

Pattern Formation and Morphogenesis
of Fungiform Papillae
during Mouse Development

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Pattern Formation and Morphogenesis of Fungiform Papillae during Mouse Development

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ABSTRACT

Pattern Formation and Morphogenesis of Fungiform Papillae during Mouse Development

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The tongue is a part of organ that plays important roles in swallowing, taste the food, and speech in human. Anatomically, the tongue is divided into an oral part and a pharyngeal part by the terminal sulcus. The following four different types of tongue papillae, which are small organs, can be found on the dorsal surface of the tongue: the fungiform, circumvallate, foliate and filiform papillae. Interestingly, development of fungiform papillae showed the stereotyped pattern on the dorsal surface of the tongue in mice from embryonic day 12 (E12) to E17. The patterned distribution and developmental processes of the fungiform papillae suggest possible similarities between the fungiform papillae and other epithelial appendages, including teeth, feathers, and hairs, which develop via epithelial mesenchymal interaction. The histological results and ultrastructural observations showed the development of specific structures in the epithelium into fungiform papillae. Many signalling factors, including signalling molecules and ECM (Extracellular matrix) molecules such as *Shh*, *Bmps*, *Fgfs*, laminin, CAM (Cell adhesion molecule) and cytokeratins, might be involved in the morphogenesis of fungiform papillae during the early development. In order to obtain direct evidence of the

epithelium and mesenchyme interaction during fungiform papillae morphogenesis, the formation of fungiform papillae like structure was examined after a recombination assay. It was conducted after the recombination assay at E12.5 and E13.5 for 2 days using an *in vitro* organ culture. From the recombination assay results, the E13.5 epithelial portion of the fungiform papillae could determine the position of the newly formed fungiform papillae with the epithelial signalling molecules. E13.5 was a critical stage for fungiform papillae morphogenesis. To understand the signalling regulations of fungiform papillae, expression patterns of *Shh*, *Bmp-2* and connexin 43 were examined. The results showed that the spatio-temporal expression pattern of signalling molecules is of evidence for proper formation of fungiform papillae. Furthermore, antisense oligodeoxynucleotide (AS-ODN) connexin 43 was treated to analyze the developmental functions of gap junction and NOGGIN protein, which is an antagonist of *Bmps*, was treated during *in vitro* organ culture. The expression patterns of signalling molecules, *Shh* and *Bmp-2* were altered by inhibition of gap junctions. *Shh*, which is known as a key molecule to determine spacing pattern, expression patterns were disturbed after the inhibition of *Bmp-2* as well. These results revealed that pathway of the signalling molecules during fungiform papillae development.

Fungiform papillae can be considered to be small epithelial appendages, which are formed with the specific patterned array via the epithelial mesenchymal interactions.

Key words : epithelial mesenchymal interactions, fungiform papilla, morphogenesis, pattern formation, signalling molecule, spacing pattern

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I. INTRODUCTION

1. Tongue and its appendage papillae

During mouse embryogenesis, the terminal sulcus divides the developing tongue into an oral part and a pharyngeal part. Four different types of tongue papillae can be found on the dorsal surface: fungiform, circumvallate, foliate and filiform papillae. These papillae are distributed in a specific pattern.¹ The fungiform, foliate, and circumvallate papillae are known as the gustatory papillae, which contain taste buds and work as sensory organs.² The gustatory papillae are comprised of an epithelial covering over a broad core of connective tissue. Taste buds, which are discrete collections of approximately 40 to 60 cells within the papilla epithelium, are an oval sensory end organ that is involved in the perception of chemical stimuli and in taste transduction.^{3,4,5} The fungiform papillae are located on the oral part of the tongue with a specific patterned array. One circumvallate papilla is located in the middle part of the terminal sulcus in rodents. In humans, about 7 to 9 circumvallate papillae are located along the terminal sulcus (Fig. 1). Foliate papillae are

found on the sides of the posterior one-third of the human tongue and are well developed at birth, but revert to a rudimentary structure in adults. The filiform papillae, which contain no taste buds, cover the entire anterior part of the dorsal surface of the tongue and consist of cone-shaped structures, each with a core of connective tissue that is covered by an epithelium expressing hair-related keratins.⁶

The information on the tongue papillae development presented in this review was obtained from previous experiments in sheep, rodents, and salamanders as well as from studies in human development.^{1,7,8} The initial study using a vertebrate tongue was aimed at examining the three-dimensional structure of the embryonic and fetal stages of the tongue and its appendages, the tongue papillae, using light microscopy and scanning electron microscopy.^{1,7} It was reported that during the development of the tongue papillae many factors might be involved in morphogenesis, including the ECM (extracellular matrix) molecules such as laminin, CAM (Cell adhesion molecule) and cytokeratins.⁹⁻¹¹ Recently, the circumvallate and fungiform papillae were examined using a variety of experimental approaches by protein expressions, signalling network and tissue interactions.¹²⁻¹⁴ In particular, the development of fungiform papillae, which are one of the gustatory papillae, was examined as an epithelial appendage.¹⁵ In this study, the formation of the fungiform papillae is discussed as a model system for pattern formation because they have a specific patterned array on the dorsal surface of the tongue.^{4,5,16}

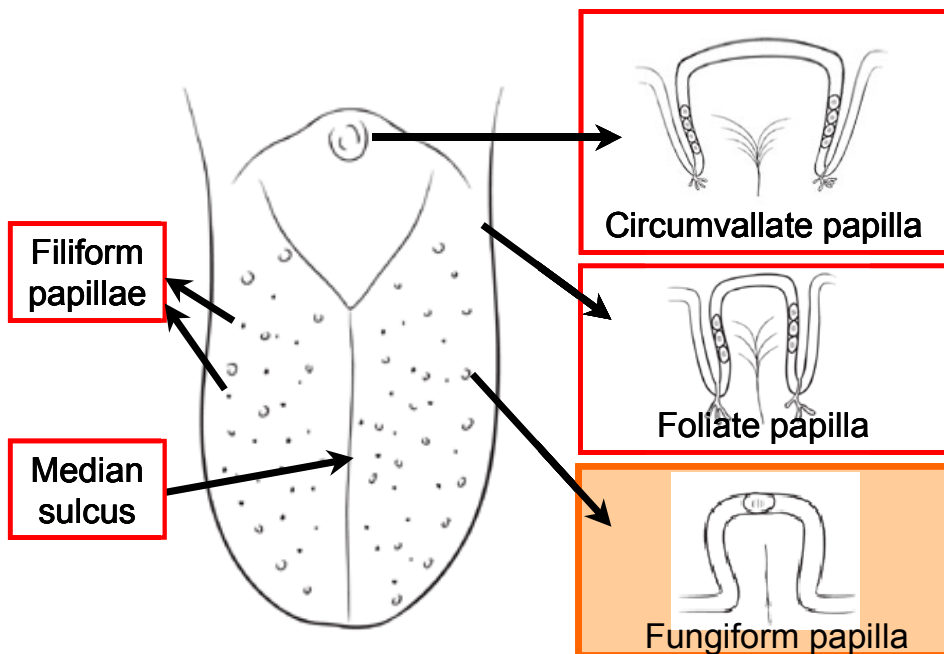


Figure 1. Schematic diagram of mouse tongue and gustatory papillae. Diagram of the mouse tongue shows general boundaries of oral part, pharyngeal part, and intermolar eminence of tongue. Mouse has a single circumvallate papilla in the middle of the posterior part. Mouse has stereotyped array of fungiform papillae on the anterior part of the tongue. Three types of papillae including fungiform, foliate, and circumvallate papillae have taste bud.

2. Outline of tongue and gustatory papillae development in vertebrate

During the initial stages of organogenesis, the induction and formation of the epithelial appendages, including feathers, hairs, mammary glands, and teeth require specific and complex epithelial-mesenchymal interactions throughout their development.¹⁷⁻¹⁹ Although the vertebrate epithelial appendages have specific structures and diverse functions, most epithelial appendages have similar developmental stages, including induction, morphogenesis, and differentiation.^{20,21}

In feathers or in scale forming regions of the skin of chick embryos, the epidermal placode is the first morphological event for the formation of an epithelial appendage. It provides a signal to the dermis, which results in the formation of dermal condensations immediately beneath the placode.²² The formation of the epidermal placodes is determined by the mesodermal signals, which was revealed by the heterospecific tissue recombinations of the ectodermal and mesodermal components of the embryonic skin.²³ Furthermore, the mesenchyme determines the number, size, location as well as the structural identity of the appendages, while the epithelium determines the zoological class type (feathers or hair) and the competence state.²⁴ Therefore, the molecular mechanisms are conserved during the evolution of the skin as well as another developmental model system such as the limbs. Moreover, similar molecules appear to be essential for its proper development.²⁵

The development of the gustatory papillae has similar pattern of formation with the other epithelial appendages. The fungiform papillae also form a repeating unit with a highly regular spatial pattern through the epithelial mesenchymal interactions over the dorsal surface of the tongue during embryonic development. A recent study reported that a reciprocal

interaction between the tongue epithelium and the underlying mesenchyme is of fundamental importance in determining the individual position of the fungiform papilla as well as its stage-dependent competency.¹⁴

In mice, the fungiform papillae begin to develop as an epithelial thickening at E13. As the tongue develops, the mesenchymal cells become more rounded and closely apposed to form a condensate. The connective tissue is overlaid by an epithelium, which is two cells thick. The periderm cells, which form a superficial thin sheet of cells, are formed over the epithelium in early stage. During fungiform papillae development, the epithelial cells elongate in the primordium and thicken.^{14,26,35} The tongue epithelium and mesenchyme undergo subsequent morphogenesis with a stereotyped pattern until E17.

Previous studies have suggested that the morphogenesis of the gustatory papillae requires a series of invaginations and evaginations, which are coordinated interactions among the lingual epithelium and mesenchyme as well as in the nerve innervation of the tongue.^{5,27} The development of the fungiform papillae can be divided into two phases. The first phase is the morphogenesis phase, in which the papillae structures are produced. The second phase is the innervation phase, in which the taste buds develop. In the innervation phase, the fungiform papillae display a different developmental procedure for complete development than the other epithelial appendages. In the case of the fungiform papilla, the nerve innervation is required to develop into sensory organs with taste buds, as shown during the development of mice.^{4,27,28} Therefore, the innervation phase represents a maturation phase or functional phase.¹⁴ Jitpukdeebodintra et al., after examining the cell adhesion molecules, reported that the formation of the circumvallate papilla initially depends on cell migration, which likely to play a major role during circumvallate placode formation.¹² During the

initial tongue innervation, the nerves follow a spatiotemporal restricted pathway, suggesting that neural guidance is governed by signalling molecules from environmental factors such as BDNF (Brain derived neurotrophic factor), NT4 (Neurotrophin-4), E-cadherin, and laminin.^{2,5,8} Indeed, as a special sensory organ, the gustatory papillae are closely associated with nerve innervation, which has been examined in detail studies on taste buds.²⁹

3. Morphogenesis and pattern formation of fungiform papillae development

Previous studies have suggested that the morphogenesis of the tongue papillae requires coordinated interactions among the lingual epithelium and mesenchyme as well as nerve innervation of the tongue.^{2,30} The development of fungiform papillae can be divided into two stages. One is the morphogenesis, which produces the papillae structures. The other is nerve innervation, which develops the taste buds.^{4,27,28,31-34} Recent studies of the tongue in developing mouse embryos emphasized that nerve innervations are needed to develop the taste bud.^{2,30} Other studies showed that the *Shh*, *Ptc*, *Gli-1*, *Bmp-2*, *Bmp-4*, and *Fgf-8* transcripts are expressed within the epithelia of the primordial tongue.^{27,35} Especially, *Shh* plays important role in establishing papillary boundaries.¹³ It was suggested that the expression of the signalling molecules is of fundamental importance during papillae morphogenesis, and occurs prior to the morphological changes.^{13,15,27,35,36} However, there is no detailed description of the series of epithelial and mesenchymal changes during the early development of the fungiform papillae. Moreover, there is no direct evidence of the epithelium and mesenchyme interactions during the early development of fungiform papillae. This study focused on the morphogenesis of the fungiform papillae structure and attempted to identify the critical stage in the position of the fungiform papillae structure. The scanning electron microscopy (SEM), histology, and gene expression results were examined collectively to comprehend the precise development mechanism of the fungiform papillae. Both an *in vitro* organ culture system and an *in vitro* recombination system were used to obtain direct evidence of the epithelium and mesenchyme interactions, which build up the fungiform papillae structure in early development, and these systems

preclude the lingual nerve innervations. The recombination assay in this study suggests that the epithelium and mesenchyme interaction plays an important role during the early development of the tongue papillae, and the E13.5 epithelium has the potential to develop newly formed fungiform papillae structures prior to nerve innervations.

4. Pattern formation and molecular studies in gustatory papillae development

BMP-2 is a member of the transforming growth factor superfamily, which is expressed in many regions of the developing embryo including the developing fungiform papillae.^{35,78,85} They have been shown to be involved in many developmental processes including epithelial mesenchymal interactions during tooth and limb development.^{79,81,82} BMP-2 also is a part of a signalling cascade that controls development of the facial primordia.⁷⁵

The SHH protein is involved in morphogenesis and pattern formation of the tongue papillae as a morphogen, which directs the cells toward a nonpapillary fate, and as a mitogen, which causes the expansion of the interplacodal epithelium.¹³ Disruption of *Shh* signalling during rat tongue development revealed that the development and patterning of the fungiform papillae depends on this signalling pathway.¹⁶

Gap junctions are specialized areas of the cell membranes that allow direct exchange of signalling molecules and metabolites between the cytoplasm of neighboring cells.⁴² They are organized collections of protein channels that allow ions and small molecules to enable communicate between adjacent cells. These protein channels that make up the gap junctions consist of two "hemi-channels" or connexons, each connexon representing an oligomeric arrangement of six polypeptides.⁴³ Each connexon is composed of six transmembrane proteins called connexins, which are localized symmetrically around a central pore. The connexin family of proteins is encoded by at least 19 genes in rodents with many homologues cloned from other species.^{44,45} They are named according to their molecular weight. Connexin 43 (Cx43), for example, is a 43 kDa protein and is widely expressed in embryo and adult tissues.^{46,47} Signals such as hormones and growth and differentiation factors are thought to perform their function through affecting levels and activities of connexins.³⁹ Gap

junctions are observed in many developing tissues including cardiac muscle, and have been suggested to participate in a variety of roles including the regulation of signal transmission, cell proliferation, differentiation, apoptosis, and tissue homeostasis.⁴³ One of the major gap junction proteins in chick and mouse embryonic limb is alpha-1 connexin or connexin-43 (Cx43). Cx43 transcript is detected both in the apical ectodermal ridge (AER) and the posterior mesenchyme by stage 20 in the chick and the AER by embryonic day 10 in the mouse.⁴⁸⁻⁵¹ A major role of connexins in the early mesenchyme may be to enable the mesenchymal cells in the polarizing region to communicate with the anterior mesenchyme.^{49,52} Expression of Cx43 in the developing limb bud is complex, and some variations exist between the mouse^{48,51,53} and the chick.⁵⁰ Expression levels of Cx43⁵⁴ and the patterning genes of *Shh*⁵⁵ and *Bmp-2*⁵⁶ are higher in the ZPA. It has been suggested that interactions between signalling molecules and junctional communication would play an important role in controlling fungiform papillae development.⁵⁴

I show here that Cx43 protein expression significantly correlates with fungiform papillae development. In order to define the relationship between *Shh* and *Bmp-2*, the expression patterns of these genes were compared by using *in vitro* organ culture. Antisense oligodeoxynucleotide (AS-ODN) Cx43 was treated to analyze the developmental functions of Cx43.⁵⁸ The monomers are deoxynucleotides like those in DNA. There are usually only 15-20 of them, hence "oligo". This sequence (3' to 5') is antisense, that is, complementary to the sense sequence of a molecule of mRNA. Physically block the ability of ribosomes to move along the messenger RNA or simply hasten the rate at which the mRNA is degraded within the cytosol.⁵⁸ I investigated the function of *Bmp-2* during tongue development by ectopically expressing NOGGIN, a potent antagonist of the BMPs.⁵⁷ The expression patterns of signalling molecules, *Shh* and *Bmp-2* were altered by inhibition of gap junctions. Expression patterns of

Shh, which is known as a key molecule to determine spacing pattern, were disturbed after the inhibition of *Bmp-2* as well. These results suggest that the signalling cascade through the gap junction is sufficient for initiating papillary pattern and morphogenesis.

II. MATERIALS AND METHODS

1. Animals

Adult ICR mice were housed in a temperature-controlled room ($22\pm 1^{\circ}\text{C}$) under artificial illumination (lights on from 05:00 h to 17:00 h) and at 55% relative humidity, with free access to food and water. mice embryos were obtained from time-mated pregnant mice. The day that the presence of the vaginal plug was confirmed was designated embryonic day 0 (E0). Embryos at development stages (E12-17, daily intervals) were used in the present study.

2. Histology

The mouse embryos were fixed in 4% paraformaldehyde (PFA) in PBS overnight at 4°C , and embedded in paraffin wax. Specimens were cut with a $5\text{ }\mu\text{m}$ thickness, and the sections were stained with both hematoxylin and eosin (H&E).

3. Immunohistochemistry

For immunohistochemistry, the specimens were fixed with 4% paraformaldehyde at 4°C . They were equilibrated in a 30% sucrose solution for cryoprotection and embedded in an OCT compound (Sakura Finetek, USA). Expression pattern of Cx43 was examined by $7\text{ }\mu\text{m}$ cryosection after whole mount immunohistochemistry. Subsequent to the whole mount *in situ* hybridization as well as the Di.I. microinjections, 20

μm serial sagittal sections were cut using a cryostat and mounted on a silane-coated slide glass slide (Muto Pure Chemicals. Co., Japan).

4. SEM (scanning electron microscope)

The tongues, which were removed from the mandible, were prefixed with 2.5% glutaraldehyde in PBS for 24 h at 4°C, rinsed with PBS for 8 hours at 4°C, and postfixed with 1% osmium tetroxide in PBS for 2 h. The samples were then dehydrated in a graded series of ethanol, followed by critical point drying using a HCP-2 apparatus (Hitachi, Tokyo, Japan) with CO₂ as the transitional fluid. The specimens, which were mounted on stubs, were coated with platinum and examined by scanning electron microscopy (SEM, S-4500; Hitachi, Tokyo, Japan).

5. *In situ* hybridization

Whole cultured tissue was washed in PBST and was treated for 10-20 minutes with proteinase K (10 $\mu\text{g}/\text{ml}$ in PBST). It was washed twice in PBST and refixed for 1 hour in 4% PFA. Embryos were then prehybridized for 2 hours at 60°C in hybridization buffer containing 50% deionized formamide. Hybridization was performed overnight at 65°C in hybridization buffer containing 0.2-0.5 $\mu\text{g}/\text{ml}$ riboprobe. Excess probe was removed by sequential washes in 2X standard saline citrate (SSC; three times at 60°C), 0.2 X SSC (three times at 60°C), 1:1 of 0.2 X SSC:0.1 M phosphate buffer (PB), and PB (twice). Nonspecific binding in the tissue was blocked for 1-2 hours in 15% goat serum, 2 mg/ml bovine serum albumin, and 0.3% Triton X-100 in 0.1 M PB. After this treatment, the specimen

was incubated overnight with anti-digoxigenin antibody conjugated to alkaline phosphatase diluted 1:2,000 in blocking solution. Excess antibody was removed by washes in 0.1 M PB, and the tissue was equilibrated with color buffer containing 100 mM Tris, pH 9.5; 50 mM MgCl₂; 100 mM NaCl; and 0.1% Tween 20. The *Bmp-2*, *Bmp-4*, and *Shh* plasmids were kindly provided by Dr. Y.-P. Chen.

6. *In vitro* organ culture

To examine the regulation of signalling molecules after micro dissecting the tongues from a E12.5 mouse mandible, the tongue was separated into the aboral and oral parts. It was then cultured in medium containing 10% fetal bovine serum for 2 days by applying a slight modification of Trowel's culture method.

7. Di.I. application and Fate Mapping

Di.I. (1,19-dioctadecyl-3,3,39,39-tetramethyl indocarbocyanine perchlorate; Molecular Probes, Eugene, OR) was used as a cell tracer to observe cell migration during tongue development. A 0.3% w/v Di.I. in DMSO was used for microinjection. The injection of Di.I. was performed using 10 cm borosilicate capillary pipettes (Sutter Instruments, BF120-94-10), pulled using a Sutter Instrument Flaming Brown micro pipette puller, and that were filled by capillary action. Using a device of electricity. The lipophilic carbocyanine dye inserts into the membrane of the cells adjacent to the injection site. The exact position of the dye can be determined by using fluorescent microscope. Injections were done onto anterior part of

tongue dorsal surface.

8. Recombination assay

Recombination of the tongue using the intact epithelium and mesenchyme was done at E12.5 and E13.5. The tongue was dissected at E12.5 and E13.5, incubated in 2.4 unit dispase II for 15 to 20 min at 37°C, and washed in medium containing 10% fetal bovine serum. The epithelium and mesenchyme were separated using a dissection microscope. The epithelia were trimmed to equal sizes beneath a transparent dish using graph paper as a guide. A lipophilic dye, Di.I. (Molecular Probes, UK; 2 mg/ml in dimethyl formamide), was injected as a marker of the epithelial thickening regions at E13.5. The epithelia remained intact in the media. The epithelium was placed on top of the mesenchyme with a 90-degree rotation and the recombinants were cultured. After 24 and 48 hours recombination, the recombinants were examined using epifluorescence microscopy (LEICA, MZ FL III). *Shh* expression was observed using whole mount *in situ* hybridization after the recombination assay to identify the fungiform papillae forming area.

9. Whole mount immunostaining

Collect embryos in ice cold PBS. The embryos were fixed in freshly prepared 4% paraformaldehyde (PFA) in PBS overnight at 4°C. Wash the embryos in PBS, three times for 15 min. The embryos were washed in PBS with 1% Triton X-100 and 10% fetal calf serum (TS-PBS) three times for 1 hour. The embryos

were incubated with primary antibody, mouse monoclonal antibody against the Connexin43, (Zymed, Cat No. 13-8300) for 24 hours. Then, the embryos were washed with TS-PBS, 6 times for 30 min. The embryos were incubated with peroxidase-conjugated goat anti-mouse immunoglobulins (diluted 1/200 in TS-PBS) overnight. After washing the embryos with PBT 6 times for 30 minutes, the antibody was localized with DAB staining kit.

10. Beads implantation

Affigel Blue beads, (BioRad) 150 μ m in diameter, were dried and then soaked in 0.5 mg/ml of human recombinant NOGGIN (Regeneron). For examination of gene regulation of fungiform papillae development, a NOGGIN soaked bead was implanted in the anterior part of the tongue at E 12.5. The tongues were used for the whole mount *in situ* hybridization, after the bead implantation. PBS soaked beads were implanted as the control experiment.

11. Antisense oligodeoxynucleotides (AS-ODN)

Cx43 specific antisense ODN was designed and shown to be effective in knocking down Cx43 protein levels (sequence 960-989; GenBank accession no. X61576).⁵⁸ Control ODN sequence was used as a sense control for the mouse Cx43 sequence ODN (Table 1). ODN were purchased from GENOTECH (KOREA).

12. Image processing and counting the *Shh* expression spots

Whole tongues were photographed and digitized by using a Dimage Scan Multi film scanner (Minolta, Osaka, Japan). Bright field and cultured tongue images were collected with digital camera (LEICA, DC-300F). Digital images of cultured tongues, which were analyzed by using whole mount *in situ* hybridization with *Shh* mRNA, were collected. Tongues were scored double blind for the presence of both large and small (<10 μm diameter) *Shh*-positive papillary spots. The mean numbers of papillae per tongue were compared for different treatment groups by ANOVA.

III. RESULTS

1. Morphological findings

In order to understand the precise development of the fungiform papillae, transverse sections of the E13 to E17 mouse embryo tongue were stained with H&E. The morphological changes of the tongue epithelium surface were examined by SEM. Epithelial thickening was detected at E13, and the epithelial cells at the basal epithelium on the anterior part of the tongue increased in height (Fig. 2A). Epithelial thickening was detected at E13.5 on the dorsal surface of the tongue with a specific pattern from the anterior to posterior (Fig. 2F and J). At E14, epithelial thickening, under the surface region, became concave as the condensed mesenchymal cells migrated upward (Fig. 2B). At E14.5, archlike structures arose on the dorsal surface of the tongue with a stereotyped pattern, and the number of these structures increased (Fig. 2G and K). The number of these regions had grown by this stage. At E15, the epithelial cells continued to elongate, but the epithelial cells in the center of the archlike structure became thinner. In addition, the underlying mesenchymal cells of the epithelial thickening region were condensed (Fig. 2C). At E16, the epithelial cells continued to increase in height, and filiform papillae began to develop. The epithelial cells in the center of the archlike structure became thinner, and the underlying mesenchyme migrated upward (Fig. 2D). At E16.5, archlike structures rose on the dorsal surface with the highest length, and the tongue grew into the shape of an adult one. The distribution pattern and number of fungiform papillae were observed using SEM (Fig. 2H and L). At E17, fungiform papillae were observed on the anterior part of the tongue. The heights between the elevation of the

underlying mesenchyme and the stratified cells became similar. The surface of the stratified regions developed into the filiform papillae (Fig. 2E). In adults, the fungiform papillae were surrounded by filiform papillae (Fig. 2I and M).

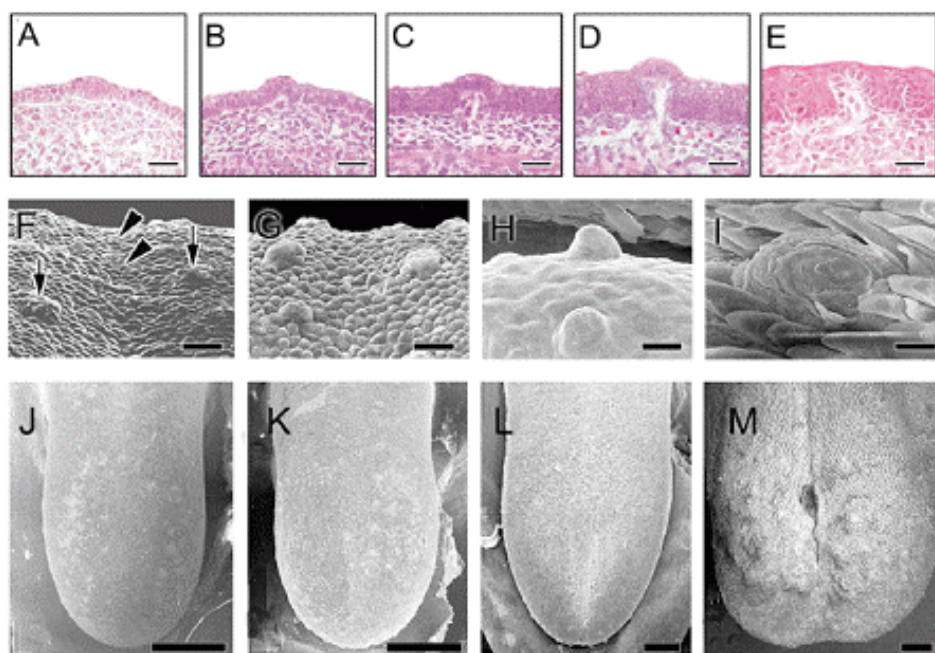


Fig. 2. Ultrastructural study of the fungiform papillae. (A-E) Histology of fungiform papillae during morphogenesis. (F-M) SEM study of mouse tongue development. (A) E13, the epithelium thickening. (B) E14, the epithelium developed into an archlike structure and the underlying mesenchymal cells were condensed. (C) E15, the epithelium of the fungiform papillae area, and their neighbor cells grew together. (D) E16, the filiform papillae began to develop. (E) E17, the development of the fungiform papillae into adult fungiform papillae (scale bar: (A-E) 25 μm). (F, J) E13.5, the fungiform papillae structures were observed on the dorsal surface, median sulcus (arrowhead), the fungiform papilla structures (arrow). (G, K) E14.5 the fungiform papillae structures raised on the dorsal surface. (H, L) E16.5 the fungiform papilla structures on the anterior part of the tongue. (I, M) PN 10 weeks, adult fungiform papillae were detected in the middle of the filiform papillae (scale bar: (F-I) 250 μm ; (J-M) 50 μm).

2. Expression patterns of signalling molecules

The *Bmp-4* and *Shh* expression patterns during the formation of the fungiform papillae in the mouse embryos from E12.5 to E14 were examined using whole mount *in situ* hybridization in order to determine the relationship between the morphological changes and the expression pattern of the signalling molecules. Transverse sections were observed after the whole mount *in situ* hybridization to confirm the regions where the signalling molecules were expressed. *Shh* expression was detected in the dorsal surface of the tongue at E12.5. This expression was broadly detected in the lateral swelling (Fig. 3A and B). *Bmp-4* had a similar expression pattern to *Shh*. However, *Bmp-4* expression was limited to a restricted area and was stronger there than in the other epithelium layers (Fig. 3I and J). At E13, the *Bmp-4* and *Shh* expression patterns were more localized and specific than at E12.5 (Fig. 3C and K). The distribution patterns of gene expression on the dorsal surface of the tongue resembled the SEM observations (Fig. 2E and J and Fig. 3C and K), and these expression patterns were restricted to the epithelium (Fig. 4D and L). At E13.5, both *Bmp-4* and *Shh* were strongly expressed in a localized spot, which appeared histologically identical to the neighboring epithelial cells (Fig. 2F and N). These gene expression patterns coincided with the distribution patterns of the elevated regions (Fig. 2E and M). At E14, the *Bmp-4* expression level was lower and broadened, but *Shh* expression was even more restricted and localized in the epithelial thickening regions (Fig. 3G and O). Both *Bmp-4* and *Shh* expression were detected in the archlike structures (Fig. 3H and P).

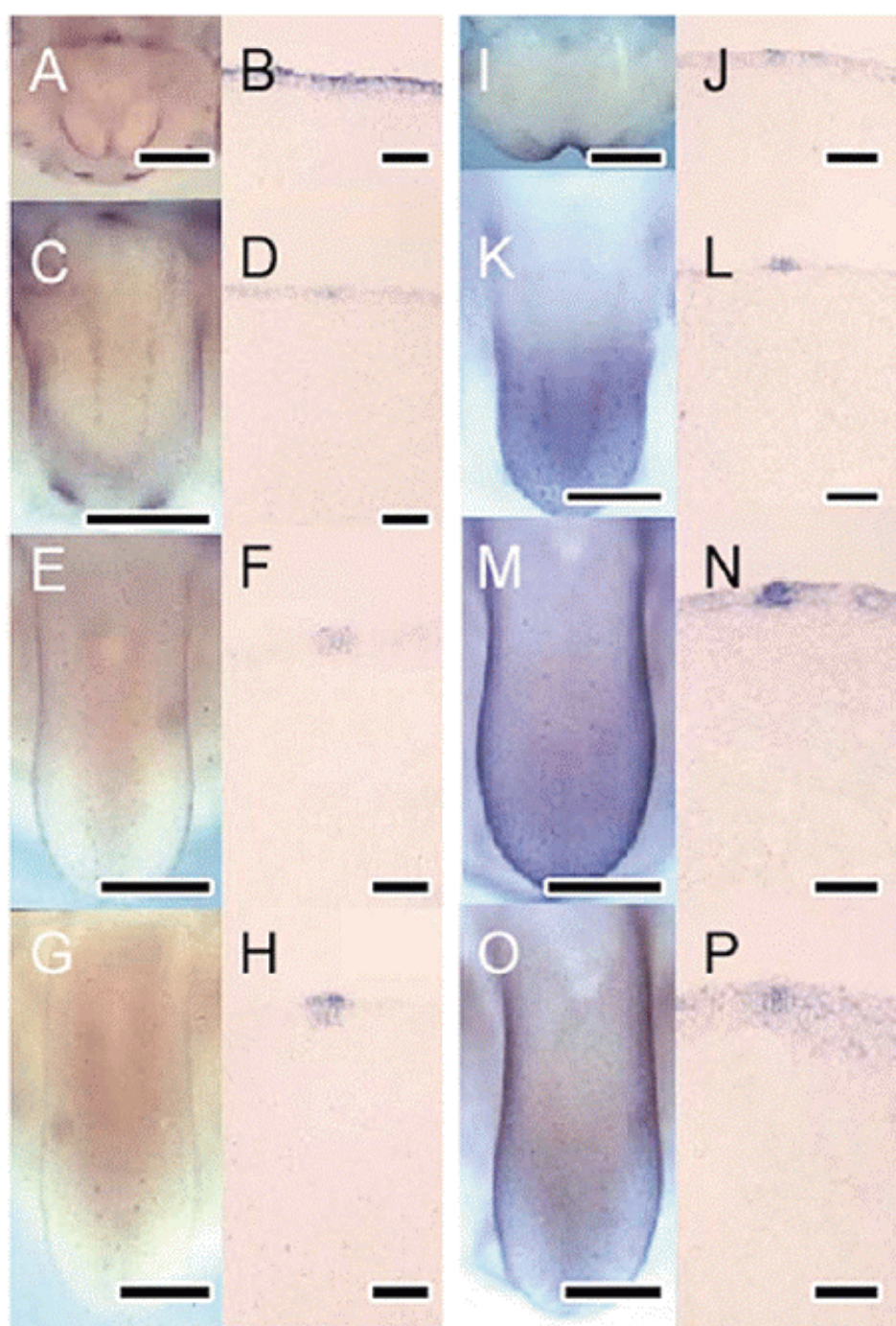


Fig. 3. Expression of signalling molecules. (A-H) *Shh*, (I-P) *Bmp-4* expression. (A, B) E12.5, *Shh* expression was detected in the epithelium. (C, D) E13, along the median sulcus, *Shh* expression with a stereotyped expression pattern and the anterior part of the mandible incisors was detected. (E, F) E13.5, *Shh* expression was restricted to certain areas of the epithelium. (G, H) E14, from the anterior to the posterior of the tongue, a stereotyped pattern expression was found on the dorsal surface of the tongue (scale bar: (A, C, E, G) 250 μm ; (B, D, F, H) 50 μm). (I, J) E12.5, *Bmp-4* was expressed broadly in the epithelium. (K, L) E13, *Bmp-4* expression was detected with a stereotyped pattern, which was restricted to the epithelium. (M, N) E13.5, the strong *Bmp-4* expression level was restricted to the archlike structures. The dorsal surface of the tongue expression spots was specified to produce a stereotyped pattern. (O, P) E14, *Bmp-4* expression changed to be broad on the dorsal surface (scale bar: (I, K, M, O) 250 μm ; (J, L, N, P) 50 μm).

3. Recombination assay

An *in vitro* organ culture was used as a control. For the *in vitro* organ culture, the E13.5 tongues were microdissected using a tungsten needle. The tongue was separated into an aboral and oral part. These microdissections allowed the fungiform papillae-like structures to develop (Fig. 4A). After the *in vitro* organ culture, the fungiform papillae-like structures were observed using the paraffin sections and H&E staining. Twenty-four hours later, the *in vitro* organ cultured tongue showed fungiform papillae-like structures. Archlike structures could be observed over the epithelium area, and there was a small amount of condensation in the underlying mesenchyme area (Fig. 4B). After 48 hours, the epithelial cells grew to make an archlike structure. Those structures were similar to the E14 fungiform papillae structure (Fig. 4C). After 72 hours, the fungiform papillae-like structures, which developed as E15 fungiform papillae, were examined. However, these structures were different from those observed *in vivo* at E15. There was no elevation above the epithelium (Fig. 4D). A recombination assay was performed to determine the role of the E13.5 epithelium and mesenchyme during early fungiform papillae development. Recombination of the tongue using intact epithelium and mesenchyme was observed using an E13.5 tongue. A Di.I. microinjection was made in the epithelium of the fungiform papillae at E13.5 in order to determine the properties of the E13.5 epithelium (Fig. 4E). All the morphological structures were disrupted after the recombination assay (data not shown). No morphological changes were observed in the recombinants of the E13.5 tongues 24 hours later (Fig. 4F). However, *Shh* expression was detected in the epithelium of the recombinants (Fig. 4F*). Cross sections of the newly formed fungiform

papillae-like structures appeared 48 hours after the recombination (Fig. 4G). Frozen sections after the whole mount *in situ* hybridization exhibited epithelial expression of *Shh* 48 hours after the recombination (Fig. 4G*). All the Di.I. microinjected epithelium of the fungiform papillae was observed to have archlike structure using fluorescence microscopy. This suggested that the E13.5 epithelium can induce newly formed fungiform papillae-like structures in the epithelium with the epithelial signalling (Fig. 4H). An additional recombination assay was done to understand the role of the E13.5 mesenchyme. Di.I. was microinjected into the underlying mesenchyme of the fungiform papillae at E13.5 (Fig. 4I). Another recombination using the epithelium of the E12.5 tongue and mesenchyme from an E13.5 tongue showed newly formed fungiform papillae-like structures 48 hours later (Fig. 4J). Newly formed fungiform papillae-like structures could not be found in Di.I. labeled mesenchyme area using fluorescent microscopy (Fig. 4K).

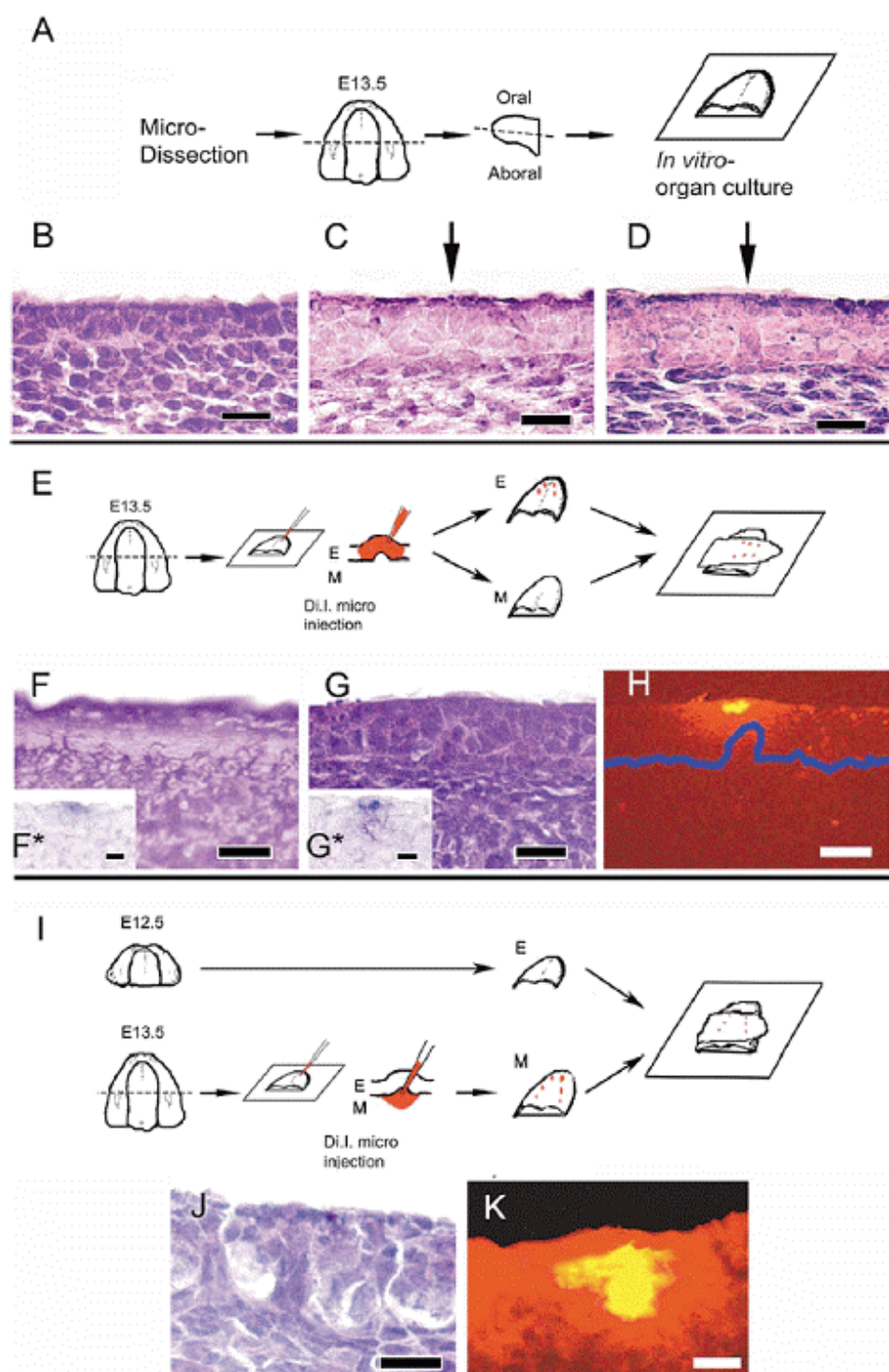


Fig 4. Recombination assay. *In vitro* organ culture of fungiform papillae at E13.5 (A, B, C, D), recombination assay of fungiform papillae at E13.5 (E, F, G, H), expression of signalling molecule after the recombination assay (F*, G*), and the recombination assays E12.5 and E13.5 (I, J, K). (A) Schematic diagram of the *in vitro* organ culture. After microdissecting the tongue from an E13.5 mouse mandible, the tongue was separated into the aboral and oral parts. It was then cultured using a slight modification of Trowell's culture method. (B) 24 hours later, it changed little from E13.5. (C) 48 hours later, the arrow indicates an archlike structure and the underlying mesenchymal cells were condensed. (D) 72 hours later, an archlike structure was detected in the epithelium and a mesenchymal core rose to the epithelium (scale bar: 50 μ m). (E) Schematic diagram of the recombination assay. After microdissecting the tongue from an E13.5 mouse mandible, the tongue was separated into an aboral and oral part. The aboral part was treated with dispase II (2.4 unit for 15 min) at 37°C and the epithelium was removed with a fine needle. The Di.I. microinjection accommodating to the epithelium, which showed a specific fungiform papillae structure. The epithelium rotated 90 degree and was then reattached to the mesenchyme (to determine the origin of that structure). It was then cultured using a slight modification of Trowell's culture method (E, epithelium; M, mesenchyme). (F) 24 hours after the recombination, most morphological structures were disrupted. (F*) *Shh* expression was restricted to certain area of the epithelium. (G) 48 hours later, it formed archlike structures in the epithelium. (G*) A strong *Shh* expression level was detected only in the archlike structure. (H) The Di.I. labeled epithelium induced a specific structure of the fungiform papillae (blue line shows basement membrane). (I) Schematic diagram of the recombination assay with E12.5 and E13.5. The Di.I. microinjection was accommodated to the mesenchyme of the E13.5 tongue (E, epithelium; M, mesenchyme). (J) 48 hours later, it formed archlike structures in the epithelium. (K) The fungiform papilla structure was not observed on the

Di.I. injected mesenchymal area (scale bar: (B, C, D, F, G, H) 50 μm ;
(F*, G*) 10 μm ; (J, K) 25 μm).

4. Whole mount immunohistostaining

In order to determine the relationship between the morphological changes and the expression pattern of Cx43. The Cx43 expression patterns during the formation of the fungiform papillae in the mouse embryos from E11 to E14 were examined using whole mount immunostaining and transverse sections were examined after the whole mount immunostaining to confirm the regions where the Cx43 was expressed.

Cx43 expression was broadly detected in the lateral swelling at E11.5 (Fig. 5A). This expression was detected in epithelium and underlying mesenchyme of the tongue at E11.5 (Fig. 5D). At E12.5, Cx43 expression patterns were more localized and specific than at E11.5 (Fig. 5B), and Cx43 expression was restricted to the epithelium (Fig. 5E). At E13.5, Cx43 was strongly expressed in a localized spot, which appeared histologically identical to the neighboring epithelial cells (Fig. 5C, F).

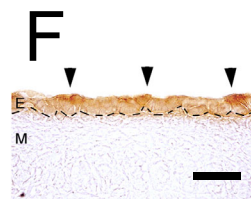
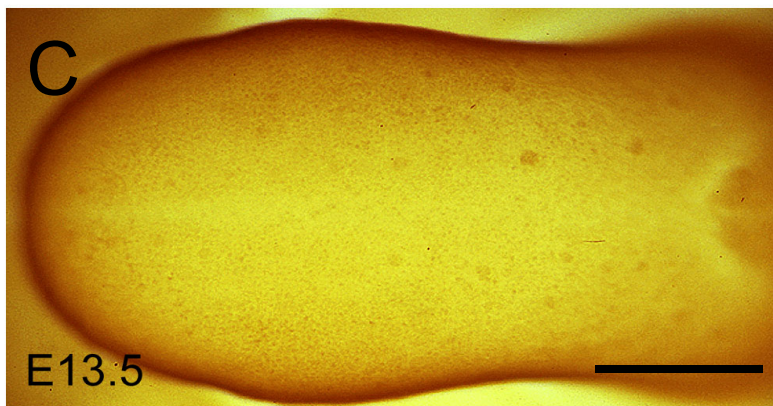
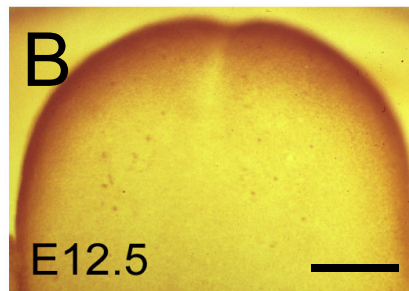
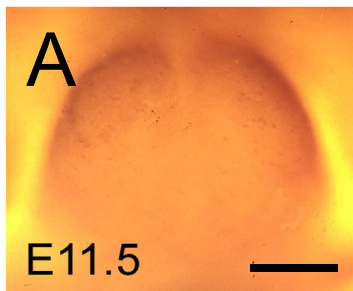
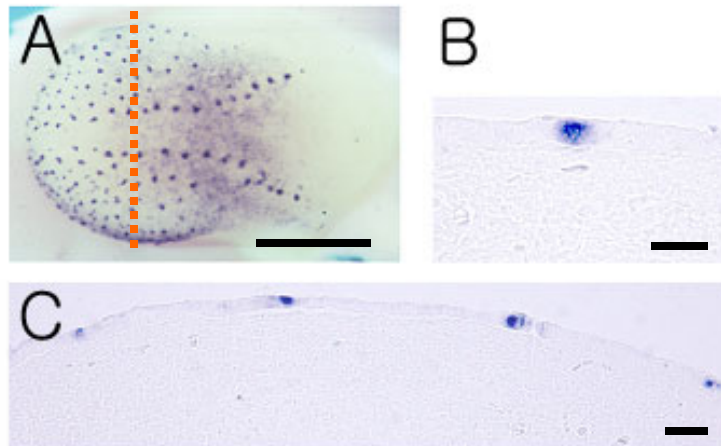


Figure 5. Whole mount immunostaining using Cx43. (A-C) Whole mount immunostaining of mouse tongue. (A) E11.5, there was a broad expression pattern of Cx43. (B) E12.5, Cx43 expression spots were observed on the dorsal surface of the tongue. (C) E13.5, Cx43 expressions were localized with specific patterned array. (D-F) Frozen sections after the whole mount immunostaining. Dotted lines indicate basement membrane. (D) E11.5, Cx43 expressions were localized in both epithelium and mesenchyme. (E) E12.5, Cx43 expressions were localized in epithelium. (F) E13.5, Cx43 expressed in the fungiform papillae forming epithelium (arrow heads indicate the strongest expression spots). (scale bar: (A, B) 100 μm ; (C) 200 μm ; (D-F) 40 μm).

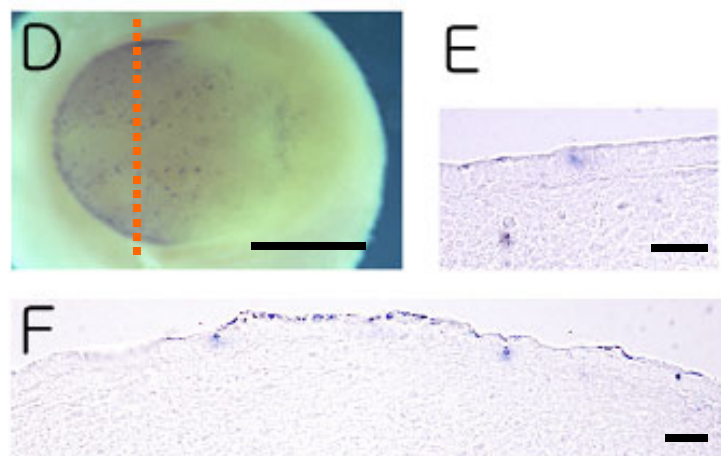
5. Gene expression pattern after *in vitro* organ culture

To examine the expression patterns of signalling molecules, E12.5 tongue was used for *in vitro* organ culture. After the micro dissecting the tongues at E12.5 mouse mandible, the tongue was separated into the aboral and oral parts. It was then cultured for 2 days in a medium containing 10% fetal bovine serum using a slight modification of Trowell's culture method (Fig. 6G). *Shh* expression was detected in the dorsal surface of the tongue after 2 days of culture at E12.5 (Fig. 6A). *Bmp-2* showed similar expression pattern to *Shh*. However, The intensity of *Bmp-2* expression was weaker than that of *Shh* expression (Fig. 6C). After whole mount *in situ* hybridization, transverse sections were performed to examine the precise expression pattern of *Shh* and *Bmp-2*. Both *Shh* and *Bmp-2* were strongly expressed in a localized spot in epithelium, which appeared histologically identical to the neighboring epithelial cells (Fig. 6B, C, E, and F).

Shh



Bmp-2



G

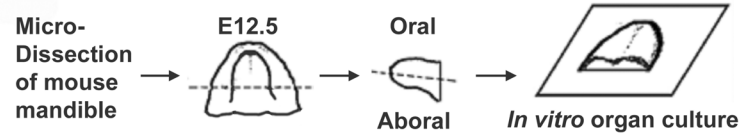


Figure 6. Signalling molecules expression pattern after *in vitro* organ culture for 48 hours at E12.5. (A-C) *Shh* and (D-F) *Bmp-2* expression patterns were observed after the whole mount *in situ* hybridization. These expression patterns are similar to the *in vivo* expressions at E14. (A) *Shh* expressed on the dorsal surface of the tongue with specific array, which were observed at E14 mouse tongue. (B, C) Transverse sections showed *Shh* expression in the fungiform papillae like structure in epithelium. (D) *Bmp-2* expressions were examined after *in vitro* organ culture. Expression patterns were similar to *Shh* expression pattern. The intensity of expressions were less than that of *Shh*. (E, F) *Bmp-2* expressions were also localized in epithelium. (G) Schematic diagram of *in vitro* culture at E12.5. (scale bar: (A, D) 250 μm ; (B, C, E, F) 50 μm). Dotted lines indicate the section level.

6. Alteration of gene expression pattern after the treatment of antisense Cx43 and implantation of NOGGIN bead to *in vitro* organ culture.

Cx43 specific AS-ODN was designed and shown to be effective in knocking down Cx43 protein levels (sequence 960-989; GenBank accession no. X61576).⁵⁸ Control ODN sequences were used as a sense control for the mouse Cx43 sequence ODN (Table 1). Both *Bmp-2* and *Shh* expressions were altered after treatment of Cx43 AS-ODN. *Shh* expression was denser than the control expression on the dorsal surface of the tongue (Fig. 7A). Otherwise, *Bmp-2* expression was decreased and restricted in the anterior part of the tongue (Fig. 7D). Expressed cell population densities were disrupted by Cx43 AS-ODN (Fig. 9). *Shh* and *Bmp-2* expression patterns were altered by Cx43 AS-ODN and confirmed with transverse section after whole mount *in situ* hybridization. Intensity of *Shh* expression was stronger than that of the control (Fig. 3B), while *Bmp-2* expression was weaker (Fig. 7E). Moreover, it is shown that antisense Cx43 AS-ODN altered expression pattern of signalling molecules (Fig. 7C, F and Fig. 9).

For examination of the gene regulation of fungiform papillae development, a NOGGIN soaked bead was implanted in the anterior part of the right side of the tongue at E12.5. PBS bead implantation was examined as the control. After the bead implantation, signalling molecules were examined using whole mount *in situ* hybridization. After NOGGIN bead implantation, *Shh* expression was altered near the bead-implanted area (Fig. 8A). Transverse sections showed that the size of fungiform papilla structure was larger than that of the control culture, and The intensity of *Shh* expression was more intense than in the control culture (Fig. 8B, C). After the PBS bead implantation, there were no critical changes from control culture (Fig. 8D, E). *Bmp-2* expressions were altered after the NOGGIN bead implantation (Fig. 8F). *Bmp-2* expressions were moved to the mesenchyme near

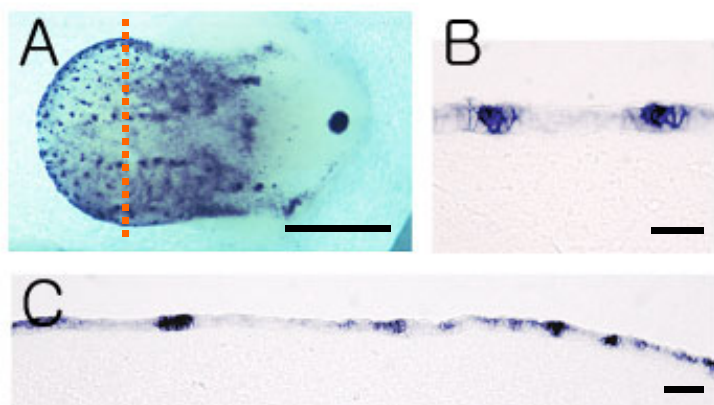
to NOGGIN bead (Fig. 8G). After the NOGGIN bead implantation, the size of fungiform papillae was larger than that of the control culture (Fig. 8H). The number of *Shh* expression spots was examined after the whole mount *in situ* hybridization as a marker for spacing pattern. Since *Shh* is well known as a major signalling molecule to set up of pattern formation and morphogenesis during fungiform papillae development (Fig. 9). Control sense Cx43 ODN treated and PBS bead implanted tongues showed similar number of *Shh* expression spots of control culture, it was 1.8 spots/100 μm^2 in the anterior part of the dorsal tongue area. However, after Cx43 AS-ODN treatment and NOGGIN bead implantation, the number of *Shh* expression spots was increased as 7.4 and 3.6 spots/100 μm^2 in the anterior part of the dorsal tongue area (Fig. 9).

Table 1. The oligodeoxynucleotide (ODN) sequences used in this study.

ODN	Sequence
Mouse Cx43 specific	GTA ATT GCG GCA GGA GGA ATT GTT TCT GTC
Sense control	GAC AGA AAC AAT TCC TCC TGC CGC AAT TAC

Cx43, Connexin43 (Law et al., 2002).

Shh



Bmp-2

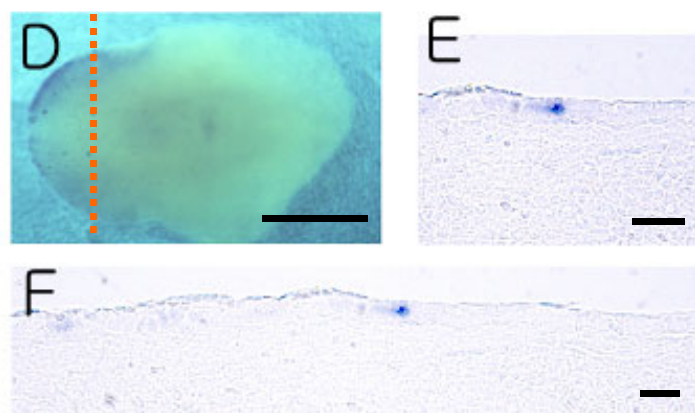


Figure 7. Alteration of gene expression pattern after the treatment of antisense Cx43 ODN. AS Cx43 ODN (Antisense Cx43 Oligodeoxynucleotide) was designed as GTA ATT GCG GCA GGA GGA ATT GTT TCT GTC. Pattern formation of fungiform papillae was disrupted following Cx43 AS-ODN treatment. (A) *Shh* expressions were altered following Cx43 AS-ODN. *Shh* expression spots were increased on the dorsal surface of the tongue. (B, C) *Shh* expressed in the fungiform papillae structures in epithelium. (D) The number of *Bmp-2* expression spots were decreased on the dorsal surface of the tongue after Cx43 AS-ODN. (E, F) Although *Bmp-2* expressed in the fungiform papillae forming epithelium, the intensity of expressions was decreased. (scale bar: (A, D) 250 μm ; (B, C, E, F) 50 μm). Dotted lines indicate the section level.

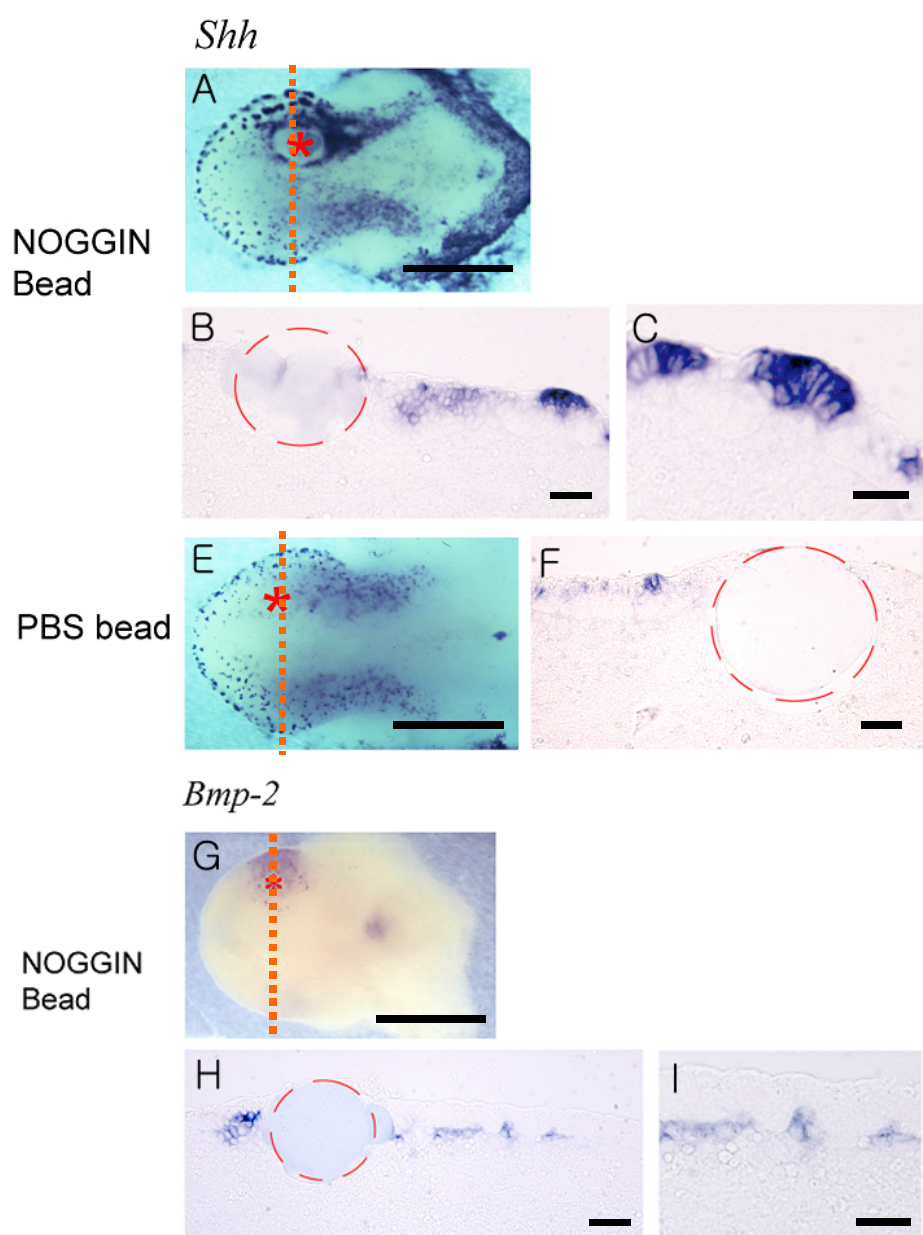


Figure 8. Alteration of gene expression pattern following the NOGGIN bead implantation (concentration of NOGGIN protein was 0.5 mg/ml). Red circles indicate beads. (A) The NOGGIN bead was implanted at right side of the dorsal surface of the tongue. The size of *Shh* expression spot was larger than that of the control. (B) Transverse section showed that *Shh* expressions were localized in epithelial cells. (C) The size of fungiform papillae like structures were increased. (D) PBS bead was implanted as a control experiment. There was no critical change from the control. (E, F) Transverse sections were also examined. (G) After the NOGGIN bead implantation, *Bmp-2* expressed near to the NOGGIN bead with broad pattern. (H, I) Transverse section showed that *Bmp-2* expressed in mesenchyme, where is the beneath to the fungiform papillae like structure. (scale bar: (A, E, G) 250 μm ; (B, C, F, H, I) 50 μm). Dotted lines indicate the section level.

The number of *Shh* expression spots

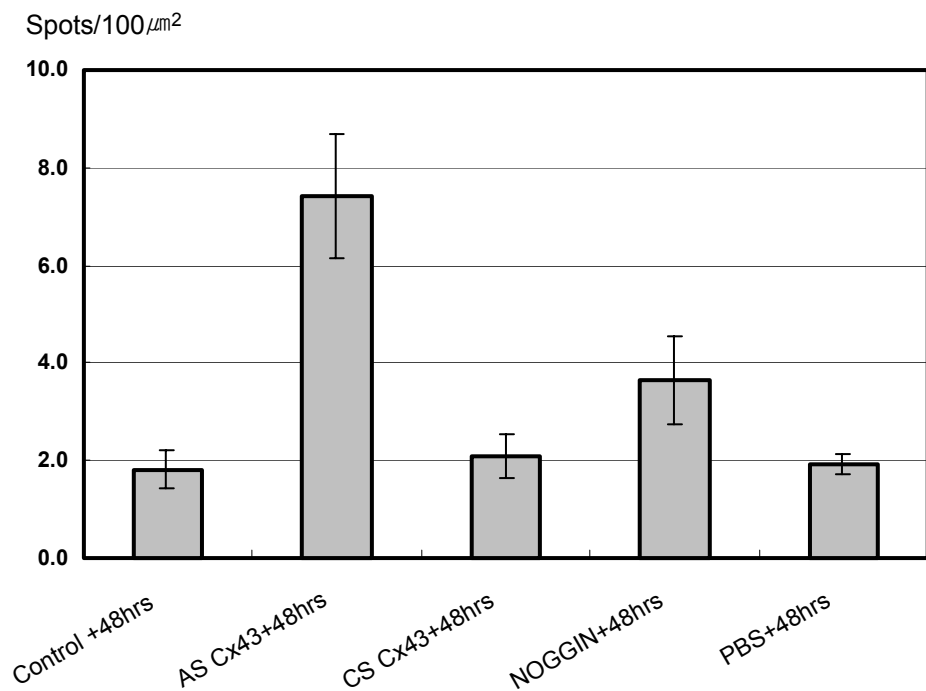


Figure 9. The number of *Shh* expression spots of tongue dorsal surface. Alteration of *Shh* expression pattern was examined by counting the expression spots after the Cx43 AS-ODN treatment and the NOGGIN bead implantation (spots/100 μm^2).

IV. DISCUSSION

1. Morphogenesis of fungiform papillae in the mouse development

Tongue papillae are types of epithelial appendages, which share a similar process to other organs, such as the hairs, kidneys, teeth, feathers, and mammary glands.¹⁵ However, fungiform papillae have different developmental procedures from other epithelial appendages for complete development. Fungiform papillae require nerve innervations to develop into sensory organs.^{4,28,30-34} Fungiform papillae begin to develop with epithelial thickening.^{7,59-61} At E12.5, cells of the epithelial layer become elongated and the periderm thickens in some restricted areas of the epithelium. From E13 to E16, the epithelium and mesenchyme undergo subsequent morphogenesis. There is a series of invaginations and evaginations, as the epithelium and mesenchyme interact to form tongue papillae with an epithelial covering over a mesenchymal core. The early stages of fungiform papillae formation resemble those of other specialized epithelial appendages, particularly the hairs, teeth, and feather buds.^{21,62-64} In those developing systems, cascades of intercellular signalling interactions specify the organ position and induce morphogenesis.^{64,65} This study showed the precise morphogenesis of the mouse tongue development. These results confirmed the previous results of mouse tongue development using SEM.⁷ At E13.5, fungiform papillae are raised on the dorsal surface of the tongue, which form two rows from the anterior to the posterior, along the median sulcus. From this stage, the fungiform papillae could be observed using a dissecting microscope. These rows had a pattern, called a "spacing pattern", on the dorsal surface.³⁷ The density of the fungiform papillae was higher in the anterior part of the tongue. At E14, fungiform papillae

developed anterior to the lateral side. The distribution pattern and density of the fungiform papillae developed to the adult one. At E16, the filiform papillae began to grow on the dorsal surface of the tongue. Therefore, the adult fungiform papillae could be observed, which were surrounded by filiform papillae. The histological results showed a similar morphogenesis pattern as that shown by the ultrastructural findings. It appears that the morphogenesis of the fungiform papillae is similar to that observed in the formation of feathers, hair, and teeth, which results from the intimate collaboration of the epithelium and mesenchyme.^{21,62-64}

2. Expression of signalling molecules prior to morphological changes

Recent studies of the tongue in developing mouse embryos have shown that *Shh*, *Ptc*, *Gli-1*, *Bmp-2*, *Bmp-4*, and *Fgf-8* transcripts are expressed within the epithelia of the tongue primordia, because these signalling molecules are of fundamental importance in papillae morphogenesis.^{27,35} In addition, this study confirmed the previous results on gene expression during tongue papillae development. These signalling molecules might regulate the early stages of organogenesis and produce signalling networks, which have been reported in many organs, including tooth buds,⁶⁶ limb buds,^{67,68} and feather buds.⁶⁹ Gene expression was explained as a morphological marker of the tongue papillae. Based on these results, the expression of these signalling molecules can only be detected in the epithelium prior to the morphological changes. Initially, *Bmp-4* and *Shh* expressions were detected in restricted area, which changed to a specific structure at E13. These signalling molecules share a similar expression pattern. However, there were differences in the intensity and the width of the expression in the epithelium. Secondly, the signalling molecules were detected as dotted patterns on the dorsal surface of the tongue. Furthermore, the specific stereotyped spacing pattern of the fungiform papillae could be observed. Overall, these results suggest that the signalling molecules, which have been implicated in the morphogenetic regulation of many other epithelial appendages, are also associated with the initial stages of tongue papillae formation.¹³ From these gene expression results, these signalling molecules are expressed in restricted area in the epithelium, which changes morphologically. It was assumed that the tongue papillae share a similar formation process as observed with other epithelial appendages.

3. E13.5 epithelium determines the position of fungiform papillae

The formation of skin appendages requires an interaction between the epithelium and mesenchyme.^{21,62-64} The mesenchyme determines the number, size, location, and structural identity of the appendage, while the epithelium determines the orientation and competence state. From the morphological results in this study, most of the signalling molecules were expressed in the epithelium. However, morphological changes were observed in the epithelium and mesenchyme. It is thought that the epithelium might play an important role during early development, as observed in hair, feather, and tooth development. In addition, the results suggest that these epithelial signalling molecules regulate the morphogenesis of the mesenchyme at E13.5. Compared to the other epithelial appendages, tongue papillae have technical disadvantages. This study could not observe the fungiform papillae on the tongue dorsal surface by a dissecting microscope prior to E13.5. Therefore, a Di.I. microinjection was available from E13.5. A previous study reported that prior to E14, the nerve does not reach the basement membrane of the fungiform papillae forming area.²⁷ Consequently, E13.5 was selected to examine the role of the epithelium and mesenchyme interaction for the development of fungiform papillae with the Di.I. microinjection. A recombination assay using an *in vitro* organ culture after the recombination assay with the intact epithelium and mesenchyme was made at E13.5 for 2 days in order to confirm the properties of the epithelium during mouse tongue development. After 24 hours, an archlike structure was observed in the epithelium. It had similar figures to the E14 fungiform papillae *in vivo*. After 48 hours, the labeled epithelium induced fungiform papillae-like structures, which were observed over the newly formed fungiform papillae

structures. These findings suggest that the epithelium can induce the fungiform papillae-like structures, and the epithelium has properties that can localize the epithelial appendages at E13.5. The signalling molecule, *Shh*, was chosen as a morphological marker of the fungiform papillae after the recombination assay to confirm the identity of newly formed fungiform papillae.¹³ *Shh* expression was also detected in the epithelium of the newly formed fungiform papillae. This suggested that the E13.5 epithelium can induce newly formed fungiform papillae-like structures in the epithelium with the epithelial signalling. This study also examined the reversal recombination using the epithelium of the E13.5 tongue and the mesenchyme of the E12.5 tongue. These observations showed the Di.I. labeled epithelium formed fungiform papillae structures, which were similar to the results of the E13.5 recombination (data not shown). However, recombination using the epithelium of the E12.5 tongue and the mesenchyme of the E13.5 tongue produced the fungiform papillae structure regardless of Di.I. microinjected mesenchyme. It suggests that E13.5 is a crucial stage for determining the position of the fungiform papillae structure and that there is mutual interaction between tongue epithelium and mesenchyme which work together for papillae development at early mouse embryogenesis.

4. Developmental role of gap junction during mouse tongue development.

Gap junctions are intercellular channels that are involved in cell-to-cell communication.⁸³ Small molecules can pass through the gap junction channels, such as ions and second messengers with molecular weights less than 1 kDa.⁸⁴ Gap junctions are considered to be important for maintaining tissue homeostasis and for communicating certain intracellular signals. Gap junctions are also observed in many developing tissues including cardiac muscle and nerve, and have been suggested in variety of roles including the regulation of signal transmission, cell proliferation, differentiation, apoptosis, and tissue homeostasis.⁴³ One of the major gap junction proteins in chick and mouse embryonic limb is alpha-1 connexin, also known as Cx43.⁴⁸⁻⁵¹ A major role of connexins in the early mesenchyme may be to enable mesenchymal cells in the polarizing region to communicate with the anterior mesenchyme.^{49,52}

I have demonstrated changing patterns of Cx43 expression and gap junction formation during mice fungiform papillae development. The patterns of Cx43 expression were examined by using whole mount immunostaining method. The Cx43 was expressed in the dorsal surface of the mice tongue from E11.5. At E12.5, expression of Cx43 was localized in the epithelium. In particular, At E13.5, expressions of Cx43 were examined in fungiform papillae forming area. These expression patterns are similar to those of the signalling molecules such as *Shh* and *Bmps*. These findings implicate that there is the relationship between gap junction and signalling molecules as it has been known in limb development.⁵⁰⁻⁵⁴

5. Expression patterns of signalling molecules after *in vitro* organ culture.

Recent studies of the fungiform papillae in developing mouse embryos have shown that *Shh*, *Ptc*, *Gli-1*, *Bmp-2*, *Bmp-4* and *Fgf-8* transcripts are expressed in the fungiform papillae forming area prior to morphogenesis, indicating that the signalling molecules are of fundamental importance in papillae morphogenesis.^{27,35} And, *Shh* is required during initiation, determination, and differentiation of fungiform papillae.¹³ In mice tongue development.^{14-16,27,35} BMP-2 has been shown to be involved in many developmental processes including epithelial mesenchymal interactions during tooth and limb development.^{81,82} BMP-2 is also a part of a signalling cascade that controls development of the facial primordium.⁷⁵

Interestingly, *in vitro* organ culture of mouse tongue was allowed to examine the regulation of signalling molecules. So I could examine the expression pattern of signalling molecules, *Bmp-2* and *Shh*, after *in vitro* organ culture at E12.5. After 2 days of culture, *Shh* expression was observed on the tongue dorsal surface with stereotyped pattern using whole mount *in situ* hybridization. Expression pattern of *Bmp-2* was also examined at fungiform papillae forming area of dorsal surface. Both signalling molecules were expressed in fungiform papillae forming area in the epithelium. These expression patterns are similar to those of the E14, *in vivo* control. This *in vitro* organ culture method would be helpful to understand the signalling pathway during early development of fungiform papillae. In particular, the regulations between activation and inhibition of signalling molecules can be examined by using various methods, such as AS-ODN and protein beads implantation.

6. Gene expression pattern in fungiform papillae following Cx43 antisense treatment.

Previously, it is suggested that interactions between signalling molecules and junctional communication would play an important role in controlling development.⁵⁰⁻⁵⁴ Here, in an attempt to understand more clearly how communication through Cx43 channels influences the cascade of gene expression involved in the patterning of the fungiform papillae. I examined that Cx43 protein expression significantly correlates with fungiform papillae development by using Cx43 AS-ODN treatment, Cx43 specific AS-ODN was designed and shown to be effective in knocking down Cx43 protein levels.⁵⁸ Control sense ODN (CS-ODN) a sequence was used as a sense control for the mouse Cx43 sequence ODN.

Shh expression pattern was altered and disrupted following Cx43 AS-ODN treatment. The number of *Shh* expression spots increased from the control. The number of *Shh* expression spots in the control specimen was 1.8 spots/100 μm^2 in the anterior part of the dorsal tongue area. While the numbers of *Shh* expression spots of Cx43 CS-ODN treated specimen were similar to that of the control, the Cx43 AS-ODN treated specimen showed much higher density of 7.4 spots/100 μm^2 . These results demonstrate that the Cx43 mediated regulation of fungiform papillae development is important in fungiform papillae patterning and that transient knockdown of Cx43 expression leads to disruption of fungiform papillae patterning. *Bmp-2* expression pattern was altered and decreased following Cx43 AS-ODN treatment. Histological sections, after whole mount *in situ* hybridization, showed that the intensity of *Bmp-2* expression was decreased from the control. In addition, *Bmp-2* expressions were decreased while *Shh* expressions were increased following Cx43 AS-ODN treatment. These results suggest that *Shh* and *Bmp-2* are downstream of Cx43 mediated signalling in the

particular cascade of fungiform papillae development. This signalling cascade in fungiform papillae development is similar to that of the limb development.^{50,52}

7. Gene expression pattern in fungiform papillae following NOGGIN bead treatment.

In order to understand the signalling regulation during early fungiform papillae development, ectopic-expressing NOGGIN, which is a potent antagonist of the BMPs, was treated to *in vitro* organ culture by using the bead implantation. *Bmp-2* expression in epithelium was blocked by BMP-inhibitor NOGGIN protein. Interestingly, localization of *Bmp-2* expressions was moved to mesenchyme. *Shh* expression was slightly altered by NOGGIN protein. The density of *Shh* expression spot was increased to 3.6 spots/100 μm^2 in the anterior part of the dorsal tongue surface.

After the NOGGIN bead implantation, a morphological disturbance in fungiform papilla was observed by transverse section during fungiform papillae development. The size of fungiform papilla was larger than that of the control. This change could be due to the effect of the regulation of the signalling molecules, such as *Bmp-2*, *Shh* and Cx43, during early development of fungiform papillae prior to nerve innervations. These altered expression patterns suggest that there would be a relationship between *Bmp-2* expression and nerve innervations, which were excluded by this *in vitro* organ culture system. These alteration could be due to the inhibitory regulation between *Bmp-2* and nerve factors.⁸⁶

To sum it up, Cx43 plays an important role during fungiform papillae development. Antisense Cx43 ODN treatment leads to a down-regulation of *Bmp-2* expression and an up-regulation of *Shh* expression. NOGGIN bead implantation leads to the alteration of size of a fungiform papilla and up-regulation of *Shh* expression. These treatments resulted in the development of abnormal fungiform papillae morphology and disrupted pattern formation of signalling molecules. It is suggested that there are regulations among *Shh*,

Bmp-2, and Cx43 mediated signalling can regulate pattern formation and morphogenesis. Transient knockdown of Cx43 leads to up-regulation of *Shh*, which in turn leads to disruption of pattern formation of fungiform papillae. I note that Cx43 is also expressed in the facial primordia and the limb during development. These regions are very similar to fungiform papillae in developmental terms. Therefore, it would be interesting to look at the role of Cx43 in these regions to determine whether it has a function similar to the one proposed here for the fungiform papillae.

V. CONCLUSION

The development of the epithelial appendages has been examined intensively over the last 30 years, mainly using a number of model systems such as feathers, hair, teeth, as well as the mammary and salivary glands.^{17-21,70-74} The fungiform papillae might be another new model system for examining epithelial appendage development. All these model systems share similar developmental processes in the early embryonic stages. In particular, fungiform papillae development shows some similarities with the development of other epithelial appendages. First, prior to the morphological changes, the signalling molecules are expressed in the fungiform papillae forming area with a specific patterned array. Second, the morphogenesis of the fungiform papillae has a specific structure such as epithelial thickening and mesenchymal condensation, which are observed in the morphogenesis of the other epithelial appendages. Third, the stereotyped pattern, which is governed by signalling molecules such as *Shh*, *Bmp-2*, and *Cx43*, is present during fungiform papillae development (Fig. 10).⁶¹ Fourth, the fungiform papillae develop through reciprocal interactions between epithelial and mesenchymal tissue. It would be interesting to know how, in the case of the gustatory papillae, individual position, size and the spacing pattern are determined (Fig. 11). Moreover, fungiform papillae have different developmental procedures from the other epithelial appendages. Nerve innervation is required for the development of mouse embryo taste buds,^{16,27} whereas the fungiform papillae can develop independently of nerve innervation in salamanders.^{4,28} This result suggested that nerve innervation is not essential for the initiation of fungiform papillae,¹⁴ but rather for the complete development of the functional and matured fungiform papillae as sensory organs in mammals. Therefore, an

investigation of the primary induction system for the individual papilla should be performed. In addition, the finding the mesenchymal factors to complete understand epithelial mesenchymal interactions, just as it revealed in limb development, early *Fgfs* signalling could be good candidates for mesenchymal signalling molecules. And the induction of the ectopic papillae as new study fields would be another challenge.

A culture system of the mouse tongue has allowed in our laboratory an investigation into the participation of three tissue types, the epithelium, mesenchyme and neural tissue during the initiation, morphogenesis, growth, and maintenance of the fungiform papillae.¹⁴ Concerning evolution, in order to understand the diversity of the fungiform papillae and how it is conserved, a comparative study using other molecular markers and available approaches is suggested.

The development of fungiform papillae represents an ideal model system for examining the epithelial mesenchymal interactions, nerve innervation and spacing pattern formation during embryonic development.

1. Prior to the morphological changes, the signalling molecules are expressed with the stereotyped pattern in the fungiform papillae forming area.
2. The morphogenesis of the fungiform papillae showed specific structures such as epithelial thickening and mesenchymal condensation.
3. Fungiform papillae develop through the reciprocal interactions between epithelium and mesenchymal tissue.
4. Development of fungiform papillae was governed by signalling molecules, such as *Shh*, *Bmp-2* and connexin-43.
5. Fungiform papillae can be considered to be small epithelial

appendages, which are formed with the specific patterned array via the epithelial mesenchymal interactions

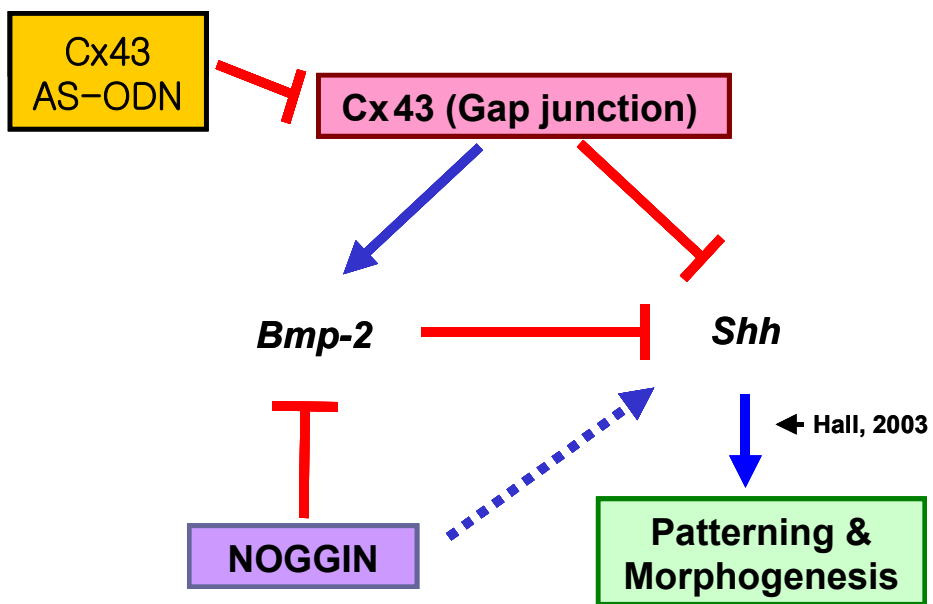


Figure 10. Schematic diagram of the regulation of signalling molecules during fungiform papillae development (Red bar; inhibition, Blue arrow; activation). Previous report showed that *Shh* is a key molecule for fungiform papillae patterning.¹³ Cx43 regulates *Bmp-2* and *Shh* with different ways. NOGGIN also would play an important role for patterning and morphogenesis of fungiform papillae.

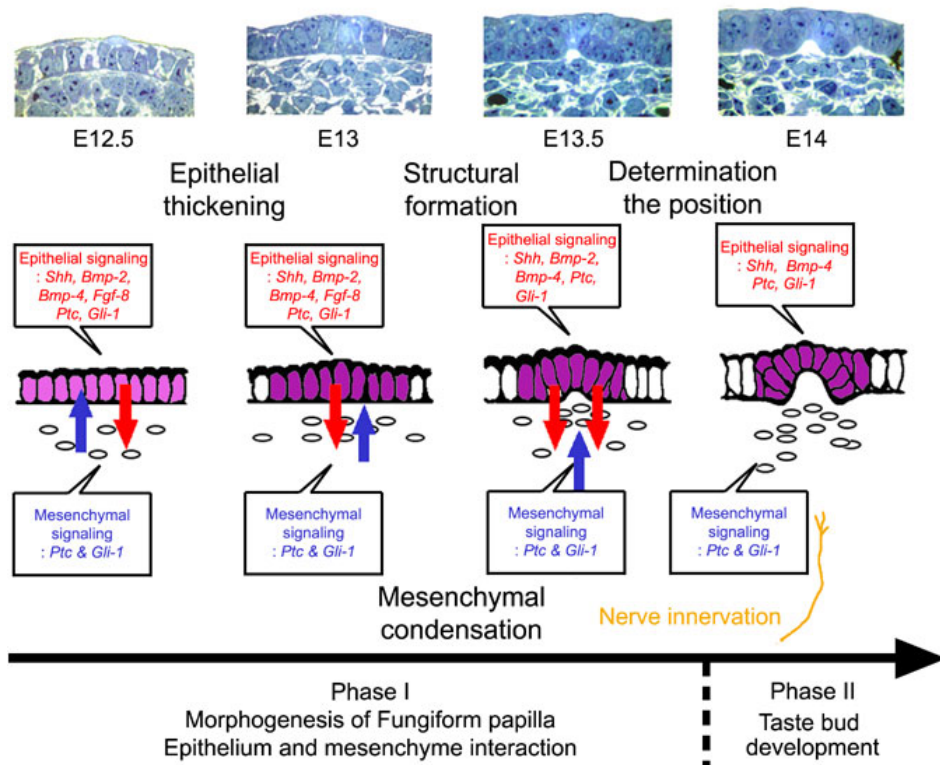


Figure 11. Schematic diagram showing the epithelial mesenchymal interactions at early morphogenesis of fungiform papillae. The development of fungiform papillae can be divided into two stages. One is the morphogenesis through the epithelial-mesenchymal interaction, which produces the papillae structures. The other is nerve innervation, which develops the taste buds. (A) At E12.5, semithin section showed no morphological changes either in the epithelium or mesenchyme. Expressions of the epithelial signalling molecules were detected in the epithelium (red arrow: *Shh*, *Bmp-2*, *Bmp-4*, *Fgf-8*, *Ptc*, *Gli-1*) and mesenchymal expressions were broadly detected in mesenchyme (blue arrow: *Ptc*, *Gli-1* purple color: epithelial expressions). (B) At E13, semithin section showed epithelial thickening, and the epithelial cells at the basal epithelium on the anterior part of the tongue increased in height. Epithelial expressions were detected in the epithelial thickening area (red arrow: *Shh*, *Bmp-2*, *Bmp-4*, *Fgf-8*, *Ptc*, *Gli-1*). Mesenchymal expressions were detected in mesenchyme broadly (blue arrow: *Ptc*, *Gli-1*; purple color: epithelial expressions). (C) At E13.5, the epithelium formed an archlike structure. Expression of the epithelial signalling molecules restricted to the archlike structure. Double arrows indicate that E13.5 epithelium can determine the position to form a fungiform papillae structure by the epithelial signalling molecules (red arrow: *Shh*, *Bmp-2*, *Bmp-4*, *Ptc*, *Gli-1*). Mesenchymal expressions were detected in mesenchyme broadly (blue arrow: *Ptc*, *Gli-1*; purple color: epithelial expressions; yellow: nerve innervations). (D) At E14, the fungiform papillae commit to the next developmental phase with nerve innervations to develop taste bud (red arrow: *Shh*, *Bmp-4*, *Ptc*, *Gli-1*; blue arrow: *Ptc*, *Gli-1*; purple color: epithelial expressions; yellow: nerve innervations)

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ABSTRACT(IN KOREAN)

생쥐의 발생과정동안 혀 유두의 형태형성과
패턴형성에 관한 연구

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혀는 구강내에 존재하는 근육기관이며, 기능적인 측면에서 볼 때 맛을 느낄 수 있고, 말을 할 수 있게 해주며 음식을 씹고 삼키게 할 수 있는 구조물이다. 생쥐의 경우, 혀에는 4종류의 lingual papillae가 있는데 fungiform, circumvallate, foliate 그리고 filiform papillae가 그것이다. 이들 중 filiform papillae를 제외한 나머지 papillae는 taste bud를 포함하고 있다. 분포지역은 혀의 양 옆 뒤쪽에 foliate papilla가 분포하고 있으며 혀의 뒤쪽 정중선쪽에는 한 개의 circumvallate papilla가 위치해 있다. Fungiform papillae는 혀의 앞쪽에서 뒤쪽으로 특징적인 배열 경향을 가지고 분포해 있다. 이러한 fungiform papillae의 경우, limb, feather, lung, kidney, hair 그리고 tooth에서 처럼 외배엽과 중배엽의 상호작용에 의해 발생하는 피부부속 기관이다. 전자현미경과 조직 절편을 이용한 형태학적인 연구결과 상피조직에서 버섯유두와 특수한 구조로 발생하는 과정을 확인할 수 있었다. 버섯유두의 초기 발생단계에는 Shh, Bmp, Fgfs 와 같은 신호전달물질, Laminin 과 같은세포결합물질과 cytokeratin과 같은 세포외기질물질들이 발생단계 동안 중요한 신호전달 요소로 관여하고 있다는 것을 확인할 수 있었다. 버섯유두 형태형성 동안의 외배엽과 중배엽의 상호작용을 확인하기 위해서 외배엽과 중배엽의 recombination 실험을 통해 버섯유두의 구조가 어느 조직에서 기원하는지 알아 보았다. 발생단계 E12.5와 E13.5에서

실험을 진행하여 2일간 체외배양 한 후 확인한 결과, E13.5의 상피조직이 외배엽의 신호전달물질 발현과 함께 새롭게 형성된 버섯유두 구조물의 위치를 결정짓는 것을 확인할 수 있었다. 결국 E13.5시기가 버섯유두의 발생에서 중요한 시기임을 알 수 있었다. 버섯유두의 발생 동안 나타나는 신호전달물질의 조절을 알아보기 위해서 Shh, Bmp-2, 그리고 Cx43의 발현양상을 확인하였다. 이 결과 신호전달물질의 시공간적인 발현경향은 버섯유두형성에 매우 중요하다는 것을 확인할 수 있었다. 생쥐 혀의 체외배양 방법을 이용하여 Bmps의 antagonist로 알려진 NOGGIN단백질과 Cx43 (gap junction)의 기능을 차단할 수 있는 AS-ODN (Antisense oligodeoxynucleotide) 등을 처리하여 gap junction과 신호전달물질의 관계를 확인해 보았다. Gap junction의 차단은 Shh과 Bmp-2의 발현양상에 변화를 주었으며, NOGGIN단백질을 처리한 결과 또한 정상 발현과는 다른 이상 발현을 확인할 수 있었다. 이러한 결과를 토대로 버섯유두 발생 동안 나타나는 신호전달 물질의 조절에 대해 확인할 수 있었다. 요컨대, 버섯유두의 초기발생 동안 나타나는 발생학적인 특징은 다른 외배엽성 기원기관에서 공통적으로 확인할 수 있다. 버섯유두의 발생은 새로운 발생생물학적 모델 시스템으로 활용될 수 있을 것이다.

핵심되는 말 : 외배엽 중배엽 상호작용, 버섯유두, 형태형성, pattern formation, 신호전달물질, spacing pattern