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REVIEW

Cell signaling regulation in salivary gland development

Akiko Suzuki[1,2] · **Kenichi Ogata**[1,2,3] · **Junichi Iwata**[1,2]

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Abstract

The mammalian salivary gland develops as a highly branched structure designed to produce and secrete saliva. This review focuses on research conducted on mammalian salivary gland development, particularly on the differentiation of acinar, ductal, and myoepithelial cells. We discuss recent studies that provide conceptual advances in the understanding of the molecular mechanisms of salivary gland development. In addition, we describe the organogenesis of submandibular glands (SMGs), model systems used for the study of SMG development, and the key signaling pathways as well as cellular processes involved in salivary gland development. The findings from the recent studies elucidating the identity of stem/progenitor cells in the SMGs, and the process by which they are directed along a series of cell fate decisions to form functional glands, are also discussed. Advances in genetic tools and tissue engineering strategies will significantly increase our knowledge about the mechanisms by which signaling pathways and cells establish tissue architecture and function during salivary gland development, which may also be conserved in the growth and development of other organ systems. An increased knowledge of organ development mechanisms will have profound implications in the design of therapies for the regrowth or repair of injured tissues. In addition, understanding how the processes of cell survival, expansion, specification, movement, and communication with neighboring cells are regulated under physiological and pathological conditions is critical to the development of future treatments.

Keywords Salivary gland · Morphogenesis · Development · Cell signaling · Submandibular glands

Abbreviations

SOX9	Sex-determining region Y-box 9
KIT	KIT proto-oncogene, receptor tyrosine kinase
FGF	Fibroblast growth factor
TGF-β	Transforming growth factor-β
SMGs	Submandibular glands
SLGs	Sublingual glands
PGs	Parotid glands
$KRT19^+$	Keratin 19 positive
MYC	Myelocytomatosis oncogene
KIT	KIT proto-oncogene, receptor tyrosine kinase
AKT	AKT serine/threonine kinase
PI3K	Phosphatidylinositol 3-kinase
EDA/EDAR	Ectodysplasin-A/ectodysplasin-A receptor
MAPK	Mitogen-activated kinase-like protein
HS	Heparan sulfate
ECM	Extracellular matrix
SHH	Sonic hedgehog
NRG	Neuregulin
HH	Hedgehog
SMO	Smoothened
PTCH	Patched
BMP	Bone morphogenetic proteins
GDNF	Glial cell line-derived neurotrophic growth factor
NRTN	Neurturin
Ach	Acetylcholine
VIP	Vasoactive intestinal peptide
Ascl3	Achaete-scute homolog 3
SCF	Stem cell factor

✉ Junichi Iwata
Junichi.Iwata@uth.tmc.edu

[1] Department of Diagnostic and Biomedical Sciences, School of Dentistry, The University of Texas Health Science Center at Houston (UTHealth), 1941 East Road, BBS 4208, Houston, TX 77054, USA

[2] Center for Craniofacial Research, UTHealth, Houston, TX 77054, USA

[3] Section of Oral and Maxillofacial Oncology, Division of Maxillofacial Diagnostic and Surgical Sciences, Faculty of Dental Science, Kyushu University, 3-1-1 Maidashi, Higashi-Ku, Fukuoka 812-8582, Japan

AQP	Aquaporin
ID	Intercalated duct
SD	Striated duct
ED	Excretory duct
NRF2	NF-E2-related factor 2

Introduction

Mammalian salivary glands comprise three pairs of major glands—the submandibular glands (SMGs), sublingual glands (SLGs), and parotid glands (PGs)—and several minor glands such as the lingual, buccal, palatal, and von Ebner's glands in the circumvallate papillae. There are multiple cell types in the salivary glands, including acinar, ductal, myoepithelial, neuronal, lymphatic, and endothelial cells [1] (Fig. 1). Complex interactions among these cell types occur during salivary gland development; however, the precise timing and mechanism of these events are not fully understood. The most lateral and cranial point of the buccal groove (the labiogingival sulcus) gives rise to PGs at the 5 – 6th weeks of gestation in humans [2], and the medial and lateral paralingual groove (the linguogingival sulcus) appears at the ectoderm-derived oral epithelial condensation at the floor of the mouth, and is separated by the basement membrane surrounding neural crest-derived mesenchymal tissue at 5.5 – 6th weeks and 7 – 8th weeks of gestation in humans, respectively. In addition, the posterior one quarter of the medial paralingual groove gives rise to the anlage of the SMGs, and the anterior three quarter gives rise to the Wharton's duct, whereas the lateral paralingual originates the SLGs [3–5].

Mice are established models used in the study of SMG development through cytological and molecular analyses. SMG development is initiated with the formation of the primitive knot and thickening of the oral epithelium at embryonic day 11.5 (E11.5) in mice, and at the 6th week of gestation in humans (the prebud stage); this primitive knot invaginates into the condensed mesenchyme containing an endothelial plexus at E12.5 in mice, and at 7–8th week of gestation in humans (the initial bud stage) [6, 7]. This single epithelial bud then undergoes several rounds of branching morphogenesis at E13.5–E14.5 in mice (the pseudoglandular stage), which is defined by multiple cycles of cleft formation, expansion of the end buds, and duct tubulogenesis [7, 8]. At E13.5, the ductal cells differentiate into two layers, the basal cell layer and luminal cell layer, and a ductal lumen is formed at the midline of keratin 19 (K19)-expressing luminal cells at E13.5 and expanded by E16.5 (the canalicular stage). This luminal formation is regulated by parasympathetic nerve transmitter vasoactive intestinal peptide (VIP) and its receptor VIPR1, but not by acetylcholine (ACh) and its muscarinic receptor 1 (M1), which are required for bud formation

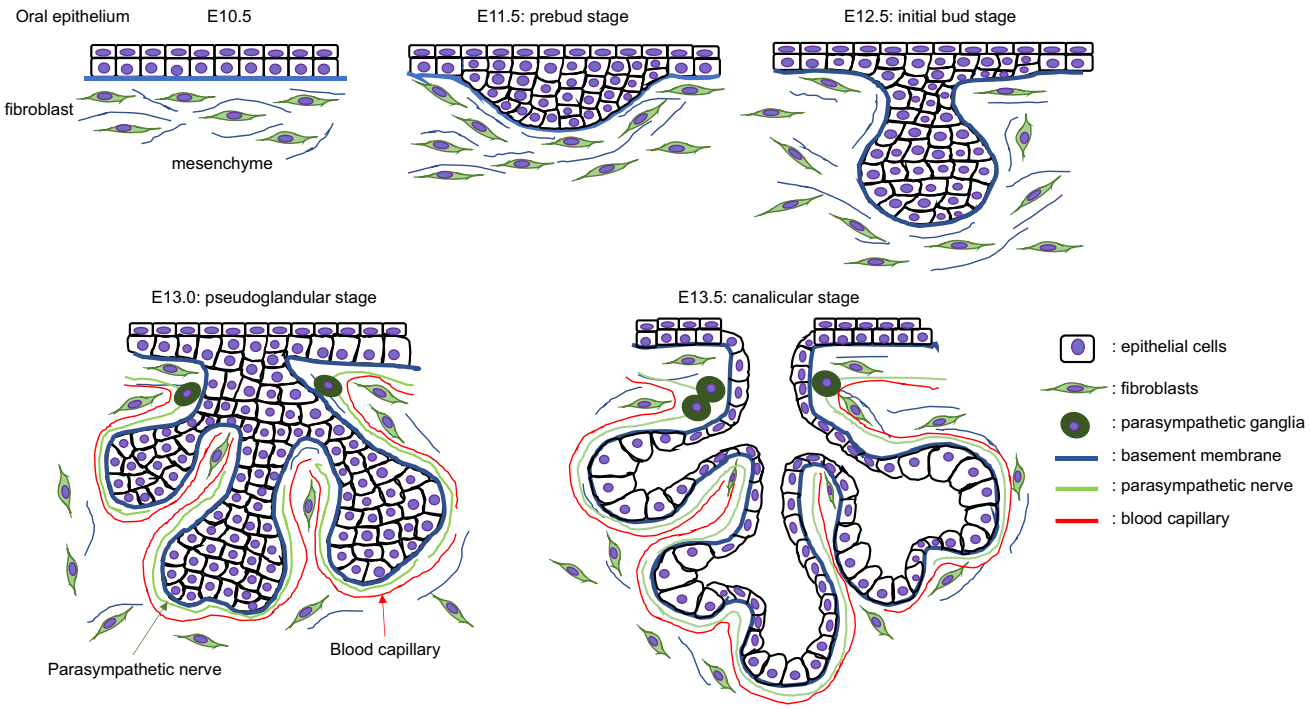

Fig. 1 Diagrams of salivary gland development. Oral epithelium is thickened (pre-bud stage) and invaginates into the mesenchyme. The initial bud further proliferates and elongates (initial bud stage) and then stars branching to form multi-end buds (pseudoglandular stage). At the ducts, lumens are formed at the center of bilayers and differentiates into terminal buds (canalicular stage and terminal bud stage)

from the oral epithelium and epithelial morphogenesis [9, 10] (Fig. 1).

The development of a lumen within the branched epithelium occurs in the following order: (1) the distal end of the main cord and the branch cords, (2) the proximal end of the main cords, and (3) the central portion of the main cord. The lumen is initially formed within the ducts before they develop into the terminal buds. In vitro studies using mammary gland epithelial cell lines and mouse SMG organ cultures suggested that apoptotic cell death in the central portion of the lumen is involved in the formation of a hollow lumen [11, 12]. Terminal differentiation of the end buds into secretory acini is apparent at the 19–24th week of gestation in humans and E17.5 in mice; this is followed by further growth and differentiation until a mature organ capable of nerve-stimulated secretion is formed (the terminal bud stage) [7, 13, 14]. Terminal differentiation of the gland then continues postnatally. These complex series of morphogenic steps suggest that multiple intrinsic and extrinsic signaling pathways are regulated in a spatiotemporal fashion. This review discusses the mechanism(s) by which salivary gland formation is affected through alterations in several cell signaling pathways.

Stem/progenitor cells in the SMGs

Multiple progenitor populations exist in both embryonic and adult salivary glands, and various nuclear, cytoplasmic, and cell surface markers have been used to characterize the salivary progenitor cells, including ASCL3, KIT, K5, K14, SOX2, and SOX9 [14–16]. c-KIT is a receptor tyrosine kinase type III that binds to a cytokine or stem cell factor to regulate cell proliferation, differentiation, and protection from apoptosis [17, 18]. It is expressed in end bud epithelial cells, but not in ductal cells, in the developing embryonic salivary glands [19], and c-KIT-expressing bud cells with high proliferation activity are subpopulated into K5-coexpressing proximal and K14-coexpressing distal progenitor cells [19]. Moreover, K5-expressing proximal progenitor cells coexpress K19 ($K5^+$;$K19^+$) during differentiation into the luminal cell layer and lose K5 expression ($K5^-$;$K19^+$) in well-developed luminal cells [9]. Interestingly, parasympathetic ACh/M1 signaling induces cell proliferation and epithelial differentiation in $K5^+$ progenitor cells via epidermal growth factor (EGF) signaling [9]. On the other hand, $K14^+$ distal progenitor cells are especially expanded by a combination of c-KIT and fibroblast growth factor (FGF) signaling mediated by receptor FGFR2b [19]. Previous transplantation studies demonstrate that c-KIT-expressing adult cells in intercalated, striated, and excretory ducts can accelerate repair after irradiation [20, 21], and a recent study indicates that these cells do not expand in cultured salivary gland organoid whereas all salivary gland cells in the organoid express K14 [22].

Progenitor cells expressing the mammalian achaete-scute homolog 3 (ASCL3), a transcription factor, exist in the striated and excretory duct cells of adult mouse salivary glands [23]. Three members of the *Ascl* gene family have been identified, and they are all expressed in tissue-specific progenitor cells and implicated in cell fate determination and differentiation events. Progenitor cells characterized by *Ascl3* expression are present in all three major salivary glands in mice [24], and lineage tracing experiments show that $ASCL3^+$ cells generate a subset of adult ductal and acinar cells in cultured sphere organoids [24–26]. Mice with a deletion of *Ascl3* ($Ascl3^{EGFP-Cre/EGFP-Cre}$) and elimination of *Ascl3*-expressing cells ($Ascl3^{EGFP-Cre/+}$;$R26R^{DTA/+}$) show hypoplastic salivary glands [26]; after ligation-induced trauma, damaged acinar cells are repopulated at the same extent as in control mice. Therefore, multiple progenitor-like cell populations that do not express *Ascl3* are present and functionally compensate for *Ascl3* absence in adult salivary glands [26].

SOX2, i.e., SRY (sex-determining region Y)-box 2, is a transcription factor that plays critical roles in embryonic development and cell fate determination and a marker of adult stem/progenitor cells [27–29]. In embryonic salivary gland development, SOX2-expressing bud cells can give rise to both acinar and ductal cells, but they are critical for acinar cell development [30]. Deletion of *Sox2* in *K14*-expressing epithelial progenitor cells ($K14\text{-}CreER^{T2}$;$Sox2^{F/F}$ induced at E10.5) results in severely hypoplastic SMGs and SLGs without affecting mesenchymal, neuronal, and ductal cell differentiation [30, 31]. Furthermore, the lack of *Sox2*-expressing progenitor cells causes failure to regenerate acinar cells after irradiation [31]. SOX9 is also expressed in epithelial progenitor cells, which give rise to both acinar and ductal cells [32], and $K14\text{-}Cre;Sox9^{F/F}$ conditional knockout mice fail to generate branches of end buds and exhibit developmental arrest at the bud stage in all three major salivary glands [32]. Moreover, prominin-1 (PROM1, a.k.a. CD133) is a transmembrane protein involved in stem cell capacity that is expressed in several stem/progenitor cells such as hematopoietic stem cells, neuronal/glial stem cells, endothelial progenitor cells, and cancer stem cells [33, 34]. PROM1-positive cells isolated from mouse SMGs can differentiate into acinar, ductal, and myoepithelial cells through regulation of *Sox9* expression [35].

It is critical to recognize the difference between stem cells and progenitor cells, which, despite being frequently mentioned interchangeably, are not equivalent and exhibit distinct properties [36]. Stem cells can replicate indefinitely and produce both undifferentiated and differentiated progeny, whereas progenitor cells undergo only a finite number of cell divisions, do not self-renew, and are often limited

in the number of cell types they can produce [37]. Long-term self-renewal and multipotent differentiation capacity are functional properties that require rigorous analysis of the cells within their native tissue niche [37]. It is known that the salivary glands contain multipotent stem cells that differentiate into either acinar cells or duct cells [38, 39], but evidence showing that differentiated cells can display cellular plasticity, particularly under stressful conditions, may help reconciling the various proposed stem cell populations in these structures [37].

Differentiation of mucous and serous acinar cells

The human salivary glands produce 0.5 – 1.5 L of saliva daily, which contains 99.5% of water, 0.3% of proteins, and 0.2% of both inorganic and organic substances [40]. Acinar cells are responsible for the production and secretion of α-amylase, mucins, and immunoglobulins essential for the digestion and taste of foods, lubrication, buffering, and prevention of dental and oral diseases [41]. There are two types of acinar cells in the salivary glands: serous and mucous acinar. Serous acinar cells are typically 8 – 12 pyramid-shaped cells with a well-developed rough endoplasmic reticulum (ER), round nuclei in the middle of the cytoplasm, and secretory granules at the apical cytoplasm; serous saliva contains a large amount of water, ions, and digestive α-amylase (AMY1A), which is crucial for food digestion [13, 42]. In contrast, mucous acinar cells have flat nuclei towards the basal cell surface with aggregated granules in the apical cytoplasm [13, 42] and secrete saliva containing mucins essential for lubrication and oral health [13, 42]. In humans, the PGs include only serous acini, but the SMGs and SLGs are a mixture of both serous and mucous acini (more serous acini in the SMGs, and predominantly mucous acini in the SLGs). In contrast, mouse PGs and SMGs are composed of pure serous acini, whereas the SLGs includes only mucous acini. Minor glands contain either mixed or mucous acini in both humans and mice [43].

The duct system and secretory duct

The mammalian duct system consists of intercalated ducts (IDs), striated ducts (SDs, a.k.a. intralobular ducts), and excretory ducts (EDs, a.k.a. interlobular ducts). Saliva produced by acinar cells in the glandular body flows sequentially through the IDs, SDs, and EDs. The IDs are lined by cuboidal to flattened single-layered epithelial cells between the acini and SDs and modify primary saliva components [44, 45]. The SDs are lined by single columnar epithelial cells, with vertical accumulation of mitochondria at the basal holding and numerous invaginations of the apical membrane, and also contain secretory granules and a smooth ER at the apical cytoplasm. They play a role in modifying the ion components of primary saliva through the secretion and resorption of ions transported bi-directionally between the ductal lumen and extracellular spaces [43, 46]. The EDs are lined by stratified epithelial cells, inner columnar to squamous cells, and basal cuboidal cells (Fig. 2).

The PGs secrete saliva via the Stensen's (a.k.a. Stenon's) duct at the parotid papillae, the SMGs via the Wharton's duct at the sublingual caruncle, and the SLGs through the Bartholin's duct, which further connects with the Wharton's duct at the sublingual caruncle or the ducts of Rivinus at the sublingual caruncle or sublingual fold [43]. In rodent males, granular convoluted tubules (GCTs), which are located between the IDs and SDs, develop in submandibular glands after sexual maturation, at 4 weeks old, in an androgen-dependent manner [43, 47, 48]. The GCTs are composed of simple columnar epithelia containing many eosinophilic secretory granules in the supranuclear cytoplasm [43]. These secretory granules contain various biologically active polypeptides such as cell growth factors EGF and nerve growth factor (NGF) and hormones, and undergo exocytosis in response to neural and hormonal stimuli [43]. Previous studies have demonstrated the role of testosterone in the development and maintenance of GCTs. For example, mice with a deficiency for the androgen receptor (*Ar*) ($Ar^{F/F}$*; CAG-Cre* mice) exhibit GCT maturation defects [49], and castrated male mice have underdeveloped GCTs that resemble those in female mice [50–54]. Moreover, GCTs in male mice with the testicular feminization mutation (*Tfm*), a spontaneous single-base deletion in the androgen receptor (*Ar*) gene, have fewer secretory granules and increased cytoplasmic vacuoles [55]. In addition, excessive cholesterol synthesis results in failed autophagy, specifically in the duct cells of the salivary glands, followed by the accumulation of NF-E2-related factor 2 (NRF2), a transcription factor known as one of the specific substrates for autophagy [56]. The accumulation of NRF2 suppresses Forkhead box protein a1 (*Foxa1*), which forms a transcriptional complex with the androgen receptor to regulate target genes, and is crucial for GCT differentiation [56].

Signaling pathways in the regulation of salivary gland formation

Fibroblast growth factor (FGF) family

FGF signaling is transduced by 23 FGF ligands and four receptors and is essential for multiple branching organs, including the lungs, pancreas, prostate, and salivary glands [57]. *Fgfr1b/2b* and *Fgf1/8/13* are expressed in the epithelium, and *Fgfr1c/2c/3/4* and *Fgf1/2/3/7/8/10/13* are expressed at the mesenchyme of SMG buds in mice [58] (Fig. 3). FGF10 is a major ligand for FGFR2B during development, and mice with loss of either *Fgf10* or

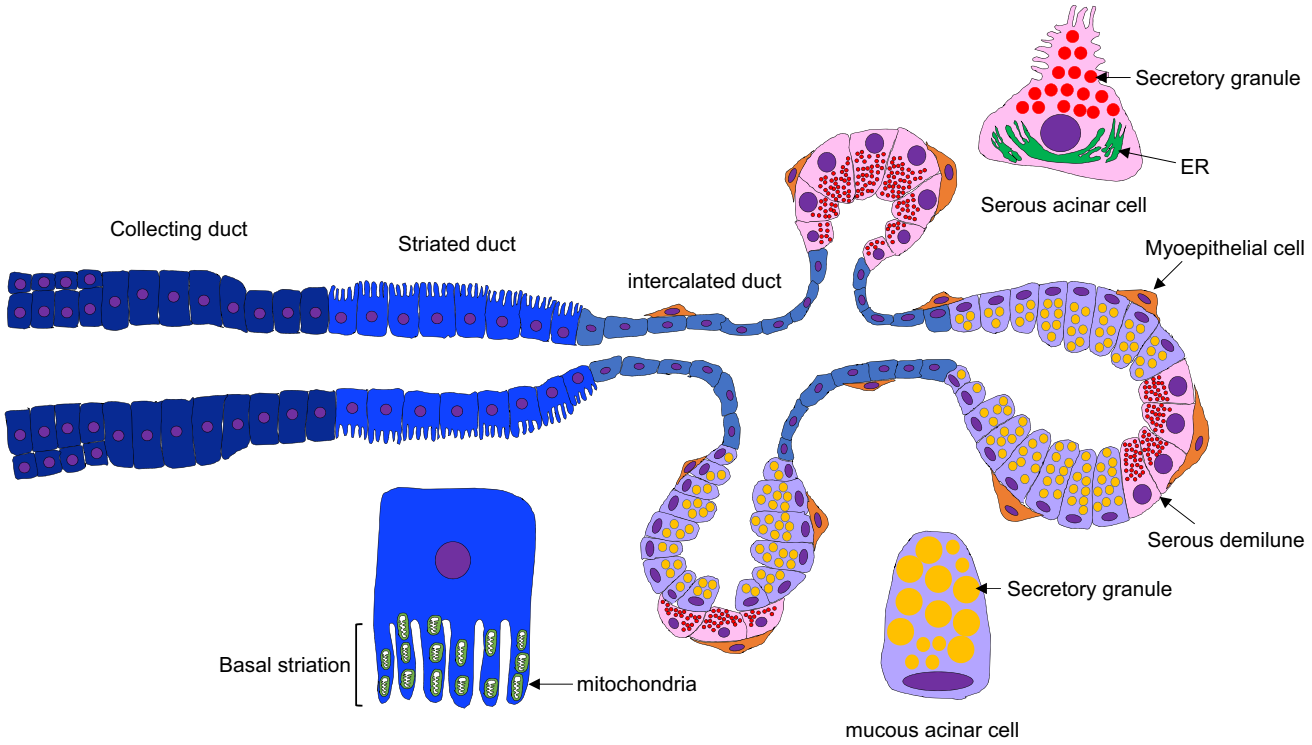

Fig. 2 A diagram of salivary glands. The salivary glands include collecting, striated, and intercalated duct cells in the duct and acinar (serous and mucous) and myoepithelial cells in the acini. Striated duct exhibit basal striation containing numerous mitochondria. Serous and mucous acinar cells contain numerous secretory granules in the cells

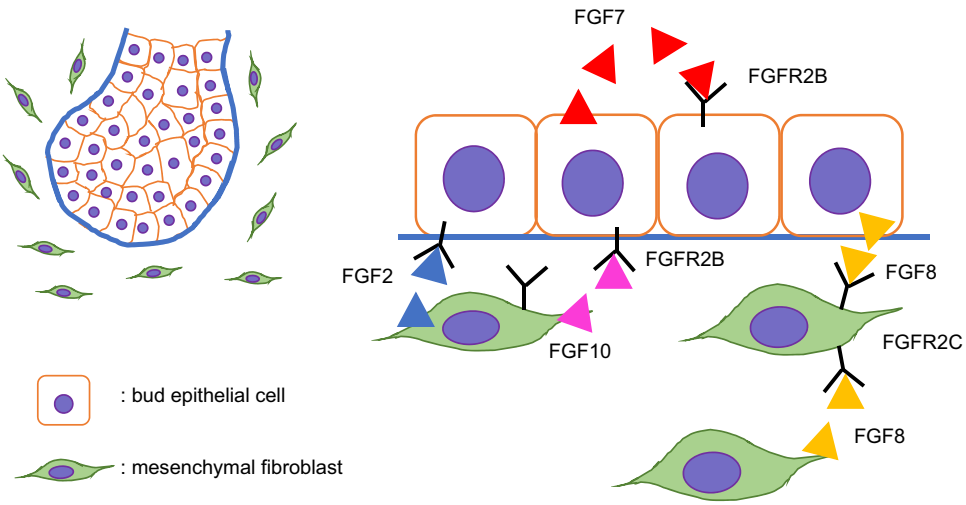

Fig. 3 Schematic images of FGF signaling during salivary gland development

Fgfr2IIIb (*Fgf10*$^{-/-}$ or *Fgfr2b*$^{LacZ/LacZ}$) exhibit similar phenotypes of dysgenesis or agenesis of the lungs, limbs, pancreas, kidney, and salivary glands that are more severe than those seen in *Fgfr2b*$^{LacZ/+}$ or *Fgf10*$^{\pm}$ heterozygous mice [59–63]. *Fgf10*$^{\pm}$ mice exhibit delayed, hypoplastic SMG development, leading to reduced saliva secretion (a.k.a. xerostomia) [59]. Mice with a cranial neural crest (CNC) cell-specific deletion of *Fgf10* (*Wnt1-Cre;Fgf10*$^{F/F}$ conditional knockout) exhibit agenesis of the salivary and lacrimal glands, which is similar to the phenotype of *Fgf10* null mice [64]. In addition, FGF signaling can induce putative progenitor cells marked with the myelocytomatosis oncogene (MYC; a transcription factor), sex-determining region Y-box 9 (SOX9; a transcription factor), and KIT proto-oncogene receptor tyrosine kinase (KIT; a receptor tyrosine kinase) in the end buds of ex vivo SMG explants

[19]. In mouse embryos with loss of either *Fgf10* or *Ffgr2b*, loss of FGF signaling leads to failure of epithelial invagination and downgrowth into the surrounding mesenchymal tissue; thus, an initial SMG placode is detectable at E12, but as a hypoplastic end bud, and then the glands are no longer detectable at E13.5 [63, 65, 66]. In addition, double heterozygous mice for the *Fgfr2c* and *Fgf10* deletions ($Fgfr2c^{+/\Delta}$;$Fgf10^{\pm}$) exhibit more severe hypoplastic SMGs than single heterozygous $Fgfr2c^{+/\Delta}$ mice [63, 67]. These phenotypic similarities seen in mice with loss of either *Fgf10*, *Fgfr2b*, or *Fgfr2c* suggest that FGF signaling is critical for exocrine gland development.

In humans, heterozygous mutations in genes related to FGF signaling are associated with autosomal dominant aplasia of the lacrimal and salivary glands (ALSG) syndrome and autosomal dominant lacrimo-auriculo-dento-digital (LADD) syndrome. ALSG is known to be caused by mutations in *FGF10* [68–71], and LADD has been associated with mutations in *FGF10*, *FGFR2*, and *FGFR3* [72–74]. Both syndromes are characterized by lacrimal and salivary glands aplasia/hypoplasia, dryness of the eye and mouth, dental caries, and oral infections. In addition, patients with LADD often display defects in the craniofacial region, in the digits, and the genitourinary system [69, 74]. FGF10/FGFR2B signaling via the mitogen-activated kinase-like protein (MAPK) and the phosphoinositide 3-kinase (PI3K) pathways is known to be involved in epithelial cell proliferation and expansion of epithelial end buds [75, 76]. In fact, the FGF10/FGFR2B complex binds to the heparan sulfate (HS) domain of extracellular matrix HS proteoglycans, such as perlecan; heparanase releases FGF10 from perlecan HS, increasing MAPK signaling activity, which is crucial for branching morphogenesis [77, 78]. LADD-related *FGF10* mutations reduce the binding affinity to FGFR2B or stability of FGF10, resulting in reduced FGF10/FGFR2B signaling [79], and LADD-causing mutations in *FGFR2* reduce receptor tyrosine kinase activity, resulting in impaired tyrosine autophosphorylation for FGF10/FGFR2B signaling [79, 80].

Another FGF ligand, FGF8, signals through FGFR2c. Mice with a conditional deletion of *Fgf8* in the 1st pharyngeal arch ectoderm ($Tfap2a^{IRESCre/+}$;$Fgf8^{F/Null}$) and mice with a hypomorphic form of FGF8 ($Fgf8^{H/Null}$) exhibit SMG agenesis with arrest at initial epithelial gland bud formation and hypoplastic SMGs with a few branched end buds with narrow luminae, respectively [81]. The branching defect caused by *Fgf8* deficiency can be rescued with exogenous supplementation of FGF10 [81]. Altogether, these results indicate that FGF10/FGFR2b and FGF8/FGFR2c signaling share common downstream targets critical for SMG development. Interestingly, mice with mouse mammary tumor virus overexpressing *Fgf8* or *Fgf8b* (*MMTV-Fgf8* and *MMTV-Fgf8b*) develop ductal hyperplasia, as well as salivary and mammary adenocarcinomas [82].

FGF7 is explicitly expressed in epithelial tissues and signals via FGFR2. The supplementation of both FGF7 and FGF1 accelerates acinar cell differentiation and branching in cultured SMG explants [83]. Interestingly, mice expressing *Fgf7* under the *Krt14* promoter (*K14-Fgf7* mice) exhibit small salivary glands with failure of duct differentiation [84]; however, *Fgf7* null mice do not have any abnormality in the salivary glands [85]. In addition, mice with an ablation of antagonists of FGF signaling Sprouty (*Spry*) 1 and 2 in epithelial cells (*Spry1/2* double null mice, *Shh-Cre;K14-Cre;*$Spry1^{F/-}$;$Spry2^{F/-}$ in which approximately 80% of the *Spry1/2* gene is deleted in epithelial cells) exhibit wide primary duct formation and abnormal branching due to reduction of $K5^+$ duct progenitor cells and lack of parasympathetic ganglia (PSGs) and innervation in the SMGs; however, *Wnt1-Cre;*$Spry1/2^{F/-}$ conditional knockout mice do not have any defect in neither SMG morphogenesis nor gangliogenesis/innervation [86]. $K5^+$ progenitor cells are known to produce and secrete WNT ligands (*Wnt4*, *Wnt5b*, *Wnt7b*, and *Wnt10a*), which activate WNT signaling in neurons of PSGs associated with neuronal survival, gangliogenesis, and innervation [86].

FGF2 is expressed in the SMG mesenchyme during development, and knockdown of *Fgf2* in mesenchymal–epithelial co-cultures isolated from gland buds reveals that expression of acinar cell marker aquaporin 5 (AQP5), a water channel crucial for saliva secretion, in the epithelial cluster and epithelial cell survival is suppressed compared with the control. Moreover, supplementation of FGF2 in epithelial clusters promotes organogenesis and expression of AQP5 [87]. These studies strongly suggest that a balance of FGF signaling activity is crucial for proper epithelial morphogenesis and progenitor cell maintenance.

Epidermal growth factor (EGF) family

The EGF family of receptor tyrosine kinases (EGFR/ErbB1, ErbB2, ErbB2, and ErbB4) and their ligands play crucial roles in cell proliferation, migration, differentiation, survival, and apoptosis in the development and maintenance of various tissues [88, 89]. During salivary gland development, all four receptors are expressed in gland buds from E12 to E15. *ErbB1* is expressed only in epithelial cells, *ErbB2* and *ErbB3* in epithelial cells and in a small population of surrounding mesenchymal cells and their ligands, and *Egf*/EGF in epithelial cells of ducts and end buds after E14, but not at E12 and E13. In addition, *Tgfa*/TGFA is expressed in epithelial cells of ducts and end buds after E12, *Hbegf* in both epithelial cells and the mesenchyme at E12 and E13, and *Nrg1* only in the surrounding mesenchyme from E12 to E15 [90–92]. EGF is a ligand for ERBB1 and an inducer of branching of end buds, and increases production of integrin alpha 6 (ITGA6) at the basal cell membrane via activation

of extracellular signal-regulated kinase (ERK) 1/2 signaling [93–97]. Heparin-binding EGF-like growth factor (HBEGF) and transforming growth factor-alpha (TGFα) are also ligands for ERBB1 that induce branching [97, 98]. In addition, stimulation of the parasympathetic nervous system via ACh/M1 signaling increases the proliferation of $K5^+$ progenitor cells in the initial buds via ERRB1/HBEGF signaling, which in turn increases differentiation of $K19^+$ luminal cells [9]. Finally, mesenchyme-specific neuregulin-1 (NRG1) is a ligand for both ERBB3 and ERBB4 and induces branching [90]. Mice with deletion of *ErbB1* (*ErbB1*$^{-/-}$ mice) exhibit slightly small-sized SMGs due to impaired terminal bud branching and reduction of epithelial cell proliferation [92, 99] (Fig. 4).

NOTCH signaling

Notch signaling is a conserved intercellular signaling pathway that regulates cell proliferation, differentiation, and cell fate determination in various tissues during development and regeneration [100–104]. Notch signaling is activated by binding of ligands Jagged 1 and 2 (JAG1 and JAG2), delta-like canonical Notch ligand 1, 3, and 4 (DLL1, DLL3, and DLL4), or delta-like noncanonical Notch ligand 1 and 2 (DLK1 and DLK2) to their receptors NOTCH1-4. In human SMGs, NOTCH1-4, JAG1/2, and DLL1 are expressed more abundantly in duct cells compared to acinar cells [105]. Moreover, ductal ligation of the rat parotid gland demonstrates that expression of NOTCH1-4, JAG1/2, and DLL1 is induced in acinar cells during regeneration [105]. DLK1 and DLK2, transmembrane proteins containing six EGF-like repeats, are secreted as soluble forms that are cleaved by a disintegrin and metalloproteinase (ADAM) 17. DLK1 and DLK2, which act as negative NOTCH signaling regulators, play roles in cell growth and differentiation in various organs, including the brain, pituitary gland, skeletal muscles, cartilages, pancreas, lung, and SMGs [106–108]. At the bud stage, DLK1 is expressed in the surrounding mesenchyme, whereas DLK2 is expressed in the epithelial cells of the end buds. At the pseudoglandular and canalicular stages, DLK1 expression is detectable in both mesenchymal cells and distal-end bud epithelial cells, but not in ductal cells, whereas DLK2 is strongly expressed in duct cells and weakly expressed in the end buds. At the terminal bud stage, the expression of DLK1 is strongly detected in myoepithelial cells [109]. NOTCH signaling is activated in the epithelial cells of the buds and ducts, the surrounding mesenchymal cells, and the parasympathetic ganglion [109]. Inhibition

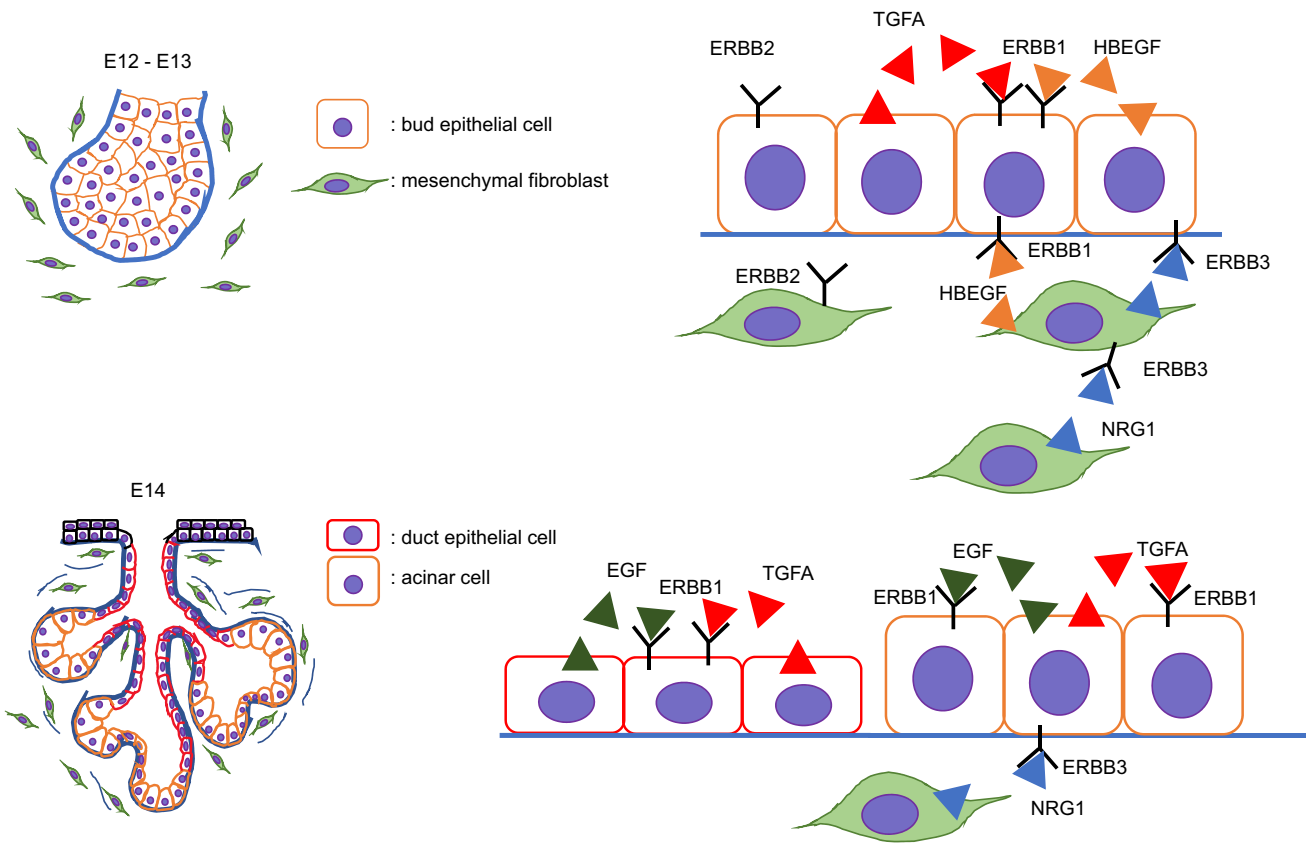

Fig. 4 Schematic images of EGF signaling during salivary gland development

of NOTCH signaling by DLK1 results in suppression of branching morphogenesis and innervation of parasympathetic nerve toward the end buds, without cell proliferation defects in bud cells [109]. As predicted, *Dlk1* null mice (*Dlk1*$^{-/-}$) exhibit small SMGs, SLGs, and PGs, resulting in reduced stimulating salivary flow without any morphological changes. However, the number of K14-expressing epithelial cells, but not K5-expressing cells, is increased in the developing SMGs of *Dlk1*$^{-/-}$ embryos [110].

WNT/β-catenin signaling

WNT signaling contributes to embryonic patterning, cell proliferation, cell fate, migration, polarization, and differentiation in multiple organisms and organ systems [111–113]. This signaling pathway is categorized into the β-catenin-dependent canonical pathway, and the independent noncanonical planar cell polarity pathway and WNT-Ca^{2+} pathway; the canonical pathway is activated by ligands WNT1, 2, 3, 3a, 7a/b, 8a, 9a/b, and 10a/b, and the noncanonical WNT/PCP and/or WNT/calcium pathways are activated by WNT4, 5a/b, 6, 7a, 11, and 16 [114–116]. These ligands bind to the receptor complex of LRP5/6 and Frizzled proteins (Fzd1 – Fzd10) [117]. Despite this abundance of ligands, WNT signaling itself is strictly regulated in a spatiotemporal manner during SMG development. Canonical WNT activity is first detectable at E11.5–E12.5 in mice at the condensed mesenchyme near the end buds and within the parasympathetic ganglia, and is restricted to ductal epithelial cells only after E14. WNT ligands are suggested to originate from $K5^+$ ductal progenitor cells [86, 99, 118, 119]. Constitutively activated canonical WNT/β-catenin signaling inhibits branching morphogenesis while inhibited canonical WNT/β-catenin signaling accelerates branching morphogenesis in SMG tissue culture at the bud stage [118].

The interactions between the FGF and WNT pathways suggest that loss- or gain-of-function mutations in the FGF genes result in altered WNT signaling, leading to changes in ductal and ganglia morphogenesis [86]; for example, stimulation of FGF signaling suppresses expression of WNT ligands. The deficient gangliogenesis and branching in the SMGs of *Spry1/2* mutant mice (*Spry1/2* double null) can be rescued by haploinsufficiency of *Fgf10* in the *Spry1/2* mutant background (*Spry1*$^{-/-}$;*Spry2*$^{-/-}$;*Fgf10*$^{\pm}$) combined with the Wnt activator [86]. Mice with a mesenchyme-specific deletion of β-catenin (*Dermo1-Cre;Ctnnb1*$^{F/-}$) exhibit small SMGs with reduced branching due to suppressed expression of the gene coding for ectodysplasin A (EDA), but exogenous supplementation of EDA can rescue the branching defect caused by WNT signaling inhibition in these mice [119]. Moreover, mice with constitutively activated canonical WNT/β-catenin signaling (*Rosa26-CreERT2;Ctnnb1*$^{Ex3F/F}$) show defects in the differentiation of acinar cells and luminal formation through suppression of KIT expression [83]. In addition, WNT3A (a ligand for canonical WNT/β-catenin signaling), but not WNT5A (a ligand for noncanonical WNT pathway), suppresses acinar cell differentiation and maintains progenitor cell status, suggesting that canonical WNT/β-catenin signaling regulates end bud differentiation [83]. In adult SMGs, a few cells among basal excretory cells co-expressing high epithelial cell adhesion molecule (EpCAM) and nuclear β-catenin are thought to constitute a stem cell population. These cells are in fact capable of forming spheres following treatment with both WNT3A and R-Spondin1. Moreover, the transplantation of these cells pretreated with WNT regenerates both acinar and ductal tissues in irradiated SMGs [120].

Hedgehog (HH) signaling

Congenital brain, limb, cochlear, neural crest, and craniofacial defects are present in individuals harboring mutations in hedgehog (HH) ligands (Sonic hedgehog, Desert hedgehog, Indian hedgehog), HH receptors [Patched (Ptch1) and Patched 2 (Ptch2), and Smoothened (Smo)], and mediators/transcription factors GLI1–3 [121, 122]. HH ligands bind to 12-transmembrane spanning receptor Ptch1 at the primary cilia, which is an antenna-like structure (described in detail below); in the absence of HH ligands, Ptch1 inhibits the activity of effector SMO, a 7 transmembrane-spanning G protein-coupled protein, in the primary cilia. Phosphorylation of SMO by the HH-Ptch1 complex activates SMO and induces translocation and accumulation of SMO in the primary cilia, where activated SMO processes GLI, and the GLI proteins then translocate into the nucleus to regulate target gene expression [121]. In general, GLI1 and GLI2 act as activators, but GLI3 has a dual function, acting as activator and suppressor depending on the presence and absence of HH ligands, respectively [122–125]. Heteromeric motor molecules (i.e., kinesins and dyneins) move in an anterograde and retrograde manner along the axoneme microtubules; intraflagellar transport (IFT) proteins bind to kinesins or dyneins to carry molecules between the cytosol and primary cilium, a microtubule-based cellular protrusion in nearly every cell except blood cells. Primary cilia play crucial role in the regulation of HH signaling; therefore, any failure in primary cilium formation and function disrupts HH signaling [126].

In the developing murine SMGs, *Shh*/SHH, its receptor Ptch1 and SMO, GLI1, and GLI3 are expressed at the ductal- and terminal-bud epithelial cells, whereas *Dhh* is expressed in the surrounding mesenchyme [119, 127, 128]. SMG development in *Shh* null mice (*Shh*$^{-/-}$) is arrested before the pseudoglandular stage, with a few undifferentiated initial buds, and a similar branching defect is seen in SMG explants treated with cyclopamine, a HH signaling inhibitor.

On the other hand, exogenous SHH increases branching [128, 129]. SHH also induces *ErbB1, −2*, and *−3* expression in the epithelium and *Egf, Nrg1*, and *Tgfα* expression in the mesenchyme in cultured mouse SMG explants, which play a role in branching morphogenesis [129]. Ectopic expression of *Gli1* in *K5-Gli1* and *MMTV-Gli1* transgenic mice exhibit compromised acinar cell differentiation, hyperplastic ductal structure, and cyst formation, suggesting that GLI1 plays a role in the regulation of both duct cell proliferation and acinar cell differentiation [130]. CNC cell-specific deletion of Shh effector *Smo* or *Gli2/3* (*Wnt1-Cre;Smo*$^{F/F}$, *Wnt1-Cre;Gli2*$^{F/F}$*;Gli3*$^{F/F}$, or *Wnt1-Cre;Gli2*$^{F/F}$*;Gli3*$^{\Delta 699/\Delta 699}$) exhibit SMG agenesis due to developmental arrest at the initial bud stage. On the other hand, transgenic mice overexpressing *Gli3* (*Wnt1-Cre;RosaGli3T*^{Flag}) display slightly hypoplastic SMGs [131]. These results suggest that SHH signaling in the surrounding mesenchyme is critical for differentiation and branching of epithelial cells in the SMGs. Moreover, mice with loss of *Kif3a* (a kinesin-2 motor protein subunit) in CNC cells (*Wnt1-Cre;Kif3a*$^{F/F}$) exhibit SMG agenesis, which is caused by arrest in development at the initial bud stage, as seen in *Smo* and *Gli2/3* mutant mice [131]. Although a relationship between SMG morphogenesis and primary cilium remains elusive, *Ift88*$^{Orpk/Orpk}$ transgenic mice (expressing a hypomorphic allele that results in reduced protein levels) display reduced duct extension and branching morphogenesis in the mammary glands [132].

Bone morphogenetic proteins (BMPs)/transforming growth factor β (TGFβ) signaling

The TGFβ superfamily is known to be involved in the development and morphogenesis of various organs [133, 134]. Ligands of the TGFβ superfamily include TGFβ, BMP, growth differentiation factor (GDF), Activin, Inhibin, Myostatin, and Nodal. The TGFβ superfamily receptors are serine/threonine kinase receptors categorized into type I, type II, and type III; type I comprises seven subtypes and type II five, where type III consist only of TGFBR3 (betaglycan). Ligands bind to type II receptors and then recruit type I receptors to form a ligand-type II receptor complex, which phosphorylates/activates the type I receptor; this phosphorylated type I receptor then phosphorylates receptor-regulated SMAD (R-SMAD; SMAD1/5/8, or SMAD1/5/9 for BMPs, SMAD2/3 for TGFβs and activin/inhibin), which then binds to common partner SMAD (Co-SMAD; SMAD4). The SMAD complex then translocates into the nucleus to regulate target gene expression [135–138]. In the developing salivary glands, various BMP signaling molecules [e.g., ligands *Bmp1-4, 6,* and *7*; receptors *Bmpr1a, 1b, 2*, and *Acvr2b*; antagonists Noggin (*Nog*) and Gremlin1 (*Grem1*)] are expressed in the SMGs and SLGs in a spatial–temporal-specific manner, whereas *Bmp8b* is expressed only in the SLGs [expression data provided by the Salivary Gland Molecular Anatomy project at NIDCR (https://sgmap.nidcr.nih.gov/sgmap/sgexp.html)] [58, 67, 127, 139–141]. For example, *Bmp1, 2, 3, 4*, and *6* and *Bmpr1b* are expressed at high levels in mesenchymal cells at E13.5, whereas *Bmp7* is expressed at higher levels in the epithelium compared to the mesenchyme. Consistent with the role of BMP7 in epithelial organ development [142], *Bmp7* null mice (*Bmp7*$^{-/-}$) exhibit aberrant salivary gland morphogenesis with fewer end buds and luminal ducts as compared to wild-type controls [67], suggesting that BMP7 regulates branching morphogenesis.

FAM20C, a Golgi kinase, phosphorylates various proteins, including BMP4. The SMGs in *Mmtv-Cre;Fam20c*$^{F/F}$ mutant mice exhibit decreased acinar lobes and defects in maturation and secretion in GCTs due to suppression of canonical BMP signaling in duct cells [143]. Interestingly, patients with Sjögren's syndrome and disease-onset NOD mice (a mouse model for Sjögren's syndrome; disease-onset after the age of 20 weeks) highly express BMP6 in the salivary glands [144, 145]. Ectopic expression of BMP6 is known to induce hypofunction in the salivary glands and lacrimal glands with increased extracellular matrix and decreased AQP5 expression. Inhibition of BMP signaling by an ALK2/3 inhibitor can restore defective salivary gland function in *NOD-Aecl1Aecl2* mice (also a Sjögren's syndrome mouse model) [144, 145].

TGFβ1, -β2, and -β3 are also expressed in the developing salivary glands. All three ligands are present in both the oral epithelium and the adjacent mesenchyme, but TGFBR1 and TGFBR2 are expressed mainly in epithelial cells at the initial bud stage. TGFβ1, TGFβ2, TGFBR1, and TGFBR2 are expressed only in the branching bud epithelium, whereas TGFβ3 is present in both the epithelium and the surrounding mesenchyme at the pseudoglandular stage. At the canalicular stage, the expression of TGFβ1, TGFβ2, TGFBR1, and TGFBR2 is restricted to the terminal buds, luminal cells, and ducts, whereas TGFβ3 is expressed only in the surrounding mesenchyme. At the terminal bud stage, TGFBR2 expression shifts to the ductal epithelial cells only [92, 146, 147]. However, *Tgfb1*, *Tgfb2*, or *Tgfb3* null mice (*Tgfb1*$^{-/-}$, *Tgfb2*$^{-/-}$, and *Tgfb3*$^{-/-}$) show normal SMG development, suggesting that TGFβ ligands are functionally redundant during SMG development [92, 148] (Fig. 5). Exogenous supplementation of TGFβ1 to mouse SMG explants can suppress branching morphogenesis as well as acinar cell differentiation in a dose-dependent manner [149], and mice overexpressing *Tgfb1* (*MMTV-Cre;Tgfb1*^{glo}) develop severely hypoplastic SMGs with acinar cell atrophy and fibrosis in the adult phase [150]. Interestingly, *Tgfb1*$^{-/-}$ mice exhibit inflammatory cell infiltration in the SMGs, similarly to Sjögren's syndrome, after postnatal day 10 due to failure in the suppression of the inflammatory response [148, 151].

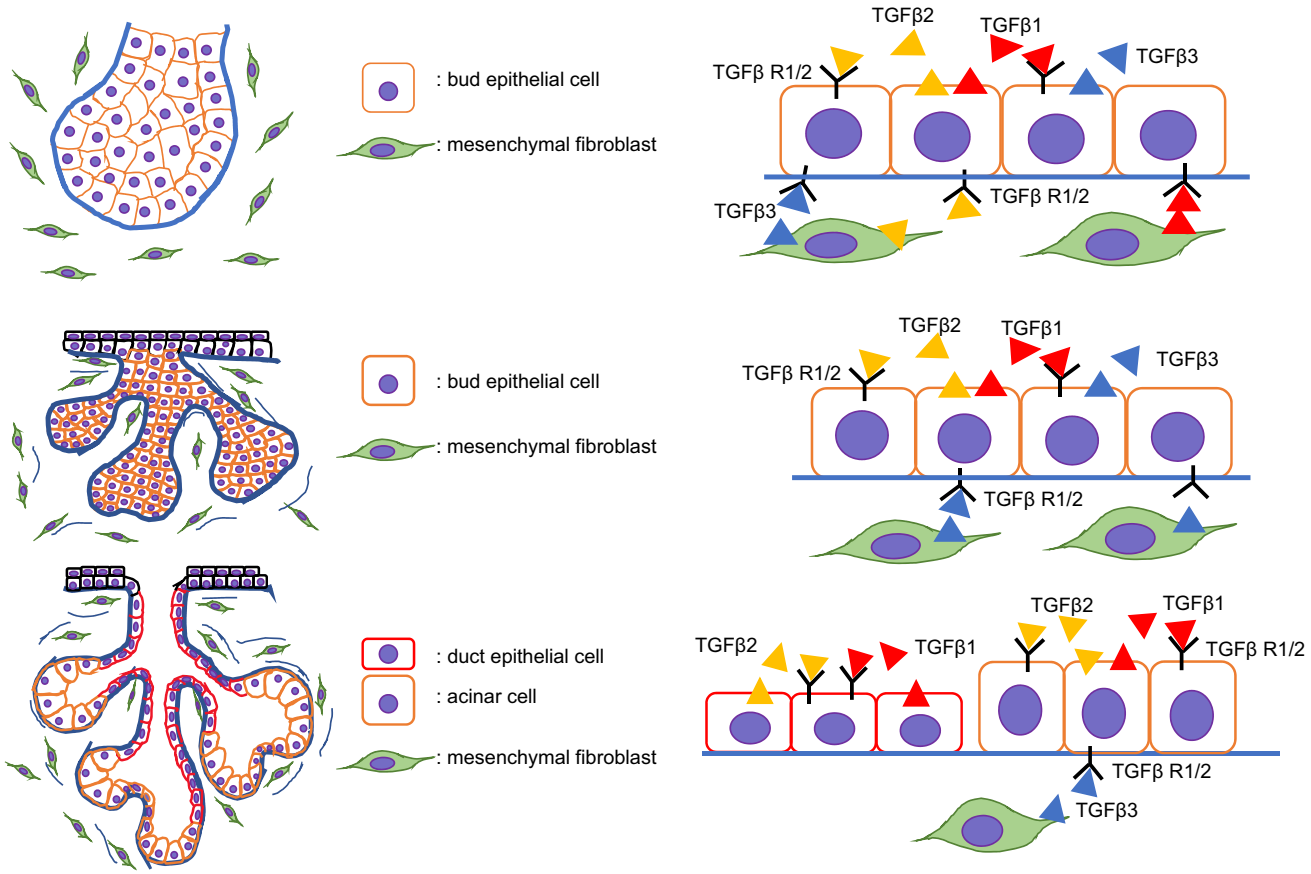

Fig. 5 Schematic images of TGFβ signaling during salivary gland development

Ectodysplasin a (EDA) signaling

EDA, a tumor necrosis factor family transmembrane protein, and its receptor EDAR play essential roles in the development of ectodermal tissues such as the teeth, skin, mammary glands, and salivary glands [152–155]. Mutations in *EDA*, *EDAR*, or *EDARADD* (an EDAR-associated death domain) cause autosomal recessive, autosomal dominant, or X-linked hypohidrotic ectodermal dysplasia (HED or XLHED), a syndrome variably characterized by tooth agenesis, hypoplastic teeth, sparse hair, dysfunction in exocrine glands, such as sweat glands, sebaceous glands, lacrimal glands, mammary glands, and mucous glands of the bronchi, and salivary glands [156–159]. Both loss- and gain-of-function studies in genetic mouse models have revealed the critical role of the EDA pathway in salivary gland ductal and acinar development. *Eda* null mice ($Eda^{-/-}$, *Tabby*) and *Edar* null mice ($Edar^{-/-}$) display defects in branching morphogenesis, resulting in hypoplastic salivary glands with a reduced number of ductal and acinar structures in the adult phase [119, 160–164]. In contrast, mouse embryos with specific overexpression of *Eda* in the epithelium under the *K14* promoter (*K14-Eda*) and *Edar* transgenic mice ($Edar^{Tg951/Tg951}$) display increased branching and number of end buds in the SMGs [119, 165]. EDA signaling is known to be mediated by transcription factor nuclear factor kappa B (NF-κB) in tooth, hair follicle, and salivary gland development [119, 166–169], and mutations in the *EDAR* gene fail to activate NF-κB signaling [170]. Previous studies demonstrated that HH and WNT signaling pathways are downstream of EDA signaling in the developing salivary glands [119, 161, 171], and the expression of *Shh* is in fact suppressed in *Eda* mutant mice and upregulated in *K14-Eda* mice when compared with wild-type controls. The branching defects in the SMG of *Eda* and *Edar* mutant mice ($Eda^{-/-}$ and $Edar^{dlJ/dlJ}$) can be rescued by treatment with SHH ex vivo [119, 163], but the excessive branching phenotype in the SMGs of *K14-Eda* mice is restored by inhibition of HH signaling with cyclopamine [119]. Despite the fact that there are interactions between the EDA and WNT signaling pathways in early SMG development (earlier than E13 in mice) (e.g., the suppression of WNT signaling in the mesenchyme reduces *Eda* expression), these two pathways appear to function independently after E13 [119]. For example, the activity of WNT and EDA/NF-κB signaling does not co-localize during duct formation/lumenization of the SMGs. WNT activity is

detectable in the surrounding mesenchyme before E15 and in the ducts after E15, whereas NF-κB signals are in fact localized only at the end buds, not the ducts [118, 172]. The mechanism of how the EDA pathway controls acinar formation in the SMGs, and whether the SMGs of individuals with HED show aberrant duct or acinar morphogenesis, remain to be determined.

Neurotrophic factors secreted by sympathetic and parasympathetic nerves

The glial cell line-derived neurotrophic factor (GDNF) ligand families (GLFs) belong to the TGFβ superfamily. GLFs comprise GDNF, neurturin (NRTN), artemin (ARTN), and persephin (PSPN), which play roles in the survival and differentiation of neurons through activation of RET, a receptor tyrosine kinase for members of GDNF family [173–175]. GLFs bind to the GDNF family receptor alpha (GFRα; GFRα1 for GDNF, GFRα2 for NRTN, GFRα3 for ARTN, and GFRα4 for PSPN), and then the complex forms a dimer with RET, resulting in the activation of tyrosine kinase for intracellular signal transduction. GDNF treatment of SMGs in irradiated mice promotes cell proliferation through activation of the GDNF-RET signaling pathway [176, 177]. Mice deficient for either *Gfra2* or *Ret* (*Gfra2*$^{-/-}$ and *Ret*$^{-/-}$) display hypoplastic submandibular ganglia and absence of parasympathetic innervation in the SLGs at birth, whereas *Gfra1* null mice show no developmental defects [178, 179]. In the adult phase, the submandibular, otic, and sphenopalatine ganglia in *Gfra2*$^{-/-}$ mice are severely atrophic, resulting in absent innervation in the lacrimal, parotid, and sublingual glands [178, 180]. A recent ex vivo study suggests that NRTN is necessary for functional innervation of the developing salivary epithelium [10]. NRTN and GFRa2 are expressed in the SMG bud epithelium and parasympathetic nerve fibers, respectively [181]. The expression of *Ret* is detectable in the parasympathetic ganglia adjacent to the ducts and is required for guiding GFRα2-expressing parasympathetic nerves toward the developing end buds [181]. NRTN treatment induces neuronal outgrowth and synapse formation in SMG explants and promotes end bud regeneration and parasympathetic nerve innervation in irradiated mice [181, 182] (Fig. 6). Therefore, bidirectional communication between the branching epithelium and the ganglia are crucial for organogenesis.

Diseases related to cranial nerve dysfunction are known to cause salivary gland dysfunction. For example, hereditary gelsolin amyloidosis, which is caused by autosomal dominant mutations in gelsolin, presents gelsolin amyloid deposition and xerostomia [183, 184]. Treacher Collins syndrome (TCS), which is caused by autosomal dominant mutations in *TCOF1*, *POLR1C*, and *POLR1D* (genes necessary for neural crest cell survival and migration), is characterized by craniofacial abnormalities such as underdeveloped zygomatic bones, micrognathia, cleft palate, and eyelid coloboma [185, 186]. Some studies suggest that salivary gland dysplasia and dysfunction are also associated with TCS [187].

The autonomic nervous system, comprising both sympathetic and parasympathetic nerves, regulates almost all organs' functions with opposite outcomes (the frequently called fight-or-flight response). In the salivary glands, the activation of the sympathetic nervous system accelerates mucous saliva secretion from mucous acini; on the other hand, the activation of the parasympathetic nervous system stimulates serous saliva secretion from serous acini. ACh binds to receptors M1 – M5, which are G-protein coupled receptors. The M3 receptor plays a role in saliva flow, whereas the M1 and M5 receptors play a role in secretion [188–190]. Moreover, the activation of M1 and M3 receptors induces the translocation of AQP5 from the apical cytosol to lipid rafts on the apical membrane via increased intracellular Ca^{2+} concentration in acinar cells of the salivary glands [191–193]. Parasympathetic nerve denervation causes salivary gland atrophy similar to that seen in irradiated salivary

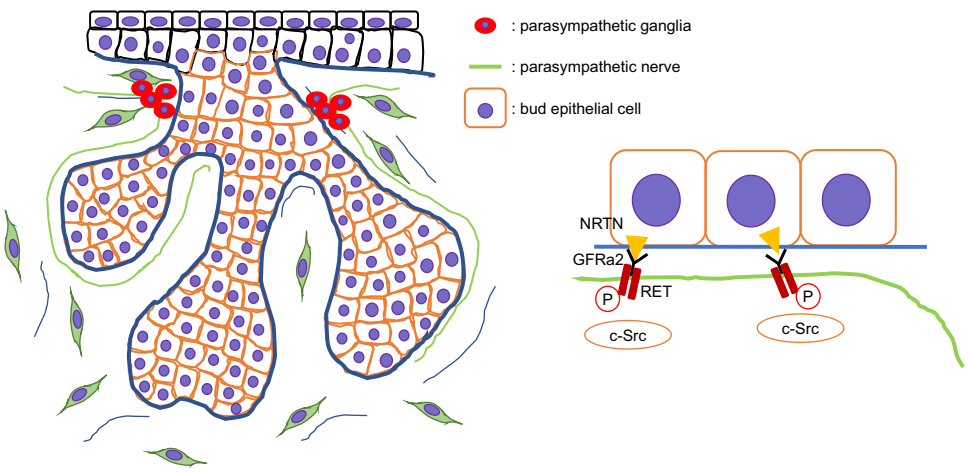

Fig. 6 Interaction of parasympathetic nerve system and salivary gland development

glands. For instance, parasympathetic nerve denervation in rat SMGs upregulates expression of M1 and M3, resulting in increased resting saliva flow, but decreased stimulating saliva flow [194]. The parasympathetic nervous system, but not the sympathetic nervous system, is essential for the maintenance of epithelial progenitor cells in an undifferentiated state required for organogenesis [9]. On the other hand, VIP, another neurotransmitter from the parasympathetic nervous system, but not ACh/M1, regulates lumen formation during duct development [10].

Conclusion

Salivary gland development involves the interactions of multiple cell types, including epithelial, mesenchymal, endothelial, and neuronal cells. Various pathways cooperate to establish acinar and ductal growth, development of the ganglia, and progenitor cell survival/proliferation. Several mouse genetic studies indicate that these molecular pathways act within complex signaling networks, which require a systematic approach to elucidate how they affect the different morphogenic processes. Further advances in human genetics and the ever-increasing number of mouse models generated will significantly increase our knowledge of the mechanisms by which signaling pathways and cells establish the tissue architecture and function during salivary gland formation.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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