Journal of Anatomy

J. Anat. (2011) **219**, pp710–721 doi: 10.1111/j.1469-7580.2011.01435.x

Development of gustatory papillae in the absence of *Six1* and *Six4*

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Abstract

Six family genes encode homeobox transcription factors, and a deficiency in them leads to abnormal structures of the sensory organs. In a previous paper, *Six1* was reported to be expressed in the taste bud-bearing lingual papillae of mice, and loss of *Six1* affected the development of these gustatory papillae. We show here that embryos lacking both *Six1* and *Six4* revealed more severe abnormalities than those lacking *Six1* alone during morphogenesis of their gustatory papillae. By *in situ* hybridization, *Six4* was shown to be broadly distributed in the epithelium of the lateral lingual swellings at embryonic day (E) 11.5, and in the tongue epithelium, mesenchyme, and muscles at E12.5. From E14, *Six4* was similar in expression pattern to *Six1*, as previously reported. In the fungiform papillae, *Six4* was expressed in the epithelium at E14–E16.5. In the circumvallate and foliate papillae, *Six4* expression was observed in the trench wall of these papillae at E15.5-P0. Although *Six4*-deficient mice had no abnormalities, *Six1/Six4*-deficient mice showed distinct morphological changes: fusion of the lateral lingual swellings was delayed, and the tongue was poorly developed. The primordia of fungiform papillae appeared earlier than those in the wild-type or *Six1*-deficient mice, and the papillae rapidly increased in size; thus fusion of each papilla was evident. The circumvallate papillae showed severe defects; for example, invagination of the trenches started asymmetrically, which resulted in longer and shorter trenches. The foliate papillae elevated initially, and showed stunted trenches. Therefore, *Six1* and *Six4* function synergistically to form gustatory papillae during development of the tongue.

Key words: gustatory papillae; *in situ* hybridization; mouse (*Six4*$^{-/-}$, *Six1*$^{-/-}$*Six4*$^{-/-}$); scanning electron microscopy; *Six4*.

Introduction

In the mammalian tongue, taste buds originate from the epithelium of three different lingual papillae – fungiform, circumvallate, and foliate. On the anterior two-thirds of the dorsal lingual surface in mouse embryos, about 70–100 fungiform papillae form in a stereotypical array. These papillae appear first at embryonic day (E) 12.5 of mouse development as localized epithelial thickenings known as primordia, which show specific expression of signaling molecules such as sonic hedgehog (Shh), bone morphogenic protein (BMP)2, BMP4, and Wnt10b (Hall et al. 1999; Jung et al. 1999; Zhou et al. 2006; Iwatsuki et al. 2007). By E14.5, these primordia begin to evaginate, forming raised papillae with mesenchymal cores. Taste buds arise from the papillary epithelia at approximately E18. Shh functions to maintain the interpapillary space and defines the papilla-free lingual regions (Hall et al. 2003; Mistretta et al. 2003; Liu et al. 2004; Mistretta & Liu, 2006). In cultures of E13 rat tongue, exogenous BMPs or noggin increases the number of papillae, whereas in E14 cultures, BMPs inhibit papillary formation (Zhou et al. 2006). In *Wnt10b* knockout mice, a reduced number and size of papillae are observed (Iwatsuki et al. 2007). The activation of Wnt signaling by the addition of lithium chloride to mouse tongue cultures leads to an increased number and diameter of papillae (Liu et al. 2007). In a previous study, we found that the *Six1* gene is expressed in the epithelium of fungiform papillae and that mice deficient in it show an increased size and number of these papillae and disturbed distribution of them at E14.5

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Accepted for publication *7 September 2011*
Article published online *6 October 2011*

in their tongue (Suzuki et al. 2010a). However, unlike the marker molecules such as *Shh, BMP4,* and *Wnt10b*, *Six1* is not expressed in the primordia, but is later expressed in the papillary epithelium. Therefore, *Six1* is suggested to regulate the formation of fungiform papillae by interacting with *Shh* or other marker molecules. Our previous study also showed that papillae and trenches of the circumvallate and foliate papillae in *Six1*-deficient mice are malformed and that some circumvallate papillae show irregularly shaped trenches (Suzuki et al. 2010a). In mouse tongue cultures, Shh regulates the formation of the trenches, where taste buds and von Ebner's glands develop (Kim et al. 2009). The *Shh* expression pattern in *Six1*-defcient mice suggests that *Six1* probably maintains the expression of *Shh* to form these papillae.

The murine Six gene family, homologous to *sine oculis*, is composed of six members (*Six1–6*). Each gene encodes a Six domain and a Six-type homeodomain, both of which are required for specific DNA binding and cooperative interactions with co-factors (Kawakami et al. 2000). *Six4* expression overlaps with that of *Six1*, but deletion of *Six4* produces no remarkable defect during development (Ozaki et al. 2001). In the development of the olfactory epithelium, *Six1/Six4* double-mutant embryos show more severe abnormalities than *Six1* mutant ones. The olfactory placode fails to form and the initiation of olfactory development is blocked in *Six1/Six4* double-mutant mice (Chen et al. 2009). In *Six1* mutant mice, the olfactory placode forms but exhibits abnormal neurogenesis (Ikeda et al. 2007; Chen et al. 2009; Ikeda et al. 2010). In the developing trigeminal ganglia, a greater number of apoptotic cells are found in *Six1/Six4* double-mutant mice than in *Six1* mutant mice (Konishi et al. 2006). *Six1/Six4*-deficient mice lack kidneys and ureter formation at birth, whereas *Six1*-deficient mice show renal hypoplasia (Kobayashi et al. 2007).

In the present study, we extended our previous work and investigated the expression of *Six4* in the developing gustatory papillae and the morphogenesis of papillae in *Six1/Six4*- and *Six4*-deficient mice. During development of the gustatory papillae in mice, nerve fibers enter at the E14.5 stage and densely innervate the papillary epithelium and mesenchyme (Mbine, 2004). Innervation by lingual gustatory and somatosensory neurons is not required for papillary formation but later development is nerve-dependent (Ito et al. 2010). *Six1*-mutant mice show malformed geniculate and petrosal ganglia during early embryonic development (Zou et al. 2004) but innervation loss is not absolute, as some neurons remain, especially those in the fungiform papillae (Suzuki et al. 2010a). *Six1/Six4* double mutant mice would be expected to display severe loss of innervation. We focused on the morphogenesis of each type of papilla at the initial nerve-independent stage and then at the subsequent nerve-dependent stage.

Materials and methods

Animals

Six4$^{+/-}$ mice were generated as described previously (Ozaki et al. 2001, 2004) and maintained by backcrossing 16–22 generations to C57BL/6 mice. The procedure for generating *Six1*$^{+/-}$*Six4*$^{+/-}$ mice was described earlier (Konishi et al. 2006). Homozygous embryos were obtained by intercrossing male and female heterozygous mice. *Six1*$^{-/-}$ mice (Ozaki et al. 2004) were also used as controls. The animals were housed in an environmentally controlled room in the Center for Experimental Medicine of Jichi Medical University, following the guidelines for animal experiments. All experimental protocols were approved by the Ethics Review Committee for Animal Experimentation of Jichi Medical University.

Tissue preparation

For immunohistochemistry, *in situ* hybridization, and histological observation, pregnant mice were killed by cervical dislocation, and embryos were fixed in 4% paraformaldehyde (PFA) in phosphate-buffered saline (PBS) at 4 °C. Each specimen was washed in PBS, cryoprotected with 25% sucrose, embedded in OCT compound (Tissue Tek; Miles, Elkhart, IN, USA), and frozen in a spray freezer (Oken, Tokyo). The tissues were sectioned sagittally or frontally at a thickness of 8–10 μm in a cryostat. The sections were then collected and placed on silane-coated slides.

Riboprobes and *in situ* hybridization

Sections were washed in PBS and treated for 10 min with 0.2 N HCl and then for 5 min with proteinase K (1 μg mL^{-1}). They were then washed in PBS and refixed for 20 min in 4% PFA. Next, the sections were prehybridized for 1 h at room temperature in a hybridization buffer containing 50% formamide, 1.3 × standard saline citrate (SSC), 5 mM EDTA, 0.5% CHAPS, 0.1% Tween-20, 1% blocking reagent, 100 μg mL^{-1} tRNA, and 50 μg mL^{-1} heparin. Hybridization was performed overnight at 65 °C in a hybridization buffer containing antisense riboprobe. For the control, other sections were hybridized with sense riboprobe. The hybridized sections were washed at 65 °C in 2 × SSC for 1 h and thereafter in 0.1 × SSC for 1 h. After a wash in PBS, they were incubated with a 1% concentration of blocking reagent (Roche Diagnostics, Mannheim, Germany) for 1 h at room temperature and subsequently incubated overnight at 4 °C with alkaline-phosphatase-conjugated anti-digoxigenin Fab fragments (Roche) diluted 1 : 500 in PBS. After three washes in PBS, chromogenic reactions were carried out by using nitro-blue tetrazolium/5-bromo-4-chrolo-3-indolyl-phosphate (Roche). We used the cDNA of the following genes as *in situ* hybridization probes: *Shh* were provided by N. Obara (Health Sciences University of Hokkaido), and *Six4* (Suzuki et al. 2010b).

Immunohistochemistry and histology

Prior to immunostaining, the sections were reacted with a protein-blocking reagent (Dako, Glostrup, Denmark) for 20 min at room temperature. For double-staining, sections were incubated overnight at 4 °C with the following commercially

obtained primary antibodies; anti-PGP9.5 polyclonal sheep antibody (Ultraclone, Wellow, UK), and anti-Sox2 polyclonal rabbit antibody (Chemicon, Temecula, CA, USA) each at a dilution of 1 : 100. After rinsing in PBS, the sections were incubated for 2 h at room temperature with one of the following secondary antibodies: donkey anti-sheep IgG labeled with Alexa Fluor 488 or donkey anti-rabbit IgG labeled with Alexa Fluor 568 (Molecular Probes, Eugene, OR, USA) at a dilution of 1 : 200. Some sections were incubated with anti- PGP9.5 polyclonal rabbit antibody (Ultraclone) and then goat anti-rabbit horseradish peroxidase (Dako). For fluorescence microscopic observation, the sections were washed in PBS, mounted on slides with PermaFluor (Thermo, Pittsburgh, PA, USA), and viewed with a Leica confocal laser scanning microscope. For light microscopic examination, the sections were visualized using diaminobenzidine tetrahydrochloride and counterstained with methyl green. Paraffin-embedded sections were routinely stained with hematoxylin and eosin (H.E.).

Scanning electron microscopy (SEM)

Tongues were fixed in 4% PFA in PBS and further incubated for 2 h in a mixture of 2% glutaraldehyde and 2% PFA in 0.1 M phosphate buffer at 4 °C. After washing with PBS, the specimens were post-fixed with 1% OsO_4 for 1 h at 4 °C. The specimens were then washed with distilled water, dehydrated and dried in CO_2 by the critical point method. Tissues were mounted, sputter-coated with gold/palladium, and examined under a Hitachi S-650 scanning electron microscope.

Quantification of taste papillae

Quantification of fungiform papillae was made in accordance with Zhou et al. (2006). SEM images of E14.5 and E19 whole tongues of wild-type mice, *Six4*$^{-/-}$, and *Six1*$^{-/-}$*Six4*$^{-/-}$, and of E12.5 tongue of wild-type mice and *Six1*$^{-/-}$*Six4*$^{-/-}$ at 70 × original magnification were used. For comparison, E12.5 and E19 whole tongues of *Six1*$^{-/-}$ were also used. Each papilla was defined as a rounded structure, raised from the surface of the tongue. The papillae were marked on a plastic overlay positioned over photographs, and their number was counted. SEM images at 200 × original magnification were used for measuring the diameters of the papillae. For each papilla, the diameter along the longest and shortest axis of each papilla was measured and averaged to calculate the diameter. For circumvallate papillae, SEM images of E14.5 and E19 ones at ×200 original magnification were used. For tongues, SEM images of E12.5 and E14.5 tongues at ×70, and of E19 tongues at ×25 magnification were used. The area of circumvallate papillae and tongues was measured using SCION image (NIH, Washington, DC).

For measurement of the height of fungiform papillae and depth of trenches of circumvallate and foliate papillae, each papilla in H.E.-stained tissue sections was photographed. The height of fungiform papillae was measured from the upper surface to the base of the epithelium of E14.5 and E19 wild-type mice and *Six1*$^{-/-}$*Six4*$^{-/-}$. The depth of trenches was measured from the upper surface to the base of the trench wall of E19 wild-type mice and *Six1*$^{-/-}$*Six4*$^{-/-}$. Quantification was performed on seven tongues at E14.5 and on four tongues at E12.5, and E19 for wild-type mice, *Six4*$^{-/-}$, *Six1*$^{-/-}$*Six4*$^{-/-}$, and *Six1*$^{-/-}$ mutants. SYSTAT 11 (Hulinks, Tokyo) was used for statistical analysis. Differences between groups were tested for significance by one-way analysis of variance (ANOVA), with a *P*-value of < 0.05. The Tukey *post-hoc* test was used to identify which group differences within the analysis were significant.

Results

Expression of *Six4* in the developing tongue and taste papillae

At E11.5 of C57BL/6 mouse embryos, the lateral lingual swellings first appeared in the mandible. *Six4* mRNA was expressed in their epithelium (Fig. 1A). Three lingual swellings, i.e. the tuberculum impar and lateral lingual swellings, fused together to form a spatula-shaped tongue at E12.5. At this stage, a weak *Six4* expression was observed in the dorsal epithelium and muscles of the tongue (Fig. 1B). The expression of *Six4* in tongue muscles was observed until E17.5 (not shown).

Fungiform papillae

At E13–E13.5, small mounds had formed on the dorsal surface of the tongue. A papillary structure was found in the epithelium, which structure developed into an arch-like one at E14. *Six4* was expressed on the surface of these papillae (Fig. 1C). At E16.5, *Six4* expression was weak and limited to the papillary epithelium (Fig. 1D). By E18.5, *Six4* was not expressed in any of the fungiform papillae.

Circumvallate papillae

A shallow invagination of thickened epithelium was observed in the center of the posterior region of the tongue at E13.5. *Six4* was expressed in the entire oral epithelium including the surface of the thickened epithelium, but was not detected in the invaginated area (Fig. 1E). At E14.5, a dome-shaped epithelial elevation, a papilla, was observed. The epithelial stalks had begun to invaginate into the underlying mesenchyme to form trenches. At E15.5, discernible trenches constituting the inner and outer trench walls were formed. Strong expression of *Six4* was observed in the trench epithelium (Fig. 1F). Invagination of the trenches became deeper at E18.5 and the primordium of von Ebner's glands appeared near the bottom of the trenches. *Six4* expression was restricted to the bottom of the trenches and to von Ebner's glands (Fig. 1G). By postnatal day 0, *Six4* expression had almost disappeared.

Foliate papillae

At E15.5, trenches were formed on both sides of the lingual margins of the posterior region of the tongue by epithelial invaginations into the mesenchyme. *Six4* was expressed in the trench epithelia, and weakly expressed in the corresponding dorsal epithelium (Fig. 1H). Each papilla consisted of four trenches and corresponding epithelial elevations (Fig. 5E). At E18.5, the expression of *Six4* was limited to the

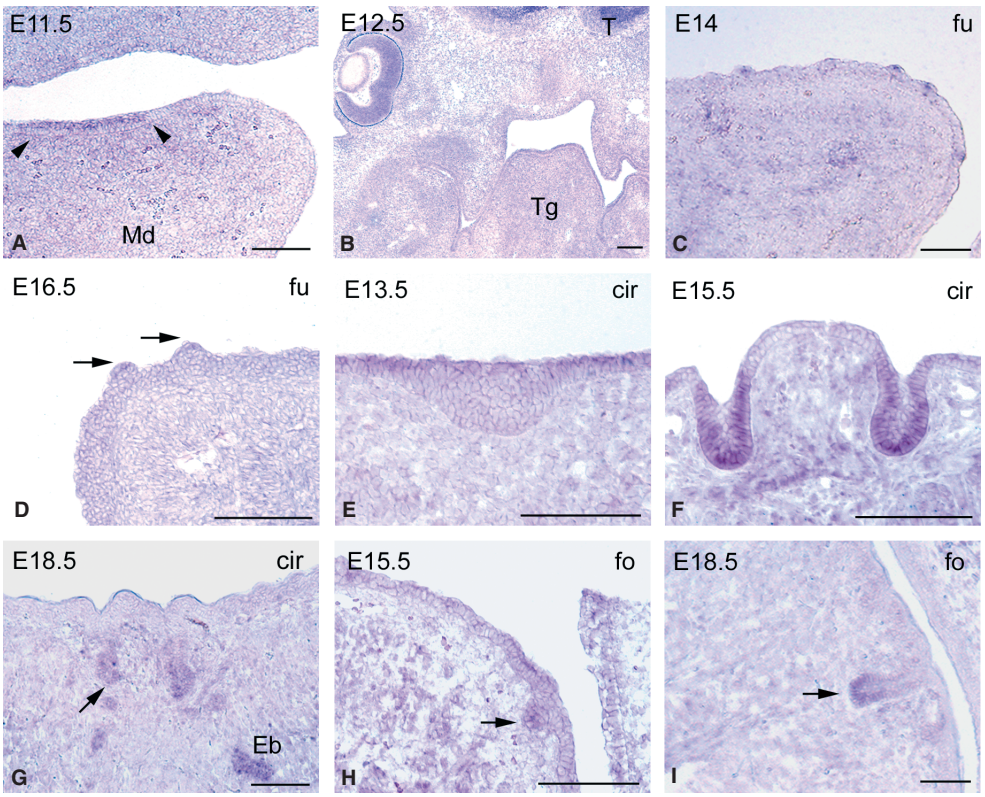

Fig. 1 Expression of *Six4* in the developing head (A,B), fungiform papillae (fu, C,D), circumvallate papillae (cir, E–G), and foliate papillae (fo, H,I), as examined by *in situ* hybridization. (A) Sagittal section of an E11.5 head; epithelial expression of *Six4* is seen in the lateral lingual swellings (arrowheads) in the mandible (Md). (B) E12.5; *Six4* is expressed in the epithelium and muscles of the tongue (Tg). Strong expression is seen in the eye and telencephalon (T). (C) E14; *Six4* is expressed in the apical epithelium of fungiform papillae. (D) E16.5; Higher magnification. *Six4* is seen in the papillary epithelium (arrows). (E) E13.5; circumvallate papilla appears as an epithelial thickening. *Six4* is expressed in the surrounding epithelium and in the surface of the thickening. (F) E15.5; *Six4* is expressed in the invaginating trenches but not in the papillary epithelium. (G) E18.5; intense staining for *Six4* in the bottom of trenches and in von Ebner's glands (Eb). (H) E15.5; *Six4* is expressed in the trenches (arrow). (I) E18.5; intense staining for *Six4* in the bottom of trenches (arrow). Scale bars: 100 μm.

bottom of the trenches (Fig. 1I). At same stage, von Ebner's glands also appeared and *Six4* was expressed in them (not shown). Sections incubated with sense riboprobe displayed no reactivity (not shown).

Morphological defects in *Six4*$^{-/-}$-, and *Six1*$^{-/-}$*Six4*$^{-/-}$-mice

Previous studies showed that *Six1*$^{-/-}$ embryos exhibit abnormal morphogenesis of their gustatory papillae (Suzuki et al. 2010a) but that *Six4*$^{-/-}$ ones appear normal (Ozaki et al. 2001). To examine whether *Six1* and *Six4* play redundant roles in the morphogenesis of the gustatory papillae, we compared the morphological consequences for papillary development at E14.5 and E19 in mice lacking *Six4* alone or at E12.5, E14.5, and E19 in those lacking both *Six1* and *Six4*.

Tongue

SEM observation at E12.5 showed the spatula-shaped tongue in the wild-type mice (Fig. 2A). *Six1*$^{-/-}$*Six4*$^{-/-}$ embryos showed a fewer fused- and shorter tongue and mandibular processes (Fig. 2B) compared with the wild-type mice. However, the area of the tongue did not differ between the mutant and the wild-type mice (Fig. 5A). Also, *Six1*$^{-/-}$ embryos had a shorter tongue, but the mandibular processes were similar to those of the wild-type mice (Fig. 2C). At E14.5, the tongue of *Six4*$^{-/-}$ embryos was similar to that the wild-type mice (Figs 3A,B and 5A), but that of *Six1*$^{-/-}$*Six4*$^{-/-}$ was small and malformed (Figs 3C and 5A). At E19, the tongues of *Six1*$^{-/-}$*Six4*$^{-/-}$ were much smaller than those of the wild-type mice (Figs 3N,Q and 5A), and their tips were firmly fixed in the mandible (Fig. 3Q). *Six1*$^{-/-}$ mice exhibited fewer defects than *Six1*$^{-/-}$*Six4*$^{-/-}$ mice (Figs 3O and 5A).

Fungiform papillae

At E12.5, the primordia of fungiform papillae, seen as slight and small epithelial elevations, appeared on the tip of the tongue in *Six1*$^{-/-}$*Six4*$^{-/-}$ embryos (Fig. 2B), but these structures were not clearly observed in the wild-type embryos (Fig. 2A), *Six1*$^{-/-}$ (Fig. 2D) or their wild-type mice (Fig. 2C).

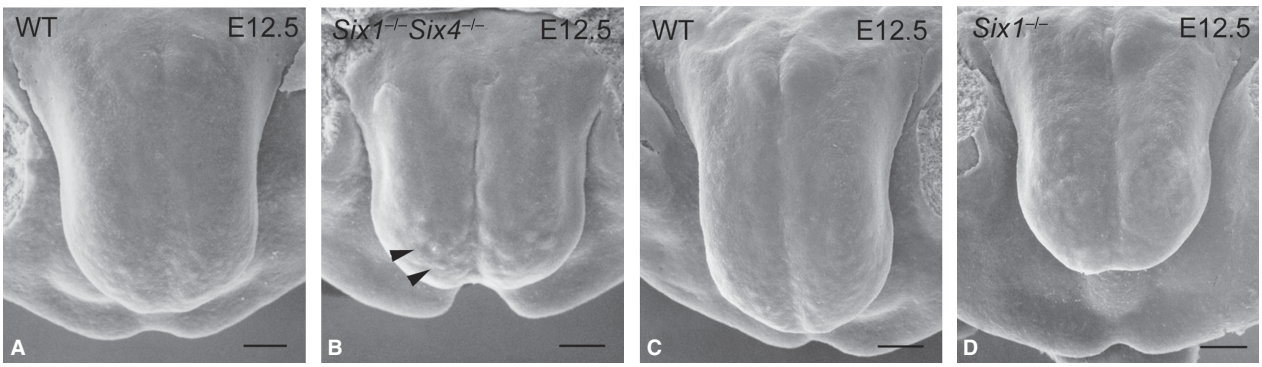

Fig. 2 Tongue of *Six1/Six4*- and *Six1*-deficient mice. (A–D) E12.5; Scanning electron-microscopic (SEM) images of the dorsal surface of the tongue of wild-type: WT (A, $Six1^{+/+}Six4^{+/+}$; C, $Six1^{+/+}$); $Six1^{-/-}Six4^{-/-}$ (B); and $Six1^{-/-}$ (D) mouse embryos. Note a less fused and shorter tongue and mandibular processes in $Six1^{-/-}Six4^{-/-}$ (B). Shorter tongue is seen in $Six1^{-/-}$ (D). Slight elevation of fungiform papillae primordia is seen in the apex of the $Six1^{-/-}Six4^{-/-}$ tongue (B, arrowheads). Scale bars: 100 μm.

At E14.5, fungiform papillae were arranged in a specific pattern, in linear rows running anterioposteriorly in the wild-type embryos (Fig. 3A) and in the $Six4^{-/-}$ embryos as well (Fig. 3B). An intermolar eminence, a papilla-free area, was evident in the posterior region of the tongue from both mice. The size of $Six1^{-/-}Six4^{-/-}$ papillae was increased compared with that of the wild-type ones (Figs 3C and 5D). The average diameter of papillae was 28.0 ± 0.9 μm for $Six1^{-/-}Six4^{-/-}$ embryos vs. 22.4 ± 1.1 μm for the wild-type ones (Fig. 5D). In the $Six4^{-/-}$ tongue, the diameter of papillae was 21.9 ± 1.5 μm, which did not differ significantly from that of the wild-type embryos (21.8 ± 1.8 μm; Fig. 5D). The number of papillae was 92.3 ± 9.4 for $Six1^{-/-}Six4^{-/-}$ vs. 80.9 ± 10.9 for the wild-type mice (Fig. 5C). However, this difference was not significant. In $Six4^{-/-}$, the papilla number was 78.4 ± 13.7, which did not differ significantly from that of wild-type embryos (78.1 ± 12.3; Fig. 5C). The height of papillae was 40. 6 ± 8.1 μm for the wild-type embryos vs. 50.2 ± 8.5 μm for $Six1^{-/-}Six4^{-/-}$ embryos. The interpapillary space of the $Six1^{-/-}Six4^{-/-}$ tongue was smaller than that of the wild-type tongue. Some papillae in the median sulcus had fused into a cluster (Fig. 3D). This pattern was conserved also at E15.5 (Fig. 3J). Papilla marker molecules such as *Shh* were more widely expressed in the $Six1^{-/-}Six4^{-/-}$ papillae than in the wild-type ones at E12.5 (Fig. 3E,F), but became focused in the papillary epithelium at E13.5 (not shown). Nerve fibers were observed in the core of fungiform papillae in both the wild-type (Fig. 3G) and $Six4^{-/-}$ specimens (Fig. 3H), but not in the $Six1^{-/-}Six4^{-/-}$ ones (Fig. 3I) at E14.5.

At late embryonic stages, many filiform papillae appeared among the fungiform papillae. At E17.5, the fungiform papillae of $Six1^{-/-}Six4^{-/-}$ were smaller than those of the wild-type mice (Fig. 3K,L). At E19, the fungiform papillae of $Six1^{-/-}Six4^{-/-}$ were significantly smaller in diameter (24.8 ± 0.7 μm) than those of their wild-type counterpart (43.0 ± 2.8 μm; Figs 3P,Q and 5D). In the $Six4^{-/-}$ tongue, the number of papillae was 99.2 ± 7.4 and did not differ significantly from that in the wild-type mice (97.0 ± 3.5). The papillary diameter was 45.5 ± 3.0 μm, again similar to the wild-type value (43.6 ± 4.1 μm). In the wild-type tongue, fungiform papillae increased in number (100 ± 6.3) compared with those at E14.5; whereas the $Six1^{-/-}Six4^{-/-}$ tongue showed a drastic decrease in papillary number from E14.5 to E19 (Fig. 5C). At E19, fungiform papillae in $Six1^{-/-}Six4^{-/-}$ showed more severe defects than those in $Six1^{-/-}$ (Fig. 3O); Most of the papillae were small and decreased in height, and thus the shape became like that of filiform papillae (Fig. 3N). Only some papillae could be distinguished from the filiform papillae (Fig. 3Q, inset). In the wild-type mice, fungiform papillae were well-developed and clearly elevated among the filiform papillae (Fig. 3M,P, inset).

Circumvallate papillae

At E13.5, the epithelial thickening in the center of the posterior tongue invaginated into the mesenchyme in the wild-type (Fig. 4A) and $Six1^{-/-}Six4^{-/-}$ (Fig. 4B) embryos. At E14.5, the dome-shaped papillae were formed; and abundant nerve fibers entered into the core of wild-type papillae (Fig. 4C). However, in the $Six1^{-/-}Six4^{-/-}$ specimens, the deeply invaginated epithelial thickenings were observed, but the nerve fibers were absent (Fig. 4D). Innervation in the $Six4^{-/-}$ papillae was similar to that in the wild-type ones (not shown). SEM images at this stage showed a single papilla in wild-type, $Six1^{-/-}Six4^{-/-}$, and $Six4^{-/-}$ tongues (Fig. 4E–H). It was calculated to be the same size in $Six4^{-/-}$ and wild-type tongues (45.2 ± 9.0 and 48.9 ± 5.5 μm^2, respectively; Fig. 5B). In the $Six1^{-/-}Six4^{-/-}$, the papillary area was 34.0 ± 9.0 μm^2 (Fig. 5B); however, this value was not significantly different from that for the wild-type embryo. The papillae in $Six1^{-/-}Six4^{-/-}$ specimens were less elevated than those in the wild-type and $Six4^{-/-}$ ones. The trench invagination was observed surrounding the papillae in the wild-type (Fig. 4E) and $Six4^{-/-}$ (Fig. 4H) tongues, but it started asymmetrically in $Six1^{-/-}Six4^{-/-}$ (Fig. 4F,G). At this

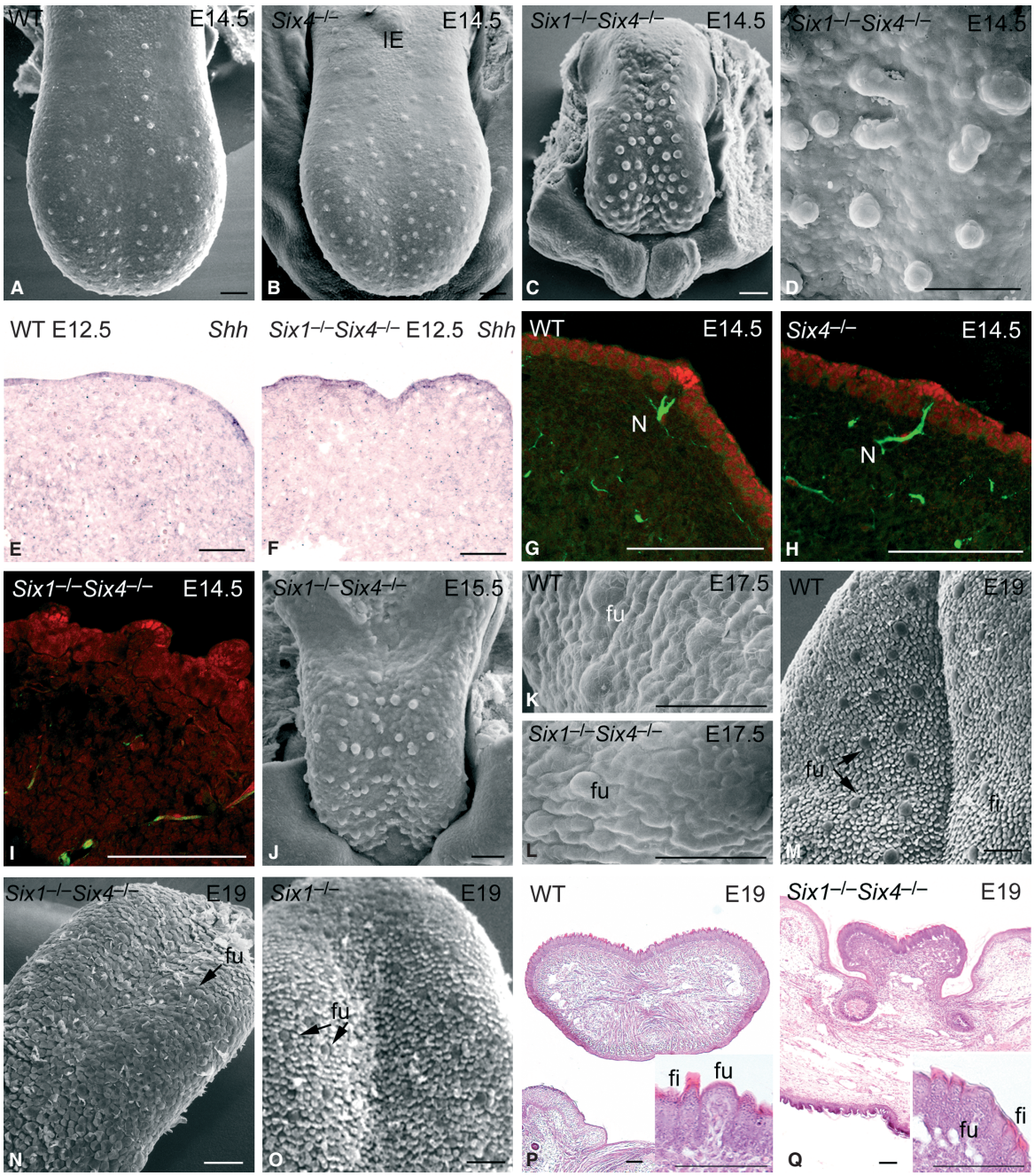

Fig. 3 Fungiform papillae of *Six4-*, *Six1-*, and *Six1/Six4*-deficient mice during development. (A–D, J–O) SEM images of the dorsal surface of the tongue of wild-type (WT, A, K, M), $Six1^{-/-}Six4^{-/-}$ (C, D, J, L, N), $Six4^{-/-}$ (B) and $Six1^{-/-}$ (O) mouse embryos. (A–D) E14.5; fungiform papillae are clearly visible anterior to the intermolar eminence (IE in B), and those in $Six1^{-/-}Six4^{-/-}$ are larger (C) than those in the wild-type (A, $Six1^{+/+}Six4^{+/+}$) and $Six4^{-/-}$ (B) embryos. (D) Higher magnification of fused papillae in $Six1^{-/-}Six4^{-/-}$. Similar pattern of papillary arrangement is seen at E15.5 (J). (E,F) Expression of *Shh* in wild-type (E) and $Six1^{-/-}Six4^{-/-}$ (F) embryos assessed by *in situ* hybridization. *Shh* is expressed in the primordial epithelium at E12.5. The primordia in $Six1^{-/-}Six4^{-/-}$ (F) are more elevated and broader than those in the wild-type embryo (E). (G–I) Double labeling immunohistochemistry with anti-Sox2 antibody (red) and anti-PGP 9.5 antibody (green) at E14.5. Nerve fibers (N) enter the fungiform papillae in wild-type (G) and $Six4^{-/-}$ (H) embryos, but there are very few in $Six1^{-/-}Six4^{-/-}$ (I). (K,L) E17.5; fungiform papillae (fu) develop in the wild-type embryo (K), but those in $Six1^{-/-}Six4^{-/-}$ (L) are smaller in size than those in the wild-type one. (M–O) E19; fungiform papillae (fu) are evident among numerous filiform papillae (fi) in the wild-type tongue (M), but are small and are embedded among filiform papillae in the $Six1^{-/-}Six4^{-/-}$ one (N). Tongue and fungiform papillae in the $Six1^{-/-}$ (O) are smaller than those of wild-type, but are larger than those $Six1^{-/-}Six4^{-/-}$. (P,Q) E19; anterior region of the tongue and mandible of wild-type (P) and $Six1^{-/-}Six4^{-/-}$ (Q) embryos stained with hematoxylin and eosin (H.E.). Insets, higher magnification of fungiform (fu) and filiform (fi) papillae. Scale bars: 100 μm.

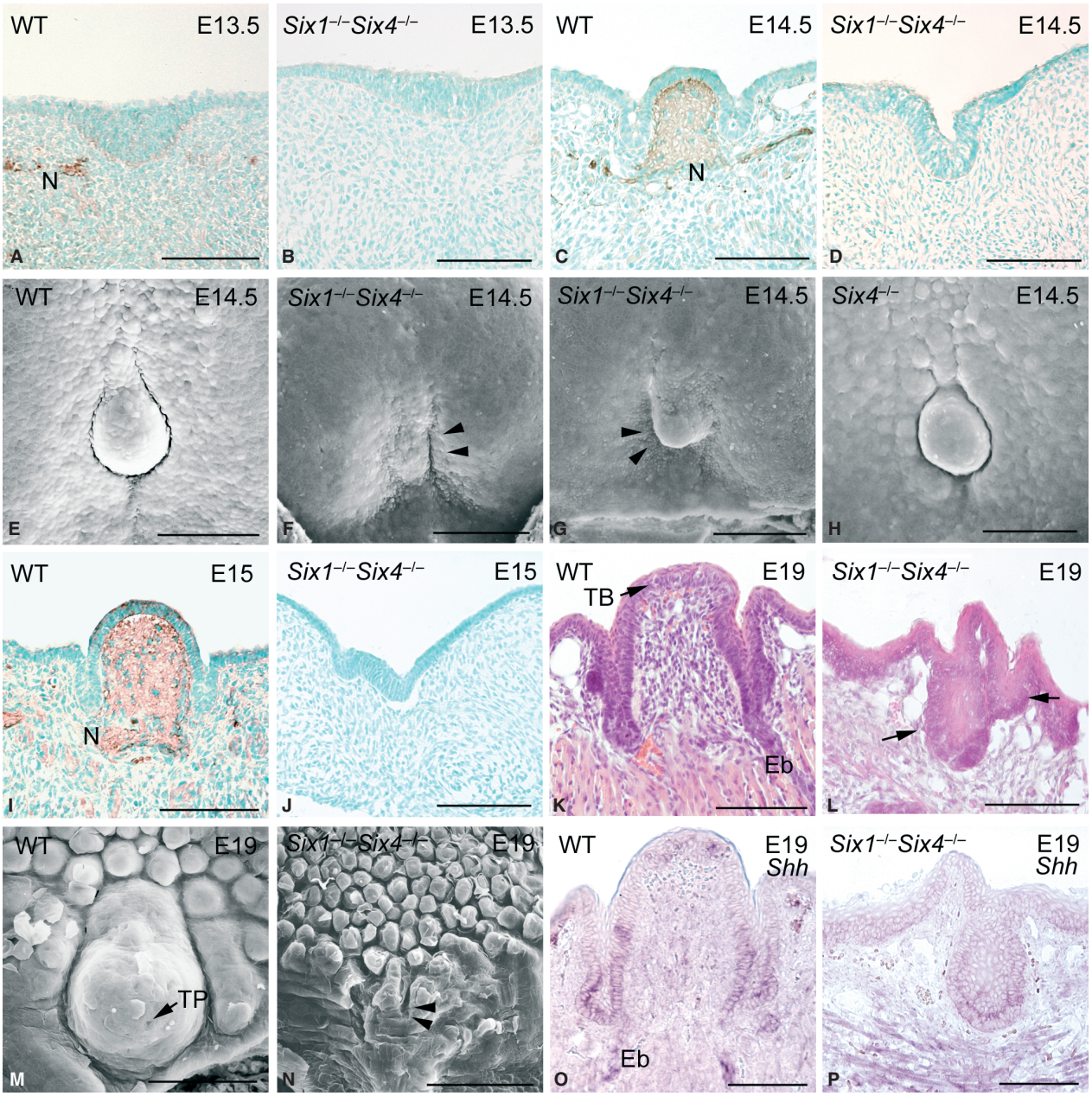

Fig. 4 Circumvallate papillae of *Six4*- and *Six1/Six4*-deficient mice during development. (A–D,I,J) Immunohistochemistry with anti-PGP9.5 antibody (brown) in E13.5 (A, B), E14.5 (C,D), and E15.5 (I,J) specimens. Nuclei (blue) are stained with methyl green. Nerve fibers (N) are seen in the wild-type (WT, A,C,I), but not in the *Six1*$^{-/-}$*Six4*$^{-/-}$ (B,D,J) embryo. Elevation of papillae is slower in *Six1*$^{-/-}$*Six4*$^{-/-}$ (D,J) than in the wild-type (C,I). SEM images of the wild-type (WT, E), *Six1*$^{-/-}$*Six4*$^{-/-}$ (F,G), and *Six4*$^{-/-}$ (H) papillae at E14.5. Dome-shaped papillae and surrounding trenches are seen in the wild-type (E) and *Six4*$^{-/-}$ (H) specimens. Asymmetrical invagination of trenches (arrowheads, F,G) and malformed papillae are seen in *Six1*$^{-/-}$*Six4*$^{-/-}$ (F,G). (K–P) E19; H.E.-stained sections (K,L), SEM images (M,N), and *Shh* expression detected by *in situ* hybridization (O,P). Papillae are well developed and round in shape in the wild-type (K, M). Taste buds (TB, K) and their pore (TP, M) are seen in the papillary surface. Well developed trenches and von Ebner's glands are seen in the bottom of trenches (Eb, K). *Six1*$^{-/-}$*Six4*$^{-/-}$ have malformed, very small papillae (L,N), and asymmetrically extended trenches (arrows, L; arrowheads, N). *Shh* expression is seen in the taste buds, trenches, and von Ebner's glands in the wild-type embryo (O) and in the bottom of trenches in the *Six1*$^{-/-}$*Six4*$^{-/-}$ ones (P). Scale bars: 100 μm.

stage, some had elevated papillae (Fig. 4G), but others did not (Fig. 4F). At E15.5, the papillae developed; and numerous nerve fibers were seen both in the epithelium and mesenchyme in the wild-type embryo (Fig. 4I). However, the *Six1*$^{-/-}$*Six4*$^{-/-}$ papillae were malformed; and no nerve fibers were seen in them (Fig. 4J). By E19 in the wild-type tongue, the trenches developed and von Ebner's glands extended from the bottom of the trenches. Also, taste buds were pres-

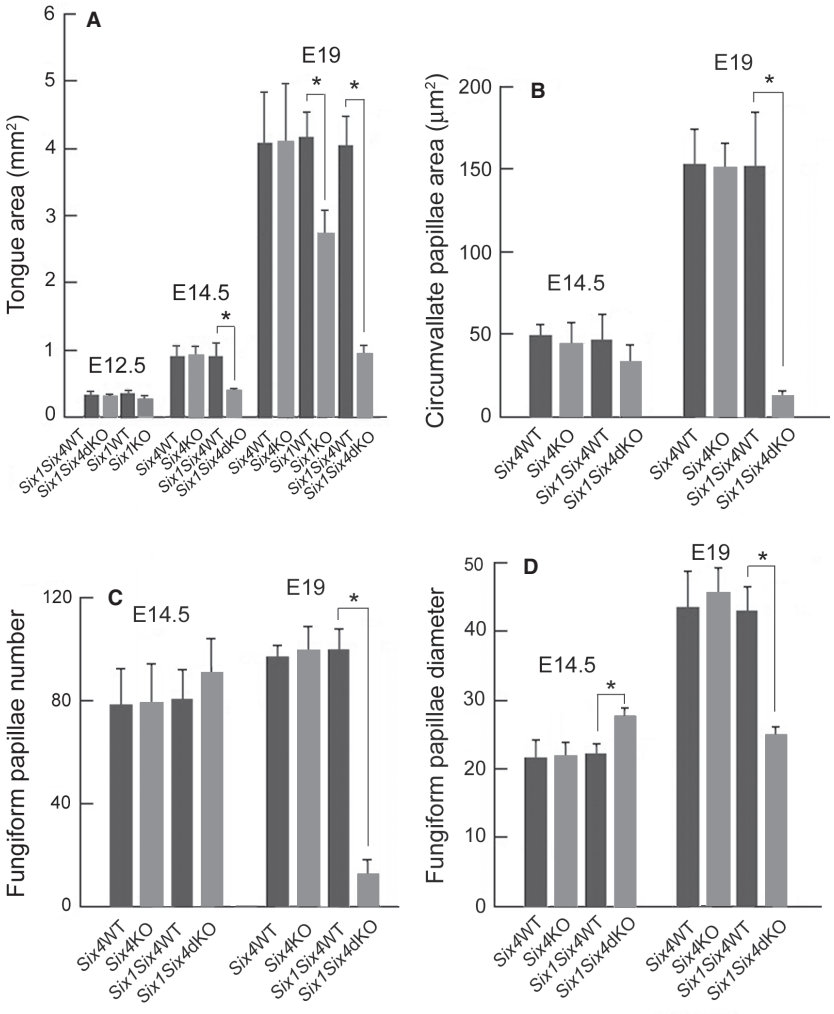

Fig. 5 Comparison of the area of the whole tongue (A), area of circumvallate papillae (B), and total number (C) and diameter (D) of fungiform papillae in the wild-type (WT), *Six4*$^{-/-}$ (KO), *Six1*$^{-/-}$ (KO) and *Six1*$^{-/-}$*Six4*$^{-/-}$ (dKO) embryos at E12.5, E14.5, and E19 (A) or E14.5 and E19 (B–D). Counts for quantification of whole tongue and papillae were taken from SEM images. Each bar presents the mean and SD. *Statistically significant difference at $P < 0.05$.

ent in the surface of the papillae (Fig. 4K). In contrast, the *Six1*$^{-/-}$*Six4*$^{-/-}$ tongue showed asymmetric extension of epithelial cords into the connective tissue, thus resulting in irregular trench length. The trench epithelium was thicker than that in the wild-type as well (Fig. 4L). The depth of the longer trenches in *Six1*$^{-/-}$*Six4*$^{-/-}$ specimens was 138.9 ± 5.1 μm; and that in the wild-type ones 148.6 ± 9.9 μm. SEM images showed that the papillae with taste pores were round-shaped, and the trenches were visible on each side in the wild-type papillae (Fig. 4M); whereas in the *Six1*$^{-/-}$*Six4*$^{-/-}$ tongue, the papillae were very small and taste pores were absent (Fig. 4N). The papillary area determined from SEM images was12.4 ± 3.4 μm^2 in *Six1*$^{-/-}$*Six4*$^{-/-}$ embryos and 152 ± 27.2 μm^2 in the wild-type ones (Fig. 5B). There was no difference between *Six4*$^{-/-}$ and the wild-type areas (151.0 ± 12.3 μm^2 vs. 153.6 ± 16.9 μm^2). At this stage, innervation was totally absent in *Six1*$^{-/-}$*Six4*$^{-/-}$ (not shown). The expression of *Shh*, a marker of trenches, von Ebner's glands, and taste buds (Fig. 4O), was observed in the bottom of the longer trenches in *Six1*$^{-/-}$*Six4*$^{-/-}$ embryos (Fig. 4P), but not in the shorter ones (not shown).

Foliate papillae

SEM examination showed epithelial elevations that appeared in the lateral margin of the posterior tongue in the wild-type embryo at E14.5 (Fig. 6A). In *Six1*$^{-/-}$*Six4*$^{-/-}$ specimens, these epithelial elevations were not obvious, but papillae elevations were observed at this stage (Fig. 6B). At E15.5, in frontal sections, papillae and trenches were observed in both the wild-type (Fig. 6C) and *Six1*$^{-/-}$*Six4*$^{-/-}$ (Fig. 6D) tongues. Nerve fibers entered into the mesenchymal core of the wild-type papillae (Fig. 6C), but not into that of the *Six1*$^{-/-}$*Six4*$^{-/-}$ ones (Fig. 6D). At E17.5, SEM observation showed that the papillae and trenches were visible as a row of four arranged in an anterior-to-posterior direction (Fig. 6E,F). In the *Six1*$^{-/-}$*Six4*$^{-/-}$ tongue, the size of papillae was much reduced (Fig. 6F). By E19, the trenches had extended deeply into the mesenchyme, and von Ebner's glands formed in the wild-type tongue (Fig. 6G). In the *Six1*$^{-/-}$*Six4*$^{-/-}$ tongue, the papillae were malformed and the trenches were devoid of glands (Fig. 6H). Also, they were shorter than the wild-type ones (66.6 ± 6.1 μm in *Six1*$^{-/-}$*Six4*$^{-/-}$ and 88.6 ± 7.5 μm in the

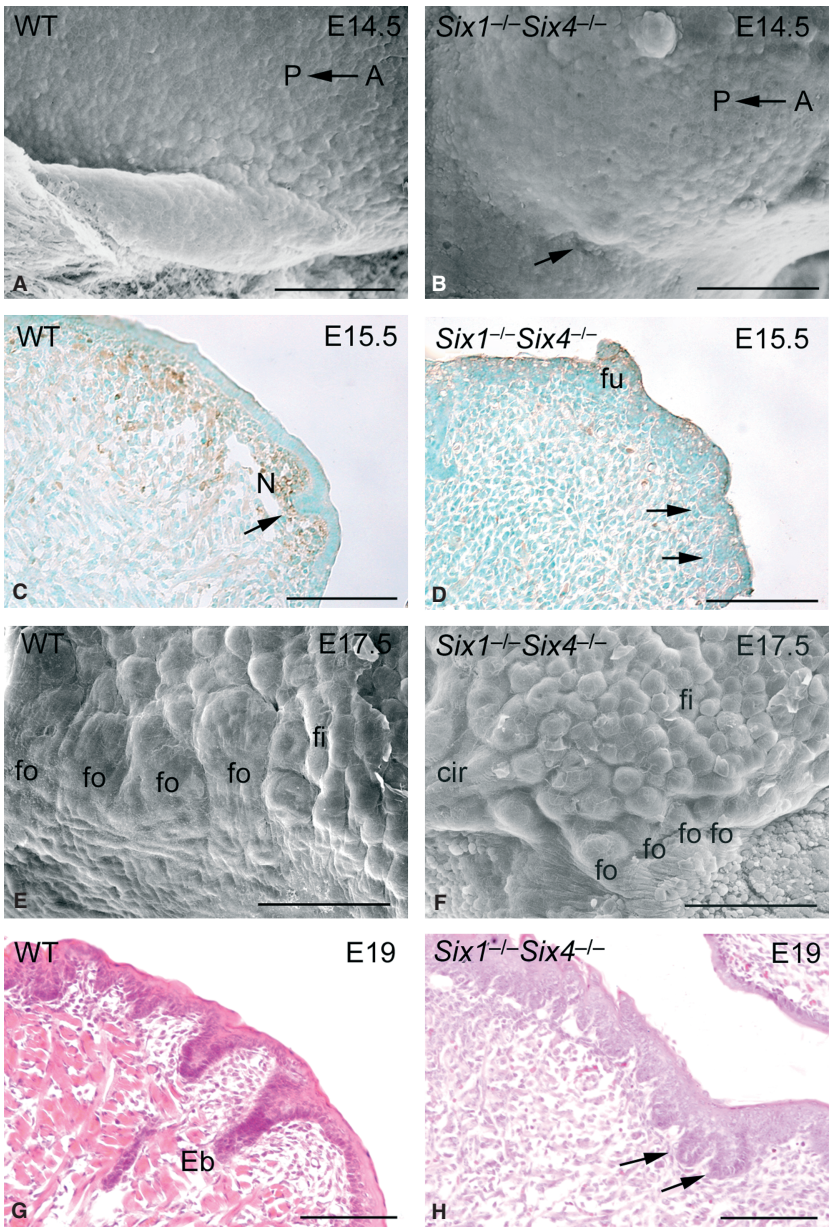

Fig. 6 Foliate papillae of *Six1/Six4*-deficient mice during development. (A,B) SEM images of wild-type (WT, A) and $Six1^{-/-}Six4^{-/-}$ (B) embryos at E14.5. (A) Epithelial elevations extend from the anterior to the posterior (P ← A) margin of the dorsal surface of the tongue. $Six1^{-/-}Six4^{-/-}$ lacks the epithelial elevations and papillae are seen in the margin of the tongue (B, arrow). Immunohistochemistry with anti-PGP9.5 antibody (brown) at E15.5. Trenches (arrows) and papillae are visible in both wild-type (C) and $Six1^{-/-}Six4^{-/-}$ (D) tongues. Nuclei (blue) are stained with methyl green. Nerve fibers (N) are seen among the trenches in the wild-type, but not in the $Six1^{-/-}Six4^{-/-}$ specimen. (E,F) SEM images of wild-type (E) and $Six1^{-/-}Six4^{-/-}$ (F) at E17.5. Four rows of papillae (fo) are visible in both wild-type and $Six1^{-/-}Six4^{-/-}$ embryos. (G,H) H.E.-stained sections at E19 show well developed trenches with von Ebner's glands (Eb) in the wild-type (G) and shorter trenches in $Six1^{-/-}Six4^{-/-}$ (arrows, H) embryos. Papillae are less elevated and the interpapillary distance is smaller in $Six1^{-/-}Six4^{-/-}$. In the lamina propria, the amount of muscle fibers is much reduced and von Ebner's glands are absent (H). cir, circumvallate papillae; fi, filiform papillae; fu, fungiform papillae. Scale bars: 100 μm.

wild-type). Throughout the stages, there was no difference between $Six4^{-/-}$ and wild-type embryos.

Discussion

Expression pattern of *Six4* in the developing gustatory papillae

Using *in situ* hybridization, the present study showed for the first time the expression of *Six4* in the gustatory papillae of developing mice. From E14 to late gestation stages, *Six4* expression was similar to that of *Six1* (Suzuki et al. 2010a) and it was observed in the epithelium of fungiform papillae, in the trenches of circumvallate and foliate papillae and in von Ebner's glands. Even in the adult, *Six1* and *Six4* are co-expressed in type-II cells in the taste buds (Suzuki et al. 2010b). At earlier stages than E14, e.g. E11.5, *Six4* was expressed in the epithelium of lingual lateral swellings. In this region, *Six1* expression is restricted to the mesenchyme (Suzuki et al. 2010a). At E12.5, weak *Six4* expression was observed in the tongue epithelium, mesenchyme, and muscles, whereas *Six1* expression was limited to the tongue muscles at this stage (Suzuki et al. 2010a). The broad expression of *Six4* in the entire tongue epithelium at E12.5 was also observed at E13.5 in the posterior region. Broader expression of *Six4* compared with *Six1* in early developmental stages is also reported to occur in the tooth germs (Nonomura et al. 2010). Moreover, similar to *Six1*, *Six4* expression was not specific to the primordial epithelium of fungiform papillae at E12.5. Other signaling

molecules, e.g. *Shh*, *Wnt10b*, and *BMP4*, are expressed in the primordium at E12.5 and in the fungiform papillae at E14.5 (Hall et al. 2003; Liu et al. 2004, 2007; Mistretta & Liu, 2006; Zhou et al. 2006; Iwatsuki et al. 2007). With regard to circumvallate and foliate papillae, although *Six1* was expressed weakly in the epithelial thickenings and papillary epithelium from E13.5 to E15.5 (Suzuki et al. 2010a), expression of *Six4* was not obvious in these papillary regions, being restricted to the trench epithelium. *Six4* may function together with *Six1* for trench formation rather than for papillary formation.

Possible role of *Six1* and *Six4* in morphogenesis of gustatory papillae

Six1/Six4-deficient mice are characterized by general muscle hypoplasia. Also, the mandible bone is drastically shortened and jugal, nasal, and premaxilla bones are absent (Grifone et al. 2005). Therefore, malformation of the tongue at E14.5 and E19 in these embryos may have been due to these reductions in intrinsic tongue muscle and mandible bone. As to the formation of lingual papillae, $Six1^{-/-}Six4^{-/-}$ embryos showed more severe defects than $Six1^{-/-}$ ones, as is the case in the development of the kidney (Kobayashi et al. 2007), olfactory epithelium (Chen et al. 2009), and trigeminal ganglion (Konishi et al. 2006), whereas *Six4*-deficient mice showed no such anomalies. The formation of fungiform papillae was observed to be earlier in $Six1^{-/-}Six4^{-/-}$ than wild-type or in $Six1^{-/-}$ mice. The elevation of primordia appeared in the tip at E12.5, and increased in size rapidly by E14.5. However, due to the presence of fused papillae, papillae number was not significantly different from that in the wild-type mice. The appearance of fused papillae was more prominent in $Six1^{-/-}Six4^{-/-}$ than in $Six1^{-/-}$ (Suzuki et al. 2010a). The molecular events leading to the formation of papillae might have occurred earlier than E12.5. *Shh*-expression in $Six1^{-/-}Six4^{-/-}$ primordia was broader than that in the wild-type ones. The mesenchymal *Six1* (Suzuki et al. 2010a) and epithelial *Six4* at E11.5 may function synergistically in initial tongue patterning. They may also interact with other key molecules that are expressed in the primordia; e.g. Wnt/β-catenin signaling and Shh are key molecules in the formation and patterning of fungiform papillae (Mistretta & Liu, 2006). Modulation of Shh signaling alters the number of papillae and their spatial distribution (Hall et al. 2003; Liu et al. 2004). Augmentation of Wnt/β-catenin signaling increases both the number and size of fungiform papillae in tongue cultures (Liu et al. 2007).

In the morphogenesis of circumvallate and foliate papillae, a clear difference between double-mutant and the wild-type papillae was noted with respect to the invagination of trenches, especially in the circumvallate papillae. In the former, it started asymmetrically at E14.5, which resulted in the irregular length of trenches seen at E19. Our present study is the first to show the asymmetrical formation of trenches of the circumvallate papillae. In our previous study, a few $Six1^{-/-}$ showed irregularly shaped trenches, but most of them did not (Suzuki et al. 2010a). Although *Six4* mutant mice appear normal, *Six4* and *Six1* may function synergistically to form a uniform length of trenches, where the numerous taste buds originate from their epithelia. Also, the length of trenches in the circumvallate papillae is changed when Shh is over-expressed or inhibited in *in vitro* organ cultures (Kim et al. 2009). *Shh* is also suggested to regulate cell proliferation, resulting in invagination of the epithelium and differentiation of von Ebner's glands (Lee et al. 2006; Kim et al. 2009). In the present study, *Shh*-expressing trenches were the longer ones, not the shorter ones.

On the other hand, our data showed that the initial papillary formation of the circumvallate and foliate papillae in the double-mutant was similar to that in the $Six1^{-/-}$ mutant; i.e. the papillary elevation was delayed. Even at E15, these papillae were hollow as they are in the *Six1* mutant (Suzuki et al. 2010a). In the formation of the foliate papillae, initial papillary elevation was observed in the $Six1^{-/-}Six4^{-/-}$ embryos as in $Six1^{-/-}$ embryos (Suzuki et al. 2010a). Other genes controlled by *Six1* and *Six4* might affect the papillary formation; in fact, a very recent report showed that *Fgf10* and *Sprouty* determine the initial size and number of the papillae during the formation of the circumvallate papillae (Petersen et al. 2011).

Moreover, at late embryonic stages the number and size of fungiform papillae, the size of circumvallate and foliate papillae, and the length of trenches of circumvallate and foliate papillae are influenced by the nerve supply. Smaller and sparser fungiform papillae compared with the wild-type ones were reported to occur in mice deficient in brain-derived neurotrophic factor (BDNF) and neurotrophin (NT), viz. $BDNF^{-/-}NT\text{-}4^{-/-}$ (Ito & Nosrat, 2009) and $BDNF^{-/-}NT\text{-}3^{-/-}$ (Nosrat et al. 2004; Ito et al. 2010), along with various degrees of geniculate ganglion cell loss. In $BDNF^{-/-}NT\text{-}3^{-/-}$, some neurons remain, one-quarter to one-third of the total number, in the cranial ganglia at P0 (Liebl et al. 1997; Ma et al. 2009). $Six1^{-/-}Six4^{-/-}$ embryos had very few neurons in their fungiform papillae, and thus these embryos showed a more severe decrease in the number and size of papillae at E17.5 and at E19 compared with $Six1^{-/-}$ (Suzuki et al. 2010a) and $BDNF^{-/-}NT\text{-}3^{-/-}$ (Ito et al. 2010) embryos. Also, the small papillae and shorter trenches in the circumvallate and foliate papillae were more obvious in the double-mutants than in $Six1^{-/-}$. $Six1^{-/-}Six4^{-/-}$ embryos lacked neurons in their circumvallate and foliate papillae. The circumvallate papillae were one-seventh to one-eighth of the surface area in $Six1^{-/-}Six4^{-/-}$ at E19. Malformed circumvallate papillae with short trenches were reported in mice with poor nerve supply (Zhang et al. 1997; Nosrat et al. 2004; Ito & Nosrat, 2009; Ito et al. 2010). *BDNF/NT3*-deficient mice at P0 have circumvallate papillae that are

one-fifth of the size of those of wild-type mice (Ito et al. 2010). Therefore, defects in *Six1*$^{-/-}$*Six4*$^{-/-}$ at late embryonic stages can be explained by the absence of or scant nerve supply.

Concluding remarks

Our findings demonstrate that *Six1* and *Six4* play redundant roles in the development of the gustatory papillae. *Six1*$^{-/-}$*Six4*$^{-/-}$ showed more prominent features than *Six1*$^{-/-}$ in terms of the abnormal morphogenesis of fungiform, circumvallate, and foliate papillae. This study also clarified that *Six* genes regulate the trench formation of circumvallate papillae independently of other molecules or of influence of innervation.

Acknowledgements

We are grateful to Nobuko Obara for kindly providing the materials listed in the text. This study was supported in part by HAITEKU from the Ministry of Education, Culture, Sports, Science, and Technology of Japan.

Author contributions

Y.S. designed experiments. Y.S., K.I. and K.K. carried out all experiments. Y.S. wrote the manuscript. Y.S., K.I. and K.K. discussed the results. All authors read and approved the final manuscript.

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