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Review Articles

Molecular Analysis of Salivary Gland Branching Morphogenesis

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Abstract: Recently, clinicians and scientists have focused on tissue engineering for regenerative medical therapy. This approach promises to provide remarkable clinical breakthroughs for the future. In oral and craniofacial medicine, most scientific approaches to tissue engineering currently involve tooth and bone, while little progress has been made toward regenerating organs such as salivary gland. To develop strategies for salivary gland regeneration, it will be important to understand the molecular mechanisms of normal salivary development. This mini-review describes a recently developed and tested set of approaches for identifying and characterizing molecules essential for branching morphogenesis and other developmental processes. It shows the value of using laser microdissection and the new process of T7-SAGE for gene discovery of putative candidate molecules that may be crucial regulators or mediators. We describe a stepwise series of associated strategies for reliable identification and functional testing of a candidate molecule, as well as its successful application to a specific candidate molecule originally identified by T7-SAGE.

Key words: branching morphogenesis, salivary gland, molecular analysis, T7-SAGE, laser microdissection

Introduction

Branching morphogenesis is a complex developmental process that is required for successful formation of many organs, such as salivary gland, lung, kidney, mammary gland, and prostate. It provides a means of generating many small substructures, e.g. acini or lobules, for effective secretion or exchange of gases or liquid. In general, it involves repeated cycles of formation of clefts (or indentations) and buds to form the three-dimensional, branched tissue pattern that characterizes of many organs[1,2]. Although studied extensively, the precise molecular mechanisms of branching morphogenesis are not well understood, and it appears likely that different degrees of active outgrowth of buds and formation of clefts contribute to the formation of different organs. Many studies have previously reported that the overall process of branching morphogenesis depends on epithelial-mesen-

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chymal interactions, growth factors and extracellular matrix (ECM) protein[1,2].

Although scientists have identified many individual molecules that are present or change in quantity as organs develop, they know little about how these molecules interact to regulate or to mediate the process. Yet such information will be vital in one day learning how to efficiently engineer replacement organs.

We will describe specific strategies to identify and to characterize the patterns of expression and the biological importance of specific molecules essential for branching morphogenesis. They depend on the existence of functionally important differences in the levels and location of expression of a specific gene needed for morphogenesis. These differential expression patterns are identified using a global approach to quantify gene expression by combining laser microdissection (LMD)[3] to isolate mRNA from potentially functionally distinct regions of tissue, followed by amplification and serial analysis of gene expression (SAGE)[4]. The tissue for laser microdissection consisted of embryonic 13-day salivary glands, which were cultured on membranes[5]. Regions corresponding to cleft and bud epithelia of frozen-sectioned salivary gland were laser-microdissected[6]. Profiles of gene expression in salivary epithelium from small amount of cells were identified by T7-SAGE[7]. In this review, we first describe the overall strategy, then a specific example of gene discovery in salivary branching morphogenesis using laser microdissection and T7-SAGE. The molecule first successfully characterized by these approaches and shown to be an essential molecule in epithelial branching morphogenesis is the ECM protein fibronectin.

Results and Discussion

A general strategy to characterize molecules essential for development

The following series of steps outlines general approaches for a project to identify and characterize crucial regulatory molecules or mediators in salivary and other developmental processes. A similar approach can be applied to characterization of pathological processes including cancer.

1. Establish preliminary evidence that the biological process is likely to involve specific changes in gene expression. For example, is the process readily and rapidly blocked by inhibition of mRNA synthesis?
2. Identify specific candidate genes regulating or mediating the biological process by determining differential gene expression patterns. A very sensitive, reliable method is T7-SAGE (described below).
3. Once a candidate molecule is identified, determine its pattern of mRNA expression. Overall patterns can be established by in situ hybridization using tissue sections. Quantification can be performed by real-time RT-PCR, e.g. using laser-microdissected samples.
4. Determine its pattern of protein localization and expression, e.g. by immunofluorescence localization.
5. Test the role of the molecule by suppressing its function, ideally at both the mRNA level (e.g. with siRNA or antisense oligonucleotides) and the protein level (most commonly using anti-functional antibodies).
6. Experimentally mimic or exaggerate the effect of the molecule by reconstitution to a deficient mutant or by over-expression or mis-expression of either the molecule or its cDNA.
7. Explore the molecule's mechanism of action, e.g. in organ culture or *in vitro*.

1. Establish preliminary evidence that the biological process is likely to involve specific changes in gene expression.

From embryonic 12.5 (E12.5) to embryonic 13.5 (E13.5) (Fig. 1a, b, c), salivary gland epithelium undergoes branching morphogenesis. This developmental process is initiated by the formation of shallow clefts in a single bud (Fig. 1b). These clefts deepen to subdivide the single bud into multiple smaller buds, freeing them to branch in different directions and form increasingly more intricate, preprogrammed three-dimensional patterns[1]. In order to determine if changes in gene expression are required for this process, we treated salivary glands isolated from E13 embryos with the transcriptional inhibitor actinomysin D and assayed for branching morphogenesis after 48hr in organ culture. After 48hr, branching was reduced by 91% in the presence of 40nM actinomysin D relative to controls (M.L., unpublished data). These data indicated that branching morphogenesis requires changes in gene transcription.

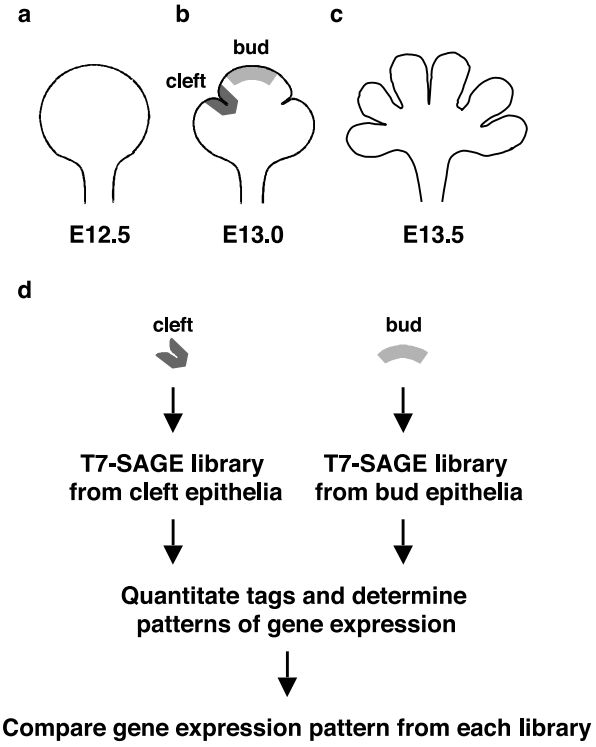

Fig. 1 Schematic representation of epithelial branching morphogenesis of mouse embryonic salivary gland. Submandibular glands from embryonic day 12.5 (E12.5) mice (a) generally consist of a single epithelial bud, which progresses to a multi-lobed structure by E13.5 (c). During this process, the gland has rounded buds separated by narrow, deep clefts that initiate branching (b).
Strategy and approach of laser microdissection and T7-SAGE (d). Salivary epithelial regions isolated by laser microdissection are shown diagrammatically from cleft epithelium (red) and bud epithelium (green). T7-SAGE libraries were produced from tissues isolated from each region. Gene expression patterns from cleft and bud libraries were compared.

2. Identify specific candidate genes regulating or mediating the biological process by determining differential gene expression patterns.

The authors were interested in identifying the genes that regulate cleft formation, the initial step of branching morphogenesis. Specifically, we wanted to identify differentially expressed genes by profiling gene expression in specific cell populations from the clefts and buds of the salivary epithelium (Fig. 1d). We used laser microdissection (LMD) of cryostat sections of developing submandibular glands to isolate the tissue that was used to prepare RNA.

While there are many techniques available for global gene expression profiling, we decided that to profile gene expression in developing salivary glands we would use a modification of the SAGE (serial analysis of gene expression) technique that we developed known as T7-SAGE[7]. SAGE has an advantage over microarray analysis in that it provides absolute transcript numbers in a digital format[4,8]. Microarrays have the advantages of being relatively easy to use and more suitable for high-throughput applications; however, mRNA quantitation is more accurate with SAGE than with microarrays[9]. A major disadvantage of SAGE is that it requires microgram quantities of starting poly(A)$^+$ mRNA, which prevents its use when mRNA is limited. Since we had to start with a limited amount of RNA, we incorporated two cycles of a high fidelity T7-based RNA amplification as the initial amplification step in the protocol[7]. We then generated two SAGE libraries from each pool and sequenced greater than 20,000 SAGE ditags from each library.

3. Once a candidate molecule is identified, determine its pattern of mRNA expression.

Unexpectedly, initial T7-SAGE data showed that cleft epithelium expressed an extracellular matrix (ECM) protein, fibronectin (FN)[10], higher than bud epithelium. While ECM proteins such as Collagen III[11] and basement membrane components such as laminin and proteoglycans are known to be required for salivary branching in general[12] we were surpised to find that fibronectin might be expressed specifically in cleft sites. To verify the T7-SAGE fibronectin expression data, we used two methods to confirm the changes in gene expression. First, we performed quantitative reverse-transcription polymerase chain reaction (RT-PCR) using the same RNA preparations that were used to generate the SAGE libraries. RT-PCR confirmed that fibronectin mRNA was expressed 16-fold higher in cleft epithelial cells than in bud epithelium. To image the changes in expression in the tissue itself, we performed in situ analysis on whole mount salivary glands. *In situ* analysis confirmed that fibronectin was expressed by the salivary gland epithelium and, further, that it was expressed higher in cleft than bud epithelium[6].

4. Determine its pattern of protein localization and expression, e.g. by immunofluorescence localization.

Immunofluorescence analysis for fibronectin with methods optimized for staining epithelium revealed intense staining for fibrils of fibronectin in narrow clefts in mesenchyme-containing cultures. This staining complemented the RT-PCR and in situ techniques that showed higher expression of fibronectin by epithelial cells adjacent to clefts. In addition, we saw staining in clefts of mesenchyme-free cultures (Fig. 2a-k), verifying that cleft epithelial cells are capable of synthesizing fibronectin in the absence of mesenchyme[6].

5. Test the role of the molecule by suppressing its function, ideally at both the mRNA level (e.g. with siRNA or antisense oligonucleotides) and the protein level (most commonly using anti-functional antibodies).

To test the hypothesis that expression of fibronectin at cleft sites was critical for branching morphogenesis, we used small interfering RNAs (siRNAs)[15] to knock-down fibronectin expression in salivary gland epithelium. SiRNAs developed against mouse fibronectin mRNA inhibited synthesis and accumulation of fibronectin in cleft sites, which resulted in a decrease in branching morphogenesis. While we found that knocking down fibronectin gene expression inhibited branching morphogenesis[6], we also wanted to verify that inhibiting fibronectin protein function could block branching. Anti-fibronectin function-blocking antibodies[16] prevented salivary cleft formation and branching in a dose-dependent manner. In addition, antibodies against β_1, α_5 or α_6 integrin subunits, the first two of which together comprise the classical fibronectin receptor, inhibited salivary branching[6]. These results implicated fibronectin function and also that of the fibronectin receptor as playing a critical function in cleft formation, the initial stage of salivary gland branching morphogenesis.

6. Experimentally mimic or exaggerate the effect of the molecule by reconstitution to a deficient mutant or by over-expression or mis-expression of either the molecule or its cDNA.

We added exogenously fibronectin to salivary gland cultures and found that it substantially promoted salivary gland cleft formation and branching in a dose-dependent manner, whereas other matrix proteins had no effect. In addition, we found that exogenous fibronectin was capable of replacing the function of endogenous fibronectin in glands depleted of fibronectin by siRNA; it restored branching in siRNA-treated salivary glands[6].

7. Explore the molecule's mechanism of action, e.g. in organ culture or *in vitro*.

We observed in cleft sites that there was a decrease in the E-cadherin[13] cell adhesion molecule in cells adjacent to nascent fibronectin fibers. Based on this observation, we hypothesized that a direct mechanism by which fibronectin might regulate cleft formation could be to induce the loss of E-cadherin cell-cell adhesions and to replace these adhesions with cell-matrix adhesions. To test this possibility, we used human salivary-gland epithelial cell cultures[14] (the HSG cell line) expressing membrane-bound cadherin and treated them with pre-aggregated cellular fibronectin. Although fibronectin-coated beads were previously found to produce only a modest (30%) decrease in global localization of cadherin[17], we found a striking decrease in local cadherin expression (Fig. 3a) associated with regions of accumulation of the pre-aggregated cellular fibronectin (Fig. 3b), suggesting that accumulation of fibronectin replaces cadherin cell-cell contacts with fibronectin-associated cell-matrix contacts[6].

This local suppression of cadherin localization by the formation of fibronectin-integrin adhesion complexes on epithelial cells provides a previously unknown mechanism for the local loss of intra-epithelial adhesion needed to form deep clefts. We speculate that the predicted resultant loss of cell-cell adhesion provides a way for deep clefts to form. Fibronectin might also be involved in maintenance of clefts through regulation of collagen deposition, which is thought to be essential for cleft stabilization.

After identifying regulation of cadherin-mediated cell adhesion as a possible mechanism of fibronectin's effects in salivary gland cleft formation, we also examined fibronectin expression in other organs to determine whether fibronectin might play a general role in branching morphogenesis. Similar observations were made in other organs, where we found that fibronectin accumulated at sites of indentation, block-

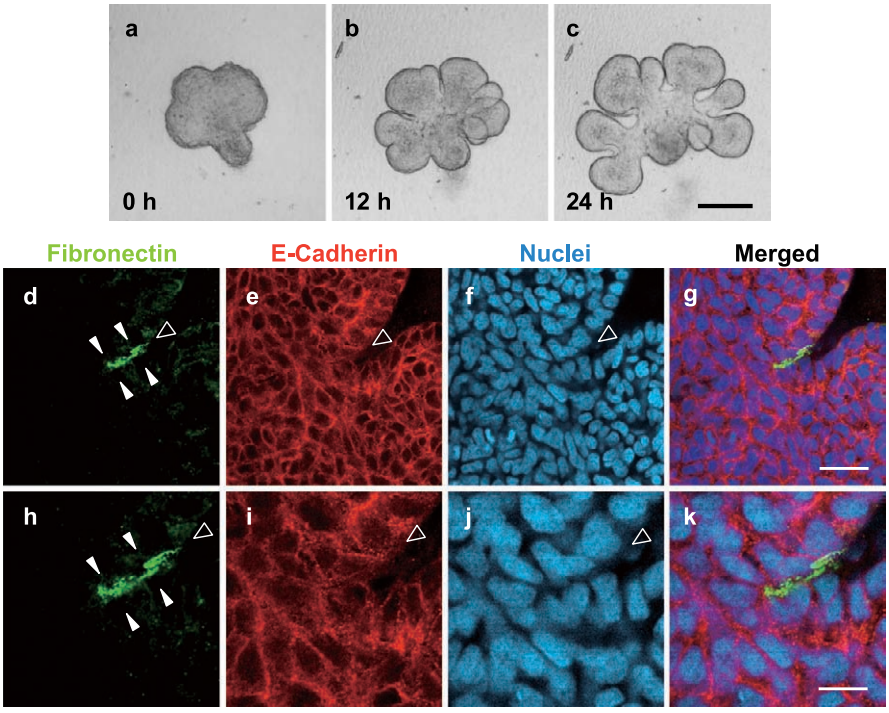

Fig. 2 Focal expression of fibronectin in isolated salivary epithelial rudiments during branching morphogenesis. Epithelia isolated from 13-day mouse embryos using dispase and mechanical separation were cultured in the complete absence of mesenchyme, similar to methods previously reported[18], on a Nuclepore membrane in Matrigel in serum-free DMEM/F12 medium with 100 ng/ml FGF7 and 1 ng/ml for 0 h (a), 12 h (b), or 24 h (c). Scale bar, 100 μm. Immunofluorescence localization of fibronectin was determined during branching morphogenesis of isolated mouse embryonic salivary epithelium cultured on membranes for 12 hours after staining with anti-fibronectin and imaging by confocal laser-scanning microscopy (d-k). Images g-I are enlarged two-fold compared to d-f for comparing location in the epithelial rudiment with localization of accumulations of fibrillar fibronectin. Triple-staining of the same confocal section with anti-fibronectin (green; d and h), anti-E-cadherin (red; e and i), or SYBR green I (blue; f, j), as well as with all three colors merged together into one image (g and k). The open triangles indicate clefts, and the filled triangles outline regions of fibronectin expression. Fibronectin accumulates focally during cleft formation. Scale bar (g), 20 μm; scale bar (k), 10 μm. Images taken from Supplementary Information provided for Sakai et al[6].

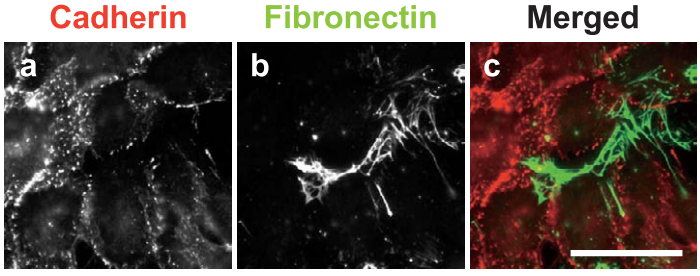

Fig. 3 Suppression of cadherin localization immediately adjacent to fibrils of exogenously added cellular fibronectin in human salivary gland cell (HSG) cells. HSG cells were treated for 24 hours with pre-aggregated chick cellular fibronectin from a suspension pre-aggregated at 25 μg/ml. After fixation with methanol-acetone, the cells were stained with anti-pan-cadherin antibody (a) or anti-fibronectin antibody (b) and photographed with a chilled CCD camera on a Zeiss Axiophot fluorescence microscope. Note the local reductions of cadherin staining in the membranes of epithelial cells immediately adjacent to the fibrils of cellular fibronectin. Scale bar, 20 μm. Images from Supplementary Information provided for Sakai et al[6].

ing fibronectin function inhibited branching, and exogenous fibronectin promoted branching. Importantly, these data indicated that our findings were not specific to salivary gland but that fibronectin is a general regulator of branching morphogenesis.

Conclusions

Following the procedure outlined above, we concluded from our studies that fibronectin expression is required for cleft formation in glandular branching morphogenesis. These studies are significant in that we demonstrated that T7-SAGE gene expression analysis of laser microdissected tissues provides new possibilities for the characterization of region-specific expression profiles in other complex, heterogeneous tissues. As outlined in this review, we believe that the combination of amplification and profiling technique of T7-SAGE with inhibition and augmentation approaches similar to those that we have used to address the mechanism of branching morphogenesis of the salivary gland will be broadly applicable to developmental and pathological processes in oral biology and other systems.

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Reference

1. Davies J. A.: Do different branching epithelia use a conserved developmental mechanism? Bioessays 24: 937–948, 2002.
2. Metzger R. J., and Krasnow M. A.: Genetic control of branching morphogenesis. Science 284:1635–1639, 1999.
3. Kolble K.: The LEICA microdissection system: design and applications. J Mol Med 78: B24–25, 2000.
4. Velculescu V. E., Zhang L., Vogelstein B., and Kinzler K. W.: Serial analysis of gene expression. Science 270: 484–487, 1995.
5. Larsen M., Hoffman M. P., Sakai T., Neibaur J. C., Mitchell J. M., and Yamada K. M.: Role of PI 3-kinase and PIP3 in submandibular gland branching morphogenesis. Dev Biol 255: 178–191, 2003.
6. Sakai T., Larsen M., and Yamada K. M.: Fibronectin requirement in branching morphogenesis. Nature 423: 876–881, 2003.
7. Sakai T., Larsen M., and Yamada K. M.: Curr Protocols Cell Biol John Wiley & Sons, New York, 2002, 19.13.11–19.13.30.
8. Ishii M., Hashimoto S., Tsutsumi S., Wada Y., Matsushima K., Kodama T., and Aburatani H.: Direct comparison of GeneChip and SAGE on the quantitative accuracy in transcript profiling analysis. Genomics 68: 136–143, 2000.
9. Polyak K., and Riggins G. J.: Gene discovery using the serial analysis of gene expression technique: implications for cancer research. J Clin Oncol 19: 2948–2958, 2001.
10. Hynes R. O.: Fibronectins (Springer-Verlag, New York, 1990).
11. Nakanishi Y., Nogawa H., Hashimoto Y., Kishi J., and Hayakawa T.: Accumulation of collagen III at the cleft points of developing mouse submandibular epithelium. Development 104: 51–59, 1988.
12. Bernfield M. R., Banerjee S. D., and Cohn R. H.: Dependence of salivary epithelial morphology and branching morphogenesis upon acid mucopolysaccharide-protein (proteoglycan) at the epithelial surface. J Cell Biol 52: 674–689, 1972.
13. Menko A. S., Zhang L., Schiano F., Kreidberg J. A., and Kukuruzinska M. A.: Regulation of cadherin junctions during mouse submandibular gland development. Dev Dyn 224: 321–333, 2002.
14. Shirasuna K., Sato M., and Miyazaki T.: A neoplastic epithelial duct cell line established from an irradiated human salivary gland. Cancer 48: 745–752, 1981.
15. Elbashir S. M., Harborth J., Weber K., and Tuschl T.: Analysis of gene function in somatic mammalian cells using small interfering RNAs. Methods 26: 199–213, 2002.
16. Yamada K. M., and Olden K.: Fibronectins-adhesive glycoproteins of cell surface and blood. Nature 275: 179–184, 1978.
17. Levenberg S., Katz B. Z., Yamada K. M., and Geiger B.: Long-range and selective autoregulation of cell-cell or cell-matrix adhesions by cadherin or integrin ligands. J Cell Sci 111: 347–357, 1998.
18. Morita K., and Nogawa H.: EGF-dependent lobule formation and FGF7-dependent stalk elongation in branching morphogenesis of mouse salivary epithelium in vitro. Dev Dyn 215: 148–154, 1999.