

Application of Developmental Principles for Functional Regeneration of Salivary Glands

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Abstract : Currently, there has been rapid increase in the studies about salivary production because of the hyposalivation, *xerostomia*, caused by radiotherapy for head and neck cancer, Sjogren syndrome and aging. An overview of anatomy and development of salivary gland is crucial to understand about the patho-physiological disorders related with saliva. For study of the morphogenesis and development of salivary glands, experiment using rodent models is widely necessary. This review wraps up the early to latest studies - the different features of each salivary gland, morphogenesis of developing salivary glands, and the comparison of human and rodent salivary glands. The goal of this review is to provide hypothesis for the further researches about differentiation of specific acinar cells, from which it is determined to be specific acini. Additionally, we discuss approaches to regenerate the function of salivary glands using environmental factor, time dependent factor and nerve factor.

Keywords : Salivary gland development, Morphogenesis of salivary gland, Rodent salivary glands

INTRODUCTION OF SALIVARY GLANDS AND SALIVA

Saliva is the complex fluid produced from salivary glands and plays crucial roles in maintaining the oral health. In

patho-physiological conditions of salivary glands, such as xerostomia, causes difficulties in swallowing, speaking, and masticating, and may result in the infection of oral mucosa or rampant caries [1-4]. There are several crucial functions of saliva executing in our body. Through clearing, lubricating, and pellicle formation, saliva protects the oral cavity. As well as it maintains pH and neutralizes acid as it buffers the foreign environment. The other important roles of saliva are organized and listed in Table 1 [5,6,13-15]. Not only confined in our oral environment, saliva exerts many influences throughout general body health. A large number of diseases and medications can affect salivary secretion, leading to salivary gland dysfunction and associated oral problems. Hyposalivation is a very common disease for patients suffering from head and neck cancer

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Table 1. Functions of saliva

Function	Effect	Active constituents
Protection [13]	Clearance Lubrication Thermal / Chemical insulation Pellicle formation Tannin binding	Water Mucins, Glycoproteins Mucins Proteins, Glycoproteins, Mucins Basic proline-rich proteins, Histatins
Buffering [5]	pH maintenance Neutralization of acids	Bicarbonate, Phosphate, basic proteins, urea, ammonia
Tooth integrity [5]	Enamel maturation, repair	Calcium, Phosphate, Fluoride, Statherin, Acidic proline-rich Proteins
Antimicrobial activity [5,14]	Physical barrier Immune defense Nonimmune defense	Mucins Secretory immunoglobulin A Peroxidase, Lysozyme, Lactoferrin, Histatin, Mucins, Agglutinins, Secretory, Leukocyte protease inhibitor, Defensins, and Cathelicidin-LL 37
Tissue repair [14]	Epithelial Wound healing	Growth factors, Trefoil proteins, Regeneration
Digestion [5]	Bolus formation Starch, triglyceride digestion	Water, Mucins Amylase, Lipase
Taste [15]	Solution of molecules Maintenance of taste buds	Water and lipocalins Epidermal growth factor and Carbonic anhydrase VI

Table adapted from Ten cate's oral histology, 8th edition; page 254.

for which radiotherapy is mandatory treatment to undergo, for those who take certain medications and for those who have Sjogren's syndrome [7-10]. In human beings, there are three pairs of major salivary glands located extra-orally which are responsible for 90% of whole salivary production - *parotid gland (PG)*, *submandibular gland (SMG)* and *sublingual gland (SLG)* [1,2]. These three major salivary glands drain secretion intra-orally through extended duct system. 10% of saliva production is contributed by minor mucosal glands which function as oral tissue protective substance [11,12].

The secretions from major salivary glands differ in the amount and constituents. In human beings, the SMG produces around 70-75% of secretion, while the PG secretes about 20-25% and small amounts are secreted from the other salivary glands [5]. Saliva is about 99.5% water. The remaining 0.5% consists of various protein, electrolytes, and antimicrobial factors. Two secretory compartments can be distinguished, the first is the fluid serous saliva, which contains mostly proteins, small amounts of carbohydrates, and zymogen granules which are precursors of the enzyme amylase involved in breaking down of carbohydrates. The second is the mucous secretion containing high amount of carbohydrates and small amount of proteins as well as a viscous substance called mucin [16].

EMBRYOLOGY AND ANATOMY OF THE SALIVARY GLANDS

In human, the three major paired salivary glands and a lot of minor salivary glands develop from the ectodermal germ layer. The PGs start to develop at 4 to 6 weeks of embryonic life, the SMGs at 6 weeks, and the SLG and minor salivary glands at 8 to 12 weeks, with the common structural development of acini and ducts. The cells of acini and ducts obtain maturity during the last 2 months of gestation. After birth, the glands gradually increase in size up to 2 years of age [5].

PG is the largest salivary gland and the first major salivary gland to develop among all human major salivary glands. Its superficial location is in front of external ears and the deeper portion lies behind the ramus of mandible [17]. PG is wedge-shaped with five processes, three at superficial and two at deep portions [18]. The duct of PG, Stensen's duct, is extended across the masseter muscle, and it runs inward at the anterior edge of masseter. The duct opens at a papilla opposite to the maxillary second molar. PG drains watery saliva, composed of enzymes such as amylase, proline-rich proteins and substances like glycoprotein [19]. SMG provides mixed secretion of serous and mucous acini [20]. SMG is placed at the posterior part of the floor of mouth, near the medial side of the mandible and

covering around the posterior border of mylohyoid muscle [17]. The duct of SMG, Wharton's duct, lies above the mylohyoid muscle and opens at the floor of the mouth, the sublingual caruncle, lateral to the lingual frenum. SLG is known to be the smallest among the major salivary glands and is also mixed acini but contains large amount of mucin which makes the saliva viscous with the presence of every component mentioned above [1,5]. SLG is situated in the anterior part of the floor of mouth between the mucosa and the mylohyoid muscle [17]. SLG secretes the saliva into the oral cavity through a series of small ducts, ducts of Rivinus, opening along the sublingual folds and also through a larger duct, Bartholin's duct, which opens with submandibular duct at the sublingual caruncle [5]. The different characteristics of each major salivary gland are presented in Table 2 [16].

Besides major salivary glands, there are numerous minor salivary glands that are smaller in size but greater in number than the major ones. The minor glands are situated throughout the oral cavity and are named for their location. The glands located at cheeks and lips are termed *buccal* and *labial* glands and contains mixture of serous and mucous secretions. Both glands at posterior hard palate and soft palate are called *palatine glands* and those of tonsillar glands are called *glossopalatine glands*, they are all pure mucous glands. Tongue has lingual glands which are mixed glands at tongue's tip. At the junction of the tongue's body and base, *serous glands of von Ebner* are located which wash out the taste buds of circumvallate papillae [5]. Tongue has also mucous glands which is located at the posterior portion under the lingual tonsillar tissues [16]. About 600-1000 minor salivary glands are thought to be present in oral cavity [7,11]. They mostly underlie at the submucosal layer and have short length of ducts that excrete directly to the surface of mucosa.

Salivary glands are composed of acinar or tubular shaped secretory end piece, *acinus*, and duct system. Secretory end pieces can be divided into serous cell and mucous cell by

its type of saliva secretion. It consists of serous acini, mucous acini and muco-serous acini which drains both serous and mucous secretion, or combination of the two acini, serous demilune [16]. Duct system - is made up of intercalated duct, striated duct and excretory duct - stimulates the changes of salivary components that secretory end pieces produce and transfer the secretion into oral cavity. In recent studies, it is discovered that some of serous cells in salivary glands also provide slight amount of mucin, as well as the mucous cells generate non-glycosylated proteins which is thought to be produced by serous cells [21]. In addition, it is proven by the development of tissue preservation procedure that serous cells and mucous cells are similar in their structures, however, it is yet to unravel what makes those two cells to secrete different forms of secretions.

COMPARISON OF HUMAN AND RODENT SALIVARY GLANDS

Various researches and studies [22-24] are currently elucidating the structures and functions of salivary glands using rodents (mice and rats). These rodent models are widely used to accumulate genetical data due to ease of access. It is also necessary to compare and understand the similarities and differences in features between human and rodent salivary glands. There are three pairs of macroscopic glandular organs in human and rodents: PG, SMG, and SLG [19,25]. The rodent salivary glands have distinguishing differences from the features of human salivary glands that mentioned above. For macroscopic anatomy of PG of rodents (Fig. 1), PG is located behind and below the ear, caudally bordering the SMG [26]. When it is separated from the skin, the gland is alike with "pancreas" in the mesentery [20]. SLG of rodent is located together with the SMG in the anterior neck spaces between the submandibular lymph nodes and the sternum (Fig. 1). Submandibular-sublingual complex is well-observed in rodent where the SLG occupies the latero-rostral one fourth of it (Fig. 1). Both glands are encapsulated with a common fascia. Main excretory ducts of the SLG and SMG are separated. Similar to human salivary glands, the duct system of rodents is consisted of the intercalated, striated, and excretory ducts. However, rodent SMG have the granular convoluted tubule located between intercalated duct and striated duct, as a segment of the duct system [1,27]. The main excretory ducts are Stensen's duct

Table 2. Minor salivary glands and contribution to saliva

Name	Location	Type secretion
Labial	Lips	Mixed [16]
Buccal	Cheeks	Mixed [16]
Palatine	Hard and soft	Pure mucous [16]
Lingual	Anterior	Mixed
	Middle	Serous
	Posterior	Pure mucous [16]

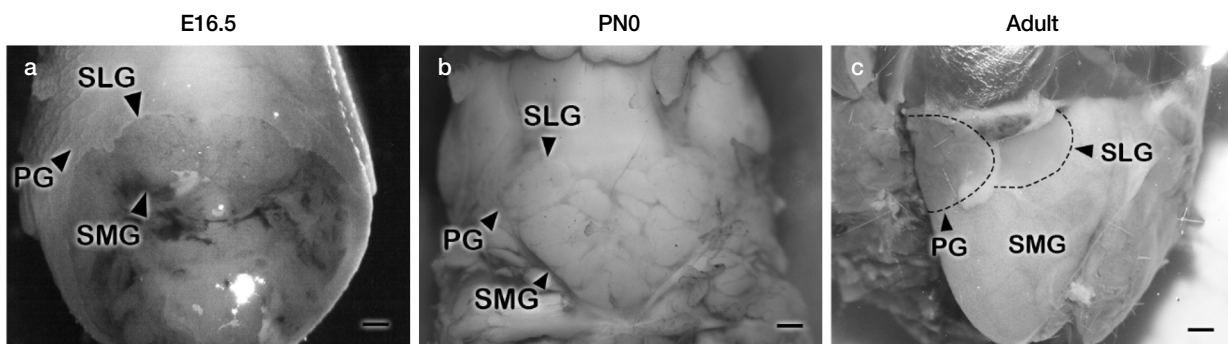


Fig. 1. Anatomy of the anterior neck portions of developing salivary glands of mice (a-c). Embryonic day 16.5 (E16.5), post-natal day 0 (PNO) and adult mice showing the location of parotid gland, submandibular gland and sublingual gland. Scale bars, 1 mm.

for PG, Wharton's duct for SMG and Bartholin's duct for SLG. Both human and rodent PG are composed of pure serous acini. The human PG is well-characterized with intralobular adipose tissues, whereas the adipocytes are not prominent in the rodent PG. SMGs of both human and rodents are mixed glands of serous and mucous acinar cells. SLGs of both human and rodents also are mixed glands, but mucous secretory cells predominate. The acini of SLG are composed of centrally-located mucous cells and peripheral serous demilunes.

SECRETION MECHANISMS OF SALIVARY GLANDS AND DEVELOPMENT

Salivary gland belongs to exocrine gland which produces and secretes through ducts, not through bloodstream, and similar with it, sweat gland and mammary gland are the typical exocrine glands whose developmental process are much identical to salivary gland. The glands originated from epithelium such as sweat gland and mammary gland have the same developmental stages with salivary glands. They all initiate as thickening of the epithelium called placodes, which invaginate into the adjacent mesenchyme to form epithelial bud-like structure [2,28,29]. The developed glands are, however, all distinct from each other in their mechanism of secretions. There are three different types of secretion: *Merocrine (eccrine)*, *holocrine*, *apocrine*. Salivary glands are termed merocrine glands due to the mode of excretion is through exocytosis which is through membrane vesicles from secretory cells into an epithelial-wall duct [16]. The merocrine glands start to form during fourth

month of gestation and first to develop on the palms and soles. The merocrine germ later forms three portions of the merocrine gland: the intra-epidermal portion, the intra-dermal duct and the secretory portion. The secretory cells are surrounded by contractile myoepithelial cells [30]. Not only salivary gland but certain sweat glands and pancreatic gland belongs to eccrine gland. For holocrine gland, the secretions in cytoplasm of cells are released by rupture of the plasma membrane resulting in the destruction of cell itself to secrete the substance into the lumen. Sebaceous gland and Meibomian gland of the eyelid are the typical holocrine glands [31]. Lastly, the secretion of apocrine gland is excreted through the plasma membrane producing membrane-bound vesicles in the lumen. The apical portion of the cell pinches off and enters the lumen. Because the part of cytoplasm is forming membrane-bound vesicles, it loses some portion of its cytoplasm for its secretion. Apocrine is less destructive than holocrine which destroys the whole cell, but it is more damaging than merocrine secretion which only uses exocytosis. The example of apocrine gland is mammary gland which is responsible for the secretion of breast milk [29,32].

The development of salivary glands in mice initiates by a thickening of the oral epithelium and the formation of placode which is induced by mesenchymal signal [33] during embryonic day 11-12 (E11-12). The epithelial placode, then, invaginates into mesenchyme which begins to condense. The formation of primary bud on a stalk starts as epithelial bud mature its feature in neural crest-derived mesenchyme [34,35]. As the primary bud forms, 'branching morphogenesis' by which a simple epithelial bud is remolded through repetitive branching and this process includes

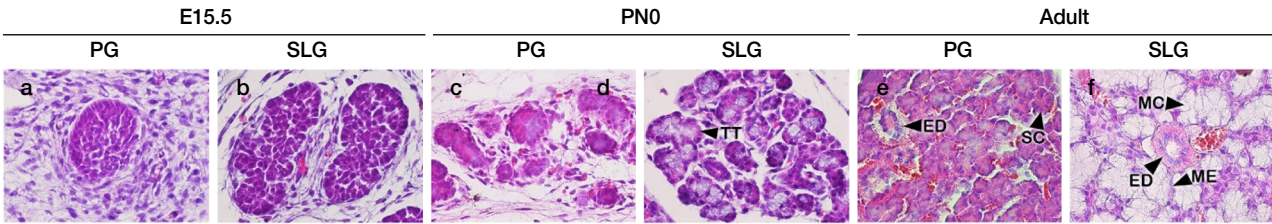


Fig. 2. Hematoxylin and eosin staining of the developing parotid gland (PG; a, c, e) and sublingual gland (SLG; b, d, f). Embryonic day 15.5 PG showing epithelial bud (a). Post-natal day 0 (PN0) PG showing terminal epithelial buds (c). Adult PG showing dark-colored acinar cells which characterizes serous acinar cell (e). E15.5 SLG with developing epithelial end buds and lumen formation (b). PN0 showing terminal tubules and differentiating acinar cells (d). Adult SLG showing light-colored acinar cells which characterizes mucous acinar cells (f). TT terminal tubule, ED excretory duct, SC serous acinar cell, ME myoepithelial cell, MC mucous acinar cell.

cell proliferation, migration, cleft formation, and differentiation. During this process, secretory organ containing thousands of acini connected to secretory duct forms [2,33,36]. Clefts in epithelium demonstrate first three to five buds which correspond to major lobules of the gland as the end buds enlarge at E13. At E14, the gland develops highly branched and the main duct begins to lumenize [36]. By E15-16, main secretory ducts nearly complete its lumenization [33] and the epithelial end buds are highly branched (Fig. 2a, b). By PN0, PG showed terminal end buds (Fig. 2c), and SLG was observed with terminal end buds forming terminal tubules (Fig. 2d). This morphogenesis and differentiation continue and is completed at birth [38]. In the adult stage, PG acini were stained with darker color which is characteristics of serous type (Fig. 2e). Adult SLG showed lighter stained acini, characteristics of mucous type (Fig. 2f).

SIGNALING PATHWAY REGULATING SALIVARY GLAND DEVELOPMENT

Complex morphological changes suggest that multiple signaling pathways are precisely regulated spatiotemporally during salivary gland development. There are many signaling pathways that regulate salivary gland morphogenesis (Table 3) [39,40]. Fibroblast growth factor (FGF) signaling is essential for branching morphogenesis of developing salivary gland [41]. FGF10/FGFR2b signaling plays an important role in increasing acinar cell proliferation and reducing lumen and duct formation [42-44]. FGFs promotes end bud development and is essential for early morphogenesis of salivary gland [45]. The epidermal

Table 3. Signaling pathways involved in salivary gland morphogenesis

End bud formation	FGF family EGF family BMP family EDA/EDAR HSPGs (Heparan sulfate proteoglycans) [43]
Cleft formation	HSPGs Collagens Laminins Fibronectin FGF family
Lumen formation	Wnt signaling EDA/EDAR HH signaling VIP (Vasoactive intestinal peptide) [44]
Duct formation	Wnt signaling EDA/EDAR EGF family HH signaling Notch signaling VIP

growth factor (EGFs) and their receptors are crucial for salivary gland morphogenesis. The SMGs of EGFR-null mice show reduced proliferation, branching morphogenesis, and maturation of the epithelium [46]. Wnt signaling is highly dynamic during SMG development. WNT/ β -catenin-dependent signaling is active in mesenchyme at the early stage of SMG development (E12-E15) and upregulated in ductal epithelium of SMG after E15 [43,47,48]. Opposing reports exist for the role of the WNT signaling pathway in salivary gland development. SB415286 that activates the β -catenin-dependent pathway [49] suppresses branching morphogenesis of SMG rudiments [43], whereas XAV939 that inhibits the β -catenin-dependent pathway also inhibits epithelial branching and growth of SMG rudiments [47].

Hedgehog (HH) signaling plays an important role in duct formation. During lumen formation, Smoothed and GLI3 acts centrally on duct and terminal bud surrounding the lumen. It suggested that Sonic Hedgehog (SHH) may be important for lumen development [50]. Ectodysplasin (EDA) pathway, another critical signaling molecule, have revealed a critical role in salivary gland ductal and acinar development [51,52]. Bone morphogenetic protein (BMP)s and their receptors are differentially expressed between the mesenchyme and epithelium in developing salivary gland. However, not all BMPs promote salivary gland morphogenesis. BMP4 has inhibitory activity that reduce end bud number and branching in the salivary gland. BMP7 regulates formation of acinar cells in a non-redundant fashion [53]. Cleft formation associates with accumulation and turnover of interstitial collagen fibers [54,55]. Early studies of salivary gland reported that degradation of collagen type I and III reduced epithelial cleft formation and consequently branching. Recently, the important roles of other extracellular matrix, such as laminins, fibronectin (FN), and perlecan, have been reported [56-58]. FN has been shown to be integral to branching morphogenesis in developing salivary gland. FN appears to be a key regulator of cleft formation controlling intracellular networks for new bud formation. Laminins display regulated expression and patterns in the developing salivary gland.

These pathways regulate the branching morphogenesis including epithelial proliferation, end bud expansion, and

cleft formation in distinct spatiotemporal patterns. Previous studies have concentrated on branching morphogenesis of mouse SMG to elucidate how these molecules act on the complex signaling pathways. We need to understand the exact mechanism of salivary gland development to achieve meaningful salivary gland regeneration. It is clear that additional research is needed, such as identifying key factors that determine the type of gland.

APPLICATION OF DEVELOPMENTAL PRINCIPLES FOR REGENERATION

For further investigation of salivary glands morphogenesis, for example how they are differentiated into specific acini structures including serous or mucous, should be studied using a range of experimental tools for proper and functional regeneration of salivary gland. For these, we propound the hypothesis: first, the spatial factors; the specific nature of salivary gland acini could be regulated and determined by environmental factors such as bone, nerve and muscle forming factors. Because the location of each major salivary glands is well-elucidated and environment of each salivary gland is very specific. For example, PG which secretes watery saliva through serous acini is placed behind and below the ear [26] where close to the bone, ramus of mandible. SMG is a mixed gland with serous cells significantly outnumber the mucous cells. It is situated

Table 4. Characteristics of major salivary glands

	Parotid gland	Submandibular gland	Sublingual gland
Developmental origin [5]	Ectodermal	Endodermal	Endodermal
Opening of the ducts	Buccal cavity across the maxillary second molar [24]	Floor of buccal cavity at the sides of lingual frenulum [17,18,24]	Floor of buccal cavity at the sides of lingual frenulum [18]
Type cells	Serous [5]	Mixed (serous and mucous) [5]	Mostly mucous [5]
Types of acini	Serous acini	Sero-mucous acini	Mostly mucous acini
Specific duct	Stensen's duct [1,5]	Wharton's duct [5]	Duct of Rivinus Bartholin's duct [1,5] - connects with Wharton's duct
Duct types	Three types intercalated, striated and excretory ducts	Three types intercalated, striated and excretory ducts and GCT in case of rodents	Three types intercalated, striated and excretory ducts
Nerve supply	Glossopharyngeal nerve (CN IX), Trigeminal nerve (CN V) [25]	Lingual nerve (Branch of CN V) & Facial nerve (CN VII) [26]	Lingual nerve (Branch of CNV) & Facial nerve (CN VII) [26]
Amount of saliva production	25% of total saliva production [16,24]	60% of total saliva production [2,27]	5% of total saliva production [16]
Size of the glands	Largest among all [18]	Intermediate	Smallest among all [18]

in the posterior part of the floor of the mouth, adjacent to the medial aspect of the mandible and wrapping around the posterior border of the mylohyoid muscle. SLG also is a mixed gland, but mucous secretory cells predominate. The gland is located in the anterior part of the floor of the mouth between the mucosa and mylohyoid muscle, where relatively free from the bony tissue. Secondly, temporal factors; we propound that there might be regulation of temporal dependent factors, involved in forming different salivary acini. In the past studies about SMG developmental stages, the solid epithelium placode of SMG occurs throughout E11.5-12, salivary branching morphogenesis is repeated several times over the following days. Single one-bud, one-duct salivary gland has both grown and branched significantly during E14 and after E15-16 the lumenization of main secretory duct is nearly completed. The acini complete lumenization at E17 and it continuously mature even after birth [37]. Even though the study about specific development of salivary gland is not yet founded in PG and SLG, there could be integral signals in determining the specific acini on each gland. Lastly, in human salivary glands, each gland is governed by different nervous system [16] as organized above in Table 4 [1,2,5,16-18,35,59-61] and this different nervous system could be the factor to discriminate between serous and mucous acini.

In this review, we examined the salivary gland development, physical functions, and signaling pathways for proper morphogenesis of functional salivary glands. In addition, we studied the different features of each salivary gland through the comparison of human and rodent salivary glands. More detailed and precise evaluation of the developmental mechanisms underlying functional morphogenesis of salivary glands would be a plausible answer for tissue regeneration.

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