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ORIGINAL PAPER

Immunohistochemical localization of Pax6 in the developing tooth germ of mice

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Abstract Recent studies have reported that supernumerary teeth were observed in the maxillary incisor area in several *Pax6* homozygous mutant mouse and rat strains. To date, it remains unknown whether Pax6 is expressed during tooth development in any species. The study aimed to analyze the expression of Pax6 during mouse incisor and molar development. C57BL/6J mouse embryos on days E12.5, E13.5, E14.5, E16.5 and E18.5 were produced. Heads from these embryos, as well as from P1.5 mice, were processed for paraffin wax embedding ($N \geq 3$ for each stage) and prepared for immunohistochemistry. Pax6 immunostaining was found in all tooth germs examined. At the E12.5 dental placode, E13.5 bud stage, E14.5 cap stage and E16.5 early bell stage, Pax6 was expressed in ectodermally derived tissues of tooth germs and oral epithelia adjacent to the tooth germs. Cells in the underlying dental ectomesenchyme that showed Pax9 expression were Pax6 negative. At E18.5 and P1.5, Pax6 was expressed in more differentiated ameloblasts and cells of the stratum intermedium and stellate reticulum that were derived from the oral epithelium, as well as in mesenchyme-derived differentiated odontoblasts. Pax6 expression was also observed in the submandibular gland, tongue filiform papilla and hair follicle at E16.5 and P1.5. The present study demonstrated that Pax6 was expressed in incisor and molar germs during mouse tooth development. The results provide a basis for exploring the function of Pax6 during tooth development.

Keywords Pax6 expression · Odontogenesis · Immunohistochemistry · Mice

Introduction

Teeth are vertebrate organs whose formation and morphogenesis are strictly regulated by the sequential and reciprocal interactions between cells of the odontogenic epithelium and the cranial neural crest-derived mesenchyme. The processes underlying tooth development are under close genetic control. Many biological molecules, such as growth factors, transcription factors and extracellular matrix molecules, are involved in regulating tooth morphogenesis and cell differentiation (Du et al. 2012; Feng et al. 2012; Hou et al. 2012; Tang et al. 2013; Thesleff 2006; Thesleff and Mikkola 2002; Zhang et al. 2012). However, supernumerary teeth, which form in addition to the normal complement found within a dentition, can occur (Cobourne and Sharpe 2010; D'Souza and Klein 2007; Fleming et al. 2010). In humans, these teeth are relatively common, occurring as an isolated finding in up to 5.3 % of the population or, less commonly, presenting as a feature of a wider developmental disorder (Wang and Fan 2011). They can present either singly or in multiples, unilaterally or bilaterally and within either the maxilla or mandible. Most of these supernumerary teeth are located in the maxillary incisor area (McDonald 2004). Compared with the mechanisms underlying the development of individual teeth, much less is known about how tooth number is controlled within the jaw.

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Mouse and rat models have provided insights into the complex genetics of tooth development in human dentition. Recent studies, and our previous work, showed that supernumerary teeth were observed in the maxillary incisor area in several *Pax6* homozygous mutant (*Pax6−/−*) mouse and rat strains (Kaufman et al. 1995; Kriangkrai et al. 2006; Lei et al. 2008; Quinn et al. 1997). In terms of tooth numbers in regions of the upper and lower molars, and lower incisors, no difference was found between wild-type and homozygous mutant mouse fetuses (Kaufman et al. 1995; Kriangkrai et al. 2006; Lei et al. 2008; Quinn et al. 1997). These findings indicated that Pax6 might be expressed during tooth development and play a role in controlling tooth number of the upper incisors. In *Pax6* homozygous mutant rats, the supernumerary upper incisors were caused by a facial cleft between the medial nasal and maxillary processes, resulting in failure of the fusion of the two primary dental placodes and the maintenance of the two distinct primary dental placodes (Kriangkrai et al. 2006). However, in *Pax6−/−* mice, the cleft was not found, and the etiology of supernumerary upper incisors has not been characterized. To date, it is not known whether Pax6 is actually expressed during tooth development in any species.

The *PAX6/Pax6* gene, a member of paired-box gene family, encodes a highly conserved transcriptional regulatory protein containing the paired and homeobox DNA-binding domains, and is essential for tissue development, including the eyes, central nervous system and endocrine glands of vertebrates and invertebrates (Simpson and Price 2002). Homozygous mutations cause early postnatal lethality and fetuses with developmental defects of the eye, nose, brain and pancreas (Hill et al. 1991; Hogan et al. 1986; Sander et al. 1997; Walther et al. 1991). Heterozygous mutation leads to aniridia in humans, and microphthalmia in mice and rats, as well as abnormal glucose metabolism and defective processing of proinsulin (Glaser et al. 1992; Jordan et al. 1992; Wen et al. 2009). The expression and function of Pax6 in optic and other organs is described in the literature in great detail.

The mammalian *Pax* gene family comprises nine members that encode DNA-binding, transcriptional regulatory proteins. *Pax9*, another member of the *Pax* gene family, belongs to a different subgroup from Pax6 (Underhill 2000). To the best of our knowledge, *Pax9* is the only member of *Pax* gene family shown to be involved in tooth development. In the mandibular arch mesenchyme, the expression of Pax9 marks the prospective sites of tooth development before any morphological signs of odontogenesis and is maintained in the developing tooth mesenchyme thereafter. In *Pax9*-deficient mouse embryos, tooth development is arrested at the bud stage (Peters et al. 1998). *Pax9* mutations in humans are involved in familial nonsyndromic oligodontia (Stockton et al. 2000; Klein et al. 2005; Suda et al. 2011).

The importance of *Pax6* gene for embryogenesis has been emphasized by existence of classical mouse mutants and human congenital malformations associated with mutations within *Pax6* gene. However, it is currently not known whether Pax6 is actually expressed during tooth development. The objective of this study was to analyze the temporal and spatial expression of Pax6 during mouse tooth development using immunohistochemistry.

Materials and methods

Animals and tissue preparation

The Peking University Animal Ethics Committee approved the study (approval number: LA2009-002). All animal experiments were performed according to the guidelines of Peking University Animal Ethics Committee.

Wild-type mouse embryos were obtained from timed matings of mice on a C57BL/6J background. The middle of the day following the detection of a vaginal plug was designated as embryonic day 0.5 (E0.5), and the middle of the day of birth was taken as postnatal day 0.5 (P0.5). Pregnant female mice were sacrificed by cervical dislocation on days E12.5, E13.5, E14.5, E16.5 and E18.5. Embryos were dissected from the uterus into cold phosphate-buffered saline (PBS, pH 7.4). Heads from these embryos, as well as from P1.5 (1.5-day old) mice, were fixed in 4 % paraformaldehyde in PBS overnight at 4 °C and then processed for paraffin wax embedding ($N \geq 3$ for each stage). Tissues were serially sectioned at a thickness of 4 μm in the sagittal or coronal plane, and prepared for immunohistochemistry.

Antibodies

The following antibodies were used for immunohistochemical experiments on paraffin embedded sections: rabbit anti-Pax6 polyclonal antibody (Millipore, CA, USA) and rat anti-Pax9 monoclonal antibody (Abcam, Cambridge, MA, USA).

Immunohistochemistry

After deparaffinization and rehydration, sections were boiled in a microwave for 20 min in Tris–EDTA buffer (pH 9.0) for antigen retrieval. Immunostaining was performed using the Polink-2 plus® Polymer HRP Detection System For Rabbit Primary Antibody (Zhongshan Golden Bridge Biotechnology, Beijing, China) and Polink-1 HRP Detection System for Rat Primary Antibody (Zhongshan Golden Bridge Biotechnology, Beijing, China), respectively, according to the manufacturer's instructions, with minor adjustment. Firstly, slices were treated with 3 % hydrogen peroxide (H_2O_2) solution for 10 min at room

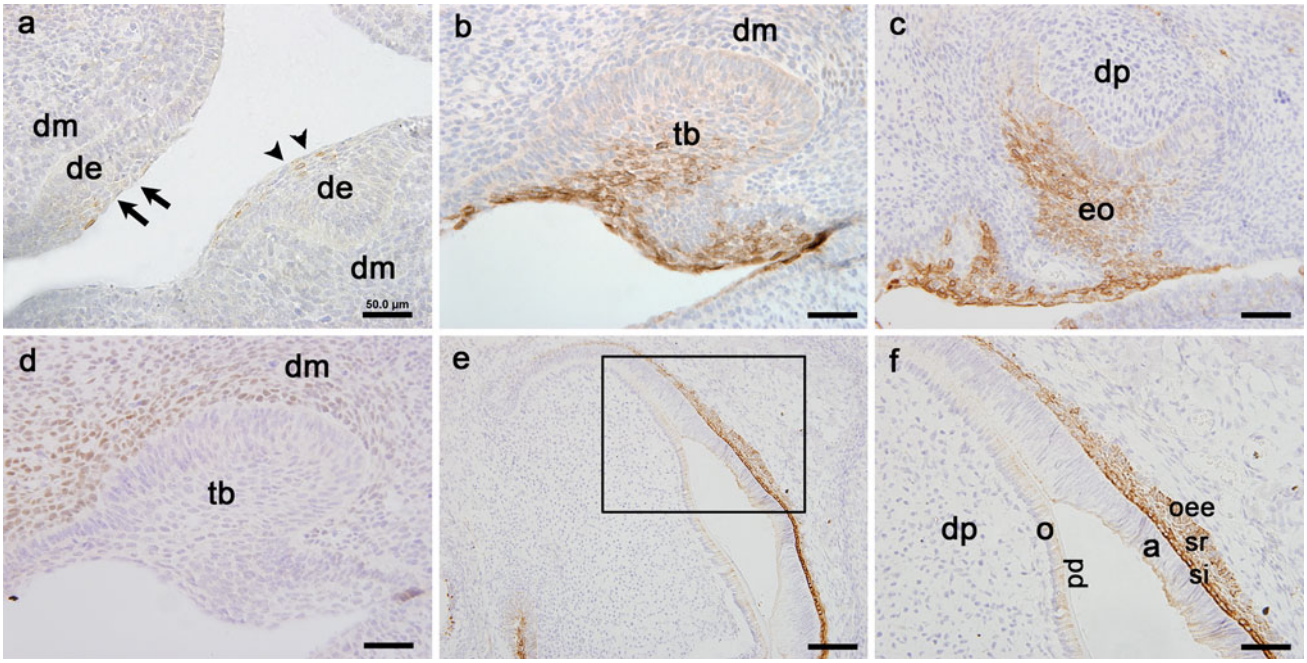

Fig. 1 Immunohistochemical localization of Pax6 in mouse upper incisor germs. **a** A subset of Pax6-positive cells was restricted to the more superficial and central part of the dental epithelium of the upper incisor germs (*arrows*) and lower incisor germs (*arrowheads*) at E12.5. **b**, **c** At E13.5 and E14.5, Pax6 was expressed in the central parts of tooth buds/enamel organs and in oral epithelia adjacent to the tooth germs at the late bud stage (**b**) and the cap stage (**c**). **d** At E13.5, Pax9 was expressed in the dental mesenchyme, and was absent from the tooth bud. **e** At P1.5, Pax6 was expressed in upper incisor germs. The *boxed area* in (**e**) is shown at higher magnification in (**f**). **f** Pax6 was expressed in morphologically distinct ameloblasts and odontoblasts, and was downregulated in those differentiating cells. Cells of the stratum intermedium, stellate reticulum and outer enamel epithelium exhibited strong Pax6 expression. *Scale bar* 50 µm (**a**–**d**, **f**), 100 µm (**e**). *a* ameloblasts, *de* dental epithelium, *dm* dental mesenchyme, *dp* dental papilla, *eo* enamel organ, *o* odontoblasts, *oee* outer enamel epithelium, *pd* pre-dentin, *si* stratum intermedium, *sr* stellate reticulum, *tb* tooth bud

temperature to block the endogenous peroxidase activity and subsequently incubated with the rabbit anti-Pax6 polyclonal antibody (1:500) or rat anti-Pax9 monoclonal antibody (1:250) overnight at 4 °C. Secondly, slices were incubated with Polymer Helper and poly-HRP anti-Rabbit IgG, or detection reagent for anti-rat IgG, respectively, for 30 min each at 37 °C. Immunostaining positive cells were then visualized using diaminobenzidine tetrahydrochloride solution (Zhongshan Golden Bridge Biotechnology, Beijing, China). Finally, the sections were counterstained with hematoxylin and mounted. The sections were washed three times for 5 min in PBS following each incubation step.

The eyes and nose tissue in the section served as positive controls for the Pax6 antibody. Negative control tests were carried out using PBS instead of primary antibody to establish the specificity of the immunostaining.

Results

Expression of Pax6 during mouse incisor development

At E12.5, tooth germs formed from the dental lamina, and the mesenchyme cells condensed around the epithelial buds. A subset of weakly-stained cells was restricted to the thickened dental epithelium in the upper incisor (Fig. 1a). Analysis of serial sections revealed that these Pax6-positive cells were localized in more superficial and central parts of the dental epithelium (Fig. 1a). At the late bud stage (E13.5) and the cap stage (E14.5), Pax6 was expressed more strongly in the central parts of the tooth buds/enamel organs and in the oral epithelia adjacent to the tooth germs (Fig. 1b, c). From E12.5 to E14.5, cells in the underlying dental ectomesenchyme that expressed Pax9 were Pax6 negative (Fig. 1a–d and data not shown).

At E16.5, the incisor germs developed to the late bell stage. The expression of Pax6 was found in the incisor germs at E16.5, E18.5 and P1.5 (Fig. 1e and data not shown). Cells in the inner enamel epithelium and dental papilla cells facing the inner enamel epithelium showed a polarized high columnar appearance, indicating distinct ameloblastic and odontoblastic differentiations (Fig. 1f). Pax6 was easily detectable in differentiated ameloblasts and was downregulated in differentiating ameloblasts. Cells of the stratum intermedium, stellate reticulum and outer enamel epithelium exhibited strong Pax6 expression (Fig. 1f). Interestingly, Pax6 was expressed in mesenchyme-derived odontoblasts. The intensity of Pax6 in

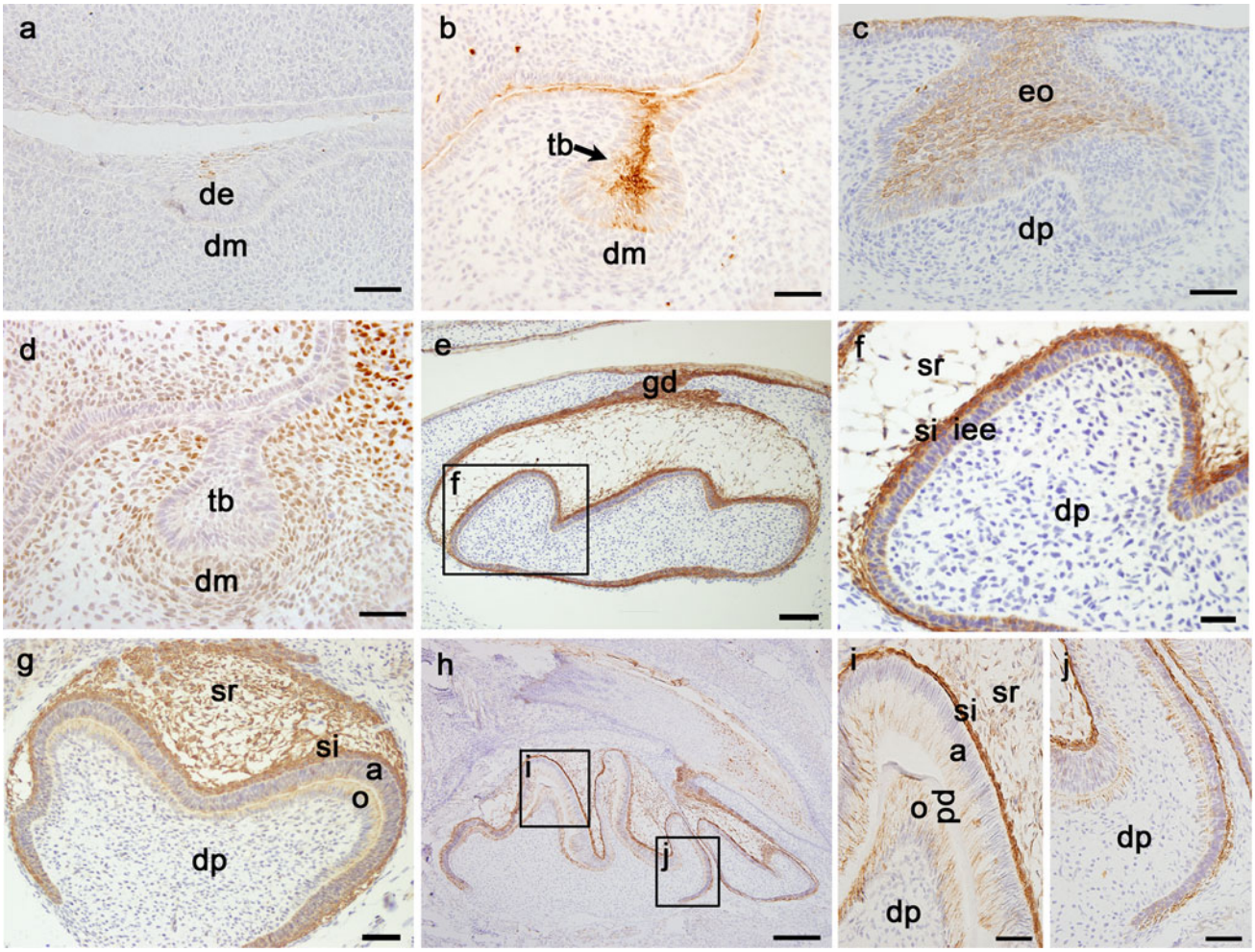

Fig. 2 Immunohistochemical localization of Pax6 in mouse first lower molar germs. **a** A subset of Pax6-positive cells was restricted to the more superficial and central part of the dental epithelium at E12.5. **b**, **c** At E13.5 and E14.5, Pax6 was expressed in the central parts of tooth buds (*arrow*)/enamel organs and in the oral epithelia adjacent to the tooth germs at the late bud stage (**b**) and the cap stage (**c**). A few cells at the tip of the tooth bud, adjacent to the dental mesenchyme, were Pax6-positive (**b**). **d** Pax9 was expressed in the dental mesenchyme at E13.5. **e**, **g** At the early bell stage (E16.5) and the late bell stage (E18.5), Pax6 was expressed in the first molar germs and gubernaculum dentis. The *boxed area* in (**e**) is shown at higher magnification in (**f**). **f** Pax6 was expressed in cells of the stratum intermedium and stellate reticulum, while the inner enamel epithelium cells showed weak staining. **h** At P1.5, Pxax6 was expressed in the first and second lower molar germs, gubernaculum dentis and oral epithelia. The *boxed areas* in (**h**) are shown at higher magnification in (**i**) and (**j**). **g**, **i** Pax6 was expressed in morphologically distinct ameloblasts and odontoblasts, and cells of the stratum intermedium and stellate reticulum. **g**, **j** Pax6 antigenicity gradually diminishing toward the cervical aspects of the dental germ. *Scale bar* 50 μm (**a–d**, **g**, **i**, **j**), 100 μm (**e**), 40 μm (**f**), 200 μm (**h**). *a* ameloblasts, *de* dental epithelium, *dm* dental mesenchyme, *eo* enamel organ, *gd* gubernaculum dentis, *iee* inner enamel epithelium, *o* odontoblasts, *dp* dental papilla, *pd* pre-dentin, *si* stratum intermedium, *sr* stellate reticulum, *tb* tooth bud

odontoblasts was in agreement with the expression of Pax6 in ameloblasts (Fig. 1e, f).

The expression of Pax6 in the lower incisors was similar to that in the upper incisors at different developmental stages (Fig. 1a and data not shown).

Expression of Pax6 during mouse molar development

At E12.5, a few weakly-stained cells were localized in more superficial and central part of the dental epithelium in molar tooth germs (Fig. 2a). At the bud stage (E13.5) and cap stage (E14.5), Pax6 was expressed in the central parts of the tooth buds/enamel organs and in the oral epithelia adjacent to the tooth germs (Fig. 2b, c). Additionally, at E13.5, a few cells at the tip of the tooth bud adjacent to the dental mesenchyme in the lower molar were Pax6-positive (Fig. 2b). From E12.5 to E14.5, cells in the underlying dental ectomesenchyme that expressed Pax9 were Pax6 negative (Fig. 2a–d and data not shown). At the early bell stage (E16.5) and late bell stage (E18.5), Pax6 was expressed strongly in the first molar germs (Fig. 2e, g). At E16.5, Pax6 was expressed in cells of the gubernaculum

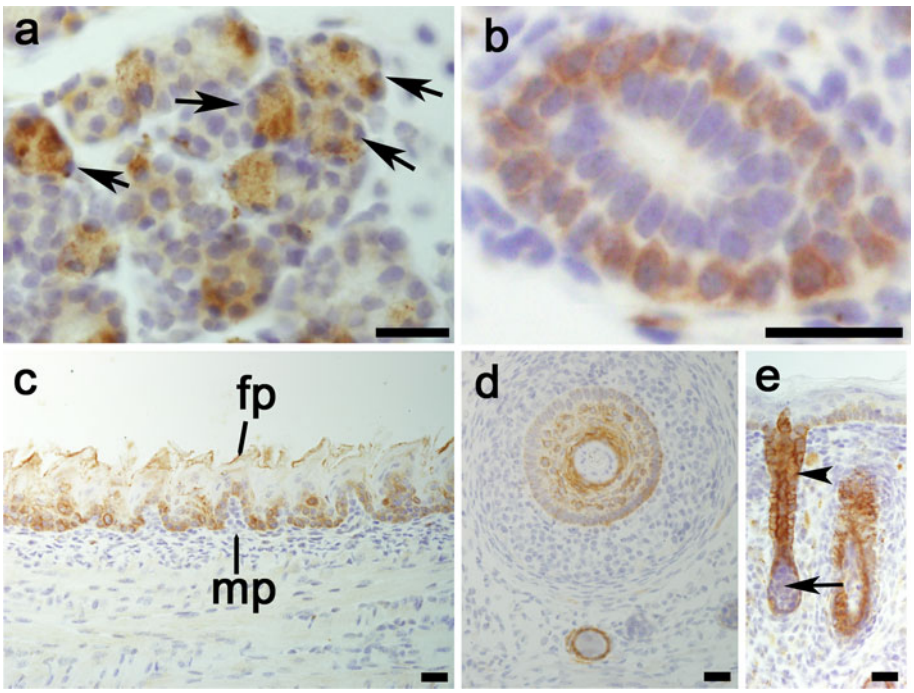

Fig. 3 Immunohistochemical localization of Pax6 in other craniofacial organs at P1.5. **a**, **b** Pax6 protein expression was observed in acinar cells (**a**, *arrow*) and the basal cells of the duct (**b**) in the submandibular gland. **c** Pax6 was expressed in cells of the tongue filiform papilla, but absent from mesenchymal cells. **d** Pax6 was expressed in the inner and outer root sheaths of the hair follicle. **e** Pax6 was expressed in the inner root sheath of the hair follicle (*arrowhead*). The mesenchymal papilla cells were Pax6-negative (*arrow*). *Scale bar* 20 μm. *fp* tongue filiform papilla, *mp* mesenchymal papilla

dentis, outer enamel epithelium, stratum intermedium and stellate reticulum, while the inner enamel epithelium cells were weakly-stained, and cells in the dental papilla were Pax6 negative (Fig. 2e, f). At E18.5 and P1.5, ameloblasts, odontoblasts, as well as cells of the stratum intermedium and stellate reticulum exhibited strong Pax6 expression (Fig. 2g, i). Pax6 antigenicity in ameloblasts and odontoblasts exhibited a gradient of staining, being stronger in the cuspal region and gradually diminishing toward the cervical aspects of the dental germ (Fig. 2g, i, j).

The expression of Pax6 in the upper molars was similar to that in the lower molars at different developmental stages examined (data not shown).

Expression of Pax6 in other craniofacial organs

Based on sagittal sections at E16.5 and P1.5, Pax6 was also localized in other craniofacial organs. Pax6 protein expression was observed in acinar cells and the basal cells of the duct in the mouse submandibular gland (Fig. 3a, b). Pax6 was expressed at high levels in the epithelial cells of the tongue filiform papilla (Fig. 3c). Pax6 was also expressed highly in the inner and outer root sheaths of the hair follicle (Fig. 3d, e). The mesenchymal cells of the tongue filiform papilla and hair follicle were Pax6-negative (Fig. 3c–e).

Discussion

The present study is the first report to describe the expression of Pax6 during mouse incisor and molar development. In general, Pax6 immunostaining was found in all tooth germs examined. The results showed that, from E12.5 to E16.5 (during active epithelial morphogenesis), Pax6 was expressed in ectodermally derived tissues of tooth germs. Cells in the underlying dental ectomesenchyme were Pax6 negative. At E18.5 and P1.5, Pax6 was expressed in both oral epithelium-derived cells and mesenchyme-derived odontoblasts. A similar expression pattern was observed for Runx2, a key transcription factor involved in osteoblast differentiation and skeletal morphogenesis (Ducy et al. 1997; Komori et al. 1997). Defects in Runx2 are responsible for the development of Cleidocranial dysplasia in humans, which is characterized by skeletal defects, supernumerary teeth, and delayed eruption (Otto et al. 1997; Wang and Fan 2011). Runx2 has a unique expression pattern in the dental mesenchyme from the bud to early bell stages, and, surprisingly, is expressed in ectodermally-derived ameloblasts during the maturation phase of enamel formation (D'Souza et al. 1999).

Pax6 expression in the dental epithelium of the dental placode, tooth bud and enamel organ suggested that Pax6 might play a role in tooth morphogenesis during early stages and in the formation of the enamel organ. Pax6 expression in more differentiated ameloblasts and odontoblasts suggested it might be involved in regulating ameloblast and odontoblast differentiation, cusp formation and the process of secreting extracellular enamel and dentin matrix. The one supernumerary incisor observed in our previous work and all the supernumerary incisors in *Pax6−/−* rats contained hematopoietic cells in the dental papilla, suggesting improper tooth development (Lei et al.

2008; Kriangkrai et al. 2006). However, in our study, Pax6 positive cells were not found in the dental papilla other than odontoblasts. In *Pax6−/−* mice, no teeth, whether the supernumerary incisors, the upper or lower incisors, or the upper and lower molars, showed any evidence of pathology or abnormality of the enamel and dentine (Lei et al. 2008; Quinn et al. 1997). Thus, it is likely that Pax6 is not a master control gene in these tooth structures. Tooth morphogenesis is regulated by signaling molecules belonging to conserved families, including TGFβ (transforming growth factor β, which includes BMPs and activins), FGF (fibroblast growth factor), Hedgehog, Wnt, Notch and TNF, as well as certain transcription factors associated with these pathways (Jernvall and Thesleff 2000; Thesleff and Mikkola 2002; Thesleff and Sharpe 1997). Alternatively, some other genes may compensate for the function of Pax6 during tooth morphogenesis in *Pax6−/−* mice. *Pax6* homozygous mutation causes early postnatal lethality, preventing analysis of the further development of teeth in situ.

Supernumerary teeth in *Pax6−/−* mice occurred only in the maxillary incisor area, not in the areas of lower incisors and molars in the maxilla and mandible. Based on this phenotype, we hypothesized that a different Pax6 expression pattern occurs in upper incisors and other tooth sites, and loss of its function resulted in supernumerary upper incisors, while the tooth number at other tooth sites was not affected. To test our hypothesis, expression of Pax6 in regions of lower incisors and molars was examined. The results revealed that the expression patterns of Pax6 in these regions were similar to those in the maxillary incisor area. These results suggested that the location of the supernumerary tooth in *Pax6−/−* mice might not to be associated with Pax6's expression in different tooth sites.

The location of Pax6 and Pax9 was different during tooth development. Pax6 was expressed in ectodermally-derived cells at all developmental stages examined, and mesenchyme-derived odontoblasts at E18.5 and P1.5. However, Pax9, which marks the prospective sites of tooth development before any morphological signs of odontogenesis, is expressed and maintained in the developing tooth mesenchyme thereafter (Peters et al. 1998). At P1.5, both genes were expressed in the epithelial cells of the tongue filiform papilla (Fig. 3c in the present study and Leon Jonker et al. 2004). Further study is needed to explore the potential relationship between the genes during organ development.

Pax6 was localized in the mouse submandibular gland and hair follicle at E16.5 and P1.5 sagittal sections. The early stages of tooth development resemble morphologically other epithelial appendages, such as hairs and glands. In all such organs, development is regulated by interactions between the epithelial and underlying mesenchymal tissues.

In conclusion, the present study indicated that Pax6 was expressed in the upper and lower incisors and molars during mouse tooth development, as well as in the submandibular gland, tongue filiform papilla and hair follicle. The results provide a basis for exploring the function of Pax6 in tooth development.

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