m/hr}$ . Besides these gaps between cells, local folding of the plasma membrane near the base of extending clefts can produce a “shelf” of membrane (Kadoya and Yamashina, 2010), suggesting dynamic interactions between matrix molecules filling the cleft and adjacent plasma membranes. Because efficient cleft formation involves these changes

located at the base of advancing clefts, the local regulatory mechanisms that govern cell motion, cell separation, and cell-matrix interactions in this region are likely to play key roles in cleft formation and progression.

### Mechanical Forces in Salivary Gland Development

The importance of the actomyosin contractile machinery of cells for cell migration and tissue morphogenesis is well known. For example, the actomyosin system is integral to cell migration (Ridley *et al.*, 2003), and actin microfilaments were implicated in salivary gland branching more than 30 years ago (Spooner and Wessells, 1972). However, development of these concepts to understand how forces drive branching morphogenesis has accelerated only recently (Michael *et al.*, 2005; Moore *et al.*, 2005; Daley *et al.*, 2009). Inhibition of Rho kinase (ROCK) and actomyosin contraction disrupts salivary gland morphogenesis; glands also begin to initiate more clefts, suggesting that ROCK/myosin may be suppressing cleft initiation by regulating tension at the periphery of the epithelial buds (Daley *et al.*, 2009; Fig. 4A). Signaling mechanisms that could locally decrease tension at certain points in the bud to allow for cleft formation have not yet been identified. After cleft initiation, ROCK-mediated actomyosin contraction is necessary for fibronectin accumulation at the basement membrane, and for subsequent cleft progression. It is possible that myosin II contractility must first be inactivated to initiate a cleft, but must then provide the mechanical force needed to pull the fibronectin into fibrils using integrin-based adhesions for cleft elongation. These studies primarily used a ROCK inhibitor and the myosin II inhibitor blebbistatin to inhibit contractility mediated by all 3 mammalian isoforms of myosin II, which have slightly different functions, regulators, and physical properties (Vicente-Manzanares *et al.*, 2009). It is possible that each myosin isoform plays a specific role in gland development. Moreover, Rho appears to have another function in addition to regulating myosin II-mediated contractility. ROCK I is also important for epithelial polarity (Yu *et al.*, 2008), and it is apparently required for Par protein localization and basement membrane deposition in embryonic salivary glands (WP Daley *et al.*, *Mol Biol Cell* 21:2350, 2010 [abstract]).

A challenge for the future will be to determine the precise mechanisms of cleft initiation and progression. Current findings suggest that cleft formation is a process with at least two steps involving contractile forces: Tension inhibiting cleft initiation must be locally released to start cleft formation, but then ROCK-regulated actomyosin contraction is needed to promote fibronectin assembly and subsequent cleft elongation. It is likely that



**Figure 4.** New pathways regulating salivary gland morphogenesis. See text and the following citations for details: **(A)** Daley *et al.*, 2009; **(B)** Rebutini *et al.*, 2009; and **(C)** Knox *et al.*, 2010.

additional regulatory factors are needed to coordinate these processes.

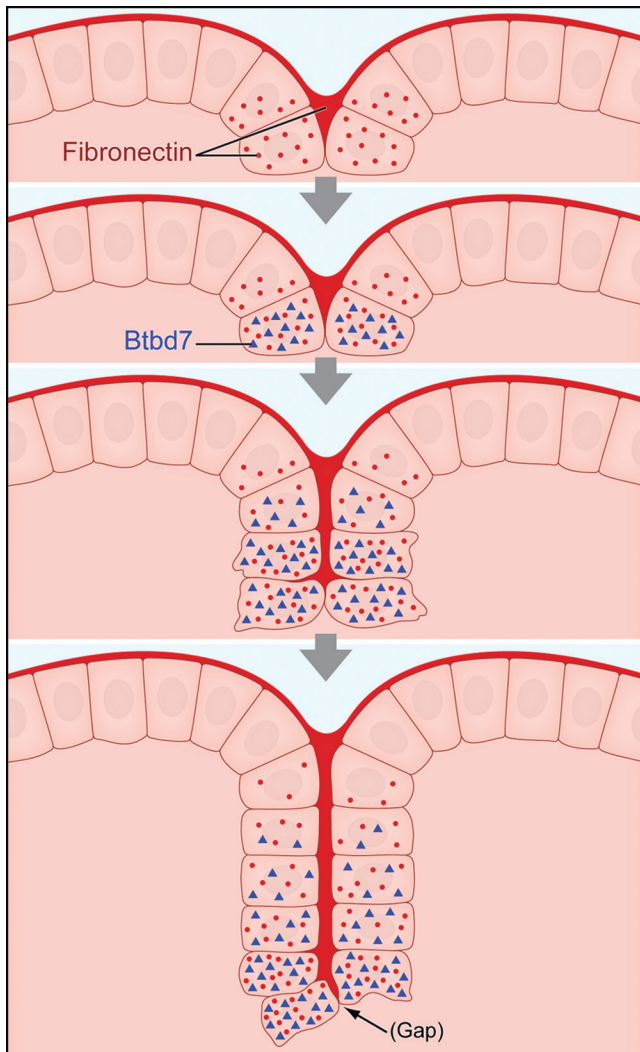
### Importance of Distinct Subsets of Epithelial Cells in Developing Glands

Even though the epithelial cells are surprisingly motile, Walker and her co-workers have established that the outer layer of bud epithelial cells adjacent to the basement membrane is morphologically distinct and different in fate from cells at the center of the buds (Walker *et al.*, 2008). The peripheral cells are more columnar than interior cells, with more organized cell-cell junctions containing the adhesion molecule E-cadherin. Interestingly, cells at the base of forming clefts lose this organization, becoming less columnar and more variable in shape, with gaps forming between these cells (Onodera *et al.*, 2010).

Outer bud cells express the B1 marker characteristic of more mature acini, and these cells contribute to acini rather than ducts later in development (Walker *et al.*, 2008). Interestingly, however, as the clefts deepen and widen, they define secondary ducts (Fig. 1). That is, clefts mature three-dimensionally to form the wide spaces around the secondary ducts connecting end buds to the primary duct. An interesting puzzle is how cells adjacent to the acinar precursors of the end buds become the external cells of ducts as clefts repetitively divide up early buds into many smaller end buds. Two possibilities are that cells change fate, or that new cells are recruited from the interior of the buds to form the external cells of ducts.

### Differential Gene Expression Patterns

Because epithelial cells at different sites in developing salivary glands appear morphologically distinct and have different differentiated fates, it is likely that they have distinct gene expression patterns. Laser microdissection provides a technology for



**Figure 5.** Schematic representation of a new pathway regulating salivary gland cleft formation. The 4 images depict a cleft that initiates and then progresses by cleft deepening. The basement membrane at the basal surface of the peripheral epithelial cells and forming clefts both contain polymerized fibronectin (dark red), and salivary epithelial cells adjacent to the cleft synthesize new fibronectin (red dots). Fibronectin induces Btbd7. As the cleft progresses, Btbd7 decreases in cells near older (upper) parts of the cleft, as Btbd7 is mainly induced at the base of forming clefts. The cleft advances through progressive extension of the zone of fibronectin filling the cleft (Larsen *et al.*, 2006) associated with the relatively stochastic formation of tiny gaps between cells expressing Btbd7. Average times between the second step (definitive cleft initiation) and the third step (advancing cleft) are approximately 2-4 hours, and approximately 3-5 hours between the third and the fourth steps depicted (deep cleft).

identifying these differences in local salivary gland gene expression (Sakai *et al.*, 2003). Epithelial cells in local regions of cryostat frozen sections of embryonic glands can be microdissected away from the rest of the gland tissue by means of a UV laser. Small numbers of cells (*e.g.*, 25-50) can be collected, and the RNA from such samples can be analyzed by SAGE or whole-genome microarrays to determine gene expression patterns at different sites and stages of development (Sakai *et al.*, 2002).

Extensive spatial-temporal gene expression data became available in March 2010 at the SGMAP (Salivary Gland Molecular Anatomy Project) Web site: <http://sgmap.nidcr.nih.gov/>. This public site provides information on both local spatial and temporal developmental expression patterns. Local site-specific gene expression patterns, as determined by Musselmann and his co-workers (submitted for publication), are provided. In addition, this site also provides extensive stage-specific expression data generated by Hoffman and his co-workers in a major extension of their previous developmental analyses (Hoffman *et al.*, 2002).

This National Institute of Dental and Craniofacial Research database can be searched for gene expression patterns of individual genes by name/symbol or description, *e.g.*, FGF or fibronectin, or by using more general gene ontology terms such as “morphogenesis” or “transcription factor”. Such searches immediately identify intriguing patterns of differential gene expression, some of which distinguish peripheral from central bud cells, developing duct from bud cells, and cells at sites of cleft progression from adjacent end bud cells. As expected, initial analyses show a considerable number of genes that are widely expressed at multiple sites (bud, cleft, and duct), comprising 79% of the genes. Interestingly, however, there was site-specific expression of 3% of the genes solely in buds, 3% only in ducts, and 6% only in cells adjacent to clefts, as well as other combinations of expression patterns (Larsen *et al.*, 2010). These distinct patterns of expression for specific genes can then be used to identify candidate gene regulators of branching morphogenesis or local cytodifferentiation, and to develop novel hypotheses in which groups of genes regulated in parallel might guide developmental dynamics or differentiation.

An example of the value of identifying spatial differences in gene expression is seen in recent studies identifying mechanisms of cleft progression. Gene expression patterns in cells immediately adjacent to advancing clefts compared with peripheral end bud cells revealed increased expression of fibronectin, Btbd7, Snail2, and TIMP3, accompanied by decreased expression of the cell-cell adhesion protein E-cadherin (Sakai *et al.*, 2003; Onodera *et al.*, 2010). These findings identified a novel regulatory pathway leading from a matrix protein to a transcription factor, and then to regulation of cell motility and cleft progression (Fig. 5). Specifically, the extracellular matrix protein fibronectin induces a previously uncharacterized gene named Btbd7 (“cleftin”), *e.g.*, within 30 minutes; Btbd7 in turn induces the well-known transcription factor Snail2 as shown by overexpression and siRNA studies, and it decreases levels of E-cadherin at the protein level. The net effect of Btbd7/cleftin function is cell separation and scattering, which appears to account for the local opening of transient gaps to promote cleft progression (Onodera *et al.*, 2010).

### Other New Pathways Regulating Branching Morphogenesis

Classic morphological and signal transduction studies using genetic mutants, organ culture, growth factors, pharmacological inhibitors, antibodies, antisense oligonucleotides, small interfering RNAs, and enzymes have previously identified numerous pathways that are important for branching morphogenesis.

These systems involve a variety of growth factors such as FGF and HB-EGF combined with various extracellular matrix molecules with a variety of downstream signaling mechanisms, including MAP kinase and phosphatidylinositol 3'-kinase pathways. These mechanisms have been the subject of excellent previous reviews (Kashimata and Gresik, 1996; Hieda and Nakanishi, 1997; Melnick and Jaskoll, 2000; Patel *et al.*, 2006). It has become increasingly clear, however, that branching morphogenesis is extremely complex; key roles continue to be identified for more and more regulators that contribute to successful morphogenesis. For example, Fig. 4 summarizes 3 newly identified pathways. As described above, one centers on Rho kinase (ROCK I), which plays roles not only in contractility, but also apparently in epithelial cell polarity. Another novel pathway links the protease MT2-MMP to collagen IV: The release of a biologically active collagen fragment activates an integrin and AKT to induce a series of genes in a feedback mechanism, promoting cell proliferation and branching morphogenesis (Rebustini *et al.*, 2009). A third pathway identifies novel external regulation by signaling from the nerves of the parasympathetic ganglion to maintain progenitor cell function for branching morphogenesis and differentiation (Knox *et al.*, 2010).

An Eda/Edar pathway was known to activate NF $\kappa$ B analogous to the TNF/TNFR pathway (Jaskoll *et al.*, 2003), but recent studies predict a second, more prominent pathway downstream of Eda/Edar in salivary gland morphogenesis through the candidate transcription factor C/EBP $\alpha$  (Melnick *et al.*, 2009). In the Eda mutant mouse, 6 genes known to be important in gland development (Edar, Fgf8, Shh, Egf, Tgfa, and Egfr) are differentially regulated, suggesting a potentially important role for Eda/Edar as an initiation point for these major signaling molecules (Melnick *et al.*, 2009). PDGF is now known to serve as an upstream regulator of FGF signaling (Yamamoto *et al.*, 2008). Involvement of neuregulin and lysophosphatidic acid in morphogenesis, and a novel repulsion phenomenon between developing buds have also been discovered recently (Okamoto *et al.*, 2010). This repulsion is promoted by FGF1, and it contributes to normal spacing between branching buds.

This tremendous regulatory complexity has precedent in other developing systems. For example, kidney development also involves a daunting number of growth factors and regulatory genes (Monte *et al.*, 2007; Brunskill *et al.*, 2008). This plenitude of new, essential regulatory pathways raises the obvious question of how they are all coordinated and interconnected, and how they guide morphogenetic dynamics. Systems analysis may help to clarify these complex interactions (Larsen *et al.*, 2010).

### Unanswered Mechanistic Questions

Many intriguing new questions remain unanswered. A particularly puzzling question involves the mechanisms of salivary gland morphogenetic self-organization and assembly. Unlike most developmental morphogenetic processes involving highly choreographed, spatially precise patterns of gene expression and movements of cells and tissues, salivary gland branching

morphogenesis is surprisingly stochastic: Besides the relatively chaotic cell movements, initiating clefts often form and disappear, and sometimes merge. How the developing gland coordinates the formation of innumerable well-formed and perfectly connected buds and ducts remains a major challenge. Important questions include: How is a cleft initially formed prior to the fibronectin-Btd7-Snail2 cascade? What drives the frenetic movements of the developing epithelial cells, *e.g.*, motility or scatter factors? How are the external cells of buds specified, and how much plasticity do they normally exhibit, *i.e.*, can they interchange with internal cells? How are the secondary ducts formed as clefts deepen to delineate new buds? Many of these questions will require increasingly sophisticated imaging and cell lineage tracking approaches. Linking the movements of single cells to local tissue organization under the regulation of gene expression, extracellular matrix, and tissue tension will require a whole new level of experimental sophistication that should have important implications for understanding the dynamic formation of other organs.

### Steps toward Salivary Gland Reconstitution or Replacement

Because losses of salivary gland function can have such severe effects on oral health, restoration of function by reconstitution/regeneration or even replacement by artificial salivary glands would be a valuable therapeutic option. Although still in the distant future, there are several different approaches that currently appear promising. One strategy is to deliver stem cells to damaged glands. Although the mechanisms of such rescue remain unclear, promising restoration of saliva flow rates has been reported in a rat model system in which glands were previously damaged by irradiation (Lombaert *et al.*, 2008). A second approach seeks to modify the remaining damaged tissue by tissue engineering, *e.g.*, using gene transfer technology to express aquaporin to enhance water transport (Delporte *et al.*, 1997; Shan *et al.*, 2005). These promising approaches that use existing damaged glandular tissue as a structural framework for regenerating a functional salivary gland have been reviewed elsewhere (Baum *et al.*, 2010; Lombaert and Hoffman, 2010; Coppes and Stokman, 2011).

A third possibility would be to try to use the dynamic cell and tissue mechanisms that underlie normal embryonic salivary gland morphogenesis to regenerate damaged tissues or to create an artificial salivary gland. Although obviously quite challenging, this approach would take advantage of the sophisticated self-assembly mechanisms used in normal development to generate the vast numbers of acini of normal glands to provide enough epithelial surface area for adequate production of saliva. Because another oral tissue, the tooth, can be regenerated in mice by transplantation of a bioengineered embryonic tooth germ (Ikeda *et al.*, 2009), it is possible that oral tissues could be replaced by dedifferentiated adult tissue that is guided to recapitulate a normal developmental program to form a functional replacement.

The intrinsic high motility of embryonic salivary gland epithelial cells during normal morphogenesis suggests that salivary

gland architecture is not fixed, but instead involves a highly dynamic, self-assembling system. Consequently, isolated dedifferentiated or progenitor/stem cells might reassemble to generate gland structures. Isolated embryonic salivary gland epithelial cells, or a mixture of epithelial and mesenchymal cells, can in fact readily self-assemble into bud-like structures that closely resemble normal developing salivary tissue and can even synthesize salivary gland differentiation markers (Wei *et al.*, 2007). Gland branching or formation of acinar structures can be stimulated by biologically active peptides from laminin or perlecan (Pradhan *et al.*, 2009; Kadoya and Yamashina, 2010), or by a biomaterial such as chitosan (Yang and Young, 2009). However, one uncertainty about this developmental recapitulation approach involves the question of how to establish the ductal system. One approach might be to bioengineer this system using biomaterials and epithelial cells (Baum, 2000). These challenging regenerative and tissue engineering approaches will benefit from a better understanding of the detailed regulatory and mechanical mechanisms of gland formation.

## CONCLUSIONS AND PERSPECTIVE

Research on salivary gland morphogenesis has advanced rapidly over the past several years with the discovery of novel regulatory pathways and direct visualization of dynamic cell motility. Besides biochemical and gene regulation, mechanical forces are also likely to play important roles in determining how glands develop. In this regard, salivary gland tissue engineering approaches will have the added benefit of providing opportunities to explore the mechanisms of gland formation. That is, experimental approaches to create glands *de novo* will allow researchers to determine which types of local physical, biochemical, and genetic inputs can produce different types of gland morphology and differentiation.

We have learned a great deal about the roles of many individual molecules and multiple specific regulatory pathways, as detailed in this review. However, it is not entirely clear how these numerous growth factors, matrix molecules, gene regulatory pathways, and mechanical forces are integrated during salivary gland morphogenesis. Further research should provide exciting new insight into this important process at the intersection of developmental biology, matrix biology, and tissue engineering.

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