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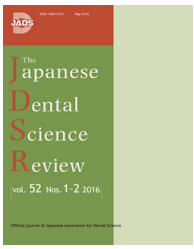

Review Article

Regulatory mechanisms of branching morphogenesis in mouse submandibular gland rudiments

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Summary Branching morphogenesis is an important developmental process for many organs, including the salivary glands. Whereas epithelial—mesenchymal interactions, which are cell-to-cell communications, are known to drive branching morphogenesis, the molecular mechanisms responsible for those inductive interactions are still largely unknown. Cell growth factors and integrins are known to be regulators of branching morphogenesis of salivary glands. In addition, functional microRNAs (miRNAs) have recently been reported to be present in the developing submandibular gland. In this review, the authors describe the roles of various cell growth factors, integrins and miRNAs in branching morphogenesis of developmental mouse submandibular glands.

Contents

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1. Introduction

Branching morphogenesis is an essential developmental process for the formation of various organs, such as the kidney, lung, pancreas, prostate and all exocrine glands, including the salivary glands [1]. Branching morphogenesis is one of the important biological events that are driven by epithelial—mesenchymal interactions [2]. However, the molecular mechanisms responsible for these inductive interactions remain largely unknown. The fundamental processes of epithelial—mesenchymal interactions depend on both the extracellular matrix (ECM) and integrins, as well as on a variety of growth factors and their receptors [1—5]. Cell growth factor receptors and integrins are located on the plasma membrane of cells, which is the point of interface between the environment immediately outside the cell and the cell interior. When receptors bind with ligands, multiple complex signaling cascades are activated, and major cellular events such as cell differentiation, proliferation, migration and formation of organ structures are elicited [6—8]. Protein expression for the signaling systems of these receptors is regulated as part of genetically controlled organ development [9—11].

The fetal mouse submandibular gland (SMG) is a well-characterized model system for *in vivo* and *in vitro* studies of the epithelial—mesenchymal interactions involved in fetal organogenesis [12—17] (Fig. 1). Over 60 years ago, a culture system for SMG rudiments was first reported as a new method for study of organ development by Borghese [18,19]. Since then, numerous reports have generated new information regarding SMG development. In particular, a study of salivary gland development identified cell growth factors and cell adhesion molecules that are involved in cell proliferation, differentiation, migration and movement [20].

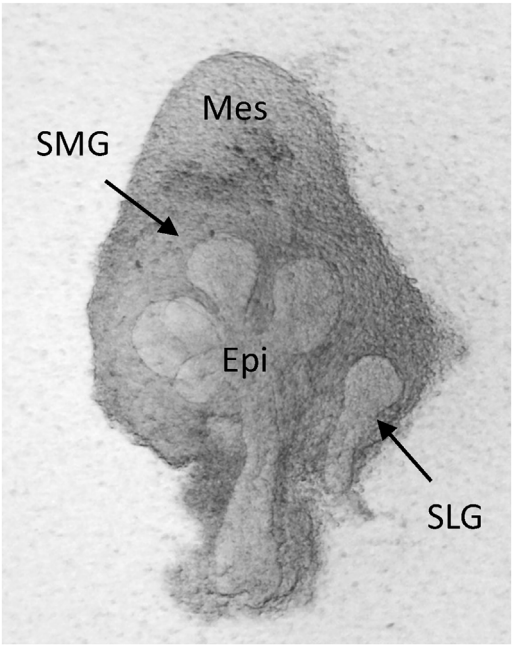

Figure 1 Submandibular gland and sublingual gland rudiments of fetal mouse at embryonic day 13. Mes = mesenchymal tissue. Epi = epithelial tissue. SMG = submandibular gland. SLG = sublingual gland.

2. Regulation by cell growth factors

In 1991, Nogawa and Takahashi [21] showed that epidermal growth factor (EGF) and transforming growth factor-α (TGFα) stimulated branching morphogenesis of cultured epithelium of submandibular gland rudiments, and those growth factors were found to substitute for the mesenchyme of the rudiments. The same research group also showed that EGF supports branching morphogenesis of SMG rudiments, especially cleft formation, and that fibroblast growth factor 7 (FGF7) is needed for stalk elongation of SMG epithelium [22]. The EGF system, including members of its ligand family such as EGF, TGF-α, HB-EGF and neureglin-1 (NRG1), and members of its receptor family such as EGFR (ErbB1), ErbB2 and ErbB3 [21—28], is one of the important regulators of branching morphogenesis of SMG rudiments. Moreover, the ligand family members activate and phosphorylate the ErbB receptor family proteins in the plasma membrane. Tyrphostin (RG 50864), a specific inhibitor of ErbB receptor tyrosine kinase, strongly inhibited branching morphogenesis of cultured SMG rudiments. Therefore, SMG endogenously expresses EGF ligands that regulate branching morphogenesis [23—28]. In addition to FGF7, FGF10 has been reported to induce morphological changes specific to stalk elongation of endpieces of SMG epithelium [29,30]. Although the EGF system is undeveloped in SMG rudiments at embryonic day 12 (E12), it becomes primed on E13 by the FGF system and plays an important role in induction of branching morphogenesis [31]. The cell growth factor systems play individual roles and stage-specific roles in regulation of branching morphogenesis in developing fetal SMG.

Intracellular signaling cascades are known to be activated by receptor tyrosine kinases, such as mitogen-activated protein kinases (MAPKs). [32] Ligand binding by growth factor receptors results in autophosphorylation of tyrosine residues in the cytoplasmic domain of the receptor molecules, and then adaptor proteins are recruited into the plasma membrane, where they link the receptors to Ras in the plasma membrane [33]. Ras in turn activates Raf, resulting in activation of MAPK kinase1/2 (MEK1/2), which phosphorylates and activates extracellular signal-regulated kinase1/2 (ERK1/2), a member of the MAPK family. Binding of complexes of growth factor receptor-bound protein 2 (GRB2) and GRB2-associated binder protein 1 (GAB1) to phosphorylated growth—factor receptor dimers leads to formation of active PI3K complexes, conversion of PIP2 into PIP3 [34] and activation of AKT signaling. Phospholipase Cγ1 (PLCγ1) can also be recruited directly through phosphotyrosine residues of growth factor receptors that serve as PLCγ1 docking sites [35], leading to PLCγ1 phosphorylation by EGFR and activation of DAG and IP3 signaling (Fig. 2).

To investigate the relationships between the cell signaling pathways and the elicited biological changes in branching morphogenesis, we compared the signaling activated in fetal mouse SMGs by EGF, FGF7 or FGF10, and correlated the findings with the specific events of branching morphogenesis [36]. Western blotting showed that EGF strongly stimulated phosphorylation of ERK1/2 [36,37] and

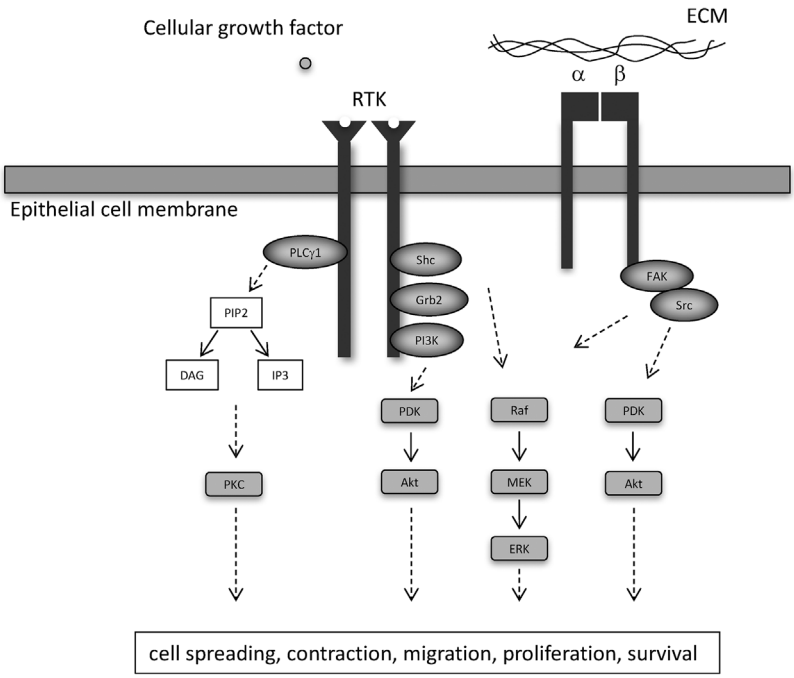

Figure 2 Signaling pathways activated by receptor tyrosine kinases (RTK) and integrins in epithelial cells of submandibular gland rudiments.

weakly stimulated phosphorylation of PLCγ1 and PI3K in cultured E14 SMG. FGF7 and FGF10 stimulated phosphorylation of both PLCγ1 and PI3K, but elicited only minimal phosphorylation of ERK1/2 [36]. Morphological studies of mesenchyme-free SMG epithelium covered with Matrigel revealed that EGF induced cleft formation, and that FGF7 stimulated both cleft formation and stalk elongation, whereas FGF10 induced only stalk elongation. These findings suggest that EGF stimulates cleft formation via ERK1/2, and that FGF7 stimulates both cleft formation and stalk elongation via PLCγ1 and partly via ERK1/2, but that FGF10 stimulates stalk elongation mainly via PLCγ1 [36]. Various investigators have reported that some other signaling systems, including sonic hedgehog (Shh) [38], platelet-derived growth factor (PDGF) [39], hepatocyte growth factor (HGF) [40], TGFβ1 [41] and Wnt [42], also regulate branching morphogenesis in developing fetal mouse SMG. Moreover, we recently reported that Shh stimulates branching morphogenesis in cultured SMG rudiments, and this stimulation is elicited by induction of synthesis of EGF ligands and EGFR proteins, resulting in activation of EGFR and ERK1/2 signaling cascades in SMG rudiments [28]. Namely, the systems described above may be cooperatively and systematically facilitating branching morphogenesis in mouse SMG rudiments through signaling cascades via epithelial-mesenchymal interactions.

3. Regulation by integrins

Many studies have demonstrated that epithelial branching depends on both the mesenchyme and the epithelial basement membrane [12]. Such epithelial—mesenchymal interactions probably involve interactions of integrins with their ligands in the ECM. The involved integrin family consists of heterodimeric α and β subunits and cell adhesion receptors and plays an important role in mediating cell adhesion to the ECM. Intracellular signals initiate ''inside-out signaling'' by inducing binding of proteins to the cytoplasmic domains of the integrin β subunits, which activates the integrins' ligand-binding function [43]. On the other hand, interactions between the integrins and their various ligands of ECM induce ''outside-in'' signals, allowing the cell to sense the extracellular environment and react accordingly [44]. ''Outside-in'' signaling induces cell spreading, retraction, migration, proliferation, and survival (Fig. 2).

Using RT-PCR analysis we detected mRNAs for α6, β1 and β4 subunits [23], and Menko et al. showed expression of α3 and α5 subunits in fetal mouse SMG [45]. We also examined the effect of GoH3 (CD49f), which neutralizes α6 integrin functions, on branching morphogenesis in cultured SMG rudiments and a mesenchyme-free epithelial culture system. In doing so, we observed that GoH3 strongly inhibited branching morphogenesis in cultured SMG rudiments and, consistent with intact culture rudiments, it also inhibited cleft formation and stalk elongation in mesenchyme-free epithelial rudiments [46]. A similar result was obtained when using anti-β1 integrin antibody [47]. Binding of integrin to ECM activates ''outside-in'' signals, including MAPK and PI3K cascades, some of which are common signaling cascades activated by cell growth factors. These cascades are cooperatively activated and regulate branching morphogenesis. However, the specifics of all the molecular mechanisms remain largely unknown.

4. Regulation by microRNAs

MicroRNAs (miRNAs) are small, single-stranded, non-coding RNAs that can potentially target a large number of genes,

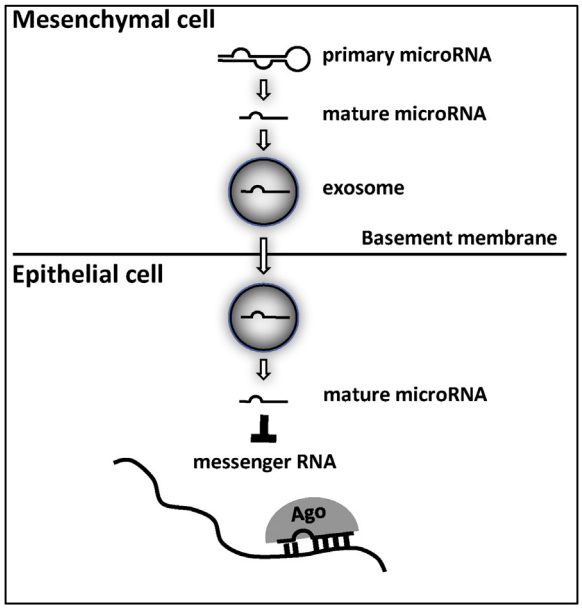

Figure 3 Epithelial—mesenchymal interactions via microRNA transport in fetal submandibular gland.

leading to translational repression and/or messenger RNA degradation [48,49]. The miRBase is an official database of miRNAs and has registered 2588 human miRNAs and 1915 miRNAs [miRBase release 21]. MiRNA genes are transcribed as hairpin-containing, long primary transcripts (pri-miRNAs). The pri-miRNAs are processed in the nucleus into double-stranded precursor miRNAs (pre-miRNAs) that are approximately 70 nucleotides in length. The pre-miRNAs are transported to the cytoplasm, where they are trimmed to shorter, single-stranded miRNAs as the mature form. Finally, the mature miRNAs are associated with Argonaute proteins in the RNA-induced silencing complex and act as guides to inhibit target mRNAs [48,49].

Hundreds of miRNAs have been detected in developing SMG [50], and a small number of miRNAs have functional roles in branching morphogenesis [17,51,52]. A mesenchymal miRNA, miR-21-3p, can repress expression of genes related to ECM degradation, leading to increased epithelial branching morphogenesis [17]. In contrast, miR-200c-3p is highly expressed in the epithelium and regulates epithelial proliferation by targeting genes that influence downstream FGF-dependent pathways [52]. Interestingly, a recent study showed miRNA transport between tissues during early SMG organogenesis [53]. A primary form of miR-133b-3p is expressed and processed into a mature form in the mesenchyme, although the miRNA is not transcribed in the epithelium. The resulting mesenchyme-specific miRNA is transported, potentially together with 80 other miRNAs, to the epithelium across the basement membrane via small extracellular vesicles called exosomes (Fig. 3). Inhibition of miR-133b-3p in the epithelium resulted in suppression of branching morphogenesis. Further analysis showed that miR-133b-3p targets a gene associated with the epigenome, leading to regulation of progenitor expansion in the epithelium [53]. It is reasonable to suppose that the other 80 miRNAs in exosomes also have functional roles in SMG development, such as proliferation, cell movement and cytodifferentiation, as well as branching morphogenesis. Therefore, these novel epithelial-mesenchymal interactions that rely on mobile genetic signals may be potential targets for RNA medicines and regeneration therapy.

5. Conclusion

Epithelial—mesenchymal interactions, which are a series of cell-to-cell communications, drive branching morphogenesis. The molecules necessary to configure epithelial—mesenchymal interactions for SMG development include at least cell growth factors such as EGF ligands, FGF ligands and TGFβ families, PDGF, HGF, Shh and Wnt signaling; integrins such as α3, α5, α6 β1 and β4 integrin subunits; and miRNAs such as miR-21-3p, miR-200c-3p and miR-133b-3p. These molecules cooperatively interact with each other during all stages of development in order to produce normal salivary glands. Elucidation of the action mechanisms of these molecules will help in the effort to develop effective regenerative medicines. SMG rudiments are well-studied and useful materials for analyzing organ development. Continued study of the SMG model will provide valuable evidence and clarification of the development of not only the salivary gland but also various other organs, and it will also lead to successful organ regeneration.

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