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Studies on the nerve-dependent morphogenesis of mouse lingual papillae and taste buds

(マウス舌乳頭および味蕾形態発生の神経依存性に関する研究)

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Contents

Contents 1-2

General introduction 3-6

Figures 7-9

Chapter 1: Analysis of the developmental mechanisms of murine tongues and appendages grafted under kidney capsule

1-1. Introduction 10-11

1-2. Materials and methods 12-14

1-3. Results 15-17

1-4. Discussion 18-20

Figures 21-28

Chapter 2: Establishment of an *in vitro* organ culture system for inducing morphogenesis of taste buds

2-1. Introduction 29-30

2-2. Materials and methods 31-34

2-3. Results 35-39

2-4. Discussion 40-45

Figures 46-51

Table 52

Chapter 3: Nerve-independent and ectopically additional induction of taste buds in organ culture of fetal tongues

3-1. Introduction 53-57

3-2. Materials and methods 58-61

3-3. Results 62-67

3-4. Discussion 68-71

Figures 72-80

Tables 81-82

General discussion 83-85

Table 86

References 87-96

General introduction

Vertebrates always gather environmental information to survive via special sensory organs. The sense of taste is essential to perceive many chemical substances as valuable nutrients or harmful toxin in foods. Taste disorder is painless, and therefore perceiving the trouble for oneself is difficult. Zinc deficiency and treatment with medical drugs are the major causes of taste disorder, but the potential patients are increasing because of changes in lifestyle including unbalanced diet. Taste buds convert food chemical stimulations into basic tastes such as sweet and bitter. Gustatory cranial nerves connect with all taste buds and the nerves transmit the taste information to the brain. Gustatory organs are found in flies, fishes, amphibians and mammals, and occur on different organs in each species; antennas and wings in flies (Matsunami and Amrein, 2003); head, lips, barbels, oral cavity, pharynx in fishes (Hasen et al., 2002); tongues, palates, and pharynx in amphibians and mammals. In addition, mammalian carnivore and herbivore have different pattern of occurrence of taste buds, suggesting that the pattern is evolutionally related to animal's food habit. Structure of taste buds is aggregates of fusiform taste cells expressing CK8, usually called as onion-like structure. One of the histological feature of taste buds is eosinophobic. Maintenance of taste buds depends on

innervation (Vintschgau and Honigschmied, 1877). Morphogenesis of taste buds is nerve-dependent in mammals, while it is autonomous in amphibians (Barlow and Northcutt, 1997). However, it is unknown in mammals how taste nerves induce the morphogenesis of taste buds.

Tongues are originated from branchial arches. First arch differentiates into anterior tongues (tongue body) and third arch differentiates into posterior tongues (tongue root). Tongues consist of three germ layers and neural crest-derived cells. Tongue epithelium is derived from ectoderm and endoderm. Tongue muscle is derived from mesoderm. Tongue connective tissue is derived from neural crest cells. While origin of taste buds is known to the same origin of tongue epithelium, one research claims that tongue epithelium and early taste buds include neural crest-derived cells (Liu, *et al.*, 2012). Gustatory ganglia are originated form ectodermal epibranchial placodal cells and neural crest-derived cells (Harlow and Barlow, 2007). The placodal neural cells innervate all taste buds (Harlow and Barlow, 2007). Gustatory innervation regulates the development of tongue papillae and taste buds (Oakley, 1998), but the mechanism remains to be elucidated. Tongue organ culture without innervation is a very useful tool to analyze nerve-independent morphogenesis. However, it has never been reported that taste buds emerge in culture (Farbman and Mbiene, 1991; Mbiene *et al*., 1997; Morris-Wiman J *et*

al., 2000; Nosrat CA *et al*., 2001; Barlow, 2003) and thus it is concluded that taste bud morphogenesis is uninducible without innervation *in vitro*.

To investigate the mechanisms of the nerve-dependent morphogenesis, the present study has sought to generate taste buds in tongue organ culture. Various organ primordia generally undergo tissue-specific reciprocal interactions between epithelium-mesenchyme (Rawles, 1963; Kollar and Baird, 1969; Umezu *et al*., 2010). Therefore, it was hypothesized that morphogenesis of tongue including taste buds would progress via only the reciprocal interactions. The development of taste buds and tongue papillae without innervation was examined by grafting under kidney capsule in chapter 1. Grafting under kidney capsule is valuable to culture organs because abundant blood vessels readily supply nutrients to grafted organs. In chapter 2, two organ culture methods for tongue were evaluated and reconstruction experiment of taste buds was performed with a taste bud-derived cell line which was isolated from *p53* deficient mice. In chapter 3, the organ culture system, which was established in chapter 2, was used for investigation of the effects of nerve-independent activation of canonical Wnt signaling pathway.

The present study demonstrates that taste buds are nerve-independently inducible in organ culture and activation of canonical Wnt signaling is important for morphogenesis

of taste buds. This discovery contributes to elucidation of developmental mechanisms of taste buds.

Finally, to make it easy to understand the present thesis, schema of development of a taste bud and specific gene expression at developmental and maintenance stages are described as Fig. 1. Canonical Wnt signaling pathway is also described as Fig. 2. Spatiotemporal pattern of Wnt signaling activity and Shh expression at E 11.5 and 12.5 is illustrated as Fig. 3.

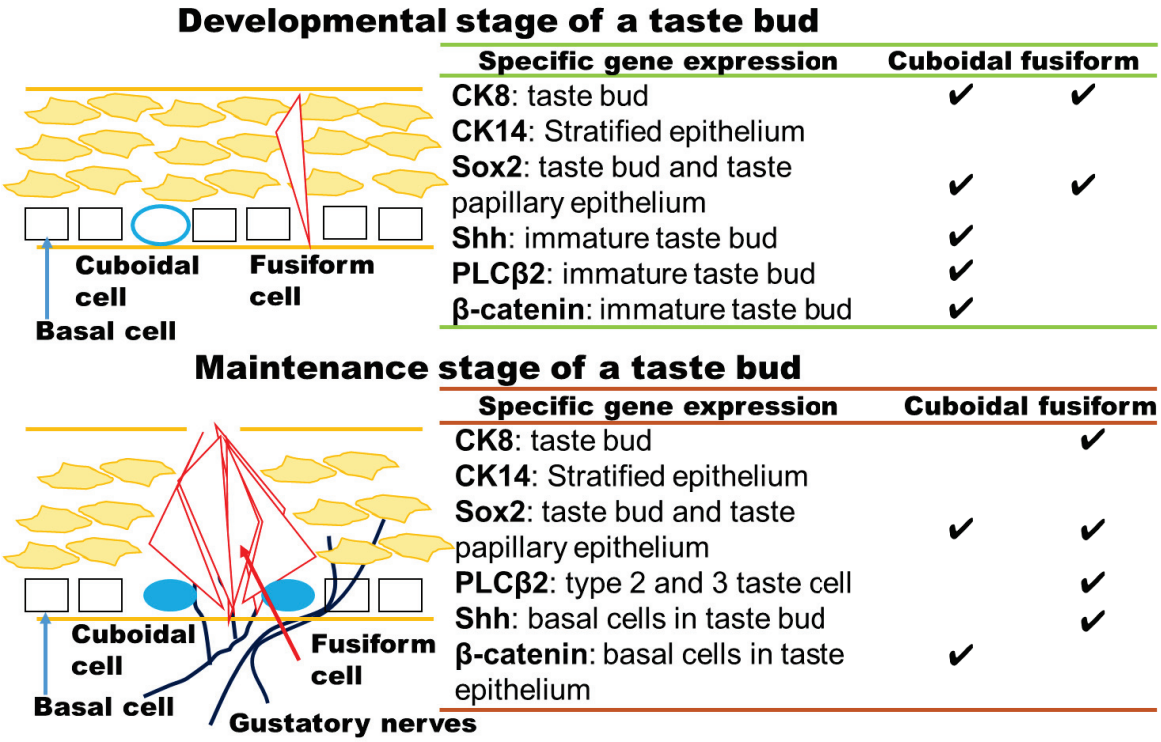

Fig. 1 Schema of development of a taste bud and lists of specific gene expression at developmental and maintenance stages

Upper: A taste bud at developmental stage. A basal cell of stratified epithelium differentiates into a cuboidal and fusiform cell. **Lower**: A taste bud at maintenance stage. An aggregate of fusiform cells and cuboidal cells is defined as a taste bud. Gustatory nerves innervate both taste buds and stratified epithelium.

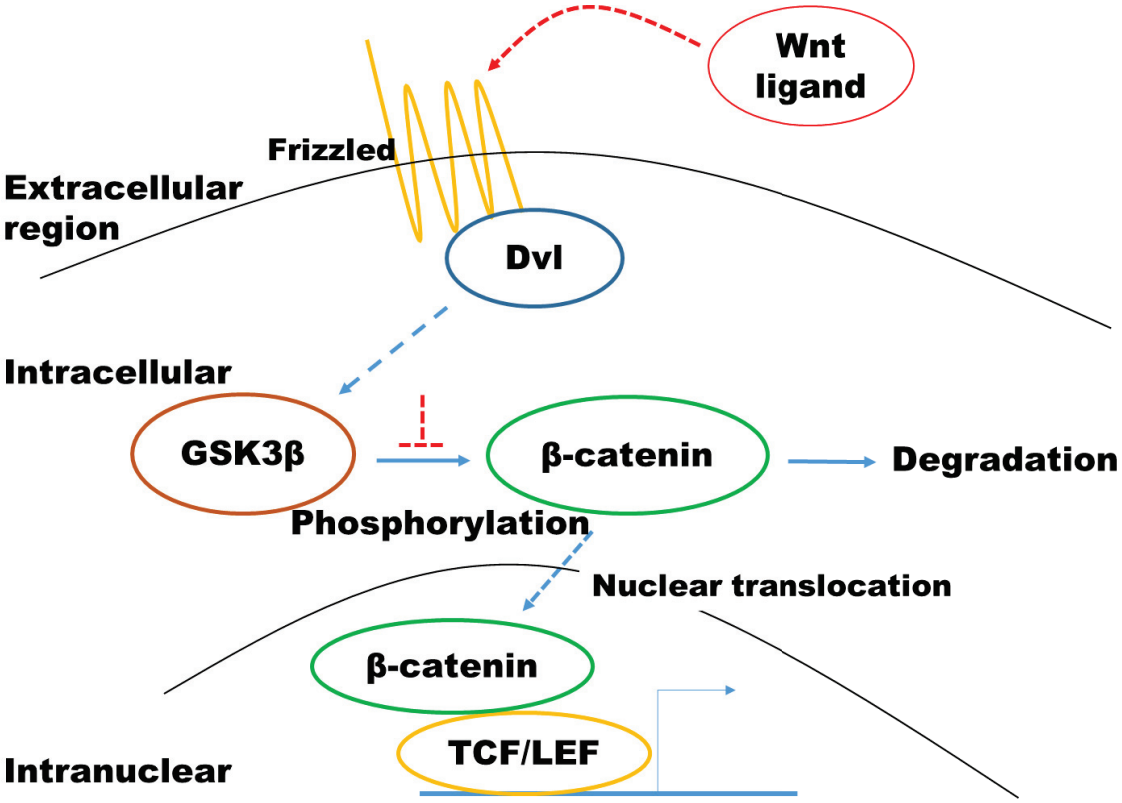

Fig. 2 A scheme of canonical Wnt/β-catenin signaling pathway

β-catenin is usually phosphorylated by GSK3β and is degraded in intracellular region. If a Wnt ligand binds a Wnt receptor Frizzled, GSK3β is inactivated by Dvl and β-catenin accumulates in the cytoplasm. Excess cytoplasmic β-catenin is translocated into the nucleus and formed a complex with TCF/LEF. The complex binds TCF/LEF binding sites and works as a transcription factor,

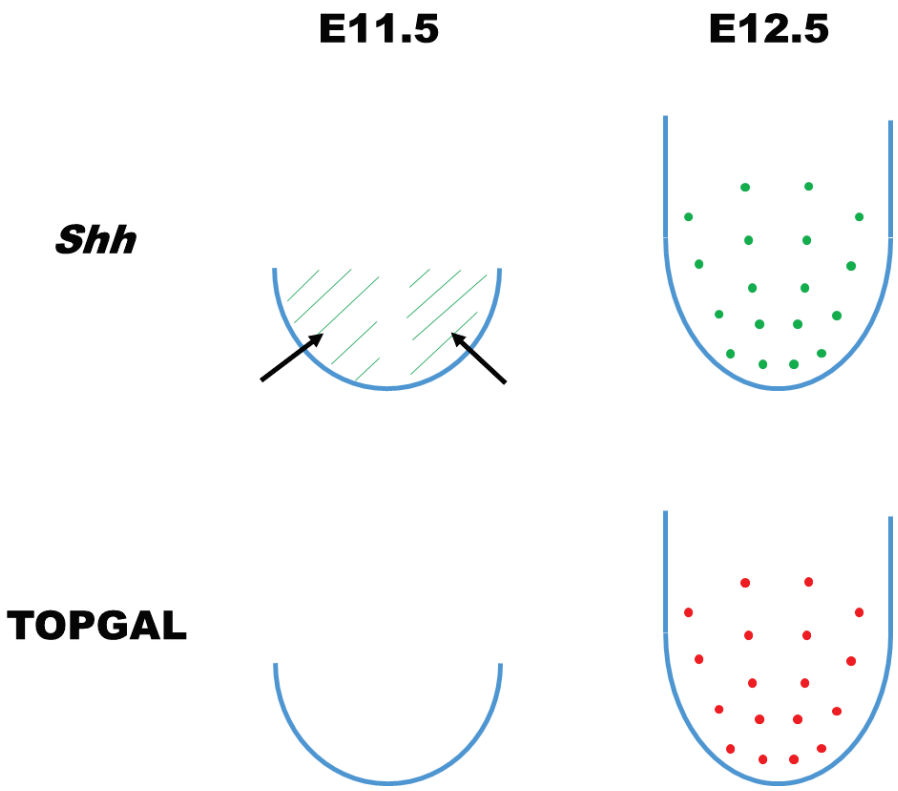

Fig. 3 Schema of the activation of Wnt signaling and the expression of Shh during early development of mouse tongues

At E11.5, lateral lingual swellings (arrows) form anterior tongues and Shh is expressed in the area (green shaded part). Expression of Shh is localized in primordial fungiform papillae (green dots) at E12.5. Activation of Wnt/β-catenin signaling is not observed by the examination of β-gal activity in TOPGAL mice at E11.5, and it is firstly observed in primordial fungiform papillae (red dots) at E12.5.

Chapter 1

Analysis of the developmental mechanisms of murine tongues and appendages grafted under kidney capsule

(腎臓被膜下移植法を用いたマウス舌および舌付属物の発生メカニズムの解析)

1-1. Introduction

The tongue includes 4 types of epithelial appendages, called filiform papillae, fungiform papillae, foliate papillae and circumvallate papillae. Filiform papillae have keratinized spines on the top of the papillae, and cover anterior tongues. Fungiform papillae occur regularly from the small swellings on the anterior tongue. A circumvallate papilla is set on the center of posterior tongue in mice, and surrounded by lateral furrows. There are serous glands at the bottom of the furrows, called Ebner's glands. Fungiform, foliate, and circumvallate papillae include taste buds in stratified epithelial layers. A taste bud is a taste sensor, and consists of about 100 taste cells. Because cutting gustatory nerves leads to disappearing of taste buds, survival of taste buds has been thought to be dependent on neural connection (Farbman, 1969). However, it is recently reported that early development of taste buds is independent of neural connection (Barlow LA, *et al*.,

1996; Ito A, *et al*., 2010). Thus, developmental mechanisms of taste papillae and taste buds are still controversial.

Mammalian organogenesis is induced by epithelial-mesenchymal interactions. In our laboratory, it was attempted to uncover the mechanisms how epithelial-mesenchymal interactions are involved in dental and female reproductive tract development with the method of long-term culture under kidney capsule (Komine, *et al*., 2007; Umezu, *et al*., 2010). Organ culture of fetal mouse tongue is used for studying early developmental mechanisms of taste papillae and a taste bud, but previous studies suggest that it is difficult to culture fetal tongue from early developmental stages to mature stage (Mbiene et al., 1997; Kim, et al., 2009). Grafting adult murine tongues into the eye chamber was used to be performed (Zalewski, 1972), but currently it is not encouraged.

Then the goal of the present study is establishment of an *in vivo* culture system eliminating the effects of nerve connection by ectopic grafting under kidney capsule. Moreover, in order to examine the effects of neural secretions without innervation into tongue epithelium, tongues and dorsal root ganglia (DRG) were co-grafted under kidney capsule.

1-2. Materials and methods

Animals

ICR mice were obtained from Sankyo Labo Service Corporation and maintained under a 12:12 h light and dark cycle at 24±1°C in the facility for rearing animal in Tokyo University of Science. All experiments were approved by the institutional Animal Care and Use committee at Tokyo University of Science.

Embryonic day 0.5 (E0.5) was defined as noon of the day on which a vaginal plug was observed.

Tissue preparation for grafting

The schema of grafting is showed in Fig. 1. Pregnant mice were killed by cervical dislocation. Embryos at E15.5 ~ E18.5 were surgically removed. Gustatory papillae regions were dissected from tongues of the embryos, and the tissues were inserted into 15 μm Cell matrix type I-A (Nitta Gelatin Inc., Osaka, Japan) including Minimum Essential Medium Eagle (MEM) Alpha Modification (Sigma-aldrich japan, Tokyo, Japan). DRG (dorsal root ganglion) was also dissected from the embryos, and inserted into a collagen drop in case of estimating the effect of neural cells. A collagen drop containing a tissue was incubated for 30 min at 37°C, placed on a cell culture insert and

cultured for 1 day in 10% fetal calf serum at 37°C in a humidified atmosphere of 5% CO_2. To bring the dissected DRG near the tongue tissues in collagen gel, each collagen drop including a tongue and a DRG were centrifuged before being incubated.

Grafting under the kidney capsule

After the incubation, two samples / kidney were grafted under the kidney capsule of an adult male ICR mouse. Mice were one or two weeks after grafting, kidneys were harvested and processed for histological and immunohistochemical staining.

Histological and immunohistochemical analyses

Harvested kidneys and tongue tissues were fixed in 4% formaldehyde at 4°C for overnight and dehydrated with graded alcohol. They were embedded in paraffin and were cut into 8 μm sections. Four sections were deparaffinized with xylene, and rehydrated with graded ethanol. Then they were stained with hematoxylin and eosin. For immunohistochemical staining, sections were deparaffinazed and rehydrated with graded ethanol. The sections were washed twice in phosphate buffered saline (PBS) with 0.05% Tween 20 (Sigma) and nonspecific binding was blocked by incubation for 1 hr at room temperature (RT) in PBS containing 1% bovine serum albumin (BSA; Trace

Biosciences Pty, NSW, Australia), 5% normal goat serum (Zymed Laboratories Inc, San Francisco, CA). And then, the sections were incubated with primary antibodies overnight at 4°C. The primary antibody was anti-trichohyalin antibody (AE15, 1/100; Abcam plc., Cambridge, UK). After washing twice with PBS, the slides were incubated with Alexa488 conjugated anti-mouse IgG (Invitrogen, Carlsbad, CA) at RT overnight. 4', 6-Diamidine-2'-phenylindole dihydrochloride (DAPI) was used for counter staining. Negative controls were incubated without primary antibody. Samples were observed with a fluorescence microscope (Carl Zeiss, Oberkochem, Germany).

1-3. Results

Development of tongue structures from E15.5 to P3

Anterior tongues, called tongue body, are covered with uniform stratified epithelium at early embryonic stage (Fig. 2a). As tongues become mature, numerous papillary structures grow (Fig. 2b-d). Keratinized spines occur at the top of the papillae, called filiform papillae. The swells of stratified epithelium become mature structure having taste buds in adult (Fig. 2d). Immature muscles differentiate into striated muscles.

At E15.5, a presumptive circumvallate papilla region forms a dorm-shaped structure from thickened epithelium (Fig. 2e). Surrounding trench is still shallow, and von Ebner's gland is not seen (Fig. 2f). After P3, taste buds become visible in stratified trench walls and the trench extends to underlying mesenchyme. After P10, von Ebner's glands developed in the muscle layer and opened to the bottom of trenches (Fig. 2f-h).

Development of presumptive taste papillae regions under kidney capsule

Anterior tongues

Anterior presumptive papillae regions at E15.5~16.5 were grafted under kidney capsule. After grafting for 1 week, formation of filiform papillae was observed (Fig. 3). Grafts had similar structures to filiform papillae observed at P3 (Fig. 3b', c'). Filiform

papilla-like structures were maintained in samples grafted for 2 weeks (Fig. 4), but the size and processes of filiform papillae-like structures were smaller than those of P10 corresponding to 2 weeks after E15.5. Expression of trichohyalin, a marker of filiform papillae and their spines, was slightly detected in samples (Fig. 4c). Almost all grafted samples developed filiform papilla-like structures, but fungiform papillae were morphologically indistinguishable. Muscle layers developed in almost all grafted samples. These results suggest that anterior tongues could be cultured under kidney capsule for 7 days.

Posterior tongues

Presumptive circumvallate papillae regions were grafted under kidney capsule. When the grafts were cultured for 1 week, stratified epithelium and papilla structures in the circumvallate papilla (secondary papillae) were observed (Fig. 5b, arrows). However, the grafts were defective in surrounding trenches and taste buds when compared to P3 circumvallate papillae (Fig. 5c). No circumvallate papillae were observed after 2 weeks under kidney capsule. Ductal structures appeared in the mesenchyme of some grafts for 1 week (Fig. 6a). In comparison with P3 tongues, mucus gland-like structures were observed in grafted samples (Fig. 6b). Serous glands-like structures also appeared in

grafted posterior tongues for 1 or 2 weeks (Fig. 7a, b). Especially a sample grafted for 2 weeks had racemose serous gland-like structures, so the racemose structures might be von Ebner's gland as compared to the gland at P10. These results indicate that immature posterior tongues develop but circumvallate papilla develop poorly under kidney capsule.

Development of presumptive taste papillae regions grafted with DRG

To examine whether morphogenesis of taste buds is nerve-dependent, tongues were grafted with DRG. Development of anterior tongues grafted with DRG was similar to those grafted without DRG. Then E15.5-18.5 posterior tongues including a presumptive circumvallate papilla region were grafted with DRG for 1~2 weeks (Fig. 8). After grafting for 1 week, the circumvallate papillae-like structures were well-organized in shape than those grafted without DRG (Fig. 8a). A circumvallate papilla survived in a sample grafted with DRG for 2 weeks, although it was undeveloped in size (Fig. 8b). However, taste bud-like structures were not found in any grafted samples. These results indicate that the development of circumvallate trenches is influenced by secretions of DRG, while differentiation of the circumvallate epithelial cells into taste buds is not induced by secretions of DRG.

1-4. Discussion

Ectopic grafting is used in embryological study. Grafting to eye chamber was previously selected as *in vivo* culture system of tongues (Zalewskiz, 1972), but this method should not be used in view of the prevention of cruelty to animals. Then we attempted to graft tongues under kidney capsule, aiming to establish the new *in vivo* culture method of tongues for long-term, expecting to make analysis of the developmental mechanisms of taste papillae and taste buds more easily.

When tongues at E11.5 were grafted, samples could be maintained but did not develop. Then tongues at E15.5 were grafted.

Fungiform and filiform papillae

Filiform papillae and their spines were formed under kidney capsule in the present study. Non gustatory papilla may appear independently of the influence of nerve fibers. However, filiform papillae of grafted samples were immature because of slight expression of trichohyalin and smaller spines than those at P10. Fibrillar connective tissues of taste papillae at P10 contain well-developed capillary vessel loops (Okada S *et al*., 1997, Hirao T *et al*., 2007), so further development of filiform papillae would be induced by further angiogenesis regulated by VEGF under kidney capsule. Fungiform

papillae were difficult to be distinguished from filiform papillae in grafted samples, because fungiform papillae without taste buds and filiform papillae without mature spines were similar in shape. Immunostaining of fungiform papillae with markers, Sox2 and keratin 14 for example, is necessary for discrimination (Okubo T *et al*., 2009).

Circumvallate papilla

In this study, circumvallate papillae grafted without DRG did not develop the lateral trenches. On the other hand, circumvallate papillae grafted with DRG could develop and elongate the trenches. These results support the observation that epithelial downgrowth is caused by loss of gustatory nerves (Oakley B, *et al*., 1998,). Circumvallate papillae of brain-derived neurotrophic factor (BDNF) deficient mice at P16 (Nosrat CA, *et al*,. 1997) are similar to grafted circumvallate papillae for 1 week, so a little effect of grafted DRG was noticed. After cutting the gustatory nerve, mature circumvallate papillae deform and taste buds in adult mice decreased the expression of BDNF (Uchida N, *et al*., 2003). And the receptors of the neurotrophic factor were expressed in connective tissues of circumvallate papillae, so maintenance and development of circumvallate papillae and taste buds may require gustatory nerves. In this study, nerve plexus in connective tissues of circumvallate papillae at E15.5 would be lost during the development under

kidney capsule, then epithelial downgrowth occurred in grafted samples without DRG. When circumvallate papillae were grafted with DRG, stability of circumvallate papillae might be brought by nerve regeneration or diffusive factors from DRG.

Salivary gland

Mucus glands and serous gland-like structures were seen in grafted samples, then these glands are probably independent of nerve connection. Especially von Ebner's glands are genuinely serous gland, and express ebnerin (Dmbt1) distinctively (Asano-Miyoshi M, *et al*., 1998). Expression analysis of ebnerin, a distinguishing marker for von Ebner's glands, is required for identification of grafted circumvallate papillae.

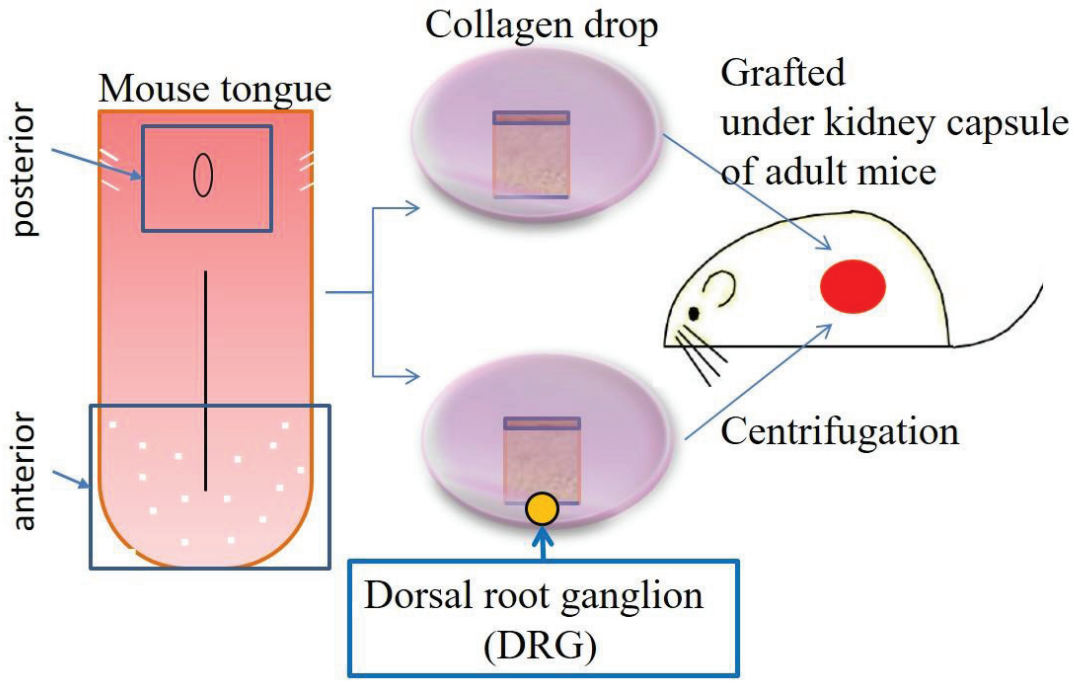

Fig. 1 Schema of ectopic grafting under kidney capsule

Immature tongues were excised and separated into anterior and posterior regions. The anterior and posterior tissues were embedded in collagen drops and grafted under kidney capsule. In case of estimating the effects of neural cells, DRG (yellow circle) was set at the bottom of tongue tissues in collagen drops. The drops were centrifuged and grafted under kidney capsule.

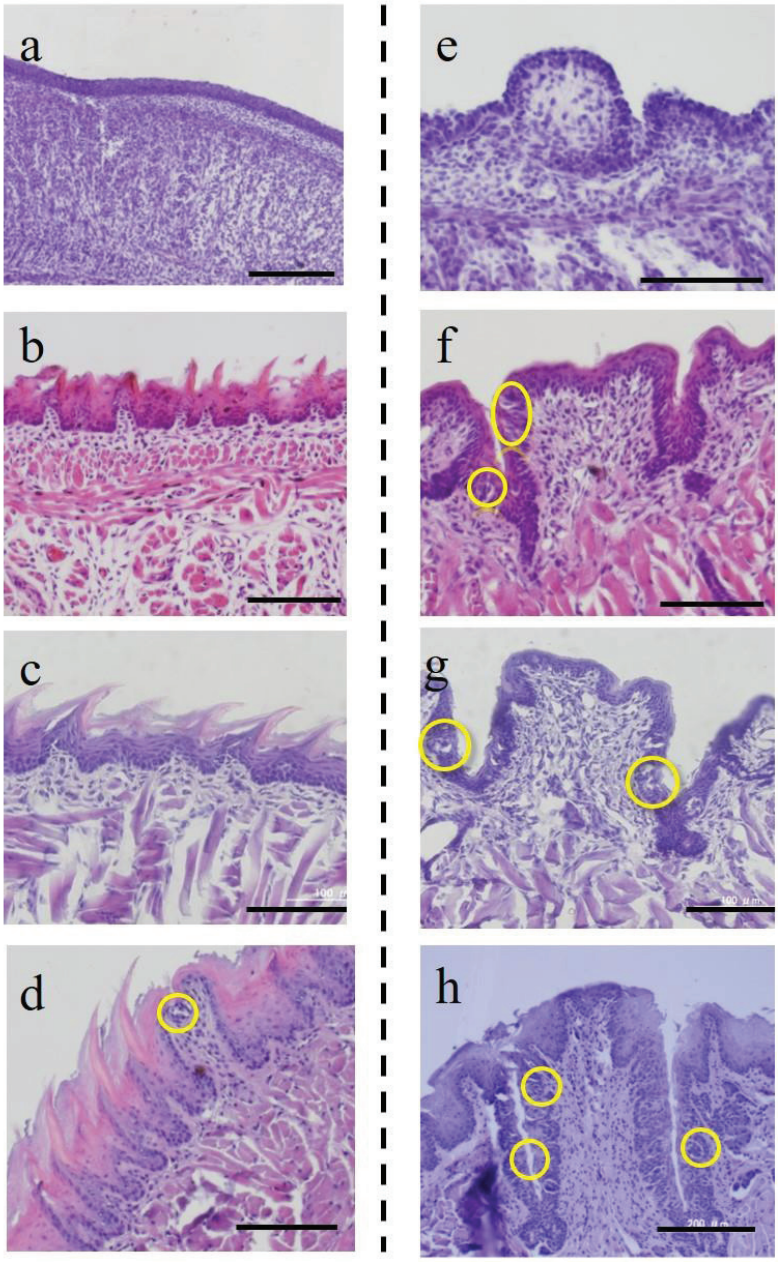

Fig. 2 Morphological change of tongue papillae

a-d: Morphogenesis of anterior tongue epithelium. **e-h**: Morphogenesis of posterior tongue. Tongues at E15.5 (**a, e**), P3 (**b, f**), P10 (**c, g**), and adult (**d, h**) were shown. Yellow circle: taste bud. Scale bar: 100 μm

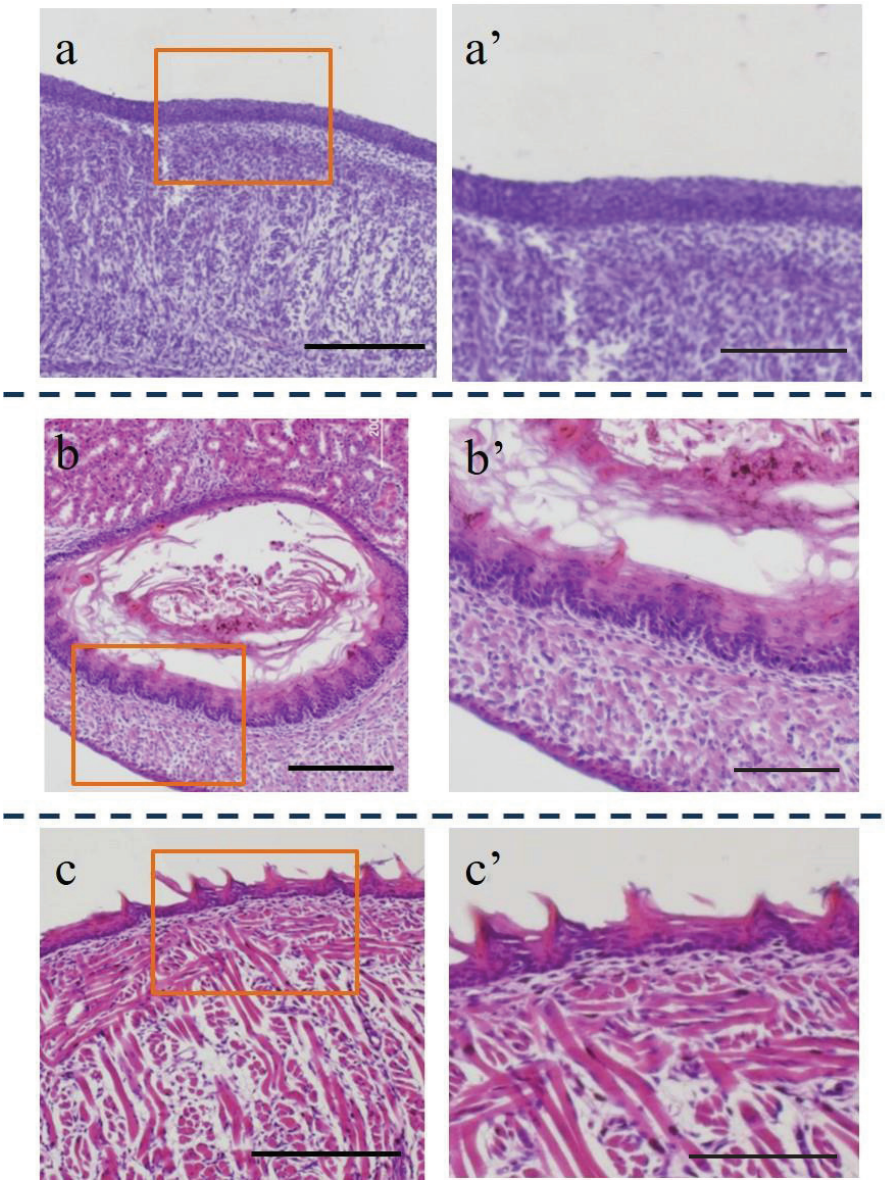

Fig. 3 Morphological changes after grafting of anterior tongues

a: E15.5 anterior tongues. **a'**: An enlarged figure of **a**. Anterior tongue epithelium at E15.5 was stratified and papillary structures were not observed yet. **b**: Anterior tongues grafted for 1 week. **b'**: An enlarged figure of **b**. The anterior tongue epithelium formed filiform papillae. **c**: P3 tongues. **c'**: An enlarge figure of **c**. Scale bar: 100 μm (**a**), 50 μm (**b'**), 200 μm (**b**, **c**), 100 μm (**b'**, **c'**)

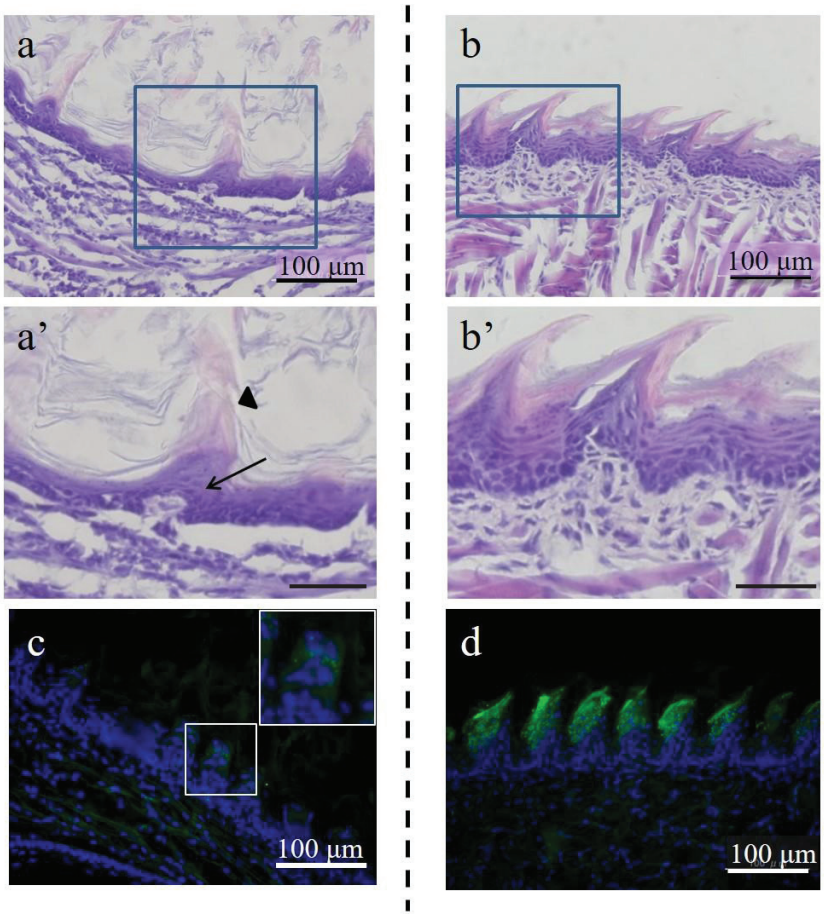

Fig. 4 Anterior tongues grafted for 2 weeks

a: Filiform papillae structures in anterior tongues grafted for 2 weeks. **a'**: Enlarged figure of A. Spine (arrowhead) and papilla (arrow) were observed. **b**: Anterior tongue at P10. **b'**: Enlarged figure of **b**. **c**: Immunostaining with anti-trichohyalin antibody for the tongues cultured for 2 weeks. The white box in the upper right corner indicates the enlarged figure of the white box in the center. A few cells expressed trichohyalin (green). **d**: Immunostaining with anti-trichohyalin antibody for tongues at P10. The epithelial cells of filiform papillae expressed trichohyalin. Green: trichohyalin Blue: nuclei Scale bar: 50 μm (**a', b'**),

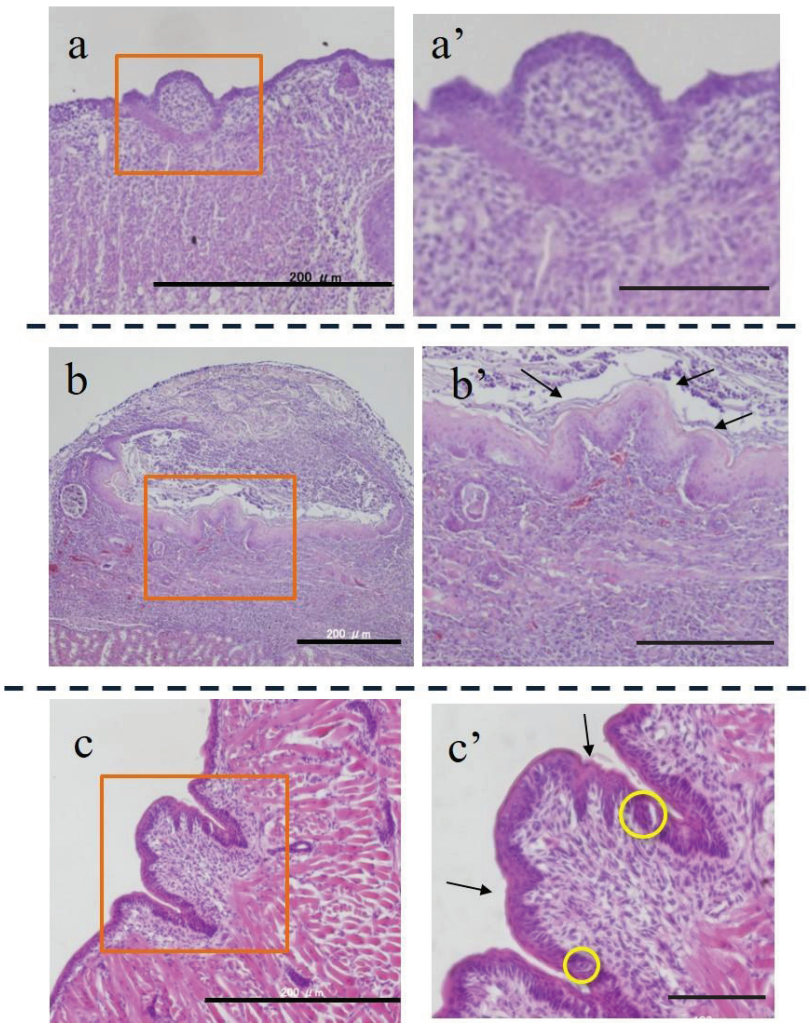

Fig. 5 Posterior tongues grafted for 1 week

a: Posterior tongues including a circumvallate papilla at E15.5. **a'**: Enlarged figure of **a**. **b**: Circumvallate papillae grafted for 1 week. **b'**: Enlarged figure of b. Secondary papillae (arrows) in circumvallate epithelium was observed. **c**: Circumvallate papillae at P3 **c'**: Enlarged figure of **c**. Lateral trenches were observed and taste buds existed in the stratified epithelium of the trenches. Arrow: secondary papilla. Yellow circle: taste bud. Scale bar: 200 μm (**a, b, c**), 50 μm (**a'**), 100 μm (**b', c'**)

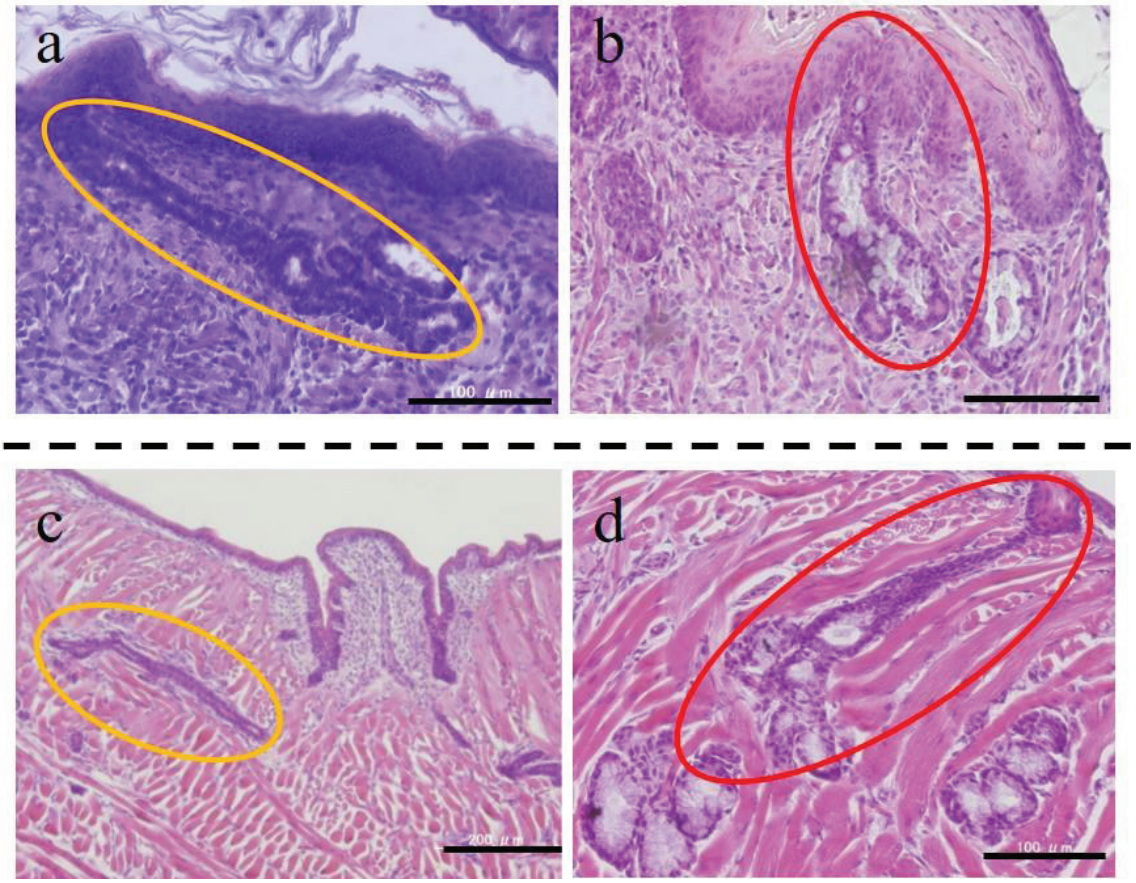

Fig. 6 Ducts and mucus glands in grafted posterior tongues

a, b: Posterior tongues grafted at E18.5 for 7 days possessed ductal structures (**a**, yellow circle) and mucus gland-like structures (**b,** red circle). **c, d:** Posterior tongues at P3. Ductal (**c**, yellow circle) and mucus gland (**d**, red circle) were shown. Ductal and mucus structures in grafted posterior tongues were similar to those of P3. Scale bar: 100 μm

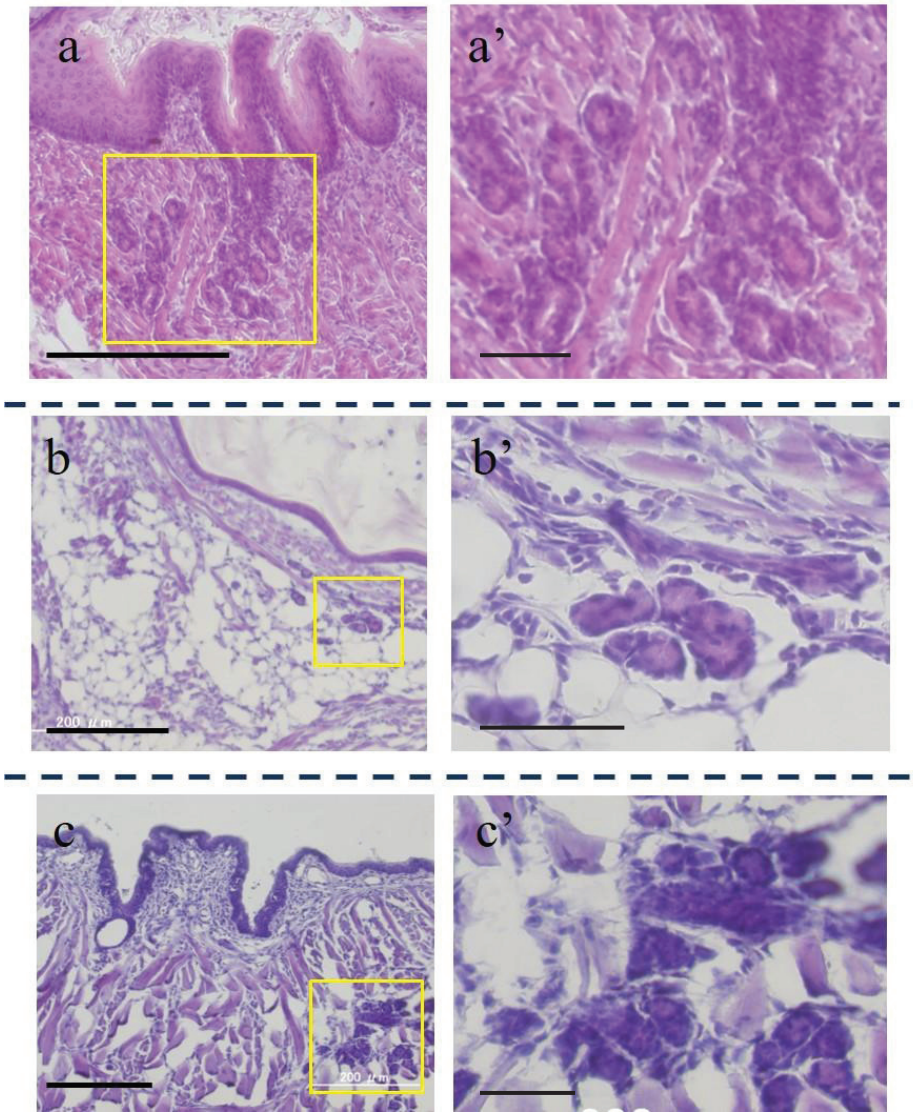

Fig. 7 Development of serous glands in grafted posterior tongues

a: Posterior tongues after grafting for 1 week. **a'**: Enlarged figure of the box of **a**. Von Ebner's glands were observed. **b**: Posterior tongues after grafting for 2 weeks. **b'**: Enlarged figure of the box of **b**. Serous gland structures were observed. **c**: Von Ebner's glands and circumvallate papillae at P10. **c'**: Enlarged figure of the box of **c**. Acinar structures were observed. Scale bar: 200 μm (**a**, **b**, **c**), 50 μm (**a', b', c'**).

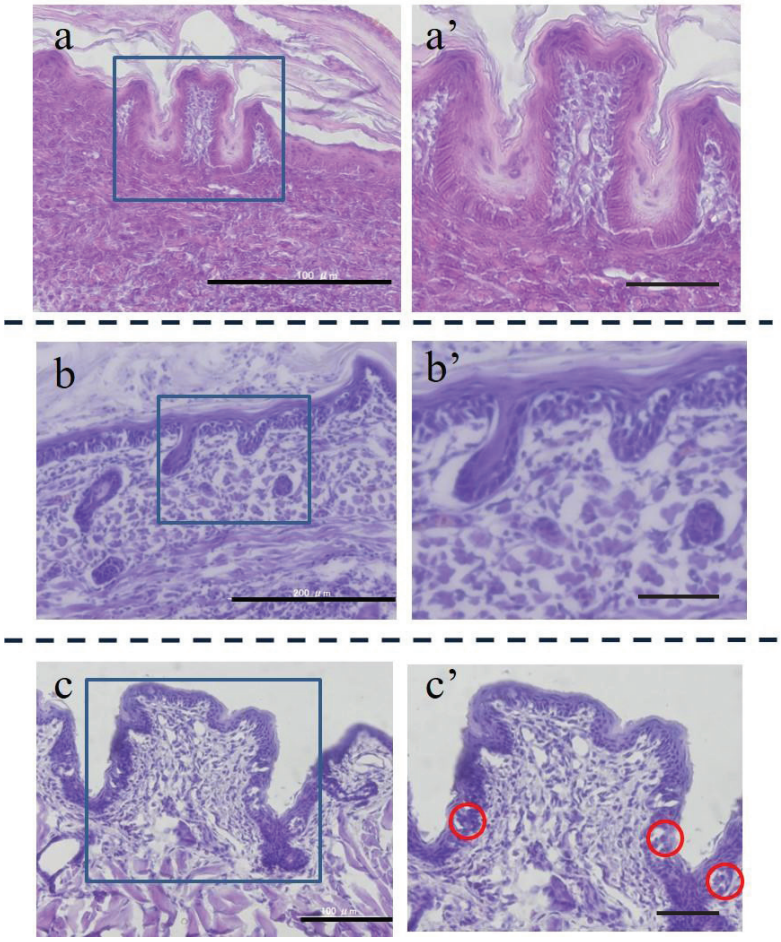

Fig. 8 Posterior tongues at E15.5 grafted with DRG

a: Posterior tongues grafted with DRG for 1 week. **a'**: Enlarged figure of the box of **a**. Circumvallate trenches were observed, but taste buds did not exist. **b**: Posterior tongues grafted with DRG for 2 weeks. **b'**: Enlarged figure of the box of **b**. Smaller circumvallate papillae was observed. **c**: Posterior tongues including a circumvallate papilla at P10. **c'**: Enlarged figure of the box of **c**. Circumvallate trenches were observed and taste buds exist in the stratified epithelium. Red circles: taste buds. Scale bar: 200 μm (**b**), 100 μm (**a**, **b'**, **c**), 50 μm (**a'**, **c'**).

Chapter 2

Establishment of an *in vitro* organ culture system for inducing morphogenesis of taste buds

(味蕾形態形成誘導を目指した *in vitro* 器官培養法の構築)

2-1. Introduction

Tongues are covered with specialized epithelial structures called “lingual papillae” and taste buds exist in the stratified papillary epithelium. Reciprocal interaction between epithelial-mesenchymal tissues is important communication process for determining cell fate and organogenesis. Epithelial-mesenchymal interactions in development of salivary gland (Patel et al., 2014), mammary gland and prostate (Cunha, 1994), tooth (Kollar and Baird, 1969), hair (Sennett and Rendl, *et al*., 2012), and oviduct (Umezu *et al*., 2010) are well known, however, the interaction has been unexplained in organogenesis of lingual papillae and taste buds.

Lingual papillae bearing taste buds are called taste papillae. Taste papillary connective tissue is invaded by abundant nerve plexus of cranial nerve VII (anterior tongue) or IX (posterior tongue). Gustatory ganglion cells and embryonic presumptive

epithelium of taste buds express brain-derived neurotrophic factor (BDNF) (Huang and Krimm, 2010). BDNF and neurotrophin 4, which are secreted from taste buds and the cells of gustatory ganglion, induce gustatory innervation to taste papillary epithelium (Huang and Krimm, 2014). Loss of nerve plexus in taste-papillary connective tissues shown in *BDNF* knockout mice is accompanied by decrease of the number of fungiform papillae and malformation of circumvallate papillae (Mistretta *et al*., 1999; Ito and Nosrat, 2009). However, a recent report reveals that early morphogenesis of taste buds is nerve-independent (Ito *et al.*, 2010). As well as the development of tooth and salivary gland, taste buds may occur through epithelial-mesenchymal interaction.

Although gustatory innervation is undoubtedly important for the development of tongue, it is difficult to examine the role *in vivo*. Innervation-independent development of lingual appendages has been examined in various organ culture systems (Mbiene, *et al*., 1997; Kim *et al*., 2003; Kim *et al*., 2009). However, no method has induced taste bud morphogenesis without innervation in tongue organ culture. The goal of the present study is establishment of tongue organ culture methods for a long period to induce taste bud without innervation.

2-2. Materials and methods

Animals

ICR mice were obtained from Sankyo Labo Service Corporation and maintained as described in Chapter 1. All experiments were approved by the institutional Animal Care and Use committee at Tokyo University of Science.

Tongue organ culture

Tongue tissues were cultured with agarose and collagen gel. A method of culturing tongues using agarose was described in Fig. 1a. DMEM/F12 medium containing 2% low melting agarose gel was heated with microwave, and liquid agarose were poured in 6 well inserts. Whole tongues or part tongues at E19.5 were embedded in cooled agarose and were cultured in 2 ml 10% FCS poured under the inserts for 5 days. A method to culture mouse tongues in collagen gel or Matrigel Matrix (BD ®Corning, corning, NY) was based on a three-dimensional organ-germ culture method for generating bioengineered tooth (Nakao *et al*., 2007) and included some alterations described as Fig. 2. The collagen for tongue organ culture was Cellmatrix type I-A (Nitta Gelatin, Osaka, Japan) prepared with 10X αMEM (M0894, Sigma-Aldrich, St. Louis, MO) and reconstitution buffer (NaOH 50 mM, $NaHCO_3$ 260 mM, HEPES 200

mM) (8:1:1). Collected immature lingual tissues were trimmed within 1 mm^3 and were embedded into 15 μl collagen gel or Matrigel Matrix drops or on a Petri dish coated with grease (Dow Corning Toray, Midland County, MI). Samples were incubated at 37°C for 30 min before they were set on 12 well inserts. The culture medium was 10% FCS containing 3D factors [transferrin 10 μg/ml (Sigma), insulin 10 μg/ml (Sigma), forskolin, 1 mM, (Sigma), hydrocortisone 0.5 mg/ml (Wako, Osaka, Japan), epidermal growth factor (EGF) 10 ng/ml (Sigma), ascorbic acid 50 mg/ml (Sigma)] and 6 supplemental factors [laminin 12.5 ng/ml (Sigma), FGF2 10 ng/ml (Sigma), IGF 10 ng/ml (Sigma), PDGF 1 ng/ml (Sigma), BDNF 10 ng/ml (R&D systems, Minneapolis, MN), NT-3 10 ng/ml (Sigma)]. After the medium was poured under inserts, samples were cultured for 6 days. To examine the effects of neural secretion on lingual papillae and taste buds, fetal tongues were co-cultured with dorsal root ganglia from fetal mice at embryonic day 15.5 or geniculate ganglia from adult mice. Collected ganglia were set adjacent to fetal tongues in collagen gel. The method of surgical dissection of geniculate ganglia was learned from Ayako Fukui at Japan Women's University. Collected samples were fixed overnight in 4% PFA/PBS at 4°C and were embedded in paraffin. Samples were sectioned and were stained with hematoxylin and eosin.

Cell culture and grafting reconstructed tongues under kidney capsule

Lines of taste bud-derived cells (TBD cell) were previously established in our laboratory (Sako, *et al*., 2011). The culture medium was a 1:1 mixture of Dullbecco's modified Eagle's medium and Ham's nutrient mixture F12 (DMEM/F12, Sigma) supplemented with 10% FCS (Life Technologies, Carlsbad, CA).

To induce morphogenesis of taste buds *in vitro*, a TBD-a5 line was reconstructed with tongue mesenchymal tissue. Tongues at embryonic day 15.5 were excited and treated with dispase (1000 U/ml, Godo syusei co. LTD, Tokyo, Japan) for 50 min at RT. Lingual epithelium was peeled from mensenchymal tissues using 25 gage needle and 1 ml syringe (Terumo, Tokyo, Japan). Collagen for culture of tongue tissue was mixture of collagen type I (Nitta Gelatin, Osaka, Japan), reconstruction buffer (HEPES 200 mM, $NaHCO_3$ 260 mM, NaOH 50 mM), and 10x α-modified MEM (Sigma) (8:1:1). These mesenchymal tissues without epithelium were put into drops of the 15 μl collagen. TBD-a5 cells at confluent in a 100 mm dish were tripsinized and collected, and centrifuged at 3000 rpm. The supernatant was removed and 2 μl of precipitation of TBD-a5 (3.5 x 10^5 cells) was draw up. The cells were set on tongue mesenchymal tissues in reconstruction collagen gel. After reconstructed samples were incubated in 37°C for 30 min, they were set on 12 well inserts and cultured with 10%

FCS medium poured under the inserts. Next day, samples were grafted under kidney capsule for 7 days as descried in Chapter 1.

2-3. Results

Tongue development in organ culture methods using agarose gel

In chapter1, nerve-independent tongue morphogenesis was examined by grafting under kidney capsule. Kidney provides a ready blood supply for grafted samples, however, it is difficult to define “the culture environment”. Therefore, the goal of the present study is to establish a tongue organ culture system for a long period.

Generally, successful culture of tissues needs the appropriate balance between nutrient and oxygen supplies to cultured tissues. Immersion of tissues in culture medium is suitable to provide nutrients to them, but less oxygen is supplied to cultured tissues because of the reduction of the surface area in contact with the air. Because all tissues produce collagenase, tissue structures are collapsing during organ culture. In the present study, agarose gel was selected for supporting whole tongue structure. In consideration of nutrition and oxygen supply, organ culture methods of embedding whole or part tongue were examined (Fig. 1a). After culturing with both methods, whole tongues were macroscopically maintained in shape and cultured tongues were fixed in 4% PFA/PBS and sectioned for HE staining. In HE sections, however, lingual epithelium cultured in both methods was detached from mesenchymal layer (Fig 1b, c). Lingual epithelial cells differentiated into cornified epithelial cells and filiform papillae were observed in

cultured tongue. These observations suggested that destruction of connective tissue and basement membrane caused epithelial detachment. Further improvement is necessary for better organ culture system that fibroblasts in connective tissue can survive for the culture period.

Tongue development in organ germ culture method.

The result of the tongue culture indicated that better nutritional supply to lingual connective tissue was needed. Therefore, the organ culture method for bioengineering tooth germ was used for tongue culture (Fig. 2). Because tongues were cut within 1 mm^2, culture medium would supply tongues connective tissue with nutrients. Embedding in collagen kept tongue tissues from collapse.

According to the results of Chapter1, DRG elongated the depth of circumvallate trenches when cultured under kidney capsule, but they had no effect on taste bud development. To investigate the effects of DRG on the development of lingual appendages, tongue tissues including circumvallae papillae and DRG were co-cultured in the organ germ culture method for 6 days (Fig. 3). As a result, the trenches of circumvallate papillae were elongated, but no taste bud developed in tongues tissues (Fig. 3a-c, Table 1).

Geniculate ganglia include some neural cells innervating taste bud in adult mice and geniculate ganglia express BDNF which taste buds also express (Patel and Krimm, 2010). Therefore, tongues were co-cultured with geniculate ganglia and were also cultured with BDNF containing culture medium (Fig 3d, e). However, in both culture conditions, development of the circumvallate trenches was undetected (Fig. 3d, e). No taste bud was induced in the cultured tongues although normal tongues at postnatal day 3 already developed taste buds in stratified circumvallate epithelium (Fig. 3f).

Moreover, the tongue organ culture method was improved by adding supplemental factors into culture medium. Transferrin, insulin, and forskolin have been used as cell growth factors for many cell lines derived from *p53* deficient mice. Hydrocortisone, ascorbic acid, and EGF have been used for 3 dimensional culture to reconstruct tongue epithelium. According to the result of agarose tongue culture, lack of basement membrane component was a problem to be solved. Therefore, these growth factors and laminin were added to the culture medium. (Fig. 4). As a result, filiform papillae were formed, but no fungiform papillae were observed in both culture conditions (Fig. 4a, b). Circumvallate papillae existed after organ culture, however, tongues cultured with supplements were under collapsing in papillary shape (Fig. 4c, d).

No taste bud developed in both conditions (Fig. 4a-d).

Besides laminin, basement membrane mainly consists of collagen IV, heparin sulfate proteoglycan, and entactin. Matrigel is basement membrane matrix which contains these basement membrane components. Matrigel was used for tongue organ culture instead of collagen I (Fig. 5). Fungiform papillae development was more clearly observed in cultured tongues embedded in Matrigel (Fig. 5c, arrow). However, the tip of epithelial cells of the fungiform papilla did not differentiate into taste buds (Fig. 5c).

Reconstruction experiment of taste buds with TBD-a5 of TBD cell lines

Taken together, these results of tongue organ culture indicated that inducing taste bud morphogenesis is very difficult without new innovation in organ culture. Maintenance of taste buds is nerve-dependent, but taste buds do not disappear immediately after the gustatory is injured (Takeda *et al*., 1996). It is presumed that innervaton to taste buds is dispensable for intragemmal cell survival and maintenance of the onion-like structure. In our laboratory, taste bud derived cell lines (TBD cell lines) are established from *p53* deficient mice and can survive on collagen I (Nishiyama *et al.*, 2011). To investigate whether TBD cell lines survive without innervation and form onion-like structures on tongue tissues, TBD-a5 cells, one of TBD cell lines, were

cultured on tongue tissues whose epithelium was removed (Fig. 6). Tongue tissues and TBD-a5 cells were grafted under kidney capsule for 6 days and were collected and sectioned for HE staining. Tongue surface is covered with epithelial cells, but taste bud-like structure was not histologically recognized in cultured tongues (Fig. 6b, c). TBD-a5 cells had been previously transfected to express GFP protein. When the cultured tongues were immunohistochemically examined with anti-GFP antibody, epithelial cells of tongue surface were positive for anti-GFP antibody (Fig 6d). These results suggested that the tongue epithelium was formed with TBD-a5 cells and the TBD-a5 cells did not form onion like-taste bud structure in the condition of only taste cell survival. (Fig. 6).

2-4. Discussion

The goal of the present study is to establish tongue organ culture methods and to examine whether taste buds occur nerve-independently in the tongue organ culture system. When a circumvallate papilla and DRG were co-cultured in the organ germ culture method, the circumvallate trenches were elongated, however, taste bud morphogenesis was not induced (Fig. 3). Supplement with growth factors had no effects on circumvallate development or taste bud induction (Fig. 4). In the experiment of taste bud reconstruction under kidney capsule, TBD-a5 cells survived on tongue mesenchyme (Fig. 6), indicating that the taste buds could survive in the environment without innervation. In conclusion, only epithelial-mesenchymal interaction is insufficient for taste bud morphogenesis *in vitro*.

Taste bud development in organ culture

Whole tongue culture methods were previously taken to examine the effects of growth factors on lingual development (Mbiene et al., 1997), but fetal tongues were too big to be cultured. A successful organ culture method for tooth germs was reported, in which small germs developed into teeth (Nakao *et al*. 2007). Therefore, it was possible that small pieces of tongues develop in the culture system.

In the study of taste bud development, it is important to understand how epithelial-mesenchymal interactions contribute to taste bud morphogenesis. Because organ culture system eliminates the effects of innervation from the complex mechanism of tongue development, the system makes it easy to observe nerve-independent development of fetal tongues. In tongue organ culture with agarose gel, tongue epithelium was detached from underlying mesenchyme, suggesting that epithelial-mesenchymal interaction did not function (Fig. 2). In tongue organ culture with collagen and Matrigel, tongue epithelium adhered to underlying mesenchyme (Fig. 5), suggesting that tongues developed through epithelial-mesenchymal interaction. However, tongue epithelium did not differentiate into taste bud, indicating that some molecular mechanisms, which are mentioned below, activated by innervation to tongue epithelium are necessary for taste bud morphogenesis.

Factors related to tongue and taste bud development

Activation of Wnt, Notch, and hedgehog singaling is important for the development of tongues and taste buds (Iwatsuki et al., 2007; Kaspsimali *et al.*, 2011), and activation of FGF, hedgehog, BMP, and EGF signaling is important for development of circumvallate and fungiform papillae (Zhou *et al.*, 2006; Liu *et al*., 2008; Liu *et al*., 2013). Taste buds

express a receptor of insulin-like growth factor, but the developmental function is unknown (Biggs *et al*., 2016). Taste buds and presumptive taste epithelium express BDNF and NT4. These neurotrophic factors are involved in induction of gustatory innervation, but other functions of the neurotrophins are unknown (Huang and Krimm, 2014). EGF is expressed in lingual epithelium except for taste buds, and is related to proliferation and differentiation of lingual epithelium (Liu *et al.*, 2008).

Growth factors, which are derived from peripheral nerve, play an important role in limb regeneration of amphibians. It is hypothesized that FGFs are released from tip of peripheral nerves to limb buds and induce dedifferentiation of fibroblasts in wound (Satoh *et al*., 2010; Satoh *et al.*, 2011) and following epithelial-mesenchymal interaction induce the limb regeneration (Makanae *et al.*, 2013). It is also known that ectopic extra limb is induced by neurite outgrowth deviation towards a wound skin in fetal axolotl (Endo et al., 2004). While taste bud occurrence and disappearance are regulated by innervation, taste buds are placed within the epithelium of taste papillae which hold much connective tissue. It is therefore hypothesized that gustatory nerve firstly induces the differentiation of taste papillary mesenchymal cells before taste buds form via epithelial-mesenhymal interaction within taste papillae.

Effects of secretion from neural ganglia on the circumvallate papillae

De-innervation causes loss of taste buds and re-innervation rebuilds taste buds, leading the conclusion that gustatory innervation maintains taste buds. However, the mechanism has been poorly understood. Co-culture with DRGs improved the development of circumvallate papillae (Fig. 3), suggesting that some diffusible factors from DRGs are involved in the papillary development. Epithelial stem cells at the bottoms of trenches of circumvallate papillae lose Lgr5 expression when gustatory innervation is injured (Takeda et al., 2013). BDNF might be one of the factors and contribute to Lgr5 expression. Secretions from DRGs might maintain stem cells at the bottom of trenches of circumvallate papillae in place of those from neural crest-derived cells of gustatory ganglia (Harlow and Barlow, 2007).

Survival of cells derived from taste buds *in vitro*

A taste bud has structural features; I. aggregation of taste cells in spindle shape; II. nuclei at different levels forming a pseudostratified epithelium; III. tight junction at the apical tip. TBD-a5 cells adhered to tongue mesenchyme when cultured under kidney capsule; however, they did not differentiate into cells in spindle shape (Fig. 6). Moreover, they formed thin stratified layer and their nuclei were at same level. These

results indicated that TBD-a5 cells only survived without forming taste bud on tongue mesenchyme.

Nishiyama et al. (2011) attempted to construct taste buds with TBD cell lines in 3-dimension collagen gel culture. TBD-a5 cells form ductal structure and some of them have taste cilia and tight-junction in collagen gel while TBD-c1 cells are incapable of making these structures. However, TBD-a5 cells have never form onion-like structure in collagen gel and they form simple monolayer when cultured on collagen gel with mesenchymal cells derived from *p53* deficient mice. Epithelial-mesenchymal interactions and innervation to TBD-a5 cells might be essential to their morphological change into fusiform shape if TBD-a5 cells are capable of differentiating into fusiform taste cells.

Differentiation into fusiform shape is important for taste cell function. To accept taste substances, all taste cells in a taste bud extend to a taste pore which is connected to out of lingual stratified epithelium. It is reported that some of taste stem cells, which express Lgr5 or 6, differentiate into fusiform cells in spheres when the stem cells are in suspension culture (Ren *et al.*, 2014). However, the induced fusiform cells elongate in disorganized direction and do not aggregate like a taste bud. This report suggests that differentiation into fusiform cells and aggregation of the fusiform

taste cells might be a discontinuous event. TBD-a5 cells would become elongated if they were cultured in sphere culture. However, induction of morphogenesis of taste buds with TBD cell lines requires further improvement of culture system because the cells in sphere culture did not aggregate to form the onion-like taste bud structure.

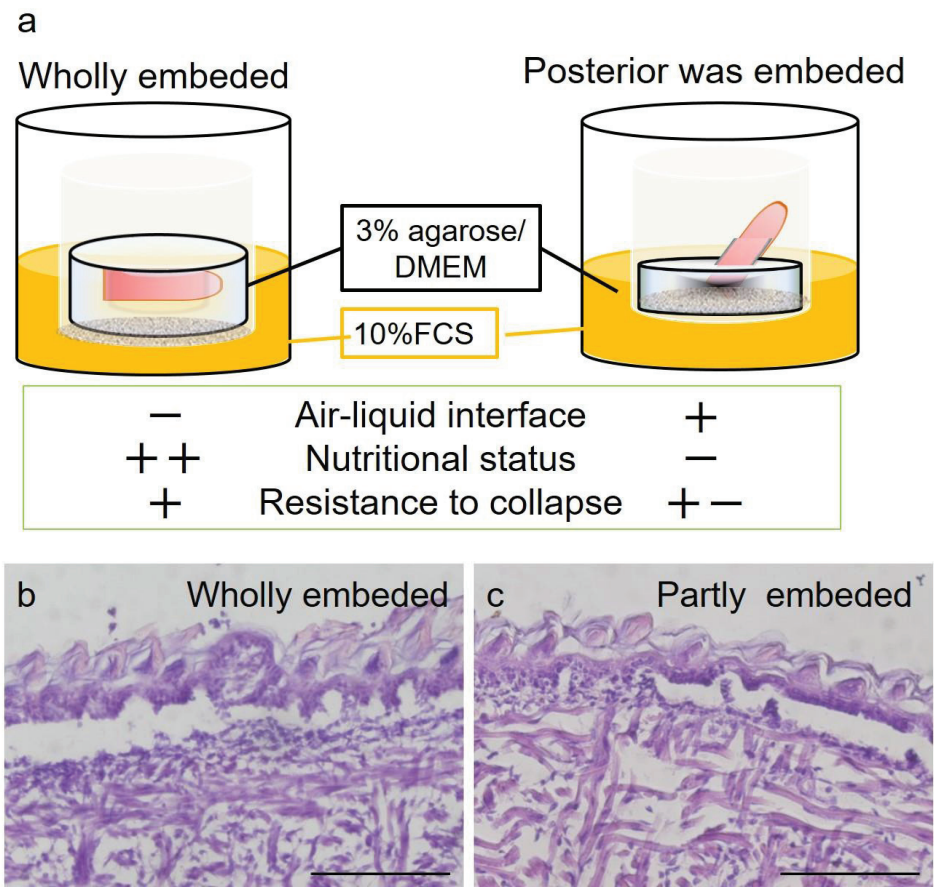

Fig. 1 Schema of tongue organ culture system using agarose gel and HE staining of cultured tongues

a: Description of culture methods using agarose gel. Collected tongues were wholly or partly embedded in agarose gel and were cultured with DMEM/F12 containing FCS at 10% for 6 days. Wholly embedding culture method (left) is superior in nutrition supply. Partly embedding culture method (right) is superior in oxygen supply. **b, c**: HE images of lingual epithelium cultured with two organ culture methods using agarose gel. Lingual epithelial layer was detached from underlying connective tissue in both culture methods. Scale: 100 μm

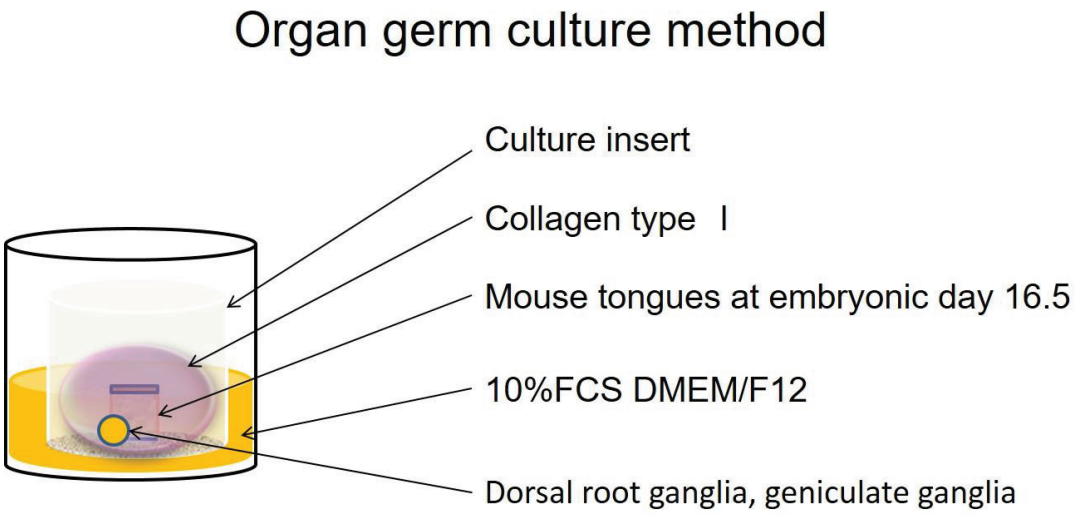

Fig. 2 Schema of organ germ culture method

A tongue tissue was embedded in a drop of collagen type I and was set in a culture insert. Culture medium was poured under the insert and tongues were cultured for 6 days.

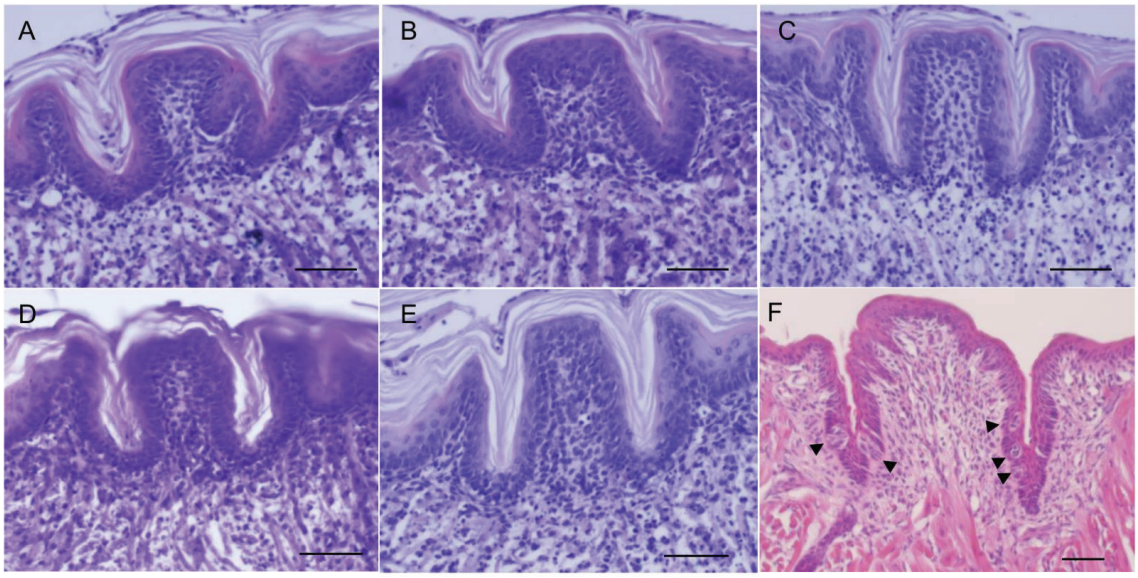

Fig. 3 Circumvallate papilla development in organ germ culture system for 6 days

A: Posterior tongues including circumvallate papillae at embryonic day 16.5 were cultured for 6 days in organ germ culture method. **B-D**: To investigate the effects of neural secretions on circumvallate papilla development, two (B) or ten (C) DRGs were co-cultured with the tongues. Circumvallate papillae at embryonic day 16.5 were also co-cultured with geniculate ganglia (D). **E**: Circumvallate papillae at embryonic day 16.5 were cultured with the medium containing BDNF 100 ng/ml. **F**: An image of circumvallate papillae at postnatal day 3. Taste buds were seen within stratified epithelium of circumvallate trenches (arrow heads). Scale: 50 μm

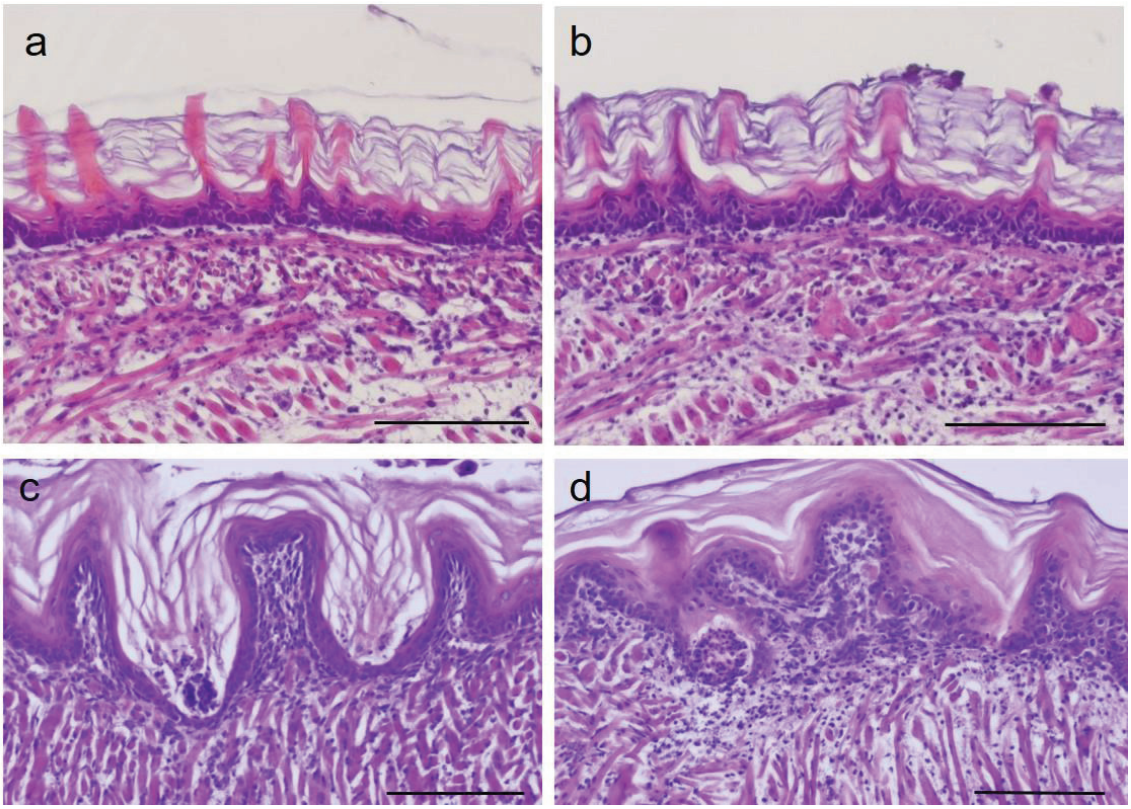

Fig. 4 Culture of tongues at E19.5 with 3-D growth factors and supplemental 6 factors.

To induce morphogenesis of taste buds, tongues at E19.5 which contain immature taste buds were cultured with 10% FCS with or without growth factors. **a, c:** Culture of anterior (**a**) and posterior (**c**) tongues with 10% FCS medium. **b, d**: Culture with 10% FCS medium containing 3-D growth factors and supplemented with 6 factors. Morphogenesis of taste buds was not induced in the conditions. Scale: 100 μm

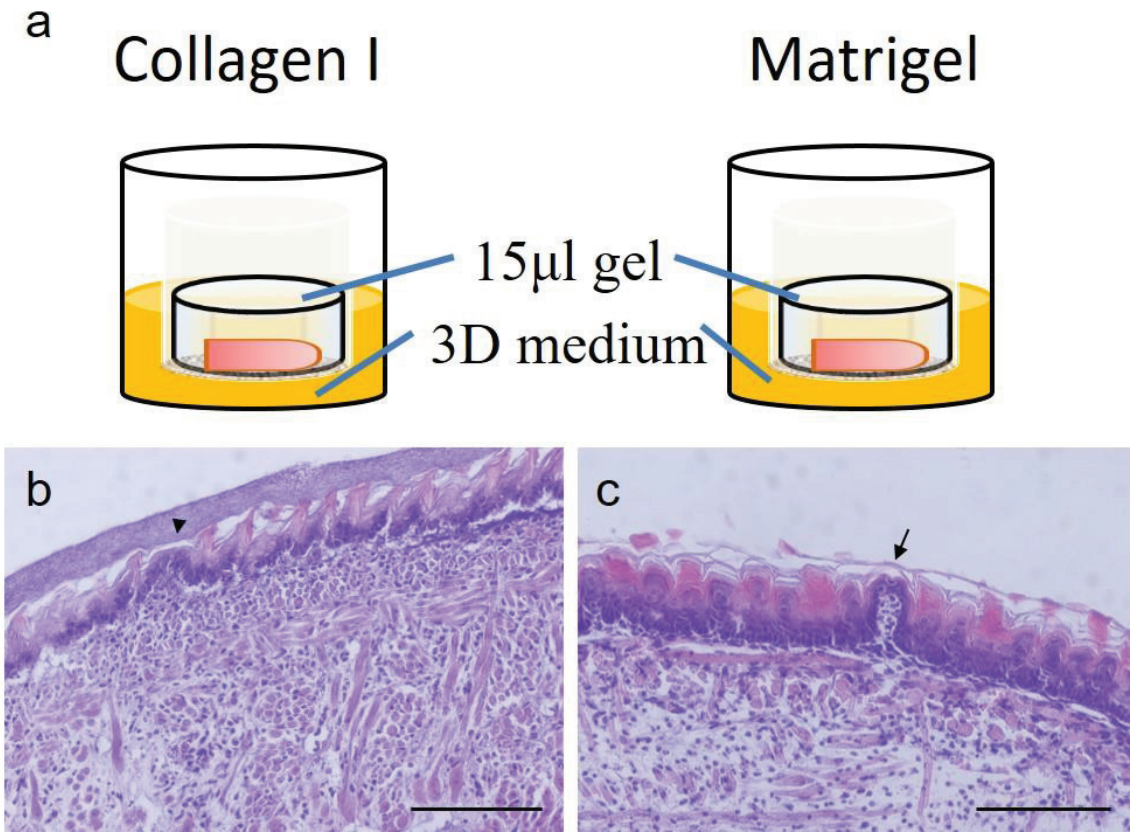

Fig. 5 Anterior tongue organ culture in Matrigel

a: The method of tongue culture with Matrigel. Tongues at E18.5 were embedded in Matrigel drops and cultured with the medium containgin 10% FCS. **b**: Anteiror tongues cultured with collagen I. **c**: Anterior tongues cultured with Matrigel. Fungiform papillae structure was observed in both culture conditions (arrow head and arrow). The fungiform in Matrigel culture (arrow) had appeared to keep more abundant connective tissues than that of **b** (arrowhead). Stratified epithelium of fungiform did not possess any taste buds. Scale: 100 µm

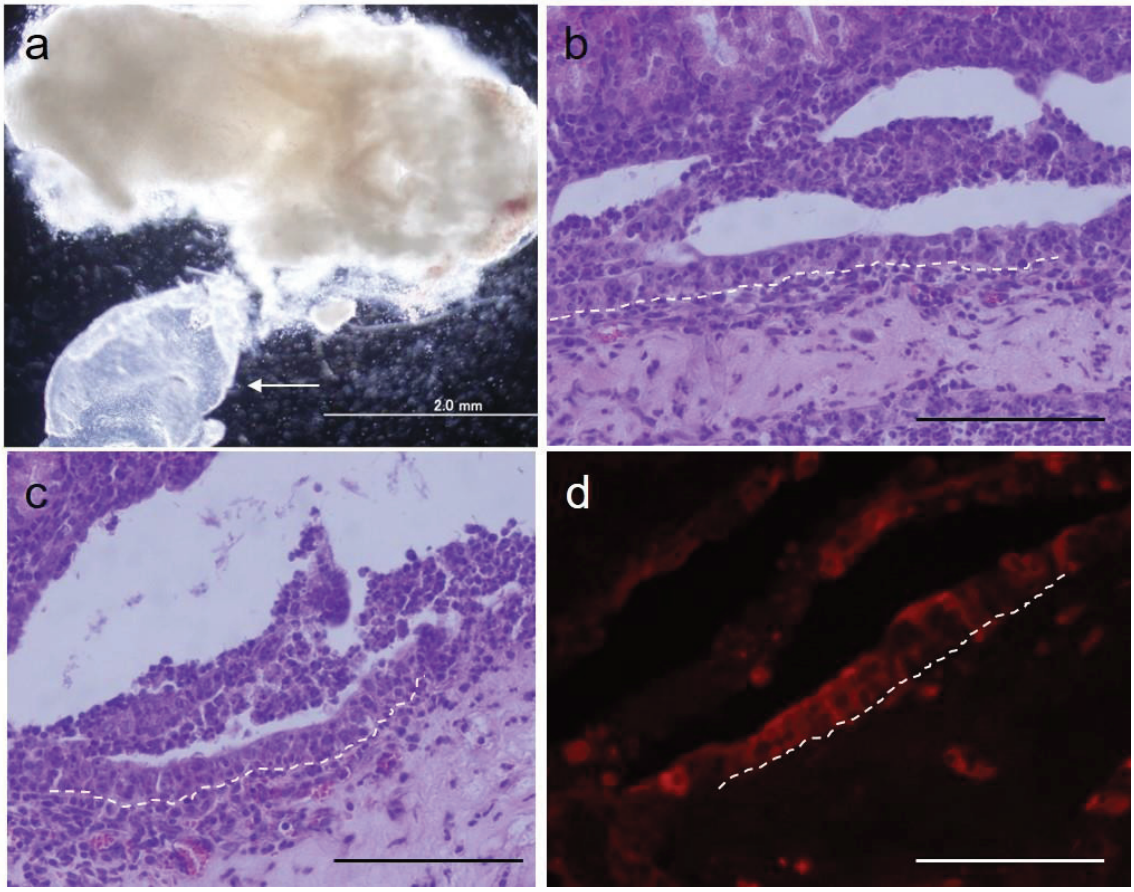

Fig. 6 Reconstruction of taste bud using TBD-a5 and tongue mesenchyme

TBD-a5 cells and tongue mesenchyme at E15.5 were embedded in collagen gel and were grafted under kidney capsule for 7 days. **a**: Tongue epithelium (arrow) at E15.5 was peeled after dispase treatment. **b**, **c**: HE image of a grafted sample. Surface of grafted tongues was covered with disorganized stratified epithelial cells. **d**: Immunohistochemical image of the same reconstructed sample as **b** and **c**. Anti GFP antibody indicated grafted TBD-a5 cells. TBD-a5 cells covered tongue mesenchyme, but onion-like structure was not observed. Dotted lines: borderlines between epithelium and mesenchyme. Scale: 100 μm

Table 1. Depth of Circumvallate trenches in cultured tongues

10FCS (n=8)	+10 DRG (n=8)
23.1+/-6.8	46.9+/-7.8*

Four antero-posterior sections of the central transverse section were selected in a circumvallate papilla. The distance (depth, μm) between the bottom of the circumvallate plate and the bottom of the trench basal cells was measured in right and left sides with Image J, and the average was calculated. Values were presented as average±standard error. A two tailed Student's t-test was used and the significant level was set at 0.05. *P<0.05.

Chapter 3

Nerve-independent and ectopically additional induction of taste buds in organ culture of fetal tongues

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3-1. Introduction

The tongue is originated from branchial arches and the lingual epithelium consists of ectodermal and endodermal epithelial tissues (Rothova et al. 2012). In adult mice, the lingual surface is covered with specialized lingual papillae; filiform (number; unreported), fungiform (number; approximately 90/tongue) (Mistretta and Liu 2006), foliate (number; 4-6/tongue) (Toprak and Yilmaz et al. 2007), and circumvallate (number; 1/tongue) papillae. Fungiform, foliate, and circumvallate papillae possess taste buds, which have receptors for taste substances. A taste bud is an aggregate of 50-150 spindle-like shaped taste cells and is embedded in multilayered epithelia of taste papillae. Cells of taste buds express CK8, whereas cells of tongue epithelium except for taste buds express CK14. Gustatory nerves innervate all taste buds and maintain

turnover of taste buds, while these nerves transmit gustatory sensation to brain.

Fungiform papillae firstly emerge at E12.5 in mice and gene expressions such as Shh and Sox2 are localized within the epithelia (Nakayama et al. 2008; Nakayama et al. 2015). At E14.5, gustatory nerves penetrate into the papillary epithelial layer and innervate immature fungiform papillae (Nakayama et al. 2008; Hall et al. 1999). Formation of filiform papillae starts at E16.5 and a distinct undulating basal layer is observed, and then, keratinized spines grow at the apical surface of filiform papillae (Jonker et al. 2004). Presumptive epithelium of circumvallate papillae is thickened at E12.5 in mice. Formation of trenches of circumvallate papillae starts at E13.5 (Kim et al. 2009) and gustatory nerves innervate the apical epithelia of circumvallate papillae at E15.5 (Hall et al. 1999). After birth, gustatory nerves innervate only the circumvallate trench epithelium and taste buds appear within the stratified epithelium (Suzuki. 2008).

Four types of taste cells compose a taste bud, in which Shh-positive basal cells differentiate into other types of taste cells (Miura et al. 2014). Intragemmal Shh expression is reduced immediately after gustatory nerve injury, suggesting gustatory innervation keeps the expression of Shh in taste buds (Miura et al. 2004). Although Sox2 is also nerve-dependently expressed in taste buds and taste epithelia in adult mice, Sox2 is nerve-independently expressed in presumptive epithelia of taste buds in

brain-derived neurotrophic factor (*BDNF)* deficient fetal mice. *BDNF* deficient mice show a significant reduction in innervation in geniculate ganglia (Suzuki 2008; Ito et al. 2010), indicating that taste buds develop without gustatory innervation at early stage (Thirumangalathu et al. 2009; Ito et al. 2010). Nerve-dependency of development and maintenance of lingual appendages is well known (Vintschgau and Hönigschmied 1877), however, it is yet unanswered how gustatory innervations contribute to the morphogenesis of taste buds.

Wnt signal pathway is involved in the early morphogenesis of taste papillae and taste buds. Wnt ligands are expressed in developing lingual epithelium and mesenchyme, and Wnt signaling pathway is activated in mouse lingual epithelium from E11.5 through developmental stages of taste papillae (Iwatsuki et al. 2007). *KRT14-Cre Ctnnb1*$^{(Ex3)fl/+}$ tongues at E18.5 show increased in number and size of fungiform papillae, suggesting that Wnt signaling regulates the early development of fungiform papillae (Iwatsuki et al. 2007; Liu et al. 2012; Liu et al. 2007). Wnt/β-catenin signaling in mature taste buds is activated but the level of the activation is reduced in older mice whose turnover rate of taste buds is reduced (Gaillard and Barlow 2011). These results suggest that Wnt/β-catenin signaling also regulates turnover of taste buds.

Receptors regulating Wnt signaling, Lgr5 and Lgr6, are expressed within taste

papillary epithelium. Lgr5+ cells located at the bottoms of trenches in circumvallate papillae work as stem cells which can differentiate into both taste cells and other papillary epithelial cells (Ren et al. 2014; Yee et al. 2013). Lgr6+ cells surrounding taste buds within fungiform papillae also differentiate into taste cells (Ren et al. 2014). When taste buds morphologically appear or regenerate after reinnervation, cytoplasmic β-catenin in taste papillary epithelial cells translocates into nuclei, suggesting that activation of canonical Wnt signal pathway in taste papillary epithelium regulates morphogenesis of taste buds (Suzuki et al. 2012).

In the present study, an *in vitro* culture system for reconstructed tooth germs (Nakao et al. 2007) is adopted for culturing fetal mouse tongues without innervation. Organ culture *in vitro* provides an environment to examine how the gustatory innervation regulates the early morphogenesis of lingual epithelial appendages (Mbiene et al.1997). The improved culture system allows to observe morphogenesis of mouse taste buds of fungiform and circumvallate papillae *in vitro*, and to examine if activation of canonical Wnt signaling without innervation induces taste bud cells from tongue epithelial cell.

Effects of activation of Wnt/β-catenin signaling with powerful inhibitor of glycogen synthase kinase 3 beta (GSK3β) were investigated on non-innervated fetal

tongues. Results of the present study demonstrated that stabilization of cytoplasmic β-catenin had significant effects on the early morphogenesis of taste buds.

3-2. Materials and methods

Mice and tissue preparation

ICR mice were obtained from Sankyo Labo Service Corporation and maintained under a 12:12 h light and dark cycle at 24±1°C in the facility for rearing animal in Tokyo University of Science. All experiments were approved by the institutional Animal Care and Use committee at Tokyo University of Science.

Embryonic day 0.5 (E0.5) was defined as noon of the day on which a vaginal plug was observed. Pregnant mice were killed by cervical dislocation and embryos were removed into phosphate buffered saline (PBS) for the following experiments.

Culture of mouse fetal tongues

Methods to culture fetal gustatory papillae were based on a three-dimensional organ-germ culture method for generating bioengineered tooth (Nakao et al. 2007) and included some alterations. Fetal tongues at E16.5 were surgically removed and washed in PBS on ice. The tongue tissues including developing circumvallate papillae were trimmed within no more than 1 mm^3. The anterior tongue tissues were obtained by transecting within 1 mm from the tip. These immature lingual tissues were embedded into 15 μl gel drops of Cellmatrix type I-A (Nitta Gelatin, Osaka, Japan) prepared with

10X αMEM (M0894, Sigma-Aldrich, St. Louis, MO) and reconstitution buffer (NaOH 50 mM, $NaHCO_3$ 260 mM, HEPES 200 mM) (8:1:1) on a Petri dish coated with grease (Dow Corning Toray, Midland County, MI). After gelation for 30 min at 37°C, tissue samples were set on cell culture inserts in 12-multiwell culture plates and cultured for 6 days with the following media; DMEM/F-12 (D2906, Sigma-Aldrich) containing 31 mg/l penicillin, 50 mg/l streptomycin (Sigma-Aldrich) and heat-inactivated fetal bovine serum (FBS) (10437-028, Gibco®Life Technologies, Carlsbad, CA) at 10% (10% FBS, v/v) or 10% FBS medium containing 3 μM CHIR99021 (Cayman Chemical, Ann Arbor, MI) and/or 10 μM cyclopamine (Cayman chemical company). These media were added under inserts and replaced every day.

Histology

All specimens were fixed in 4% paraformaldehyde in PBS, pH 7.4, at 4°C for 1.5–3.5 hours, then dehydrated with graded tissue dehydration solution (Wako Pure Chemical Industries, Ltd., Osaka, Japan). These samples were embedded in paraffin and serially sectioned at 6–8 μm for hematoxylin and eosin (HE) staining or immunohistochemical analysis.

The HE stained images were used for quantitative analysis of the spines,

papillae, and eosinophobic cells. A filiform papilla consists of a papilla structure and a spine. The papilla is the stratified epithelium invaded with thread-like connective tissues and the spine is keratinized and protruding into oral cavity. The HE staining delineates these characteristic morphological features. Each structure was counted separately in tongues cultured with or without CHIR99021.

Immunohistochemistry

Primary antibodies were as follows: anti-cytokeratin8 (1:20, mouse, 61038, Progen, Heidelberg, Germany); anti-cytokeratin14 (1:200, mouse, MCA890, AbD Serotec, Oxford, UK); anti-Sox2 (1:200, rabbit, ab5603, Abcam, England, UK); anti-Shh (1:40, goat, AF464, R&D systems, Minneapolis, MN); anti-β-catenin (1:200, rabbit, Abcam); anti-IP3R-3 (1:100, mouse, 610312, BD Biosciences, San Jose, CA); anti-PLCβ2 (1:50, rabbit, sc-206, Santa Cruz Biotechnology, Dallas, TX) antibodies in blocking solution (PBS containing 5% donkey serum (S-30, Chemicon®Merck Milipore, Darmstadt, Germany)). These primary antibodies were detected using secondary antibodies as follows; Alexa Flour 488 donkey anti-mouse IgG (1:400, Life Technologies, Carlsbad, CA); Alexa Flour 594 donkey anti-mouse IgG (1:400, Life Technologies); Alexa Flour 594 donkey anti-rat IgG (1:400, Life Technologies); Alexa Flour 488 donkey anti-goat

IgG (1:400, Life Technologies); Alexa Flour 594 donkey anti-goat IgG (1:400, Life Technologies); Alexa Flour 488 donkey anti-rabbit IgG (1:400, Life Technologies); Alexa Flour 488 goat anti-rabbit IgG (1:200, Jackson immunoresearch, west grove, PA), in blocking solution.

All sections for immunostaining were rehydrated and immersed in PBS, and some rehydrated sections were processed for antigen retrieval with microwave at 85–95°C in 10 mM citrate buffer at pH 6.0 for 5 minutes or electric pot at 93°C in immunosaver (Nissin EM, Tokyo, Japan) for 45 minutes. Sections were then covered with blocking solution for 1 hour at room temperature and incubated with primary antibodies overnight at 4°C. After rinse in PBS, sections were reacted with fluorescently labeled secondary antibodies for 2 hours at room temperature and were counterstained with DAPI to visualize nuclei. Confocal images were photographed with LSM510 (Carl Zeiss, Oberkochen, Germany). All image resolution was improved with Gimp2 (version 2.8.14).

3-3. Results

GSK3β inhibitor induced morphogenesis of taste buds without innervation through activation of Wnt/β-catenin signaling

Taste buds were observed in the tip of fungiform papillae and the trenches of circumvallate papillae of adult mice (Fig. 1). CK8 and Sox2 were expressed in the intragemmal cells and Sox2 were also expressed in perigemmal taste epithelial cells (Fig. 1a-c). Shh was expressed in the basal cell of taste buds without CK8 expression (Fig. 1e-g). Lack of affinity for eosin (eosinophobic) was characteristic feature of taste buds in the lingual epithelium (Fig. 1d, h). They are described as “The taste buds are seen in sections under low power as pale“ (Bloom and Fawcett 1971; Leeson and Leeson 1976). These features are useful to identify taste cells in the course of lingual morphogenesis *in vivo* and *in vitro*.

LiCl is known as one of GSK3β inhibitors but could not induce taste buds in organ culture of fetal tongues (Iwatsuki et al. 2007). In this study, tongues were cultured with more selective inhibitor of GSK3β CHIR99021 to clarify the effects of Wnt signaling activation without innervation on the early morphogenesis of lingual appendages in organ culture.

Tongues were cultured in 10%FBS containing CHIR99021 for 6 days and were

immunohistochemically analyzed (Fig. 2). Anterior tongues displayed a decrease in number of filiform papillae (Table 1) and a prominent appearance of eosinophobic epithelial cells in cluster in stratified lingual epithelia (Fig. 2a, c, g, k, o). Then, serial sections including eosinophobic epithelial cells were immunohistochemically examined. Eosinophobic epithelial cells expressed Sox2 (Fig. 2b), and they also expressed CK8 (Fig. 2d-f, l-n), indicating that they were taste cells. Eosinophilic epithelial cells expressed Sox2 and CK14 (Fig. 2h-j), and few cells expressed CK14 in eosinophobic epithelial cells (Fig. 2o-r). Some of induced taste cells co-expressed Shh and CK8 (Fig. 3a-d) and some of CK14 expressing cells were negative for Shh (Fig. 3e-h, arrowheads). In anterior tongues cultured without CHIR99021, all lingual epithelium was eosinophilic and no expression of CK8 and Sox2 was detected (Fig. 2s-v). Eosinophobic cells were always localized at the basal cell layer and the appearance frequency was estimated as ratio (%) of length of basal lamina linings covered with eosinophobic basal cells to length of the total basal lamina lining covered with both eosinophobic and eosinophilic epithelia of the cultured anterior tongues. Eosinophobic basal cells covered the basal lamina at 28.8~53.6% (Table 2).

It is known that PLCβ2 and IP3R3 are expressed in type II taste cells in adult mice. However, embryonic taste buds also express them (Ota et al. 2009). To investigate

differentiating stages of eosinophobic epithelial cells, tongues cultured with CHIR99021 were immunostained with anti-PLCβ2 and -IP3R3 antibodies. As a result, eosinophobic epithelial cells were negative for IP3R3 and some of eosinophilic epithelial cells were positive for PLCβ2 (Fig. 4). These results indicated that CHIR99021 treatment caused decrease of the number of filiform papillae, and induced both PLCβ2-expressing eosinophilic cells and CK8-expressing eosinophobic cells in anterior cultured tongues.

Ectopical induction of taste buds in the non-gustatory area

Posterior tongues including circumvallate papillae were also cultured with CHIR99021 for 6 days (Fig. 5). A circumvallate papilla existed in the center of the cultured tongues (Fig. 5a). A few epithelial cells in a circumvallate papilla expressed Shh (Fig. 5b, white arrowhead). The adjacent section stained with HE demonstrated that the Shh-expressing cells were eosinophobic (Fig. 5c, black arrowhead). These taste cells are possibly primordial cells of taste buds. Unexpectedly, the epithelial cells in non-gustatory area (Fig. 5a, arrow) surrounding circumvallate papillae also expressed Shh. Non-gustatory areas were further subjected to observation in the following.

Eosinophobic epithelial cells were also observed in the non gustatory area surrounding circumvallate papillae (Fig. 6a, e, i, k). Interestingly enough, these

eosinophobic cells often took an “onion like” structure characteristic of the mammalian taste bud (Fig. 6i, k, arrowheads). Epithelia in the non gustatory area were analyzed by immunohistochemical staining (Fig. 6b-d, f-h, j, l). Eosinophobic epithelial cells expressed both CK8 and Sox2 (Fig. 6a-d) and not expressed CK14 (Fig. 6e-h). A few eosinophobic cells expressed CK14 (Fig. 6e-h, arrows). The onion-like structure lost CK14 expression, a marker of stratified epithelial cells (Fig. 6i, j, arrowheads). These cells in the non-gustatory area expressed Shh as well as cells in the gustatory area did (Fig. 6k, l, arrowheads). Eosinophobic epithelial cells were never observed in posterior tongues including circumvallate papillae cultured without CHIR99021 (data not shown).

β-catenin localization in cultured fetal tongues

To examine whether GSK3β inhibitor stabilizes β-catenin in epithelial cells of cultured tongues, β-catenin localization was visualized by immunohistochemistry with anti-β-catenin antibody (Fig. 7). In anterior tongues cultured with GSK3β inhibitor, a majority of eosinophobic cells were CK14 negative and the rest of them were CK14 positive (Fig. 7a-d). The CK14 positive cells localized β-catenin in nuclei (Fig. 7b-d). Among eosinophobic epithelial cells in posterior tongues cultured with GSK3β inhibitor,

a CK14-negative cell (arrow head) strongly displayed nuclear localization of β-catenin and the neighboring cell expressed CK14 without nuclear localization of β-catenin (arrow) (Fig. 7f-h). When anterior tongues were cultured with only 10FBS as a control, β-catenin was undetected in nuclei of lingual epithelium (Fig. 7i-l). Both β-catenin and CK14 were co-expressed in all basal epithelial cells. Thus, GSK3β inhibitor caused an induction of multiple signal pathways and eosinophobic epithelial cells were singly or in cluster on the sequential events.

Differentiation of eosinophilic cells into eosinophobic cells was prevented by inactivation of Smoothened.

In the present study, GSK3β inhibitor CHIR99021 induced expression of Shh in eosinophobic epithelial cells. Previously, ectopic taste buds are observed in transgenic adult mice in which lingual epithelial cells conditionally express Shh by tamoxifen treatment. To investigate the role of hedgehog signaling in tongue organ culture, anterior tongues at E16.5 were cultured with cyclopamin, which causes inactivation of hedgehog signaling through inhibition of Smoothened, and CHIR99021 (Fig. 8). When anterior tongues were cultured in 10% FBS medium containing 10 μM cyclopamine, papillary structures were observed without eosinophobic epithelial cells (Fig. 8a). When

anterior tongues were cultured with both cyclopamine and CHIR99021, almost all papillae disappeared but eosinophobic epithelial cells were not observed (Fig. 8b). These results suggested that hedgehog signaling mediated induction of eosinophobic epithelial cells caused with CHIR99021 treatment.

3-4. Discussion

The aim of the present study is to observe morphogenesis of taste buds in organ culture for relatively longer period and to examine if a powerful GSK3β inhibitor induce taste bud cells. The present study successfully induced morphogenesis of taste buds in tongue organ culture. Moreover, it was shown that β-catenin nuclear localization was correlated to cytoskeletal transition from CK14 to CK8 in the tongue organ culture.

Taste cells and buds are "eosinophobic"

In standard text books of histology, taste buds are described as "being pale" because they are written in black & white (Bloom and Fawcett 1971; Leeson T and Leeson C 1976). The histological sections are commonly stained with hematoxylin & eosin and taste buds look "eosinophobic" under light microscope. Electron microscopic observations reported that a taste bud consisted 50~150 cells and 60% were dark cells and 30% were light cells. At lower magnification, however, a taste bud looks pale (samples were stained with uranyl acetate) (Kinnamon et al. 1985; Kinnamon et al. 1988). "Eosinophobic" might be better than "pale" in recent color printed publications including textbooks of histology.

Addition of GSK3β inhibitor CHIR99021 to culture medium caused loss of affinity for eosin in 28.8~53.6% of cells in the basal layer. "Eosinophobic" is " in some

condition" and not well defined biochemically. Stem cells/progenitor cells locate at the basal layer in stratified epithelium, to which mitosis is restricted. The high coincidence raised a question why and how CHIR99021 specifically induced the change in cells at the basal layer. The question was discussed below.

Wnt/β-catenin signaling controls the development of lingual appendages

Wnt/β-catenin signaling plays an important role in lingual epithelial development as well as hedgehog signaling (Iwatsuki et al. 2007). Expression of Shh and β-catenin is at the apex of fungiform papillae during tongue development (Thirumangalathu and Barlow, 2015). For instance, inhibition of hedgehog signaling before E16.5 increased the number of fungiform papillae in anterior tongues (Liu et al. 2004), and dominant negative mutation of phosphorylation site of β-catenin caused fungiform hyperplasia (Liu et al. 2007). In addition, LiCl, when added to culture medium, activated Wnt/β-catenin signaling and increased the number of fungiform papillae at early developmental stages, but the effect was lost in E17.5 fetuses (Okubo et al. 2006). In the present study, β-catenin stabilization by CHIR99021 caused a decrease in number of papillary structures. Thus, modification in Wnt/β-catenin and hedgehog signaling causes various effects on the development of lingual appendages (Thirumangalathu and Barlow

2015), although confirmation of the effect is needed with other drugs.

The present study demonstrated that treatment with CHIR99021 induced β-catenin nuclear localization in the lingual epithelium. As discussed above, the treatment resulted in high appearance of eosinophobic epithelial cells at 28.8~53.6%. β-catenin nuclear localization was recognized in CK14 negative cells and some CK14 positive cells showed weak or strong nuclear localization of β-catenin (Fig. 6), suggesting that cytoskeleton subtype transition from CK14 to CK8 (Mbiene and Roberts 2003; Asano-Miyoshi et al. 2008) is following after the loss of affinity for eosin in the lingual epithelium. It needs to be further explored whether clusters of the eosinophobic cells were clonal and derived from a single stem cell. *In vivo*, gustatory innervation was shown to induce nuclear β-catenin localization in basal cells surrounding taste buds (Suzuki et al. 2012). The critical point of the present study is that the induction of taste cells and buds was observed in organ culture without innervation.

It is well accepted that type IV cells in taste bud produce other three types of taste cells and type IV cells are supplied from stem cells/progenitor cells lying in surrounding epithelia (Miura et al. 2014), and the stem cells/progenitor cells are able to produce both gustatory and non-gustatory epithelial cells (Yee et al. 2013). These stem cells/progenitor cells are believed to be dwelling in specific areas such as trenches of

circumvallate papillae. The ectopical induction of taste cells and buds in non-gustatory epithelium proposes another cell lineage of taste cells. In the present study, taste cells are defined as eosinophobic epithelial cells expressing CK8 and Sox2. Therefore, “taste cells” may include all cells from immature (primordial cells observed in a circumvallate papilla) to mature taste bud cells. Further study provides information to classify taste cells into specific category.

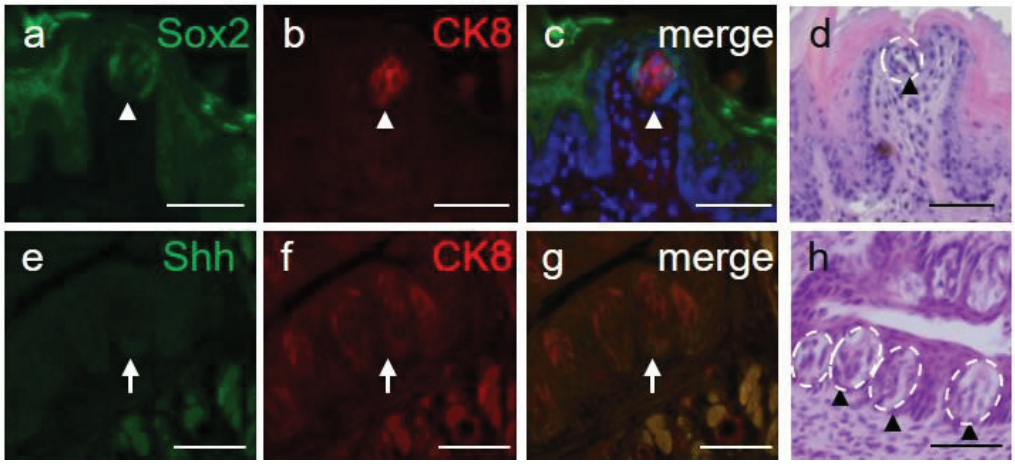

Fig. 1 Features of taste buds displayed by HE and immunohistochemical staining

Taste buds of fungiform (**a-d**) and circumvallate (**e-h**) papillae were immunostained with anti-Sox2 (**a**), -CK8 (**b, f**), and -Shh (**e**) antibodies or stained with H&E (**d, h**). a and b, and e and f were merged in c and g, respectively. Taste buds expressed Sox2 and CK8 (**a-c**) and basal cells expressed Shh (**e-g**). Taste buds were eosinophobic and surrounding stratified epithelial cells were eosinophilic (**d, h**). Arrowhead: a taste bud. Arrow: Shh expressing basal cell. Blue: DAPI. Scale bar: 50 μm. Dotted line: taste bud.

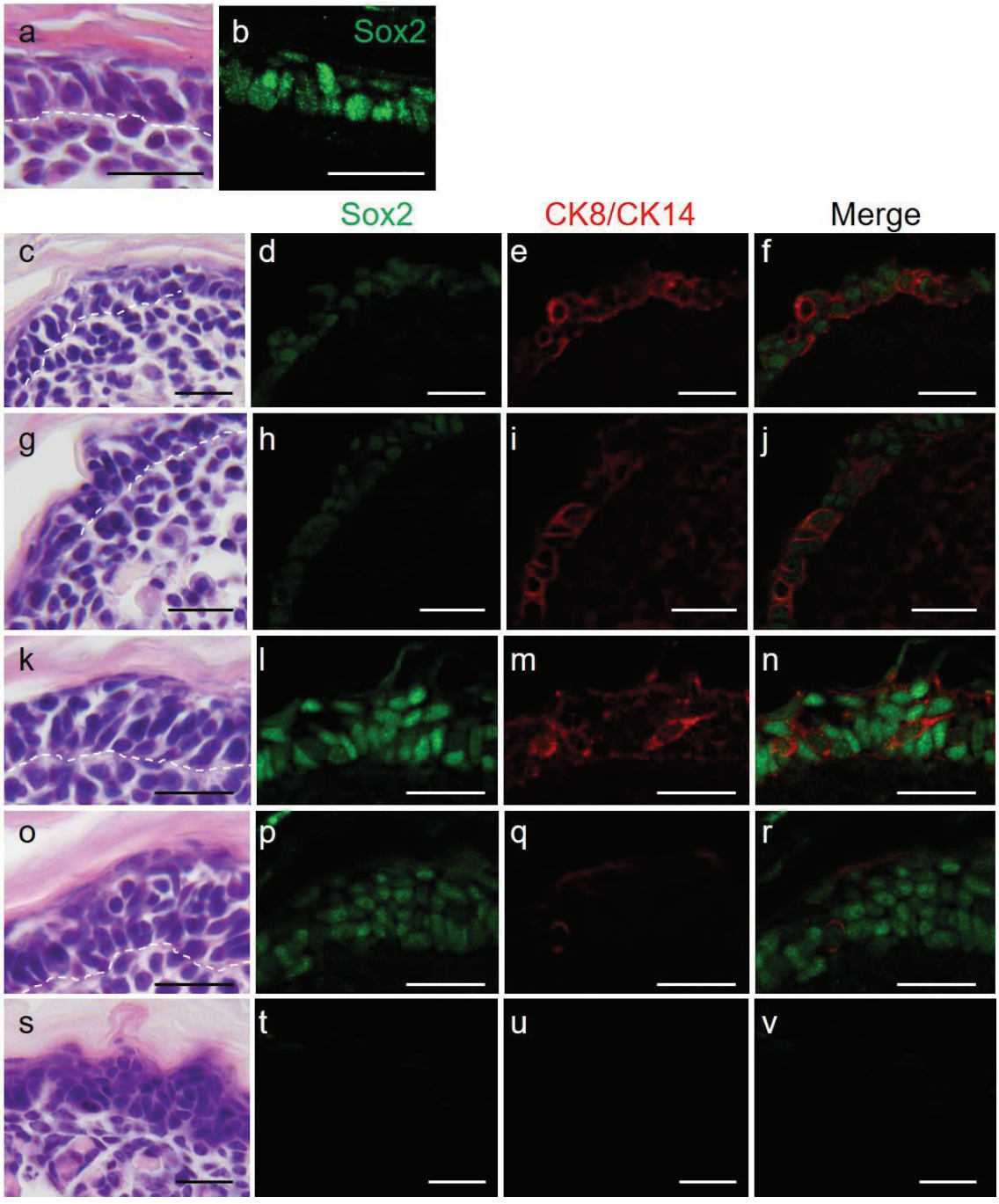

Fig. 2 Anterior tongues at E16.5 were cultured with 10FBS containing CHIR99021 for 6 days

a, b: Sox2 expression in eosinophobic epithelial cells. **c-r**: Expression of Sox2 and CK8 (**c-f** and **k-n**) or Sox2 and CK14 (**g-j** and **o-r**). H&E images (**c**, **g**, **k**, **o**). Expression of

CK8 and Sox2 was broadly detectable (**d-f** and **l-n**), and cells in spindle shape expressed CK8 (**m**). Eosinophilic cells express CK14 (**g-j** and **o-r**). There were a few CK14 expressing cells among eosinophobic cells (**q**). **s-v:** Cultured tongues with only 10FBS as a control. Scale bar: 20 μm. Dotted line: eosinophobic epithelial areas.

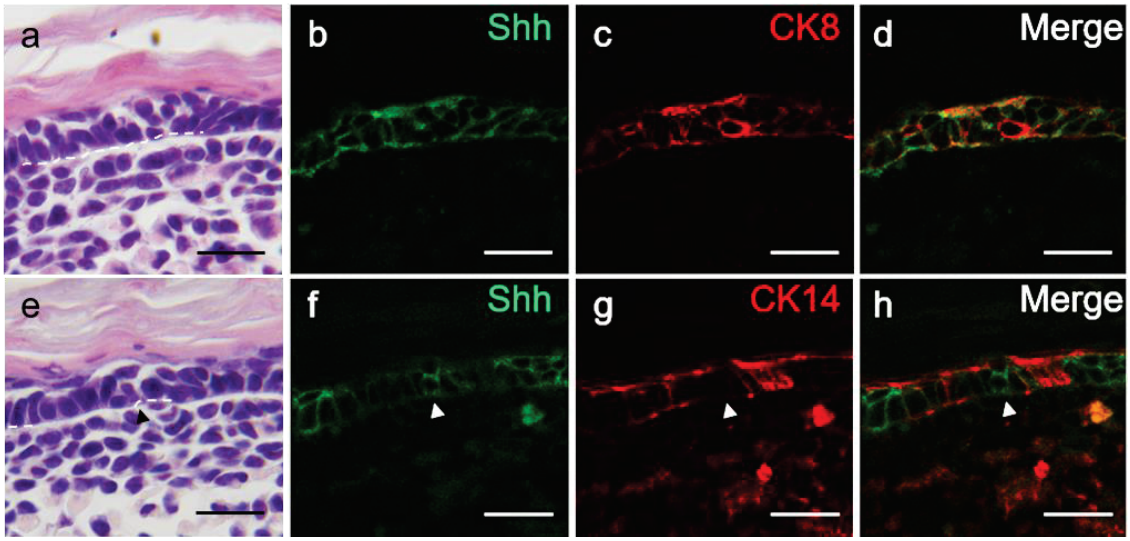

Fig. 3 Shh expression in eosinophobic epithelial cells in E16.5 anterior tongues cultured with CHIR99021

Eosinophobic epithelial cells were detected by H&E staining (**a, e**) and each adjacent section was immunostained with anti-Shh, -CK8, and CK14 antibodies (**b-d**, **f-h**). Cells expressing Shh were detected (**b**, **f**) and they also expressed CK8 (**c**, **d**) but not expressed CK14 (**e-h**, arrowheads). Scale bar: 20 μm. Dotted line: eosinophobic epithelial areas.

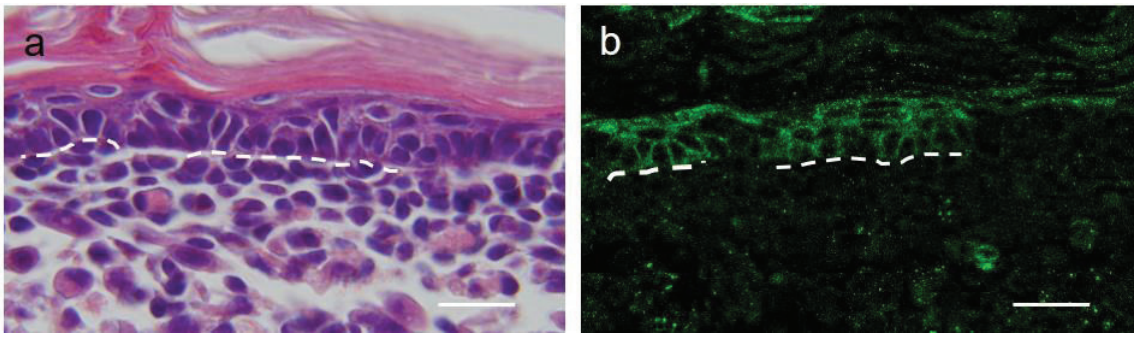

Fig. 4 PLCβ2 expression of eosinophilic epithelial cells in CHIR99021-treated anterior tongues

a: Eosinophilic cells in CHIR99021-treated anterior tongues. **b**: PLCβ2 expression in an adjacent section of **a**. Scale bar: 20 μm. Dotted line: areas of PLCβ2 expressing cell

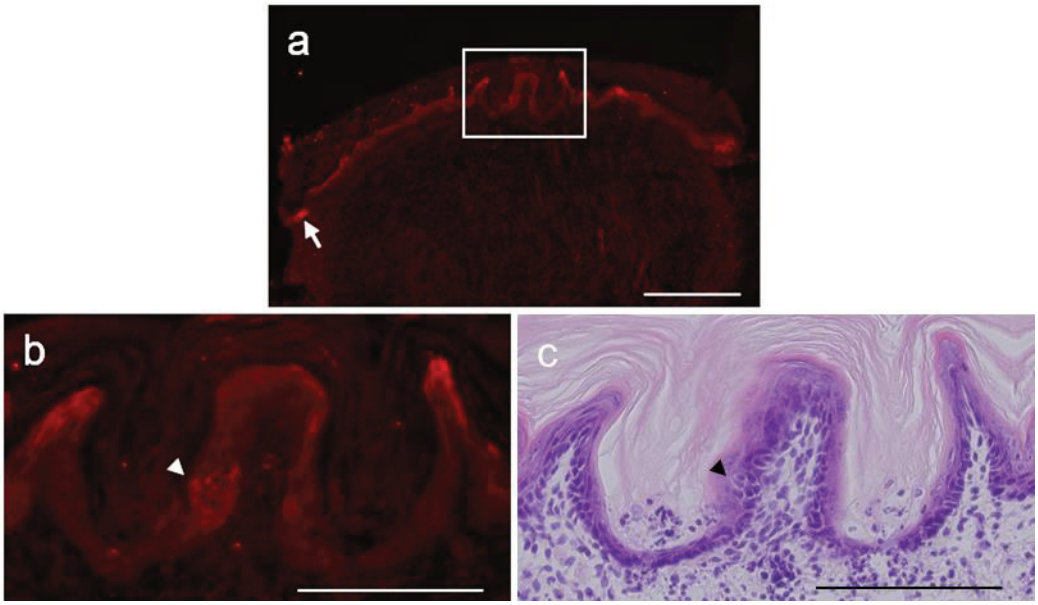

Fig. 5 Shh expression in a posterior tongue cultured with CHIR99021

a; A posterior tongue including a circumvallate papilla cultured with CHIR99021 was immunostained with anti-Shh antibody. **b**; Higher magnification of white box in **a.** A few epithelial cells of circumvallate papillae expressed Shh (white arrowhead). **c**; A HE image of the adjacent section of **b**. Shh exressing cells were eosinophobic cells (black arrowhead). Note that an arrow (**a**) indicates Shh expression in non-gustatory epithelium. Scale bar: 250 μm in **a**, 100 μm in **b**, **c**.

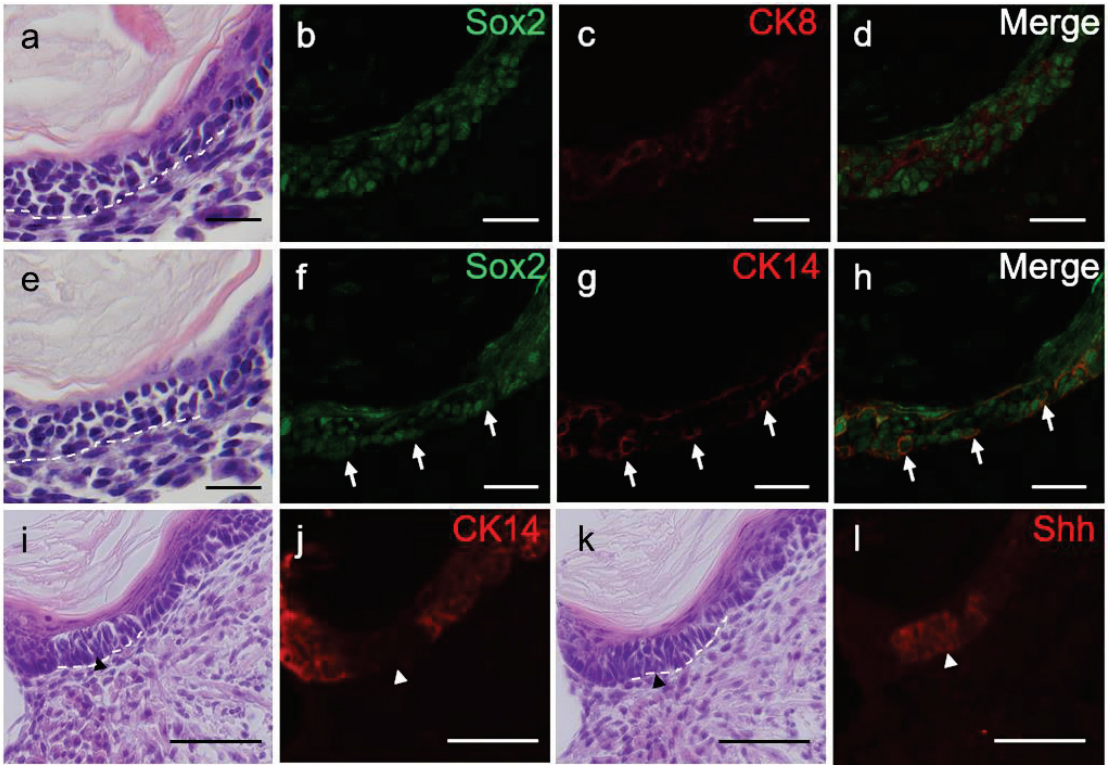

Fig. 6 Taste buds emerged in non-gustarory epithelium surrounding circumvallate papillae in posterior tongues cultured with CHIR99021

a-h: Ectopic eosinophobic epithelial cells expressed Sox2 (**b, f**) and clusters of eosinophobic epithelial cells expressed CK8 (**c**). Almost all eosinophobic cells did not expressed CK14 (**g**) and some cells expressing CK14 were detected (**f-h**, arrows). **i-l**: Shh expression was examined using four serial sections. Ectopic eosinophobic epithelial cells were negative for CK14 (**j**) and expressed Shh (**l**). Scale bar: 20 μm in **a-h**, 50 μm in **i-l.** Dotted line: eosinophobic epithelial areas.

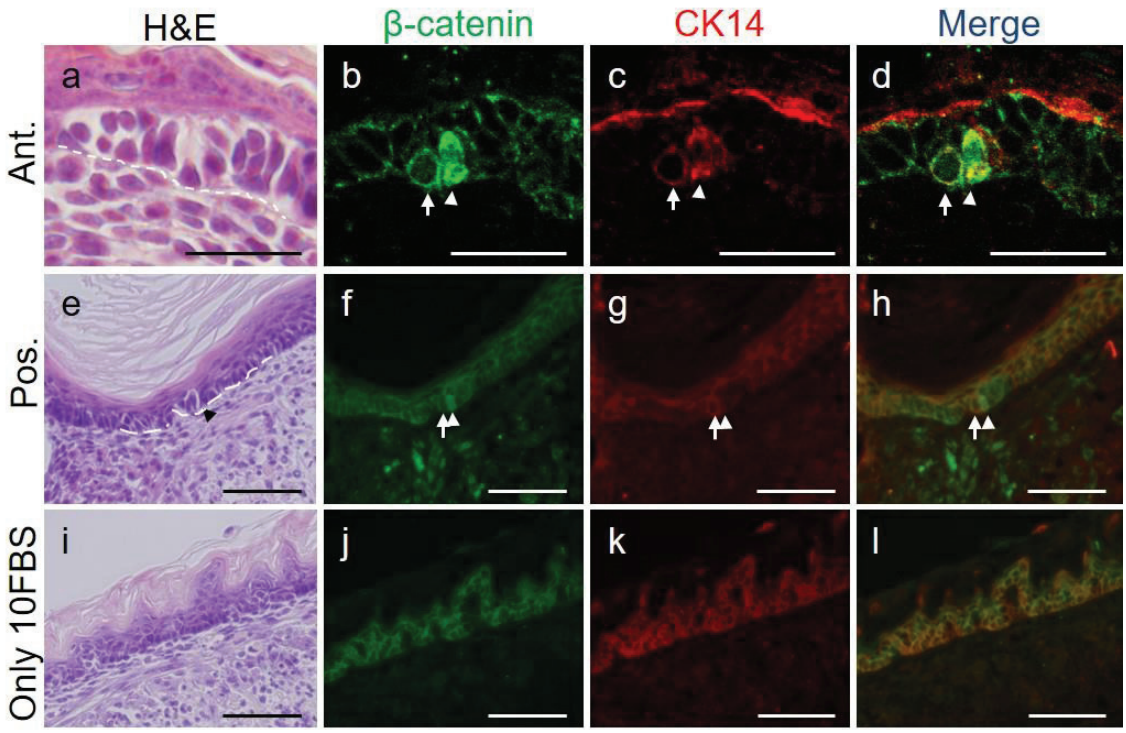

Fig. 7 CHIR99021 caused β-catenin nuclear translocation in eosinophobic epithelial cells

Ant.: A few eosinophobic basal cells expressed CK14 (arrow and arrowhead) (**c**). Weak (arrow) or strong (arrowhead) expression of β-catenin was detected in nuclei (arrowhead) (**b**). **Pos.**: There was a CK14 expressing cell (arrow) (**g**) and β-catenin was strongly localized in nucleus of the neighboring cell (arrowhead). **Only 10FBS**: Anterior tongues were cultured with only 10FBS as control. Scale bar: 20 μm (**a-d**), 50 μm (**e-l**). Dotted line: eosinophobic epithelial areas.

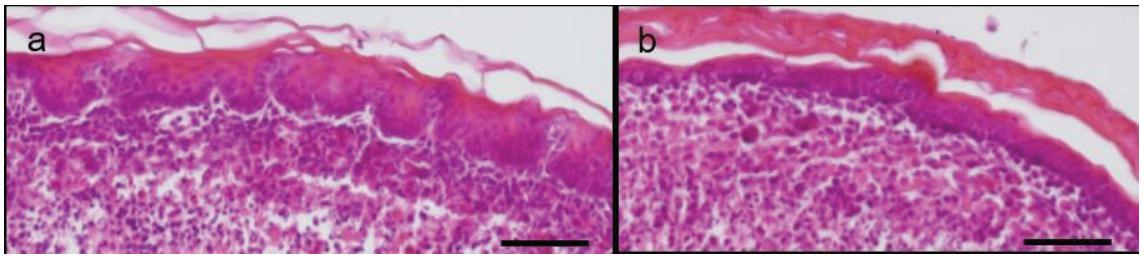

Fig. 8 Inhibition of hedgehog signaling in anterior tongues cultured with or without CHIR99021

The cultured tongues were sectioned and stained with hematoxylin and eosin. **a**: Anterior tongues at E16.5 were cultured with 10 μM cyclopamine. **b**: Anterior tongues were cultured with CHIR99021 and cyclopamine. Scale bar 50 μm

Table 1 Number of filiform papillae and spines

Spine (n=3)		Papilla (n=3)	
10FCS	+CHIR99021	10FCS	+CHIR99021
30.7+/-6.9	8.6+/-2.2*	37.2+/-3.8	2.8+/-1.4**

Anterior tongues at E16.5 were cultured with or without CHIR99021 for 6 days and sectioned. 4 or 5 sections at every 50 μm were selected and number of filiform papillae and spines was counted. Values were presented as average±standard error. A two-tailed Student's t-test was used and the significant level was set at 0.05. *P<0.05, **P<0.01

Table 2 Ratio (%) of length of basal lamina linings covered with eosinophobic basal cells to length of the total basal lamina lining

Sample number	Ratio of eosinophobic cells (%)
1	28.8+/-4.3
2	47.8+/-4.9
3	53.6+/-6.7

Length of basal lamina linings covered with eosinophobic basal cells was measured by tracing and represented by the number of pixel in 4 or 5 sections selected at every 50 μm. Anterior tongues were cultured with CHIR99021 or vehicle (DMSO, controls) for 6 days. No eosinophobic cells were observed in control samples (ratio: 0%). Values were presented as average±standard error.

General discussion

The present study is based on an idea that taste buds could be generated nerve-independently in culture system. It has been considered impossible that taste buds occur morphologically *in vitro*, because of loss of innervation. Therefore, analysis of the developmental mechanisms of taste buds has been performed only *in vivo*. Some studies on the tongue development using organ culture systems are reported. However, none of organ culture methods is established to generate taste buds *in vitro*. The present study focused on the establishment of the culture system that taste buds occur without innervation. Results of tongue organ culture are summarized below in Table 1.

Reciprocal interactions between tissues are important for organ development. The reciprocal interaction and gustatory innervation regulate the development of tongue appendages, therefore, the developmental mechanisms are likely complex than that of other tissues. Tongues development without innervation was analyzed in this thesis as follows; In chapter 1, culture of tongues under kidney capsule; In chapter 2, culture of tongues in *in vitro* organ culture systems and reconstruction of taste buds using TBD cells under kidney capsule; In chapter 3, culture of tongues with forced activation of canonical Wnt signaling in the organ culture system. As a result of this thesis, nerve-independent generation of taste buds is possible in tongue organ culture. Strong

inhibition of GSK3β induces β-catenin nuclear localization and Shh expression in tongue epithelium. β-catenin nuclear localization correlates with cytoskeletal change from CK14 to CK8. Nerve-dependency of morphogenesis of taste buds is distinctive, however, the mechanisms have been unknown. The present study firstly reveals the importance of canonical Wnt signaling in morphogenesis of taste buds without innervation. The result indicates that β-catenin nuclear localization is beginning of the start of development of taste cells at the early stage and maintenance. Therefore, innervation to gustatory epithelium probably plays an important role in inducing β-catenin nuclear localization within innervated epithelial cells. However, the tongue organ culture had never produced an aggregation of fusiform cells like a taste bud although CK8-expressing eosinophobic cells emerged in cluster. Three-dimensional structural analysis and physiological test for taste substances are needed to in future. Because treatment of CHIR99021 extinguished papillary structures in anterior tongues, developmental mechanisms of taste buds of fungiform papillae remains unsolved.

The sense of taste is indispensable for living animals. Vertebrates are able to discriminate better eatable foods with the sense. Humans enjoy eating foods containing more varieties of taste stimuli. Taste perception, as well as other special senses, is subjective, supports to communicate with oneself and sharing what you perceive with

anybody is impossible. Therefore, it is difficult to understand those who suffer taste disorder. For example, cancer patients lose the sense of taste caused by side-effect of cancer treatment. Dietary habit and one's health affect the sense and taste disorder is one of zinc deficiency diseases. However, the mechanisms which lead to difference of the taste sense among each individual is still unexplained well. Generation of taste buds *in vitro* will accelerate the elucidation of the mechanisms of taste sense. Galileo Galilei said "Wherever the senses fail us, reason must step in". The science, which is based on reason, help us better understand the sense of taste, because only the senses of individuals are unable to understand each individual taste sense.

Table 1. Summary of the development of lingual papillae and taste buds

	Graft	Graft DRG	O.C.	O.C. DRG	O.C. CH	O.C. Cy	O.C. CH.Cy
Filiform	○	○	○	○	×	○	×
Fungiform	×	×	○(Matrigel)	×	×	×	×
Circumvallate	×	○	△	○	△	-	-
Taste bud	×	×	×	×	○	×	×

In the present thesis, fetal tongues were cultured in the organ culture system and under kidney capsule. Taste buds occurred and filiform papillae disappeared only in the organ culture system with CHIR99021. Fungiform papillae were detected when fetal tongues were cultured in Matrigel drops. The organ germ culture method suited culturing circumvallate papillae better than grafting under kidney capsule, and DRG influenced their trenches to be elongated.

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