Region specific morphogenesis of tongue papillae

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Region specific morphogenesis of tongue papillae

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ABSTRACT

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The tongue of most vertebrates is muscular organ, and is used to manipulate and swallow food. It plays an important role in the digestive system and acts as the main organ of taste in the gustatory system. The human tongue is divided into the oral region of the anterior 2/3 part and the pharyngeal part of the posterior 1/3 part by the terminal sulcus, which is a V-shaped groove. The dorsal surface of the tongue is covered with a masticatory mucosa with keratinized stratified squamous epithelium. There are a number of papillae that contain taste buds and taste receptors. These lingual papillae have four forms, circumvallate, foliate, fungiform and filiform, but filiform papillae do not have a taste bud.

Taste disorder associated with taste sensation occurs in various causes in life. Therefore, in this study, when the taste disorder such as decease or loss of taste occurs, it is interested in how to regenerate the taste bud in tongue in order to regain taste. To regenerate these lingual papillae, it is necessary to understand the development of lingual papillae.

Regarding the development of lingual papillae, the interaction of epithelial and mesenchymal tissues, which can be observed in the development of most epithelial appendages such as feathers, lungs and teeth, is also necessary in the development process of lingual papillae. However, each of the other organs has different signaling pathways and regulatory factors for development.

In the case of circumvallate papillae, morphology of embryonic day 13.5, 15.5, 17.5 (E13.5, E15.5, E17.5), post-natal 1 (PN1) and adult was confirmed by histological results. Various signaling molecules may be involved in the formation of the circumvallate papillae during the early developmental process. Among various signals, the location of *Lgr5* was confirmed through *in situ* hybridization during the development of circumvallate papillae from E13.5 to adult, moreover, the distribution of FGF10 was confirmed in development process of circumvallate papillae through immunohistochemistry at E15.5 and E17.5. To obtain direct evidence of the interaction between epithelium *Lgr5* and

mesenchymal *Fgf10* during the morphogenesis of circumvallate papillae, the formation of a circumvallate papillae-like structure was examined after a reverse (180-degree rotation) recombinant. It was performed after *in vitro* recombination assay for 72 hours using *in vitro* organ culture. When the epithelium of circumvallate papillae positioned to the absence of *Fgf10*, the structure of circumvallate was not observed. When the implanted bead soaked in FGF10 was located below the epithelium of circumvallate papillae that was the absence area of *Fgf10*, the form of circumvallate papillae could be formed. After 6-bromoindirubin-3′-oxime (BIO) treatment, abnormal cell proliferation and cell death were observed by a decrease in mesenchymal *Fgf10*. These results suggest that the communication between epithelial *Lgr5* and mesenchymal *Fgf10* is necessary for proper formation of circumvallate papillae in the process of tongue development.

Key words : tongue papillae development, circumvallate papillae, epithelial mesenchymal interactions, signaling molecule, *Lgr5*, *Fgf10*, morphogenesis

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I. INTRODUCTION

1. Tongue and lingual papillae

The embryonic origin in the tongue comes from all pharyngeal arches that contribute to other components. Since the tongue is formed from the floor of the oral cavity, it is not easily seen from an external point of view during the embryonic stage. During mouse embryo generation, the terminal sulcus divides the tongue into the oral and pharyngeal part[1]. The dorsal tongue is covered with stratified squamous epithelium with different papillae and taste buds, and there are four types of papillae. These papillae are circumvallate (or vallate), foliate,

fungiform and filiform, with have different structures and each papillae is distributed in a specific pattern [1]. Among these are circumvallate, foliate, and fungiform papillae except filiform papillae, which is known to work as sensory organ of taste [2]. Taste buds, a composite cell of about 40 to 60 cells in the papillae epithelium, are oval sensory end organs involved in the recognition of chemical stimuli and in delivery of tastant [3-5].

These taste buds are the essential senses for ingesting nutrients and maintaining good health[6]. However, the taste disorder can occur due to various causes, and taste disorder can have a major impact on life. Therefore, in this study, it was thought that the method of regenerating the taste bud in the tongue could be a fundamental solution to recover the taste when the taste was decreased or lost due to the taste disorder. And in order to regenerate the taste bud, it is necessary to understand and study the development of lingual papillae with taste bud firstly.

The circumvallate papillae is dome-shaped structure and varies in the human tongue from 8 to 12, but in rodents, one is located in the median posterior one third area of the tongue (Fig. 1)[7]. Foliate papillae are short vertical folds that

exist on both posterior sides of the tongue, have 4 or 5 vertical folds, are of various sizes and shapes, and are usually symmetrical bilaterally. The fungiform papillae are usually red processes on the tongue, shaped mushroom, and they are usually found on the dorsal surface of the tongue and spread out between the filiform papillae, and are mostly located at the sides and end of tongue. The filiform papillae are tiny, small, conical papillae, mostly covering the upper part of the tongue and covering front two-thirds of the tongue's surface. Though the largest number of papillae is tongue papillae, unlike other papillae, filiform papillae do not include taste buds.

The report about the development of tongue papillae, as suggested by this review, was derived from previous studies on sheep, rodents, and salamanders and from human development[1,8,9]. The early study with vertebrate tongue examined the three-dimensional structure of tongue and tongue papillae at the embryo and the fetus stage, using a light microscopy and a scanning electron microscope[1,8]. However, during the development of tongue papillae, many factors, including ECM (extracellular matrix) molecules such as CAM (cell-attached molecules) and cytokeratins, were reported to be able to determine its

shape[10-12]. In recent years, several methods have been studied for protein expression, signal networks and tissue interactions in circumvallate and fungiform papillae[13-15]. In this study the circumvallate papillae formation is discussed because it has a specific shape and a defined location on the dorsal surface of the tongue[4,5,16].

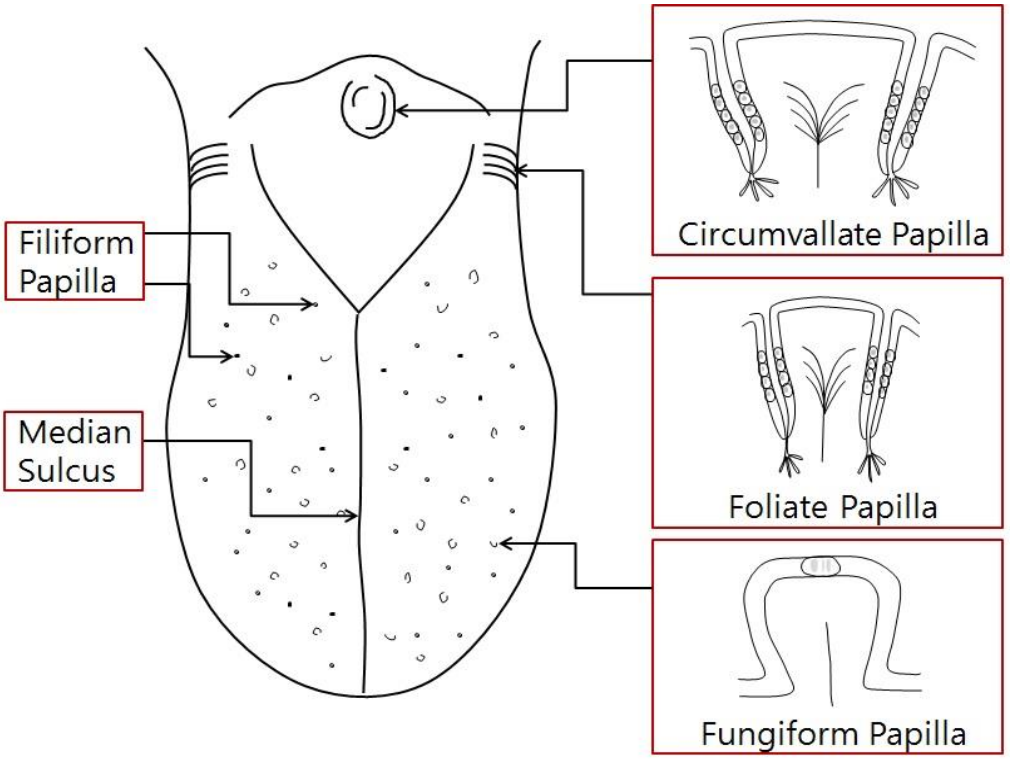

Figure 1. Schematic diagram of mouse tongue and gustatory papillae.

Diagram of the mouse tongue shows general boundaries of oral part, pharyngeal part, and intermolar eminence of tongue[7]. The mouse has only one circumvallate papillae on its tongue and is located in the mid-posterior part. The fungiform papillae are fixedly arranged in the anterior part of the tongue. Three types of papillae, circumvallate, foliate and fungiform, contain taste bud except filiform papillae.

2. Outline for development of the epidermal appendages in vertebrate

Epidermal appendages of vertebrates have a specific structure and various functions, but the development of tongue papillae and other organogenesis systems, such as feathers, hair, lungs, mammary glands and teeth, are similar, and circumvallate papillae is no exception[17-19]. The induction, morphogenesis, and development of these epidermal appendages are carried out by a special and complicated sequential interaction between epithelium tissue and mesenchymal tissue[20-23].

In the development of feather or scales on the skin of a chick embryos, the formation of an epidermal placode first occurs in terms of the formation of an epithelial appendage and the dermis receives a signal to form the dermal condensation directly below the placode[24]. The mesodermal signal, known by the heterospecific tissue recombination of the ecto and mesodermal components of the embryonic skin, determine the formation of epidermal placode. Among them, the number, size, position, and structural identity of the appendages are determined by the mesenchyme, and the type of zoological species and ability status are controlled by the epithelium[25,26]. Thus, molecular mechanisms are

maintained throughout the evolution of the skin, as well as several development model systems, and similar molecules are identified as essential for each appropriate development[27]. In other words, the placode induced structures are all similar in the order of development from initial placode formation through cell differentiation to final structure formation[28,29].

However, each of the other organ is different in the programs that are designed to development, and the resulting regulatory factors and signaling pathways are not the same[30,31]. In particular, unlike other gustatory papillae, the circumvallate papillae have progenitor cells that can differentiate into taste buds and serous cells in von Ebner's gland. Because of these different pathways, the circumvallate papillae become a model for patterning and cell differentiation during organogenesis. The number of circumvallate papillae is nine to thirteen in human, one in mouse and rat, two to three in rabbit, pigs and horse, depending on the species.

Jitpukdeebodintra et al. reported that cell migration may play an important role in the formation of circumvallate papillae through experiments with cell adhesion molecules[13]. In the initial tongue nerve distribution, the nerves follow

a pathway that is dominated by signaling molecules, and in fact, through the studies of taste buds, it has been confirmed that the gustatory papillae, the special sensory organ, is closely related to the innervation of the nerve[2,5,9,32].

3. Morphogenesis and pattern formation of circumvallate papillae development

In the case of circumvallate papillae formation in mouse, the dorsal epithelium begins to thicken in the middle part of the posterior one third of the tongue on embryo days 11 and 12 (E11 and E12). The circumvallate papillae epithelial invagination in E13.5 is derived from the mesenchyme of adjacent tongue. Two adjacent epithelial stalks are used to form a "dome-shaped" structure on E15 to E16. At E17, these stalks infiltrate the underlying mesenchyme and cause a small salivary gland of E18. In order to form a taste cell by terminal cell differentiation, the gustatory papillae needs a nerve innervation not possessed by other placode-dependent organs. In circumvallate papillae of mouse, at E13, innervation and morphogenesis begin[2,33-37].

Many studies have reported that the morphogenesis of tongue papillae requires not only the nerve innervation of tongue, but also the signaling molecules for interactions between the lingual epithelium and mesenchyme[2,14,30,31,38-40]. Recent reports have suggested that nerve innervation is necessary for the development of taste buds[2,39]. Other studies suggest that the

initiation of development in gustatory papillae is independent of nerve innervation but maintenance of form is nerve dependent[2,34-37].

For the development of circumvallate papillae, Jitpukdeebodintra et al. reported that laminin distribution may be related to localization, guiding innervation of epithelial taste buds, and that the morphogenesis of circumvallate papillae is dependent on innervation[13].

However, understanding of the mechanism for morphogenesis of the circumvallate papillae is still insufficient. Previous studies have suggested that certain gene expression patterns may modulate morphogenesis for circumvallate papillae and pattern formation of taste buds[1-3].

This study examined morphogenesis for circumvallate papillae and attempted to identify important factors for the position of the circumvallate papillae. Histology and gene expression have been used to understand the mechanism of the development for circumvallate papillae. *In vitro* organ culture systems and *in vitro* recombination systems were used to establish evidence of epithelial-mesenchymal interactions during the formation of circumvallate papillae during early development.

4. Pattern formation and molecular studies in circumvallate papillae development

Leucine-rich repeat-containing G-protein coupled receptor 5 (*Lgr5*) is a protein encoded by the *Lgr5* gene in humans and is expressed in a wide range of tissues such as muscle, placenta, spinal cord, and brain, especially as a biomarker of adult stem cells in certain types of tissues[41-43].

Lgr5 in circumvallate papillae expresses the taste bud stem and progenitor cells, and *Lgr5*-positive cells induce all major types of taste bud cells identified by *Lgr5*-EGFP-IRES-creERT2 mice[44,45]. *Lgr5*-positive stem cells have been reported to be present in various organs such as small intestine, colon, stomach, hair follicle, liver, pancreas, cochlea, etc., and various organoids are produced by *Lgr5*-positive stem cells containing taste bud organoid[46,47-50]. In particular, in the taste bud of the circumvallate papillae in the posterior region of the adult mouse tongue, *Lgr5*-positive cells expresses a population of the active stem cell in the taste bud of trench area and the weak *Lgr5*-positive cells are located at the base of the taste buds rather than the anterior part of the tongue[50,51]. In contrast to *Lgr5*-positive cells, Leucine Rich Repeat Containing G protein-coupled

receptor 6 (*Lgr6*), like *Lgr5*, is expressed as a taste stem and progenitor cells marker in the taste buds of circumvallate papillae and fungiform papillae, and in the anterior part of tongue with many fungiform papillae Lgr6-positive cells are mostly observed[50,52]. However, *Lgr5* expression pattern during circumvallate papillae development was still uncertain.

Fibroblast Growth Factor (FGF) family members have a wide range of cell divisional and cell viability activities and participate in a variety of biological processes such as embryonic development, cell growth, morphogenesis, tissue repair, tumor growth and invasion[53]. Fibroblast Growth Factor 10 (FGF10) is a protein made by the *Fgf10* gene and is part of a family of proteins, which is FGFs that are involved in processes such as prenatal development[54]. When the FGF10 protein attaches to the receptor protein, triggering a cascade of chemical reactions within a cell signaling the cell to make certain changes, such as maturation, to perform a particular function. FGF10 initiates the development of limbs and participates in the branching of morphogenesis in various organs such as lung, skin, ear, and salivary glands[54]. In developing tongue, the expression patterns of various FGFs and their receptors were confirmed[55-57]. In

particular, the expression of *Fgf10* was confirmed in the development process of circumvallate papillae mesenchyme, and it was shown that *Fgf10* knockout mice were abnormal in the morphology of circumvallate papillae[58].

An important factor in the regulation of epithelial stem cells in the mouse incisors is mesenchymal FGF10. To maintain *Lgr5* positive epithelial stem cells in an apical bud, *Fgf10* is needed around the mesenchyme[59]. In the tongue, *Fgf10* is expressed in different parts of each embryonic day before birth. *Fgf10* was found at the lingual boundary of the tongue at E11.5, at the anterior tongue at E12.5, and highly in the longitudinal muscles from E13.5 to E14.5 [56].

Thus, it has been hypothesized that the communication between the epithelial *Lgr5* and the mesenchymal *Fgf10* signaling may form the circumvallate papillae, and may be a necessary interaction of components, particularly for epithelial invagination.

II. MATERIALS AND METHODS

All experiments were performed according to the guidelines of the Yonsei University College of Dentistry, Intramural Animal Use and Care Committee

1. Animals

Adult Institute of Cancer Research; Caesarian Derived-1 (ICR; CD-1) mice were housed in a temperature-controlled room (22°C) under artificial illumination (lights on from 05:00 to 17:00) and 55% relative humidity. The mice had access to food and water ad libitum. Embryos were obtained from time-mated pregnant mice. E0 was designated as the day a vaginal plug was confirmed. Embryos at developmental stages E13.5, E15.5, E17.5, PN1 and adult mice were used in this study.

2. *In situ* hybridization

All samples were fixed in 4% paraformaldehyde in phosphate buffered saline (PBS) and 4μm thickness paraffin sections under RNase free circumstance were

prepared for section *in situ* hybridization. *In situ* hybridization for *Lgr5* were performed using RNAscope 2.5 Assay (Advanced Cell Diagnostics, ACD, USA) according to manufacturer's protocols[60]. RNAscope *Lgr5* probes were designed and validated by ACD. Paraffin sections were deparaffinized and heated in boiling target retrieval buffer and pretreated with protease prior to hybridization with target oligo probes. After hybridization, following steps of signal-amplification steps were hybridized to the target probes. Color development with Fast Red substrate is following steps. Intracellular red punctate dots are considered as positive results while negative control probes DapB (dihydrodipicolinatereductase) and positive control probe PPIB (peptidylprolylisomerase B) were also tested for each slide for quality control.

3. Histology and immunohistochemistry

Samples were fixed in 4% paraformaldehyde in PBS and then embedded in paraffin using standard procedures. Serial paraffin sections (4-μm thickness) were prepared for Hematoxylin and eosin stain (HE), Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) and

immunostaining. Antigen retrieval was achieved by citrate buffer, pH 6.0. After antigen retrieval, immunohistochemistry analyses were performed using the Dako Cytomation Envision System (using horseradish peroxidase with diaminobenzidine enhancer) (Dako, USA) according to the manufacturer's instructions. FGF10 (Santa Cruz, USA), Caspase3 (Cell Signaling Technology, USA) and Ki67 (abcam, UK) primary antibody was used for immunostaining.

4. Recombination assay

Recombination of the tongue using the intact epithelium and mesenchyme was done at E15.5. The tongue was dissected and approximately 0.05 ml of Indian ink (Royal Talens, Holland) was injected using a 25-gauge needle into circumvallate papillae regions. Dissected tongues were incubated in 2.4 unit Dispase II (neutral protease, grade II) (Roche Applied Science, Switzerland) for 30 to 50 minutes at 37 °C, and washed in medium containing 10% fetal bovine serum. The epithelium and mesenchyme were separated under a dissection microscope. Separated epithelia remained intact in the media. The epithelium was placed on top of the mesenchyme with a 180-degree rotation, from anterior

to posterior, and the recombinants were cultured for 72 hours using Trowell's method.

5. Bead implantation

Heparin for mate-derived beads (Sigma, USA) were incubated in 50 μg/ml recombinant human FGF10 (R&D systems, USA) for 1 hour at room temperature. FGF10-soaked beads were implanted between Indian ink labeled circumvallate papillae epithelium and tongue mesenchyme at E15.5 and cultured for 72 hours. 0.1% BSA in PBS-soaked beads was implanted as controls.

6. BIO treatment

The developing tongues were dissected at E15.5 in PBS and cultured using Trowell's method for 72 hours with media containing 1ng/ml BIO (GSK inhibitor, Sigma-Aldrich, USA). Dimethyl sulfoxide (DMSO) was used for control.

7. RNA preparation and real-time quantitative polymerase chain reaction analysis (RT-qPCR).

Total RNA of cells were extracted using Trizol reagent. The extracts were reverse transcribed using Maxime RT PreMix (#25081; iNtRON, Korea). RT-qPCR primer sets designed using Primer Express software (Applied Biosystems, USA) and RT-qPCR was performed using Step OnePlus Real-Time PCR System (Applied Biosystems, USA). The expression levels of each gene are expressed as normalized ratios against glyceraldehyde-3-phosphate (*Gapdh*) house keeping gene. The oligonucleotide RT-PCR primers for *Fgf10*, *Lgr5* and *Gapdh* are as follow table:

	The oligonucleotide RT-PCR primers	
Lgr5	Forward	AGC ATG CTT CTG GCA AGA TGT TC
	Reverse	GAC TTA ACG CCC TGC GTT TGA
Fgf10	Forward	CAT CTG CGG AGC TAC AAT CA
	Reverse	CCC CTT CTT GTT CAT GGC TA
Gapdh	Forward	GTC ATC ATC TCC GCC CCT TCT G
	Reverse	ATG CCT GCT TCA CCA CCT TCT TG

Table 1. The oligonucleotide RT-PCR primers for *Lgr5*, *Fgf10* and *Gapdh*

III. RESULTS

1. Morphogenesis and expression pattern of *Lgr5* during mouse circumvallate papillae development.

HE staining method was applied to circumvallate papillae from embryonic day 13.5 to adult (E13.5, 15.5, 17.5, PN1 and adult) to observe the morphology of circumvallate papillae during development (Fig. 2A-E). At E13.5, epithelial invagination was confirmed in the adjacent mesenchyme of circumvallate papillae (Fig. 2A). At E15.5, trenches of the circumvallate papillae got deeper on both sides of the circumvallate papillae (Fig.2B). At E17.5, the epithelium of circumvallate papillae formed epithelial crypts by making both trenches deeper (Fig. 2C). At PN1, the deeper epithelial invagination of the circumvallate papillae formed deep trenches, which were connected to the von Ebner's glans (Fig. 2D). In adult circumvallate papillae, normal epithelial trenches with the circular furrows of the circumvallate papillae epithelium and various taste buds in the circumvallate epithelium were observed (Fig. 2E).

In situ hybridization was performed during development of circumvallate papillae to identify the location of *Lgr5* (Fig. 2F-J). At E13.5, *Lgr5* was

expressed in the epithelium of circumvallate papillae but not in the entire epithelium of the tongue and in the adjacent mesenchyme (Fig. 2F). At E15.5, *Lgr5* was expressed in the trench area of epithelial invagination and in the ventral epithelium of circumvallate papillae (Fig. 2G). At E17.5, *Lgr5* was strongly expressed in the trench region of epithelial invagination, but not in ventral region of epithelium (Fig. 2H). At PN1, *Lgr5* was expressed in the area where the taste buds were being made, except for the area where the circular furrow was being created. In addition, *Lgr5* was expressed in the partial ventral epithelium of circumvallate papillae (Fig. 2I). In adult, *Lgr5* was expressed in regions of taste buds and bottom area of the trenches of epithelial invagination (Fig. 2J).

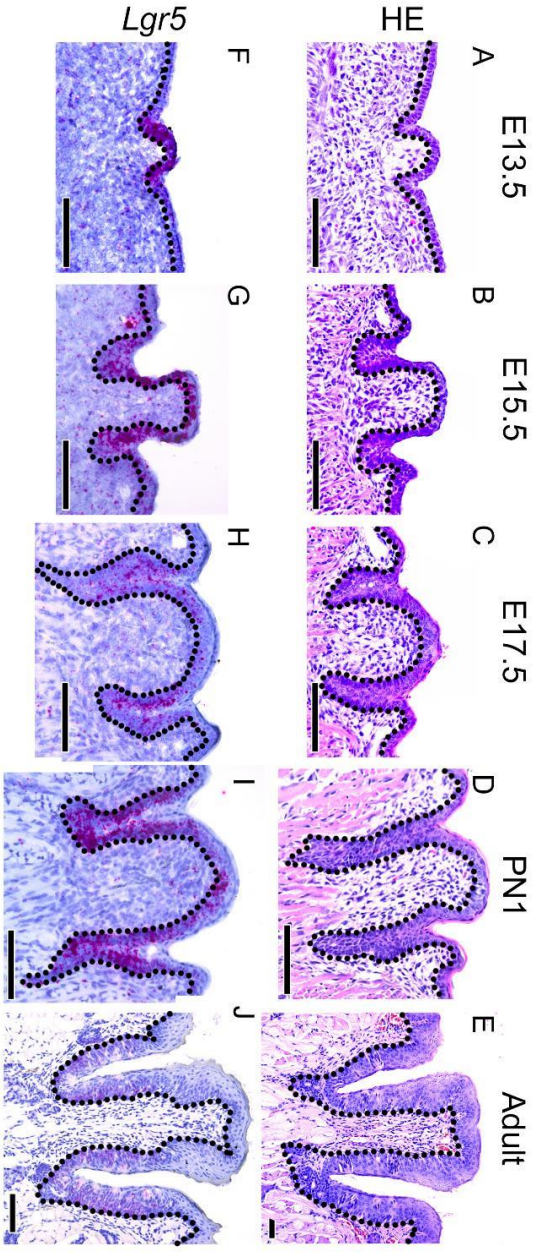

Figure 2. Morphology of developing circumvallate papillae and expression patterns of *Lgr5* during circumvallate papillae development.

(A-E) HE staining shows the dynamic morphological changes during circumvallate papillae development. (A) The epithelial invagination of circumvallate papillae was observed into adjacent mesenchyme at E13.5. (B) At 15.5, epithelial invaginations at both sides of circumvallate papillae are deeper. (C) At 17.5, deep trenches are formed resulted from epithelium invagination of circumvallate papillae. (D) At PN1, von Ebner's gland are connected to the invaginated epithelium of circumvallate papillae which getting deeper. (E) At adult stage, circular furrows and taste buds were observed in well-formed epithelial trenches of circumvallate papillae. (F-J) *Lgr5* expression pattern is confirmed by *in situ* hybridization. (F) *Lgr5* expression is observed in the epithelium of circumvallate papillae, but not in tongue epithelium and adjacent mesenchyme at E13.5. (G) At 15.5, *Lgr5* is strongly expressed in epithelial invagination region and ventral epithelium of circumvallate papillae. (H) At 17.5, strong *Lgr5* expression is detected in epithelial trenches of circumvallate papillae but weakly observed in ventral epithelium of circumvallate papillae. (I)

Expression of *Lgr5* is detected through taste buds forming region but not in circular furrow forming region while partial expression was detected in ventral epithelium of circumvallate papillae at PN1 stage. (J) At adult stage, the expression is only detected around taste buds and bottom region of epithelial trench.

2. FGF10 localized in developing circumvallate papillae mesenchyme.

Immunohistochemistry analysis was applied to identify the distribution of FGF10 in developing circumvallate papillae at E15.5 and E17.5, the critical periods of epithelial invagination in circumvallate papillae. The developing tongue was sagittal sectioned to compare the mesenchyme of the areas where fungiform papillae and circumvallate papillae were being formed (Fig. 3A-F). At E15.5, FGF10 was strongly localized in mesenchyme of the circumvallate papillae but not in the fungiform papillae formed in the anterior mesenchyme (Fig. 3A). In high magnification results, FGF10 was clearly identified only in the mesenchyme of the circumvallate papillae compared to the mesenchyme of the fungiform papillae (Fig. 3C, D). At E17.5, as at E15.5, FGF10 was identified only in the mesenchyme of circumvallate papillae (Fig. 3B). In high magnification results, FGF10 was strongly detected in the mesenchyme of the circumvallate papillae, but not in the mesenchyme of the fungiform papillae (Fig. 3E, F).

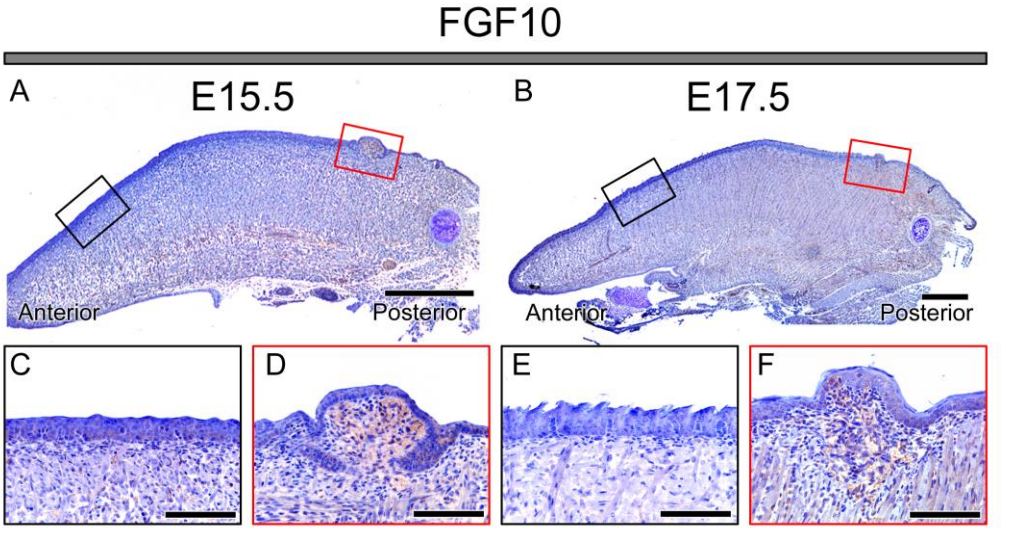

Figure 3. Expression patterns compared to the forming region of fungiform papillae for FGF10 during development of circumvallate papillae.

(A, B) At E15.5 and E17.5, FGF10 localizes strongly in the mesenchyme of circumvallate papillae forming region but not in fungiform papillae forming region. (C-F) Higher magnification results indicated localization of FGF10 is strongly detected in circumvallate papillae mesenchyme but not in fungiform papillae mesenchyme at E15.5 and at E17.5. Scale bars; 500μm.

3. Morphological analysis after reverse recombination of tongue epithelium.

In order to identify site for morphogenesis of specific lingual papillae, E15.5 mouse tongue epithelium was reversely recombined (Fig. 4-6). In the schematic diagrams, three different experiments for the recombination of the tongue epithelium were designed. In the first experiment (Fig. 4), control recombination was an experiment design that the tongue epithelium separated with Dispase II was recombined at the same position for the tongue mesenchyme and cultured for 72 hours (Fig. 4A). In the second experiment (Fig. 5), reverse recombination was an experiment design that the epithelium of the tongue separated by the same method as the first experiment was rotated 180-degrees from anterior to posterior for the tongue mesenchyme and then recombined (Fig. 5A). Indian ink was marked on the ventral side of the circumvallate papillae before the tongue epithelium was separated, to confirm the epithelial location of circumvallate papillae after recombination. In order to identify the function of mesenchymal FGF10 (Fig. 6), the bead soaked in FGF10 was placed under the epithelium of the circumvallate papillae that was reverse-recombined (Fig. 6A).

According to results of HE staining, the circumvallate papillae in the control recombinant group (n=9/10) was properly formed (Fig. 4B). However, the circumvallate papillae structure was not found in the epithelial region of the circumvallate papillae in the reverse recombination group (n=20/20), but structure such as fungiform papillae was observed (Fig. 5B). To induce the epithelial morphogenesis of circumvallate papillae, bead soaked in FGF10 was implanted under the epithelium of the circumvallate papillae after reverse recombination. The implanted bead soaked in FGF10 induced epithelial invagination of the circumvallate papillae in the reverse recombination (n=9/15) (Fig. 6C). In contrast, the implanted control bead soaked in PBS could not induce morphology of circumvallate papillae (n=10/10) (Fig. 6B).

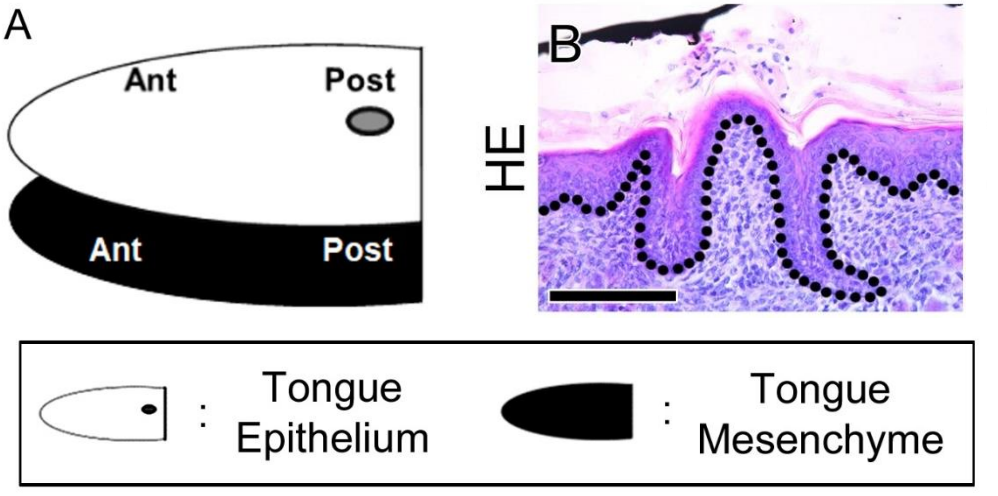

Figure 4. Control Recombination of tongue epithelium : Schematic diagram of experimental design and morphology of circumvallate papillae.

(A) Control recombination indicate that separated epithelium from mesenchyme recombined to same position and cultured for 72hr. (B) HE staining shows proper morphogenesis of developing circumvallate papillae in control recombination (n=9/10). Scale bars; 500μm.

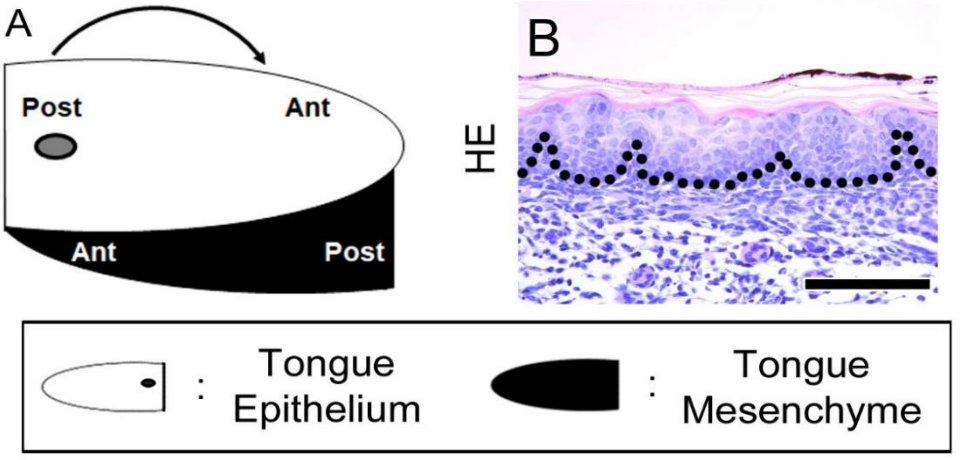

Figure 5. Reverse Recombination of tongue epithelium : Schematic diagram of experimental design and morphology of circumvallate papillae.

(A) Reverse recombination indicates that 180-degrees rotated tongue epithelium refer to anterior-posterior axis then recombined to tongue mesenchyme. To indicate the circumvallate papillae region, Indian ink is marked the ventral surface of circumvallate papillae epithelium. (B) In reverse recombination tongue, fungiform papillae like structure was observed in circumvallate papillae epithelium (20/20). Scale bars; 500μm.

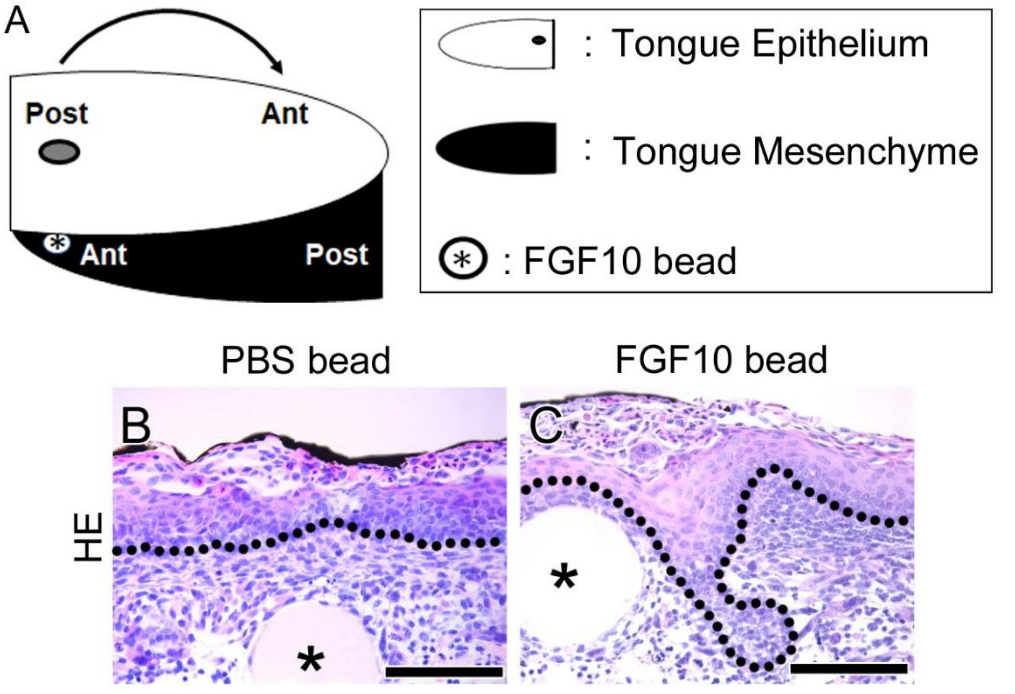

Figure 6. Bead Implantation after reverse recombination of tongue epithelium : Schematic diagram of experimental design and morphology of circumvallate papillae in implanted PBS and FGF10 bead.

(A) Bead implantation was performed under Indian ink leveled circumvallate papillae epithelium of reverse recombination. (B, C) Implantation of FGF10 soaked beads rescue epithelial invagination of circumvallate papillae in reverse recombination while PBS soaked beads could not rescue the morphology. Scale bars; 500μm.

4. Disruption of circumvallate papillae morphogenesis by inhibition of *Fgf10* signaling

Previous reports suggested that mesenchymal *Fgf10* is an essential factor to maintain epithelial *Lgr5* positive stem/progenitor cells in the developing mouse incisor [61]. To confirm the morphogenesis of circumvallate papillae, which is affected by the interaction of *Fgf10* and *Lgr5*, BIO treatment was applied on mouse tongue at E15.5 (Fig. 7). As a result of HE staining, the morphology of the circumvallate papillae in the DMSO control group (n=10/10) was well formed (Fig. 7A). However, the form of circumvallate papillae applied with BIO treatment collapsed (n=20/20) (Fig. 7C). To identify information on the destruction of circumvallate papillae morphogenesis, apoptotic cell was confirmed by Caspase 3. Caspase 3-positive apoptotic cells were not found in the circumvallate papillae epithelium of the DMSO control group (Fig. 7E). However, a large number of Caspase 3-positive apoptotic cells were found in collapsed epithelium of circumvallate papillae treated with BIO (Fig. 7G). After processing with BIO treatment, immunohistochemistry for expression of Ki67 was applied to evaluate cell proliferation. In the basement membrane of

circumvallate papillae and tongue epithelium, Ki67-positive cells were found (Fig. 7F). However, after BIO treatment, cell proliferation in the epithelium of circumvallate papillae and tongue was significantly reduced. (Fig. 7H) RT-qPCR was applied to confirm the activity of BIO. After BIO treatment, expression of *Fgf10* and *Lgr5* was decreased in the developing tongue (Fig. 7B, D).

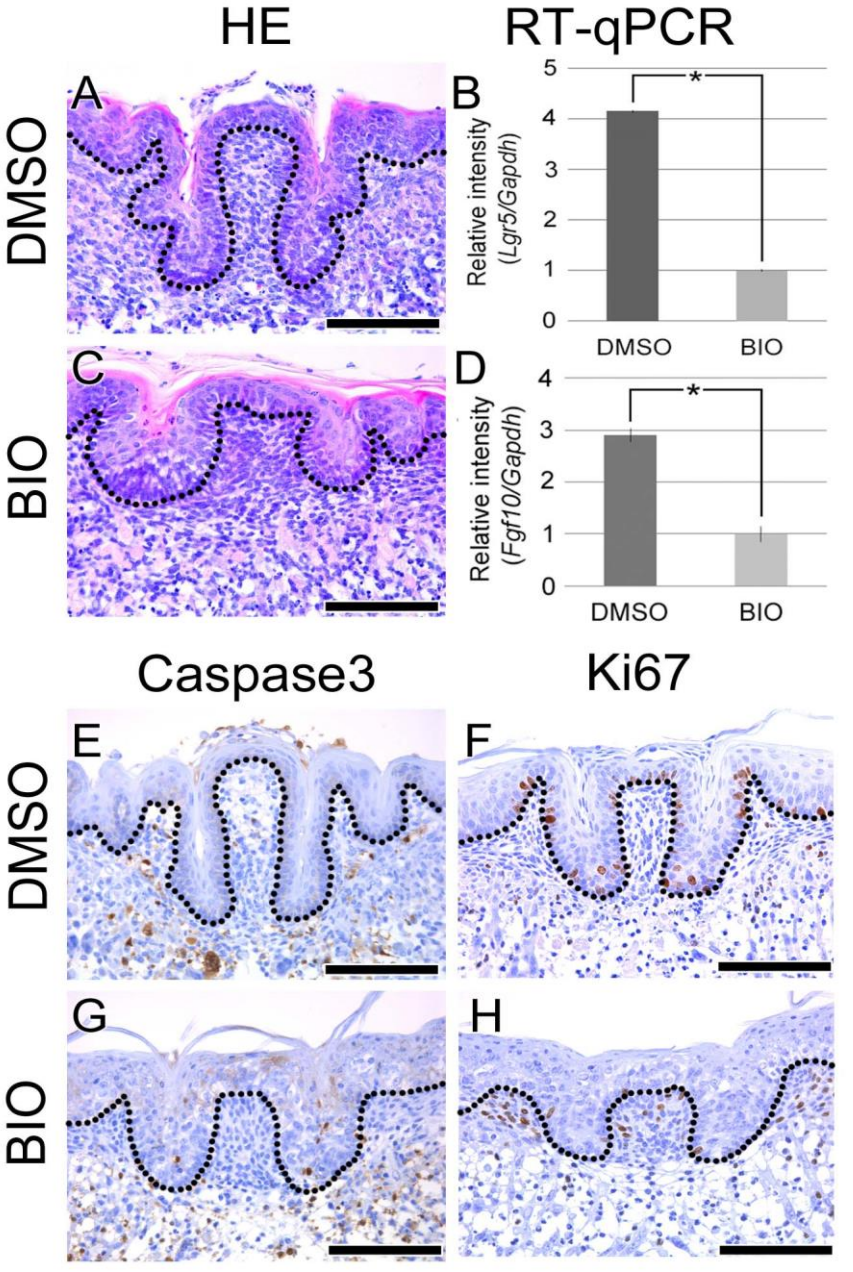

Figure 7. Morphogenesis and cellular events analysis after BIO treatment.

BIO (1ng/ml) is treated at E15.5 mice tongue and cultured for 72hr. (A) In control group, normal morphology of circumvallate papillae is observed. (C) BIO treated group, significantly disrupted circumvallate papillae structure with reduced epithelial invagination depth and different papilla shape is observed. (B, D) To confirm the efficiency of the BIO, RT-qPCR is performed. *Fgf10* and *Lgr5* are significantly reduced after BIO treatment compared to control group. (E) No Caspase3 positive circumvallate papillae epithelial cell is detected in control group. (G) In BIO treatment group, apoptotic cells were detected at the crypt region of disrupted circumvallate papillae epithelium where stem cells normally located. (F) Numerous number of Ki67 positive proliferating cells are observed in not only circumvallate papillae epithelium but also tongue epithelium. (H) Proliferating cells are reduced after BIO treatment both in circumvallate papillae epithelium and tongue epithelium. Scale bars; 500 μm.

IV. DISCUSSION

1. Morphogenesis of circumvallate papillae in the development of mouse

Lingual papillae have four types, circumvallate papillae (vallate papillae), foliate papillae, fungiform papillae and filiform papillae. These are distributed on the surface of the tongue. The taste papillae contain taste bud but not in the filiform papillae. All four types of lingual papillae have specific regions of formation, and circumvallate papillae are distributed in the posterior region of the tongue[2,7,62,63].

The development of circumvallate papilla is similar to that of other organs such as feather, lung, mammary gland and teeth[17-19]. Complex and sequential interactions occur between tissues of the epithelium and the mesenchyme during developmental processes. However, the program required for the development of each distinct organ is driven by tissue-specific regulatory factors and signaling pathways. The circumvallate papillae of mouse begins to form thickening of the epithelium in the mid-region of the posterior one third of the dorsal tongue at the E11.5 and E12.5. The invagination of epithelium in the process of morphogenesis of circumvallate papillae is one of the key factors[13].

Thus, the interaction between epithelium and mesenchyme is an essential issue for complex molecular and cellular event[64].

This study showed the morphogenesis stage of circumvallate papillae development in mouse. The morphology of developing circumvallate papillae from E13.5 to the adult was confirmed by HE staining. At E13.5, the epithelium of circumvallate papillae was invaginated in the adjacent area to the mesenchyme (Fig. 2A). At E15.5, the trenches of circumvallate papillae in both sides of the circumvallate papillae became deeper (Fig. 2B). At E17.5, the epithelium of circumvallate papillae formed deep trenches which were epithelial crypts (Fig. 2C). At PN1, epithelium of circumvallate papillae forms deeper trenches and were connected to the Von Ebner's glands (Fig. 2D). Circumvallate papillae of adult showed well-formed epithelial trenches and the epithelium of circumvallate papillae contained circular furrows and various taste buds (Fig. 2E).

2. Expression of signaling molecules and morphological changes for development of circumvallate papillae

Previous report has suggested that *Lgr5* and *Lgr6*, stem/progenitor cell markers, are expressed during the development of circumvallate papillae, whereas *Lgr5* in fungiform papillae is not expressed or almost not expressed[50]. This study confirmed the expression pattern of *Lgr5* by *in situ* hybridization. The expression of *Lgr5* in the early developmental stage of the tongue was observed in the epithelium of developing circumvallate papillae (Fig. 2F, G). In the late developmental stage, the expression of *Lgr5* was partially appeared in the epithelium of the circumvallate papillae, in particular, in the area where taste buds forming (Fig. 2H, I). In adult, the expression of *Lgr5* was confirmed only in the deep trench area of the epithelium of circumvallate papillae (Fig. 2J). These results suggested that the induction of invagination of the circumvallate papillae and the formation of the taste bud may be related to *Lgr5*.

Previous studies have suggested that the expression of *Fgf10* appears in the mesenchyme of tongue directly below the epithelium of the circumvallate papillae and that the *Fgf10* KO mouse shows loss of the circumvallate papillae[58].

Immunohistochemistry was applied to the sagittal sectioned developing tongue to compare the expression site of FGF10 in forming areas where the fungiform papilla and circumvallate papillae. At E15.5 and E17.5, the expression of FGF10 was detected only in the mesenchyme below the circumvallate papillae but not in the fungiform papillae formation region (Fig. 3A-F). These results suggest that the signaling of mesenchymal FGF10 may play a key role in the development of circumvallate papillae.

Expression of mesenchymal FGF10 is essential factor for maintaining epithelial *Lgr5*-positive cells by inhibiting apoptosis in mouse incisor[59]. In the process of tongue development, epithelial *Lgr5*-positive cells and mesenchymal FGF10 are essential signaling molecules for circumvallate papillae (Fig. 2, 3).

3. At E15.5, the developmental role of mesenchymal FGF10 during the development of circumvallate papillae

Interaction between epithelium and mesenchyme is necessary for the formation of epithelial appendages[17,23,65,66]. In this study, to confirm the necessity of mesenchymal FGF10 during the development of circumvallate papillae, the tongue epithelium of E15.5 mouse was reversely recombined (Fig. 4-6).

In the epithelium of circumvallate papillae marked with Indian ink, epithelial invagination occurred in the control recombination group with FGF10 positive mesenchyme, but no epithelial invagination in the reverse recombined group with FGF10 negative mesenchyme (Fig. 4B and 5B). In addition, expression of *Lgr5* was reduced in mesenchyme of anterior tongue of reverse recombination group compared to control recombination group (Fig. 4C and 5C).

To confirm that the supply of FGF10 could reactivate the morphogenesis of circumvallate papillae after reverse recombination, FGF10 soaked bead was implanted between the epithelium of circumvallate papillae and anterior

mesenchyme. Implanted bead soaked in FGF10 reactivated the epithelial invagination of the circumvallate papillae around it (Fig. 6B and C).

These results suggest that mesenchymal FGF10 plays an important role in the morphogenesis of circumvallate papillae, especially for epithelial invagination mesenchymal FGF10 is essential.

4. Morphology change and gene expression pattern of the circumvallate papillae following BIO treatment.

BIO, which is glycogen synthase kinase 3 inhibitor, stimulates Wnt signaling by blocking degradation of β-catenin[67]. BIO treatment at the incisor of the mouse inhibits the anti-apoptotic effect of FGF10, resulting in a decrease in *Lgr5*-positive stem/progenitor cells[59]. BIO treatment was applied on the E15.5 mouse tongue to confirm the inhibition of anti-apoptotic effect of FGF10 in the developing mouse tongue. In this study, as a result, after the BIO treatment, destruction of the morphology of the circumvallate papillae and reduced *Lgr5* and *Fgf10* expression level were observed. In addition, decreased proliferating cells, and increased of apoptotic cells were confirmed after BIO treatment (Fig. 7). These results suggest that mesenchymal FGF10 plays a key role in the morphogenesis of the circumvallate papillae because it induces an anti-apoptotic effect on epithelial *Lgr5*-positive cells during circumvallate papillae development.

5. Further consideration

The development of epithelial appendages has been experimented using many model systems such as feathers, teeth, hair, wire and salivary glands[21-23,25,68-72]. Studies on the development of lingual papillae are also increasing. Most of these organs are similar in the developmental process observed in the early embryonic stage. In particular, the development of circumvallate papillae is similar to that of other epithelial appendages. First, for the morphological changes during the development of the circumvallate papillae, the expression of specific signal molecules and specific expression patterns are found in the region where circumvallate papillae are formed. Second, in the morphogenesis of circumvallate papillae, structures such as epithelial thickening and mesenchymal condensation are found, as in the morphogenesis of other epithelial appendages. Third, the development of circumvallate papillae is caused by the interactions between epithelial tissue and mesenchymal tissue.

On the other hand, circumvallate papillae, unlike other epithelial appendages, has other factor that affect the developmental process. In order to form a taste cells by terminal cell differentiation, the gustatory papillae needs a nerve innervation not possessed by other placode-dependent organs. In circumvallate

papillae of mouse, at E13, nerve innervation and morphogenesis begin[33-36]. Many studies have reported that the morphogenesis of lingual papillae requires not only the signaling molecules for interactions between the lingual epithelium and mesenchyme, but also the nerve innervation of tongue[2,14,30,31,38-40]. In the initial tongue nerve distribution, the nerves follow a pathway that is dominated by signaling molecules, and in fact, through the studies of taste buds, it has been confirmed that the gustatory papillae, the special sensory organ, is closely related to the innervation of the nerve[2,5,9,32].

Although this study focused on signaling molecules, nerve innervation is a factor to consider, and further investigation of the effect on morphogenesis of circumvallate papillae by nerve innervation will be significant. It would be also important to find signaling molecules other than the signaling molecules shown in this experiment to understand that it affects morphogenesis during the development of circumvallate papillae.

V. CONCLUSION

Summarizing the results of this study, signaling molecules involved in interactions between epithelium and mesenchyme during the morphogenesis of circumvallate papillae were identified. The following facts are known from the study of these signaling molecules.

1. Signaling molecules involved in epithelium-mesenchyme interactions are *Lgr5* and FGF10.

2. Expression of *Lgr5* was found in the epithelial tissue of circumvallate papillae during circumvallate papillae development.

3. Localization of FGF10 was observed in the mesenchymal tissue of the tongue just below the area where circumvallate papillae were formed.

4. When the epithelium of developing circumvallate papillae was moved to the area without FGF10, the structure of circumvallate papillae was not found.

5. In situation 4, the bead soaked in FGF10 could activate the morphogenesis of circumvallate papillae.

6. After BIO treatment, mesenchymal *Fgf10* was reduced, resulting in abnormal cell proliferation and apoptosis.

These results indicate that crosstalk between epithelial *Lgr5* and mesenchymal FGF10 is key issue for normal morphogenesis of circumvallate papillae during tongue development, because mesenchymal FGF10 induces the epithelial invagination and anti-apoptotic effect on epithelial *Lgr5*-positive cells during circumvallate papillae development (Fig. 8).

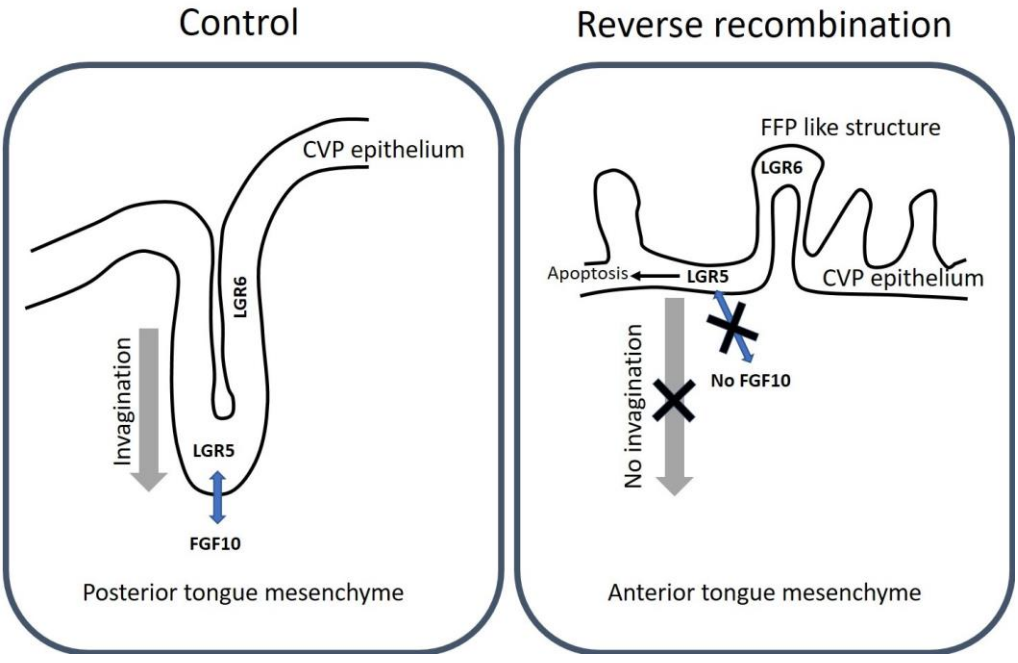

Figure 8. Schematic representation of the circumvallate papillae development.

The molecular interactions between FGF10 and *Lgr5* modulate various cellular events including apoptosis and cell proliferation to ensure correct circumvallate papillae development. Arrows; positive modulation, CVP; circumvallate papillae, FFP; fungiform papillae.

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ABSTRACT(IN KOREAN)

혀 발생 중 영역에 따라
혀 유두의 특정 형태형성을 조절하는 신호전달 체계

<지도교수 정 영 수>

연세대학교 대학원 치의학과

이 규 태

대부분 척추 동물의 혀는 근육 기관으로 이루어져 있으며, 음식을 조작하고 삼키는 데 사용된다. 혀는 소화 체계에서 중요한 역할을 하며, 미각 체계에서도 맛을 느끼는 주요 감각기관 역할을 한다. 사람의 혀는 V 자 모양의 고랑에 의해 전방의 구강 부분과 후방의 인두 부분으로 나누어진다. 혀의 등쪽 면은 각화된 중층 편평 상피의 저작성 점막으로 덮여있고, 미뢰와 맛 수용체를 포함하는 수많은 유두가 있다. 이러한 혀 유두는 성곽(circumvallate), 엽상(foliate), 용상(fungiform) 및 사상(filiform)의 네 가지 형태가 존재하고, 사상유두를 제외한 나머지 유두에는 미뢰가 있다. 이러한 미뢰에 문제가 생겨 나타나는 미각과 관련된 미각 장애는 다양한

원인에 의해서 발생할 수 있다. 하지만 미각 장애에 대한 여러 치료 방법 중 미각 개선에 대한 치료는 아직까지는 쉽지 않은 상황이며, 근본적인 미뢰 재생에 대한 치료는 없다. 따라서 이번 연구에서는 미뢰 재생을 위해서는 미뢰를 포함하고 있는 혀 유두의 발생에 대한 이해가 필요하다고 판단하여 혀 유두 중 성곽유두의 발생에 대해서 연구하였다.

대부분의 피부 부속 기관인 깃털, 폐 및 치아 등의 경우 발생 과정에서 상피와 간엽의 상호 작용이 관찰된다. 그러나 각각의 기관들은 발생을 위한 신호 전달 경로와 조절 요소들이 다르다. 성곽유두의 경우 발생 과정 동안 줄기세포 표지자인 *Lgr5* 의 발현을 *in situ* hybridization 을 통해 확인하였고, 배아 E15.5 와 E17.5 시기에는 immunohistochemistry 를 통해서 FGF10 의 발현도 확인하였다. 성곽유두의 형태형성 과정 동안 상피의 *Lgr5* 와 간엽의 FGF10 사이에서 상호 작용이 이루어지는 지에 대한 직접적인 증거를 확보하기 위해, 혀 상피를 간엽에서 완전하게 분리하여 역방향(180 도 회전)으로 재조합(recombination)하고 *in vitro* organ culture 에서 72 시간 동안 배양 후 분석을 통해 성곽유두 구조의 형성 여부를 확인하였다. 성곽유두의 상피는 *Fgf10* 이 없는 간엽 위로 재조합 되면, 성곽유두의 구조가 관찰되지 않았다. FGF10 이 적셔진 구슬을 재위치된 성곽유두 상피 아래인 *Fgf10* 이

없는 간엽 위에 위치시키면, 정상적인 성곽유두의 형태가 형성됨을 확인할 수 있었다. BIO 처리를 통해 Wnt 신호를 활성화시키면 상피의 *Lgr5* 와 간엽의 *Fgf10* 감소로 인해 비정상적인 세포 증식 및 세포 사멸이 관찰되었다.

이러한 결과들은 상피에서 발현되는 *Lgr5*와 간엽에서 발현되는 *Fgf10* 사이의 상호작용은 혀 발생 과정에서 성곽유두의 적절한 형태형성을 위해서는 반드시 필요하다는 것을 시사한다.

핵심 되는 말 : 혀 유두 발생, 성곽 유두(circumvallate papillae), 상피 간엽 상호작용, 신호전달물질, *Lgr5*, *Fgf10*, 형태형성