Histol Histopathol (2014) 29: 305-312
DOI: 10.14670/HH-29.305

http://www.hh.um.es

Histology and Histopathology
Cellular and Molecular Biology

Review

Molecular cues for development and regeneration of salivary glands

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Summary. The hypofunction of salivary glands caused by Sjögren's Syndrome or radiotherapy for head and neck cancer significantly compromises the quality of life of millions patients. Currently no curative treatment is available for the irreversible hyposalivation, whereas regenerative strategies targeting salivary stem/progenitor cells are promising. However, the success of these strategies is constrained by the lack of insights on the molecular cues of salivary gland regeneration. Recent advances in the molecular controls of salivary gland morphogenesis provided valuable clues for identifying potential regenerative cues. A complicated network of signaling molecules between epithelia, mesenchyme, endothelia, extracellular matrix and innervating nerves orchestrate the salivary gland organogenesis. Here we discuss the roles of several cross-talking intercellular signaling pathways, i.e., FGF, Wnt, Hedgehog, Eda, Notch, Chrm1/HB-EGF and Laminin/Integrin pathways, in the development of salivary glands and their potentials to promote salivary regeneration.

Key words: Salivary glands, Development, Regeneration, Xerostomia, Molecular cues

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Introduction

Salivary gland hypofunction or Xerostomia, as an inevitable consequence of Sjögren's Syndrome or radiotherapy for head and neck cancer, significantly compromises the quality of life of millions patients through associated poor oral health (Nederfors, 2000). Salivary glands are super-sensitive to irradiation (IR), and the irreversible hyposalivation after radiation is caused by loss of functional salivary stem/progenitor cells (SSPCs) that normally continuously replenish aged saliva producing cells (Konings et al., 2005). Current treatments such as artificial saliva and saliva secretion stimulators can only temporarily relieve the symptoms. Regenerative strategies targeting SSPCs have shown promise for functional restoration in animal models (Coppes and Stokman, 2011; Lombaert et al., 2011), but little is known about the molecular control of adult SSPCs. Meanwhile, great advances in mechanisms of salivary gland morphogenesis have been achieved in recent years. Considering the similarities between morphogenesis and regeneration in many other organs, the potentials of using molecular cues in salivary gland development to promote salivary regeneration are worth careful exploration. The development of salivary glands is orchestrated by interactions between epithelia, mesenchyme, extracellular matrix and innervating nerves via a complicated network of molecular cues. Recent advances in molecular control of salivary gland branching morphogenesis have been reviewed comprehensively elsewhere (Harunaga et al., 2011). Here we will focus on the roles of several cross-talking intercellular signaling pathways in salivary gland morphogenesis and their potentials to promote

regeneration of salivary glands.

PDGF-FGF pathway

Fibroblast growth factors (FGF) signaling is essential for submandibular salivary gland (SMG) branching morphogenesis *in vivo* and *ex vivo* as indicated by major SMG phenotypes caused by knockout of FGF8 (Jaskoll et al., 2004a), FGF10 (Ohuchi et al., 2000), FGFR2b (De Moerlooze et al., 2000) or FGFR2c (Jaskoll et al., 2002), as well as human salivary gland aplasia associated with FGF10 and FGFR2 mutations (Entesarian et al., 2005; Shams et al., 2007). The roles of the FGF pathway in salivary gland branching morphogenesis have been the subject of an excellent previous review (Patel et al., 2006). Briefly, multiple FGF ligands expressed by either mesenchymal or epithelial cells were required for SMG branching morphogenesis, whereas the expression of FGFR1b and FGFR2b were found in the epithelium (Hoffman et al., 2002).

Recently, Platelet-derived growth factor (PDGF) signaling was found to promote FGF expression in neural crest-derived SMG mesenchymal cells and SMG branching morphogenesis. PDGF-A is expressed in SMG epithelium, whereas PDGF-B, PDGFRα, and PDGFRß were expressed in mesenchyme and PDGFRα is a marker of neural crest-derived cells, which suggested that the PDGF-FGF cascade is a possible mechanism involved in the interaction between epithelial and neural crest-derived mesenchyme (Yamamoto et al., 2008).

In adult mouse SMG, FGFR2IIIb is exclusively expressed on intercalated and excretory duct cells, as well as in salispheres formed by SSPCs, and FGF7/Keratinocyte Growth Factor (KGF) protein treatment prevents irradiation damage to salivary glands by expansion of the SSPC pool (Lombaert et al., 2008). Consistently, human KGF gene delivery to murine SMG prevented salivary hypofunction caused by single or fractionated irradiation without affecting the growth of squamous cell carcinoma (Zheng et al., 2011). However, KGF treatment after irradiation only slightly recovered salivary function after radiation in a mouse model (Lombaert et al., 2008). In a Phase II study of palifermin (a recombinant human KGF, ΔN23-KGF) and concurrent chemoradiation in head and neck squamous cell carcinoma, palifermin appeared to reduce xerostomia and other morbidities during hyperfractionated radiotherapy but not standard radiotherapy (Brizel et al., 2008), suggesting that either a higher dose of palifermin or combination with other strategies are needed to prevent or rescue IR-induced hyposalivation.

Wnt pathways

Wnt (wingless/int) signals are transduced through the canonical pathway via stabilization of ß-catenin or several other noncanonical pathways. These pathways are highly conserved during evolution and play essential roles in embryonic development and regulating many types of adult stem cells. During salivary gland organogenesis, the Wnt/ß-catenin pathway is activated firstly in the mesenchyme and later, at the time of lumen formation, in the ductal epithelium cells, but is never activated in endbuds (Patel et al., 2011). Mesenchymal Wnt/ß-catenin signaling induces expression of ectodysplasin-a (Eda) to trigger activation of the Edar/NF-κB pathway in the epithelium, whereas inhibition of mesenchymal Wnt/ß-catenin signaling impairs SMG branching morphogenesis (Haara et al., 2011). On the other hand, ectopic activation of epithelial Wnt/ß-catenin signaling blocks branching morphogenesis, whereas non-canonical Wnt signaling promotes ductal maturation possibly by regulation of ductal maker Cp2l1, and the lack of both Wnt/ß-catenin signaling and noncanonical Wnt signaling activities in endbuds is mediated through FGF-mediated upregulation of SFRP1, a secreted inhibitor of Wnt signaling (Patel et al., 2011).

In adult salivary gland Wnt/ß-catenin signaling is marginal but is activated significantly in the duct epithelium during functional regeneration after ligation of the main excretory duct, and ectopic activation of epithelial Wnt/ß-catenin signaling promoted expansion of c-Kit$^+$/Sca-1$^+$ SSPCs (Hai et al., 2010). Interestingly, IR does not significantly affect the activity of the Wnt/ß-catenin pathway in salivary glands, while concurrent transient activation of epithelial Wnt/ß-catenin pathway prevented IR-induced salivary gland dysfunction, likely by suppressing apoptosis and preserving functional SSPCs (Hai et al., 2012). However, similar Wnt activation 3 days before or after IR did not show such beneficial effects, possibly due to intensive induction of mitosis in epithelial cells or by missing the critical treatment window right after IR to inhibit apoptosis (Hai et al., 2012). In addition, ectopic Wnt activation in the epithelium after IR may also impair acini differentiation during tissue regeneration similar to that during embryonic branching morphogenesis.

Hedgehog pathway

Hedgehog (Hh) signaling is initiated through derepression of Smoothened (Smo), a G-protein coupled transmembrane protein, by interaction between Hh liagnds and their receptors Patched (Ptch), and is mediated by Gli transcription factros (Jiang and Hui, 2008). In the epithelium of embryonic salivary glands, the expression of Sonic Hedgehog (Shh) is induced by Edar/NF-κB pathway downstream of mesenchymal Wnt-Eda pathways (Haara et al., 2011), and the epithelial localization Ptch and Smo suggested that Shh may act within the epithelium in a juxtacrine manner to promote proliferation and differentiation of epithelial cells (Jaskoll et al., 2004b). Branching morphogenesis of SMG is promoted by Hh activation *ex vivo*, and is impaired by Hh inhibition *in vivo* and *ex vivo* (Jaskoll et al., 2004b). In addition, Hh activation promotes cell

polarization and lumen formation in developing SMG *ex vivo* (Hashizume and Hieda, 2006).

In adult salivary glands Hh signaling is marginal but is activated significantly during functional regeneration after duct ligation (Hai et al., 2010, 2013). Targeted expression of Gli1 in salivary glands promoted epithelial proliferation, and the salivary glands become histologically normalized after withdrawal of Gli1 expression (Fiaschi et al., 2011). We found recently that transient Hh activation in salivary glands by epithelial Shh over-expression expanded c-Kit$^+$/Sca-1$^+$ SSPCs. On the other hand, IR does not significantly affect Hh activity in salivary glands, whereas transient Hh activation after IR significantly rescued salivary gland function, SSPC maintenance, parasympathetic innervation and expression of related genes, including those in Bmi1 and Chrm1/HB-EGF pathways (Hai et al., 2013). These data suggested that transient Hh activation is a promising strategy to promote salivary gland regeneration after radiotherapy.

Eda/Edar/NFκB pathway

Eda is a type II membrane protein of the TNF (Tumor Necrosis Factor) superfamily, and shed from the cell membrane to bind as a trimer to its trimerized cognate receptor Edar and activate canonical NFκB pathway (Cui and Schlessinger, 2006). Eda signaling is critical for branching morphogenesis: Eda mutations in mouse and human are associated with absence or hypoplasty of almost all exocrine glands, including salivary glands (Jaskoll et al., 2003). As mentioned above, Eda signaling is downstream of Wnt signaling and upstream of Hh signaling in salivary gland development (Haara et al., 2011), and Shh treatment can rescue defects of SMGs branching morphogenesis caused by Eda mutation *ex vivo* (Wells et al., 2010), but not the induction of minor salivary glands (Wells et al., 2011). Although FGF8 is a potential target of Eda signaling in the SMGs (Melnick et al., 2009), and FGF8 peptide supplementation can rescue defects of SMGs branching morphogenesis caused by Hh inhibition (Jaskoll et al., 2004b), it could not rescue SMG defects caused by Eda mutation, suggesting that mechanisms of the Eda pathway in SMG morphogenesis may be much more complicated than currently understood. No application of modulating Eda pathway for salivary gland regeneration has been reported, but human bone marrow-derived mesenchymal stem cells transfected with Eda have been successfully used for regeneration of sweat glands to improve treatment of severe full-thickness burn injury (Cai et al., 2011), suggesting that modulation of Eda signaling activity may be a possible strategy to promote salivary gland regeneration.

Parasympathetic innervation and Chrm1/HB-EGF pathways

Parasympathetic innervation is required for salivary organogenesis by maintenance of the epithelial progenitor cell population (Knox et al., 2010). Muscarinic M1 receptor (Chrm1) is the major muscarinic receptor in the embryonic SMG epithelium, and acetylcholine signaling via Chrm1 promotes epithelial morphogenesis and proliferation of Keratin5$^+$ progenitor cells by transactivating the Heparin-Binding Epidermal Growth Factor (HB-EGF) pathway during salivary organogenesis (Knox et al., 2010). Chrm1 signaling transactivates HB-EGF pathways by matrix metalloproteinase (MMP)-mediated cleavage of proHB-EGF to release the N-terminal ectodomain HB-EGF (Prenzel et al., 1999) and the carboxyl-terminal fragment (CTF) of HB-EGF from epithelial membrane. HB-EGF induces membrane type 2 (MT2)–MMP and FGF receptor (FGFR) expression via the EGFR pathway, and MT2-MMP-dependent release of bioactive NC1 domains from collagen IV promotes SMG branching morphogenesis via Integrin ß1 and PI3K-AKT signaling pathway (Rebustini et al., 2009). Meanwhile, HB-EGF-CTF moves into the nucleus to promote cell proliferation by binding and exporting nuclear promyelocyticeukemia zinc finger (PLZF) protein, a transcriptional repressor, to allow expression of target genes such as cyclin A (Tanida et al., 2010).

Parasympathetic innervation is also essential for regeneration of adult salivary glands after duct ligation (Proctor and Carpenter, 2007). In adult SMG acini cells Chrm1 is not ubiquitously expressed and only plays a minor role in cholinergic stimulation of salivary flow, which is mainly mediated by ubiquitously expressed CHRM3 under physiological conditions (Nakamura et al., 2004). Keratin5$^+$ cells increase during regeneration of adult SMGs after duct ligation when the innervation to the gland is intact (Hai et al., 2010), and postnatal epithelial regeneration of salivary glands may require muscarinic stimulation of the Keratin5$^+$ progenitor cells in a similar manner, as indicated by the *ex vivo* culture of denervated lobules of adult SMGs with muscarinic agonist or antagonist or EGFR antagonist (Knox et al., 2010).

In SMGs of both mouse and human, IR significantly impaired parasympathetic innervation and downregulated Chrm1 expression, which may contribute to IR-induced hyposalivation, whereas restoration of parasympathetic innervation and Chrm1 expression by treatment of neurotrophic factor Neurturin improved regeneration of mouse embryonic SMGs after IR *in vitro* (Knox et al., 2013). The downregulation of Chrm1 expression by IR is likely related with miR-107 which targets Chrm1 (Scarr et al., 2013) and is upregulated by IR (Chaudhry et al., 2013).

Notch pathway

Expression of activated Notch4 (int-3) transgene interferes with epithelial differentiation in mouse salivary glands, and results in the accumulation of proliferating immature ductal cells and multiple poorly differentiated adenocarcinomas (Jhappan et al., 1992). On the other hand, during development of the drosophila

salivary gland, Notch ligand Serrate directs formation of actin rings in the salivary duct (Haberman et al., 2003). In normal human SMGs, the Notch signaling is active as indicated by the expression of Notch 1 to 4, Jagged 1 and 2, Delta 1, and HES1 with nuclear localization; in human salivary gland cell line HSG, the Notch signaling is critical for growth and differentiation; in rat SMG, the Notch pathway was activated during regeneration after duct obstruction (Dang et al., 2009). These data suggested that proper differentiation of salivary epithelial cells relies on tightly controlled Notch activity.

Laminin/Integrin pathway

The branching morphogenesis and differentiation of salivary epithelial cells depends on interactions between extracellular matrix (ECM) proteins and their integrin receptors similar to that in other branched organs (Pozzi and Zent, 2011). Laminin-111 (laminin-1/LM-111), a heterotrimeric ECM protein essential for basement membrane formation, and its receptor Integrin α6ß1, are required for branching morphogenesis of mouse SMG *ex vivo* (Kadoya et al., 1995). Consistently, interaction between laminin-1 and Integrin α6/ß1 are required for *ex vivo* differentiation of human salivary gland cell line HSG (Hoffman et al., 1996), which functions through upregulation of metallothioneins, a group of cystin-rich proteins with various functions including metal metabolism and homeostasis (Hecht et al., 2002). Other laminins and integrin receptors are also important for salivary gland development and homeostasis. Mutation of Integrin α3 leads to defects in the apical-basal polarity axis and in the basement membrane of mouse SMG epithelial cells, which may be through regulation of Cdc42 and RhoA (Menko et al., 2001). Laminin α5 controls SMG epithelial morphogenesis through ß1 integrin signaling by regulating FGFR expression, which also reciprocally regulates the expression of Laminin α5; interestingly, the loss of both integrin α3 and α6 resulted in a similar phenotype to that of Laminin α5 knockout, suggesting that interactions between α3ß1 and α6/ß1 integrins with laminin 511 are required for SMG development (Rebustini et al., 2007).

In adult salivary gland, rodent and human salivary progenitor cells express intracellular laminin and its receptor integrins such as α6 (CD49f), ß1(CD29) and ß4 (CD104) (Sato et al., 2007); and integrin α6ß1-expreessing cells isolated from rodent salivary glands have stem cell capabilities, including colony formation and multipotent differentiation (Okumura et al., 2003; David et al., 2008). Sjögren's syndrome is characterized by low levels of acinar compartment-specific laminin α1 (Laine et al., 2004), and signaling mediated by integrin α1ß1 and α2ß1 is necessary for salivary gland remodeling by inducing differentiation of intercalated duct progenitors to acinar cells (Porola et al., 2010). These data suggested that the Laminin/Integrin pathway is also essential for homeostasis of salivary glands, and provides a promising target for regenerative therapy to restore salivary function.

Cross-talk between the above pathways

The above signaling pathways interact at multiple levels during the development and regeneration of salivary glands. The cross-talk between Wnt, Eda and Hh pathways and that between Fgf and Wnt pathways in branching morphogenesis have been mentioned above. In addition, in embryonic salivary glands, Shh expression is modulated by Fgf8 levels and Shh peptide supplementation *in vitro* partially rescued the abnormal SMG phenotype associated with decreased FGF8 signaling (Jaskoll et al., 2004); conversely, Shh signaling regulates FGF8 protein expression too and the abnormal SMG phenotype caused by Hh inhibition could be rescued by FGF8 peptide supplementation *ex vivo* (Jaskoll et al., 2004a), which demonstrated that FGF and Hh pathway function in an coordinated manner in this process. In adult SMGs, Wnt activation upregulated Shh expression (Hai et al., 2010), whereas Hh signaling promoted Chrm1 expression and parasympathetic innervation, probably through miR-107 and Brain-derived neurotrophic factor respectively (Hai et al., 2013).

More interactions of these pathways have been reported in other tissues. For instance, Hh signaling activates Lama1 expression during assembly of the myotomal basement membrane (Anderson et al., 2009), and activates Integrin α2 (Itga2) expression during osteoblast differentiation of human bone marrow Mesenchymal stem cells (Oliveira et al., 2011); Notch ligand Jagged2 (Jag2) and Notch target gene HEY2 are both direct target genes of the Hh pathway (Rabadan et al., 2012; Wu et al., 2012), whereas muscarinic activation via Chrm1 increases expression of Notch receptor Notch1 in oligodendrocytes (De Angelis et al., 2012) and activity of ADAM17 protease (Alfa Cisse et al., 2007), which cleave Notch1 to facilitate Notch signaling (Bozkulak and Weinmaster, 2009). Whether such interactions exist in development and regeneration of salivary glands is worthy of further investigation. The roles and cross-talks of these signaling pathways during morphogenesis and regeneration of salivary glands are summarized in Figs. 1 and 2 respectively.

Gene therapy for hyposalivation

Since abbrent activation of the above signaling activities is closly related with tumorigenesis, their activation for regeneration or fuctional restorsation of salivary glands in survivors of head and neck cancer must be done with great caution. Hence, local gene transfer into salivary glands seems to be a much safer strategy than systemic delivery of protein or small molecule agonists. Major salivary glands have several features that make them attractive targets for gene transfer. Firstly, they are easily accessible, since the ductal orifices of all major salivary glands open directly

into the oral cavity. Secondly, essentially all epithelial cells (acinar and ductal) in these glands have their plasma membranes (apical surfaces) directly in contact with the ductal tree, and thus with the oral cavity. Thirdly, they are well encapsulated, which should eliminate or at least minimize the concern about vector spread beyond the salivary gland tissues. Finally, salivary gland epithelial cells are capable of producing considerable amounts of proteins, especially for export, mostly in an exocrine direction (O'Connell et al., 1996). Conventional cannulation techniques can be applied to introduce viral or non-viral vectors into the gland (Delporte et al., 1998). While no animal model is entirely representative of human physiology, large animals can provide a more appropriate size target, and often a better predictive result, for many potential therapies than rodents (Casal and Haskins, 2006). The primate is the best model (Price et al., 1995), but for most labs it is too expensive. The miniature pig has been increasingly used as a large animal model in a variety of biomedical studies, and the parotid glands of miniature pigs are almost identical to those of humans in terms of their volume and morphology (Wang et al., 1998).

Transfer of adenovirus or adeno-assoicated virus encoding human aquaporin-1 (AdhAQP1, AAVAQP1) has successfully rescued radiation-induced hyposalivation in rodent and miniature pig models (Baum et al., 2006, Gao et al., 2011). In human, AdhAQP1 vector delivery to a single parotid gland was safe and transfer of the hAQP1 cDNA increased parotid flow and relieved symptoms in a subset of subjects (Baum et al., 2012). In addition, local gene delivery of KGF (Zheng et al., 2011), vascular endothelial growth factor (VEGF) (Cotrim et al., 2007) or tousled kinase (Palaniyandi et al., 2011) has shown protective effects on salivary glands from IR. Our two labs are collaborating on testing the potential of modulating Wnt or Hedgehog signaling activities by gene delivery to prevent or rescue radiation-induced hyposalivation.

Conclusions and prespective

Our knowledge on molecular controls of salivary gland morphogenesis have advanced signifcantly during

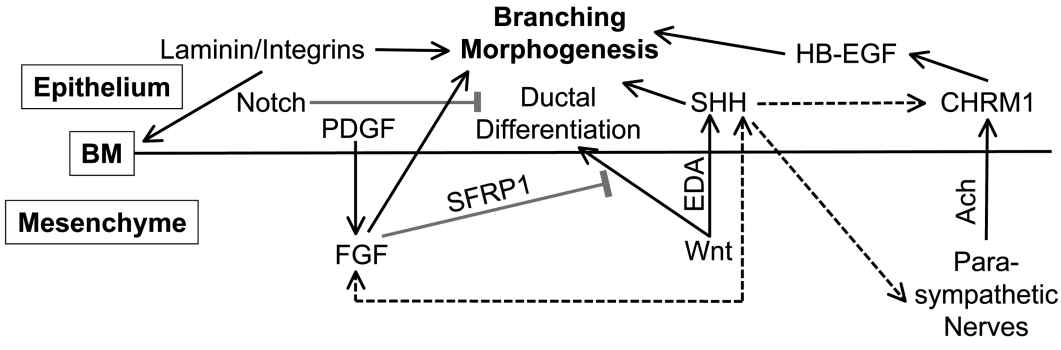

Fig. 1. Roles and interactions of signaling pathways during salivary gland morphogenesis. BM, basement membrane; Ach, Acetylcholine; Black arrow, promoting effects; Gray blunt connector, inhibitory effects, Dashed line, Non-determined interactions.

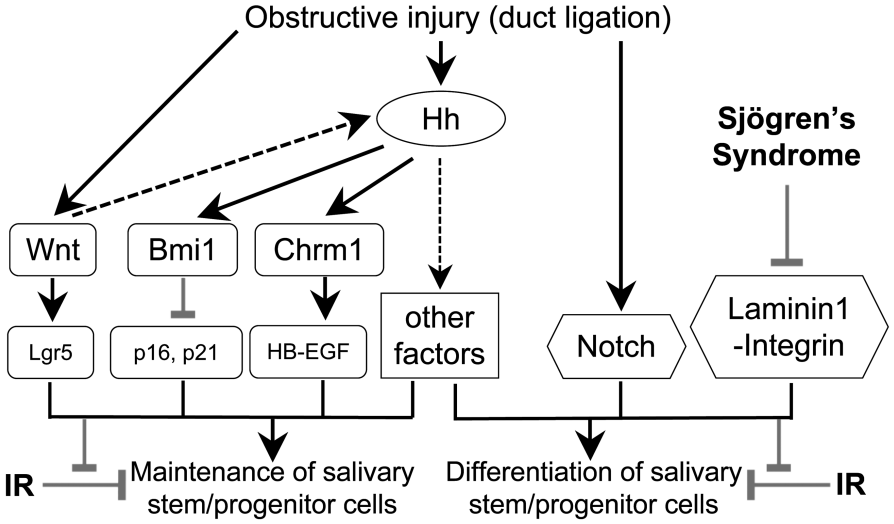

Fig. 2. Roles and interactions of signaling pathways during salivary gland regeneration after injury. IR, Irradiation; Dashed line, Non-determined interactions.

the last decades, while research on the molecular cues of salivary gland regeneraiton is still at the very early stages. With the insights and research tools from salivary gland morphogenesis, exciting findings on the mechanisms of salivary regeneration should be expected. However, the roles of the same molecule cues may be very different during morphogenesis and regeneration, and in addition to the theraputic potential for salivray gland regeneration, the roles of candidate molecues in regulation of salivary stem/progenitor cells need to be extensively studied.

Acknowledgements. The work on the roles of Wnt and Hedgehog signaling pathways in salivary gland regeneration is funded by NIH/NIDCR 1RC1DE020595-01 (FL) and 1R01DE022975-01 (FL).

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Accepted October 30, 2013