

Morphogenesis and Branching of Salivary Glands: Characterization of New Matrix and Signaling Regulators

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Summary: Salivary glands develop from a simple epithelial bud to a complex organ during embryonic development by the process of branching morphogenesis. Extracellular matrix molecules and signal transduction pathways regulate the dynamics of this complicated process. Fibronectin, laminin $\gamma 2$, and tissue inhibitor of metalloproteinases-3 are differentially expressed in developing clefts or buds of submandibular glands. Integrin receptors for fibronectin and laminin cooperate with site-specific, developmentally regulated gene expression of fibronectin in this process. Fibronectin-mediated crosstalk between integrin cell-to-matrix and cadherin cell-to-cell adhesion systems promotes the formation of clefts. A particularly important intracellular signaling regulator of branching morphogenesis is phosphatidylinositol (PI) 3-kinase, which generates signaling lipids such as PIP_3 . This enzyme and several different growth factors regulate branching. Experimental manipulation of either the fibronectin/integrin or the PI 3-kinase/ PIP_3 systems can control branching morphogenesis.

Conclusion: Progress in understanding the mechanisms of salivary branching morphogenesis promises to provide novel approaches to future tissue engineering or regeneration of damaged salivary glands.

Key words: salivary gland, branching morphogenesis, extracellular matrix, signal transduction, PI 3-kinase, T7-SAGE

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INTRODUCTION

The development of salivary glands from simple precursor epithelial buds to functional glands containing thousands of acini feeding efficiently into duct systems for delivering saliva to the mouth is a highly complex and dynamic process. The mechanism by which a simple bud becomes such a complicated final structure is termed branching morphogenesis, where complicated tissue rearrangements generate the branched structures that subsequently differentiate into mature glands.

Understanding the mechanisms of branching morphogenesis will be essential for developing replacement salivary glands or regenerating damaged tissue. Restoration of salivary function is important to alleviate the suffering of patients with xerostomia. The suffering from dysphagia, mucosal infections such as candidiasis, and rampant caries accompanying loss of salivary function after damage by Sjögren's disease or radiation therapy for head-and-neck cancer affects many patients; for example, there are ~30,000 new cases of the latter

yearly in the US alone. Regardless of whether replacement of damaged salivary glands is ultimately accomplished by implanting bioartificial salivary glands or by regenerative therapy, it will be essential to understand and to control the formation of the complex branched structures that are required to provide adequate fluid-secreting epithelial area in a functional gland.

In the following sections, we will first briefly review the process of branching morphogenesis. We then describe current concepts and new findings concerning the extracellular matrix proteins that regulate or are otherwise required for successful branching, and also describe the crosstalk between cell adhesion systems that contribute to this process. We then turn to a variety of studies on intracellular signaling and review how several growth factors and the PI 3-kinase pathway are particularly important for branching morphogenesis. We conclude by considering ongoing approaches to understanding the dynamics of branching morphogenesis and questions that still need to be answered before considering therapeutic applications.

MATERIALS AND METHODS

Salivary Gland Organ Culture

Salivary submandibular glands (SMG) were dissected from embryonic day 12.5 (E12.5) ICR mice (Harlan). For organ cultures, SMG were cultured on Nuclepore filters (Whatman; 1 μ m pore size) in serum-free Dulbecco's MEM/F12 medium as previously described (Hoffman et al, 2002).

Laser Microdissection

Embryonic SMG were cultured for 10 hours, washed with phosphate-buffered saline (PBS), embedded in Tissue-Tek OCT compound (Sakura) in cryomolds (Lab-Tek), and frozen in dry-ice methanol. All steps were performed under RNase-free conditions. Cryostat sections of SMG were mounted on LMD foil slides (Leica). The sections were stored at -80°C . SMG sections were stained with Toluidine blue after ethanol fixation to visualize epithelial cleft regions as described (http://www.arctur.com/research_portal/resources/protocol_toluidine.htm).

A laser microdissection system (Leica LMD) with a UV laser mounted on an upright automated microscope was used for excision of cleft and end bud epithelial cells. Approximately 500 cells were laser-microdissected with a UV laser from each of ~ 20 SMG cryostat sections (20–30 cells per cleft or end bud region) as depicted diagrammatically in Fig 2. The microdissected samples were transferred to 0.5 ml PCR tubes (Eppendorf).

T7-based Serial Analysis of Gene Expression (T7-SAGE)

T7-SAGE was performed as described (Sakai et al, 2002). This technique is optimized for small numbers of cells, such as from laser-microdissected samples. T7-based RNA amplification was performed on total RNA from approximately 500 cells using the RiboRNA

Amplification kit (Arcturus) according to the supplier's instructions. DNA sequence files were analyzed using SAGE 2000 Software version 4.12 (<http://www.sagenet.org>). SAGE provides 10 bp expressed sequence tags (EST) termed 'SAGE tags' that can be used to identify and quantify transcripts expressed in a tissue when matched against the NCBI SAGEmap database (<http://www.ncbi.nlm.nih.gov/SAGE>). Assessment of significant differences between two SAGE libraries was performed using Monte Carlo simulation analysis (Zhang et al, 1997). A P-value of 0.05 or less was considered significant.

RESULTS AND DISCUSSION

Branching Morphogenesis

The defining characteristic of salivary branching morphogenesis is its dramatic dynamic reorganization of salivary tissues into complex structures. Within days, simple buds undergo complex, choreographed restructuring to form complicated branched structures, which eventually differentiate into a final system of many thousands of acini and ducts. Branching is essential for adequate expansion of the surface area of salivary epithelium to provide the enormous volumes of fluid needed for adequate salivary function. This general biological strategy of expanding the surface area of an epithelium by generating many small epithelial structures such as acini or alveoli is a central feature of the development of a number of organs. Salivary glands, lungs, and kidneys require this process, as do secretory organs including the pancreas, mammary glands, and prostate. In all of these systems, embryonic development requires morphogenesis of new buds separated by clefts during repeated cycles of sequential branching. Fig 1 shows this process in which a tiny initial bud is transformed into a large, highly branched structure.

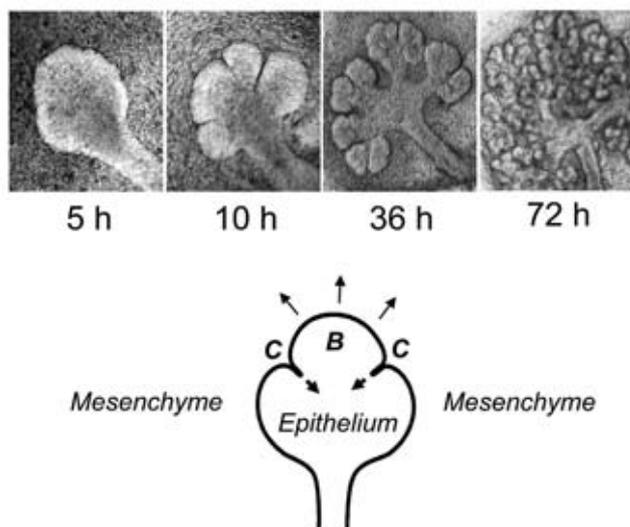


Fig 1 Examples and key topological features of salivary branching morphogenesis. Salivary gland development starts with a single epithelial bud that undergoes cleft formation delineating the end buds. The clefts **C** move inward between epithelial cells, and the buds **B** expand outward. The process repeats over and over to form a multi-lobed structure. Although the surrounding mesenchyme is supportive, branching can proceed in the complete absence of mesenchyme if the epithelium is provided with appropriate matrix and growth factors. Modified from Sakai et al (2003).

Although tissue growth of developing salivary and other glands by extensive cell division is essential to generate the final organ, a noteworthy feature of salivary gland branching is that the individual steps of cleft and bud formation do not require cell division (Nakanishi et al, 1987). Instead, studies in the 1960s suggested a key initial role for local contraction of the cytoskeleton to form indentations and clefts (Wessells et al, 1971; Spooner and Wessells, 1972). Disruption of the actin-based cytoskeleton or inhibition of cellular actomyosin contractility will halt salivary branching (Spooner and Wessells, 1972); Larsen, unpublished data). At the tissue level, however, the salivary gland is actually a multi-layered epithelium, so models postulating local contraction of a single layer of epithelial cells (Spooner and Wessells, 1972) are not likely to explain the process beyond the initial formation of an external indentation in early cleft formation. Instead, other crucial processes are likely to include the formation of fissure-like clefts and outward expansion of buds (Fig 1).

Classical studies of branching morphogenesis identified key roles for extracellular matrix (Bernfield and Wessells, 1970). Proteoglycans and the basement membrane were implicated by initial studies using enzymatic treatment (Bernfield et al, 1972; Bernfield and Banerjee, 1982; Bernfield et al, 1984). Later studies showed that laminin-1 and its $\alpha_6\beta_1$ integrin receptor are important in salivary branching morphogenesis (Kadoya et al, 1995; Hosokawa et al, 1999). Collagen is also involved in branching, perhaps to stabilize clefts, and collagen III has a particularly close association with formed clefts (Nakanishi et al, 1988). These and other findings suggest that various extracellular matrix molecules play significant roles in these early developmental events.

Broad-Based Quest for Roles of Matrix Molecules in Branching

In order to identify novel extracellular matrix proteins that mediate branching morphogenesis or to characterize further molecules thought to contribute to this process, we developed the T7-SAGE procedure for ultra-micro analysis of gene expression of both known and entirely novel genes (Sakai et al, 2002). We compared the expression of genes using libraries of SAGE tags generated from the cells located immediately adjacent to clefts versus those at the tips of buds (Fig 2). Preliminary T7-SAGE data using a total of approximately 40,000 tag sequences from a cleft epithelial library and an end bud epithelial library permitted the identification of 10,000 genes expressed at either or both of these sites using the NCBI SAGEtag database to identify individual genes (see MATERIALS AND METHODS).

Searching for extracellular matrix proteins that were strongly differentially expressed in clefts versus end buds identified the three interesting differences shown in Table 1. The laminin γ_2 chain is a subunit of the basement membrane protein laminin-5, and is expressed in epithelial cells of many embryonic tissues including skin, teeth, and collecting tubules of developing kidney (Sugiyama et al, 1995; Lu et al, 2001). Studies have also revealed that laminin γ_2 is expressed at the edges of malignant carcinomas, consistent with a possible role in inducing cell motility (Katayama et al, 2003). Laminin γ_2 is normally expressed in the epithelial cells of many different embryonic tissues, including the collecting tubules of developing kidney (Sugiyama et al, 1995). Our initial T7-SAGE analyses reveal that laminin γ_2 is expressed highly in the epithelial end bud regions of embryonic day 13 (E13) salivary glands; the relative number of SAGE tags (proportional to gene expression) in cleft sites was 4 compared to 22 in end buds (Table

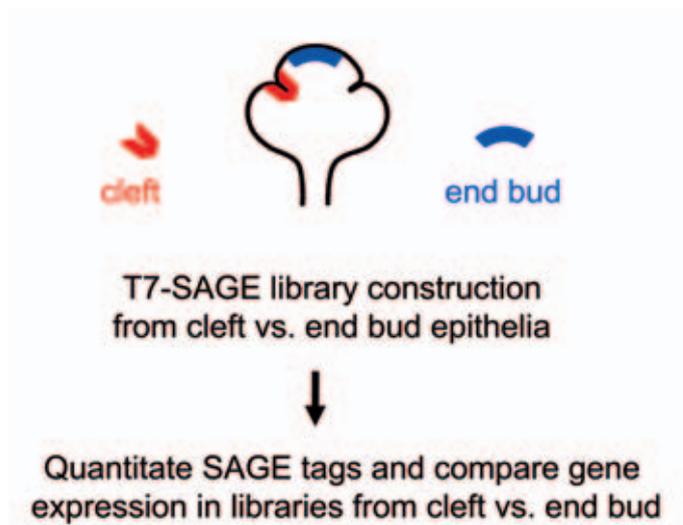


Fig 2 Discovery of differentially expressed extracellular matrix molecules from embryonic submandibular gland utilizing laser microdissection and T7-SAGE. Submandibular glands from embryonic day 13 (E13) mice form narrow, deep epithelial clefts to initiate branching. T7-SAGE libraries were constructed from epithelial cells laser microdissected from clefts (red) or from end buds (blue).

Table 1

Sage tag	UniGene	Name	C*	B*	Total	P
GATGTGTATT	329272	LN γ 2	4	22	26	0.00027
CCAACGCTTT	193099	FN1	124	4	126	<0.00001
TGTAGGCTGT	4871	TIMP3	27	0	27	<0.00001

*C, cleft cells of SMG epithelia; B, end bud cells; LN γ 2, laminin γ 2; FN1, fibronectin
 Preliminary T7-SAGE data showing extracellular matrix genes that are highly differentially expressed in cleft or end bud regions of developing embryonic salivary epithelium.

1). There are at least 16 types of laminin, which differ in their subunit chains, cell-type specificity, and biological activities (Miner and Yurchenco, 2004; Aumailley et al, 2005). Our initial finding suggests that laminin-5 is involved selectively in some end bud function. Because it has been shown to regulate cell migration *in vitro*, laminin γ 2 might regulate the development of embryonic epithelium in various organs by stimulating cell motility; in the salivary gland, it might be involved in outward bud expansion.

Fibronectin is an extracellular matrix (ECM) glycoprotein involved in a diverse range of important biological processes including wound healing, tissue development, and cancer invasion (Yamada and Olden, 1978; Mosher, 1989; Hynes, 1990). According to our T7-SAGE analysis, fibronectin was highly expressed in cells from epithelial cleft regions but not end buds (clefts: 124 tags; end buds: 4 tags; see Table 1). This result directly supports our previous report identifying fibronectin as an essential regulator of branching morphogenesis in embryonic salivary gland, kidney, and lung (Sakai et al, 2003). Using quantitative PCR analyses of laser-microdissected cells, we observed a burst of developmentally regulated fibronectin expression near clefts but little expression in end buds. These findings were corroborated by *in situ* hybridization for fibronectin gene expression. Branching morphogenesis was strongly inhibited by experimental suppression of fibronectin expression by RNA interference using siRNA and by inhibition of fibronectin function using monoclonal or polyclonal anti-fibronectin antibodies. Interestingly, the process of salivary gland branching could be accelerated two-fold by the addition of exogenous purified fibronectin to salivary gland organ cultures. Either plasma fibronectin added to organ cultures or cellular fibronectin added to isolated cultured salivary epithelia dramatically stimulated branching, whereas other matrix proteins including collagen III did not (Sakai et al, 2003). Stimulation of branching morphogenesis also occurs after the addition

of exogenous fibronectin to developing kidney and lung (Sakai et al, 2003; De Langhe et al, 2005).

Extracellular matrix proteins undergo degradation and turnover by matrix metalloproteinases (MMPs). Tissue inhibitors of metalloproteinase (TIMPs) are endogenous protein inhibitors of MMPs. Previous studies have shown that ECM remodeling results from a change in the balance between active MMPs and TIMPs (Leco et al, 2001), allowing TIMPs to regulate ECM degradation by MMPs. A recent study shows that inactivation of TIMP3 impairs lung branching morphogenesis (Gill et al, 2003); in support of our study implicating fibronectin expression in branching morphogenesis, the authors note that fibronectin levels are abnormally low in their system lacking TIMP3, and branching morphogenesis is defective. Our initial T7-SAGE data shown in Table 1 reveal that TIMP3 is highly expressed in epithelial cleft regions compared to end buds of developing salivary gland (clefts: 27; end buds: 0). This increased expression of TIMP3 in the clefts of salivary epithelium undergoing branching morphogenesis could serve to protect locally expressed fibronectin from degradation by MMPs. These findings support the concept that local regulation of expression and stability of specific ECM molecules plays important roles in the mechanisms of branching morphogenesis of salivary and other tissues. In addition, the capacity to stimulate this complex process by the addition of a single purified protein, fibronectin, suggests that tissue engineering with fibronectin and other regulators may become practical.

Matrix Receptors Essential for Morphogenesis

As indicated in the introduction, a previous study implicated the major laminin receptor $\alpha_6\beta_1$ in salivary branching morphogenesis. The relative contributions of specific laminin- and fibronectin-binding integrin receptors to salivary branching morphogenesis were compared by monoclonal antibody analysis (Sakai et al, 2003). Since the major laminin receptor $\alpha_6\beta_1$ and the

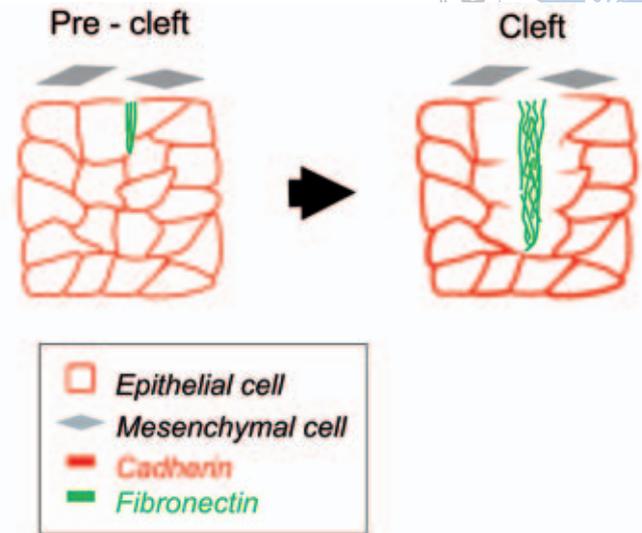


Fig 3 Summary diagram of cell-cell and cell-matrix interactions that mediate salivary branching morphogenesis. Local, developmentally regulated induction of fibronectin is followed by assembly of a fibronectin matrix. Cell-matrix adhesions form to this three-dimensional fibronectin matrix, and they replace cadherin cell-cell adhesions. Modified from Sakai et al (2003).

major fibronectin receptor $\alpha_5\beta_1$ have distinct α subunits and yet also use the same β subunit, specific antibodies can be used to test for distinct or parallel functions. As previously reported (Kadoya et al, 1995), inhibition of α_6 function with anti- α_6 antibodies partially inhibits branching morphogenesis (Sakai et al, 2003). Interestingly, inhibition of $\alpha_5\beta_1$ fibronectin receptor function with anti- α_5 was as least as effective, but the effect was again partial. Combining the two antibodies produced synergistic inhibition, indicating that these integrin receptors and their target ECM ligands have distinct functions. Inhibition of the β_1 subunit present in each of these receptors produced the same effects as the double β -subunit inhibition (Sakai et al, 2003). The fact that there was no further inhibition beyond that produced by combining anti- α_5 and anti- α_6 antibodies indicates that none of the other possible β_1 integrin receptors, including collagen receptors, plays an additional role. Consequently, branching morphogenesis appears to depend primarily on these two ECM systems.

Crosstalk between Adhesion Systems Regulating Cleft Formation

The accumulation of aggregates and fibrils of fibronectin at developing clefts is associated with a concomitant local decrease in E-cadherin localization. This protein mediates cell-to-cell adhesion, and its loss is known to promote cell separation in other biological systems. Although culturing of human salivary HSG cells on tissue culture substrates coated with purified fibronectin does not down-regulate cadherin localization to cell junctions (Yamada, unpublished data), the addition of exogenous cellular fibronectin capable of forming fibrillar matrix induces local loss of cadherin localization (Sakai et al, 2003). The mechanism of this intriguing local loss of cadherin from the side of a cell

adjacent to fibronectin remains to be determined, but it may include local competition for cytoskeletal factors needed for forming cell adhesions or some local signaling process. Whatever the mechanism, these findings provide a simple model for the ability of local expression of an ECM protein to down-regulate local cell-cell adhesion and thereby promote cell separation from adjacent cells to form a cleft (Fig 3).

Signaling Pathways in Branching Morphogenesis

Branching morphogenesis is also known to be regulated by growth factors, and current concepts in the field are summarized diagrammatically in the model shown in Fig 4. There are significant contributions to morphogenetic branching from at least three distinct growth factor signaling pathways. A key pathway involves fibroblast growth factor receptors (FGFRs). In fact, the FGFRs (isoform containing exon 9 IIIc), or FGFR2IIIc, appears to be particularly important for initiation of salivary gland development, since the knockout mouse does not develop salivary glands (De Moerloose et al, 2000). FGFR1 is important for subsequent branching; inhibition of FGFR1 inhibits branching morphogenesis by affecting proliferation but not apoptosis (Hoffman et al, 2002).

Salivary gland branching morphogenesis can be stimulated in organ culture by several different isoforms of FGF that act through the FGFRs receptors (Steinberg et al, 2005). Different FGF isoforms appear to promote different aspects of morphogenesis while interacting with the FGFR1b and especially FGFR2b; FGF7 and FGF10 appear to be particularly important regulators. FGF7 is especially effective in stimulating bud formation, acting through a downstream combination of another FGF isoform, FGF1, and matrix metalloproteinase-2

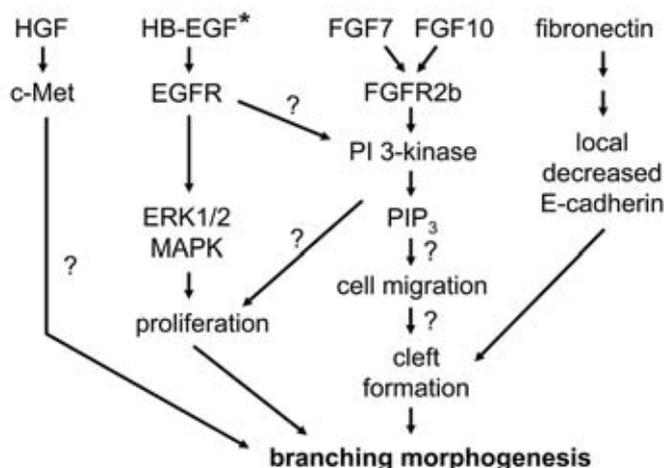


Fig 4 Schematic diagram of signaling pathways in salivary gland branching morphogenesis. Question marks indicate connections that are not yet well-defined. See text for discussion of each pathway.

(Steinberg et al, 2005). FGF7 and its mediators stimulate cell proliferation and produce expansion of buds. In contrast, FGF10 primarily targets the ducts, which elongate in response to this particular isoform (Steinberg et al, 2005). Because heparan sulfates are important for regulation of binding of FGFs to their receptors in other branching organs (Izvolosky et al, 2003), they are likely to be critical for regulating growth factor binding in the salivary gland as well.

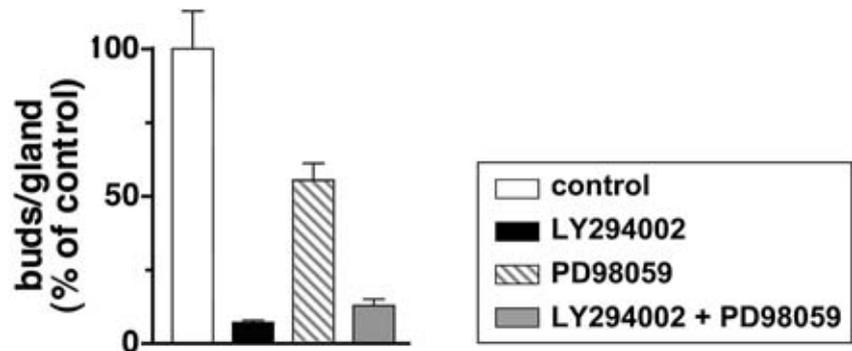
Consistent with the role of FGFRs and other tyrosine kinase receptors in branching morphogenesis, the broad spectrum tyrosine kinase inhibitor genistein was a particularly effective inhibitor of branching morphogenesis (Larsen et al, 2003a). Consistent with the role of FGFRs and other tyrosine kinase receptors in branching morphogenesis, the broad spectrum tyrosine kinase inhibitor genistein was a particularly effective inhibitor of branching morphogenesis (Larsen et al, 2003a). Protein phosphatases often play important biological roles in opposing or modulating the actions of kinases, and various phosphatase inhibitors were also found to inhibit branching morphogenesis (Larsen et al, 2003a, 2003b). These findings suggest that the balance between specific kinases and phosphatases is important during branching morphogenesis, but their exact roles remain to be elucidated.

Downstream effectors of FGFRs receptors include phosphatidylinositol 3'-kinase (PI3K) and ERK1/2 (Steinberg et al, 2005). In a broad screening of a variety of pharmacological inhibitors, a particularly sensitive central regulator was found to be PI3K (Larsen et al, 2003a). Inhibiting this enzyme targeted the process of branching downstream of FGFR2IIIb (Steinberg et al, 2005). The high sensitivity of branching to inhibition by PI3K (Fig 5) indicates it could function downstream of more pathways than simply FGF signaling. In fact, PI3K

also functions downstream of the epidermal growth factor receptor (EGFR) (Koyama et al, 2003, Larsen et al, 2003a). A major product of PI3K is the lipid mediator PIP₃, and exogenous restoration of PIP₃ levels suppressed by PI3K can partially reverse its inhibition of branching morphogenesis by stimulating cleft formation, the initial step in branching morphogenesis (Larsen et al, 2003a). The downstream molecules beyond PIP₃ involved in branching remain to be identified, though Akt is a logical molecule to test due to the correlation between its activity levels, as measured by phosphorylation status, with inhibition by the PI3K inhibitor (Larsen et al, 2003a). PI3K and PIP₃ are well-known regulators of migration, including chemotactic migration in which PIP₃ accumulation at the leading edge of migrating cells promotes directional migration in response to chemotactic agents (Srinivasan et al, 2003; Sasaki et al, 2004; Van Haastert and Devreotes, 2004). It is possible that PI3K also plays a migration-related role in salivary morphogenesis.

As mentioned previously, another pathway involves activation of the EGFR since inhibition of EGFR signaling inhibits branching morphogenesis (Kashimata et al, 2000), and the EGFR1 null mouse shows reduced branching. Since levels of the EGFR ligands, EGF and transforming growth factor- β (TGF- β) are both very low at early stages of salivary gland development (Kashimata et al, 2000), another ligand may be involved. Accordingly, the heparin-binding EGF-like growth factor, HB-EGF, may be the more physiologically-relevant EGFR ligand at this early stage of development. HB-EGF binding to EGFRs receptors, following release from its membrane-bound form by a metalloproteinase, stimulates branching morphogenesis (Umeda et al, 2001). Inhibition of HB-EGF release with either a protease inhibitor or a peptide corresponding to

Fig 5 Crucial role of PI 3-kinase in salivary branching morphogenesis. Inhibition of PI 3-kinase by LY294002 markedly inhibits branching and the formation of buds in SMG organ culture, whereas inhibition of the MAP kinase (ERK) pathway by PD98059 has only modest inhibitory effects. The inhibition of PI 3-kinase was statistically significant at the $P < 0.0001$ level. Modified from Larsen et al (2003a).



the heparin-binding domain inhibits branching morphogenesis (Umeda et al, 2001). Between E14 and E15, expression levels of both EGF and TGF- β increase (Kashimata et al, 2000), at which time activity of these EGFR ligands may become predominant. Inhibition of EGFRs function by PD98059 partially inhibits branching morphogenesis in organ culture. The downstream ERK1/2 pathway plays a moderate role in branching morphogenesis according to one assay, yet little role when assayed in another (Kashimata et al, 2000; Larsen et al, 2003a). Given that expression profiles of growth factors and their receptors change throughout development, it is possible that different pathways differ in importance depending on specific time of development and on the specific assay conditions used.

A third pathway that can contribute to early salivary morphogenesis involves the binding of the highly morphogenic growth factor hepatocyte growth factor (HGF) to the c-Met receptor (Ikari et al, 2003). HGF is a mesenchymal-derived factor that is produced not only by the salivary mesenchyme but also by the nearby tongue muscle to promote branching morphogenesis, acting through the epithelial-expressed c-Met receptor (Ikari et al, 2003). Interestingly, the effects of HGF seem to be required after the initial rounds of branching, since salivary epithelium at embryonic day 14 (E14) is quite responsive to exogenously added HGF, while E13.5 epithelium is not (Ikari et al, 2003). Thus, the tongue may regulate the rapid branching that occurs *in vivo* after E14 via expression of HGF.

The existence of so many growth factor-based regulatory pathways might be expected for such a complex process that is likely to require careful coordination between growth and expansion of individual forming buds and ducts, as well as between different segments of the developing embryonic gland. Precise control of

temporal and spatial expression patterns of growth factors, their receptors, and their modulators appears to be critical for control of branching morphogenesis.

ONGOING APPROACHES AND FUTURE PROMISE

A central future objective of studies of salivary branching morphogenesis is obviously to make progress towards clinical applications using either regenerative or replacement approaches. One approach will be to produce a bioartificial salivary gland that can be implanted in patients with xerostomia to restore salivary fluid flow sufficient to relieve the myriad symptoms and improve quality of life (Baum et al, 1999). Studies testing potential biomaterial and matrix components of such a first-generation system showed that fibronectin is an excellent substrate for salivary epithelial cell attachment (Wang et al, 1999; Aframian et al, 2000). Although issues of host response and safety could be addressed (Aframian et al, 2001, 2002b), a stumbling block involves the type of cell to be used (Aframian et al, 2002a). Current thinking suggests that the use of epithelial cells from each patient or stem cells may be necessary in order to preserve crucial cell junctional stability involving tight junctions, which are essential for a water-tight seal when water is pumped into the lumen of such artificial glands. In addition, such tissue engineering approaches will require further research on engineered scaffolds or matrices to support and organize the epithelial cells.

An alternative or potentially overlapping approach involves cell-based regeneration strategies. Seeding of damaged salivary glands with morphogenesis-capable cells is an attractive option. However, these cells would need to be activated and able to undergo appropriate local organization and branching morphogenesis. Thus,

a crucial goal will be to tissue engineer cells so that they can move and rearrange themselves to organize local tissue structures; they should ideally also be able to undergo a process similar to normal embryonic branching morphogenesis in order to expand the epithelial surface area sufficiently to provide adequate fluid production. Thus, studies of cell motility of developing salivary tissues, the capacity of these cells to self-organize, and their ability to produce buds and undergo effective branching morphogenesis will also be important. Although ongoing studies are encouraging in these areas, future work will require extensive investigation to control these cellular functions sufficiently to make regenerative approaches practical at a clinical level.

An understanding is also needed of the regulatory steps that govern local formation of clefts, i.e. the molecular mechanism that specifies the site destined to become a cleft. The striking induction of local fibronectin expression we have described is likely to be controlled by specific transcription factors that need to be identified. Conversely, bud-specific gene expression is likely to be important for bud expansion. It should be possible to identify candidate regulatory molecules by T7-SAGE and microarray approaches that further compare gene expression patterns in clefts compared to bud regions.

By understanding the molecular and biological mechanisms that initiate and control branching morphogenesis, the promise of new therapeutic approaches for gland replacement or cell-based regeneration may eventually become a reality.

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