

Wnt signaling in the murine diastema

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SUMMARY The correct number and shape of teeth are critical factors for an aesthetic and functional dentition. Understanding the molecular mechanisms regulating tooth number and shape are therefore important in orthodontics. Mice have only one incisor and three molars in each jaw quadrant that are divided by a tooth-less region, the diastema. Although mice lost teeth in the diastema during evolution, the remnants of the evolutionary lost teeth are observed as transient epithelial buds in the wild-type diastema during early stages of development. Shh and Fgf signaling pathways that are essential for tooth development have been shown to be repressed in the diastema. It remains unclear however how Wnt signaling, that is also required for tooth development, is regulated in the diastema. In this study we found that in the embryonic diastema, *Wnt5a* expression was observed in mesenchyme, whereas *Wnt4* and *Wnt10b* were expressed in epithelium. The expression of *Wnt6* and *Wnt11* was found in both tissues. The Wnt co-receptor, *Lrp6*, was weakly expressed in the diastema overlapping with weak *Lrp4* expression, a co-receptor that inhibits Wnt signaling. Secreted Wnt inhibitors *Dkk1*, *Dkk2*, and *Dkk3* were also expressed in the diastema. *Lrp4* mutant mice develop supernumerary teeth in the diastema that is accompanied by upregulation of Wnt signaling and *Lrp6* expression. Wnt signaling is thus usually attenuated in the diastema by these secreted and membrane bound Wnt inhibitors.

Introduction

An abnormal dentition could be caused by many factors such as micrognathia, macrognathia, abnormal tooth shape including microdontia and macrodontia, and abnormal tooth number including fused teeth, missing teeth, and extra teeth. The correct position, shape, and number of teeth are thus vital factors for establishment of aesthetic and functional dentitions. Since the number and shape of teeth are genetically determined, understanding the molecular mechanisms regulating tooth number and shape is of fundamental importance to orthodontics.

Teeth develop through sequential and reciprocal interactions between oral epithelium and neural crest-derived mesenchyme. The first morphological sign of tooth development is a narrow band of thickened epithelium on the developing jaw primordia. The thickened epithelium progressively takes the form of the bud, cap, and bell configurations as differentiation proceeds. Subsequently, epithelial cells and mesenchymal cells (dental papilla) differentiate into enamel-producing ameloblasts and dentin-producing odontoblasts, respectively. It is known that many signaling pathways such as Bmp, Fgf, Wnt, and Shh play critical roles in regulating tooth position, number, and shape (Thesleff and Sharpe, 1997; Thesleff and Åberg, 1999).

Mice are the commonly studied mammals for investigating the mechanisms of tooth development; however, they have a highly derived dentition; one incisor

and three molars in each jaw quadrant that are divided by a tooth-less region, the diastema. It is known that mice lost teeth in the diastema during evolution approximately 100 Mya since modern placental mammals have evolved from a common ancestor with three incisors, one canine, four premolars, and three molars (Butler, 1939; Peyer, 1968; Meng *et al.*, 1994; Ji *et al.*, 2002). In the diastema of mice, rudimentary tooth primordia develops through the initial stages of tooth development as remnants of the evolutionary lost tooth but cease before the cap stage and regress by apoptosis (Peterkova *et al.*, 2000; Figure 1).

Negative feedback regulators of Fgf signaling, *Sprouty2*, and *Sprouty4* are expressed in the embryonic diastema and mutant mice show supernumerary teeth in the diastema (Hacohen *et al.*, 1998; Kim and Bar-Sagi, 2004; Klein *et al.*, 2006). The Shh inhibitor, *Gasl*, is expressed in the diastema and *Gasl* mutant mice also have extra teeth in the diastema (Ohazama *et al.*, 2009). Fgf and Shh signalings are thus inhibited in the diastema to prevent embryonic tooth buds from developing into the teeth. The data from these mutants also suggest that mice have retained the genetic potential for the development of the evolutionary lost teeth.

Wnt signaling plays critical roles in many biological processes such as cell fate determination, cell polarity, cell proliferation, cell differentiation, and apoptosis in many organs including liver, kidney, brain, and bone during development. It has been shown that canonical Wnt

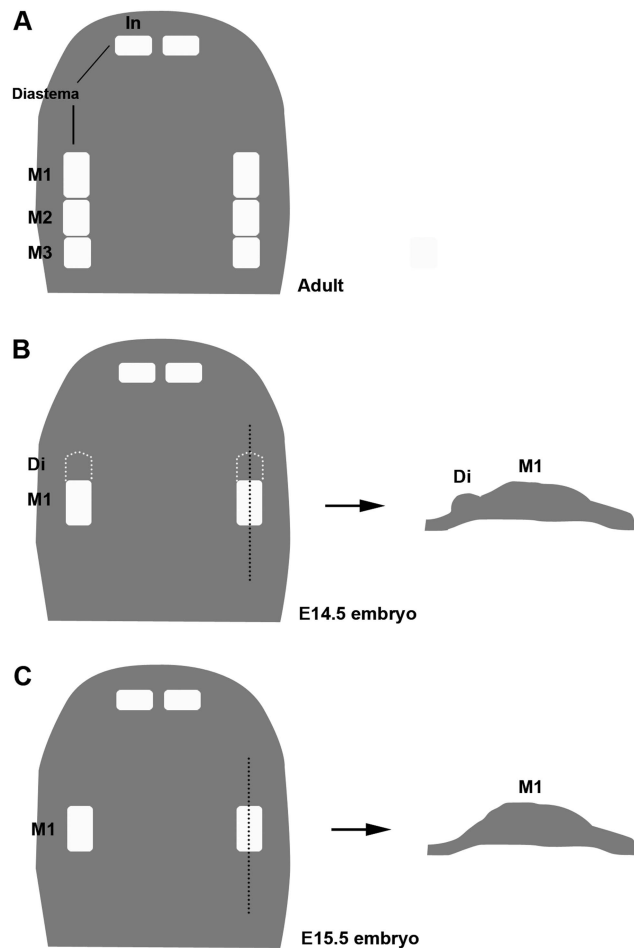


Figure 1 Schematic diagrams showing tooth buds in rodent maxilla. Schematic representations of maxilla from wild-type mouse at adult (A), E14.5 (B), and E15.5 (C). Right diagrams of B and C showing sagittal view of tooth germs. In, incisor; M1, first molar; M2, second molar; M3, third molar; Di, rudimentary tooth bud.

signaling is one of major signaling pathways regulating tooth development. Transgenic mice ectopically expressing the Wnt inhibitor, *Dkk1*, in epithelial cells show arrest of tooth development (Andl *et al.*, 2002). Mutation of *Lef1* (a central transcription mediator of Wnt signaling) also causes arrested tooth development (van Genderen *et al.*, 1994). Conversely, the constitutive activation of β -catenin in embryonic oral epithelium results in continuous supernumerary tooth formation from multiple regions of the jaw (Järvinen *et al.*, 2006; Wang *et al.*, 2009). Upregulation of *Lef1* in the oral epithelium shows numerous tooth-like epithelial invaginations in non-tooth regions of the mouth (Zhou *et al.*, 1995). Canonical Wnt signaling thus regulates initiation of tooth development, but it is unclear how Wnt signaling is inhibited in the diastema.

We show here expression in the diastema of Wnt ligands and Wnt co-receptors known to be expressed during tooth development. *Lrp4* mutant mice showed supernumerary tooth formation in the diastema that was accompanied by

upregulation of canonical Wnt signaling. Wnt signaling is thus attenuated in the diastema by secreted and membrane bound inhibitors.

Materials and methods

Production and analysis of transgenic mice

Lrp4 hypomorphic mice (*Lrp4*^{hypo/hypo}) were generated from the mouse line produced by Johnson *et al.* (2005). *Lrp4* null mice were generated by deletion of the transcription start site at exon 1, which encodes the signal peptide and the initiating ATG. To trace canonical Wnt activity, BAT-gal mice were crossed into the *Lrp4*^{hypo/hypo} line. BAT-gal mice were produced as described by Maretto *et al.* (2003).

The day on which vaginal plugs were found was considered as embryonic day (E) 0.5. To accurately assess the age of embryos, somite pairs were counted and the stage confirmed using morphological criteria, such as relative size of maxillary and mandibular primordia, extent of nasal placode invagination, and the size of limb buds. Harvested embryos were genotyped using polymerase chain reaction and Southern blot analysis using standard techniques. *Lrp4* mutant and wild-type mouse heads were fixed in 4 percent paraformaldehyde (PFA), embedded, and serially sectioned at 8 μ m. Sections were split over 4–10 slides and prepared for histology and radioactive *in situ* hybridization. Decalcification using 0.5 M EDTA was performed after fixation of newborn mice.

In situ hybridization

Radioactive *in situ* hybridization with ³⁵S-UTP-radiolabelled riboprobes was performed as described previously by Ohazama *et al.* (2008).

Wnt activity detection

Tissues were fixed in 1 percent PFA and 0.2 percent glutaraldehyde for 1 hour at 4°C. Explants were then assayed for β -gal activity by staining with X-Gal staining solution overnight at 37°C.

Results

Tooth-related gene expression in the diastema

Vestigial tooth primordia are observed in the diastema from E12 to E14.5 (Peterkova *et al.*, 2006). *Sprouty2* mutant mice show extra teeth in the diastema and the tooth germs of the extra teeth are distinct from E14.5 (Peterkova *et al.*, 2009). Tooth-related gene expression has been seen in the diastema at E12 and E13, but it is unclear whether these molecules are expressed in the diastema at E14.5 (Tureckova *et al.*, 1995; Keränen *et al.*, 1999). We examined *p63*, *Msx2*, *Pitx1*, and *Notch1* that are known to be expressed in tooth epithelium and *Runx2* and *Msx1* that have been shown to be

expressed in tooth mesenchyme (MacKenzie *et al.*, 1992; Jowett *et al.*, 1993; Mitsiadis *et al.*, 1995; D'Souza *et al.*, 1999; St Amand *et al.*, 2000; Laurikkala *et al.*, 2006). The expression of *p63*, *Msx2*, *Pitx1*, and *Notch1* was observed in diastema epithelium, whereas *Runx2* and *Msx1* were expressed in diastema mesenchyme, suggesting that tooth-related genes retained their expression in the diastema at E14.5 (Figure 2). Their expression patterns were similar to those in the first molars.

Expression of Wnt ligands in the diastema

A comparison of the gene expression of Wnt signaling-related molecules including ligands and their receptors is necessary as a starting point to understand how Wnt signaling is regulated in the diastema. We examined the expression of Wnt ligands that have been shown to be expressed in tooth development as well as in the development of other craniofacial ectodermal organs, such as palate and tongue papillae (Sarkar and Sharpe 1999; Lee *et al.*, 2008; Kim *et al.*, 2009). *Wnt6* and *Wnt11* were expressed in both diastema epithelium and mesenchyme, whereas weak *Wnt4* and *Wnt10b* expression could be detected in diastema epithelium at E14.5 (Figure 3A, 3A', and 3C–3E'). The expression of *Wnt5a* was observed in diastema mesenchyme at E14.5 (Figure 3B and 3B').

Lrp4 and *Lrp6* expression in the diastema

The Wnt co-receptor, *Lrp6*, is an essential molecule in canonical Wnt signaling and was found to be weakly expressed in both mesenchyme and epithelium of the diastema at E14.5 (Figure 4B and 4B'). *Lrp4*, a co-receptor that inhibits Wnt signaling, was also weakly expressed in both mesenchyme and epithelium in the diastema at E14.5 (Figure 4A–4A'; Ohazama *et al.*, 2008). The expression of *Lrp4* overlapped with *Lrp6* expression in the diastema at E14.5. In common with *Lrp4*^{Hypo/Hypo} mice, *Lrp4* null mutant mice also showed extra teeth in the diastema. The mutant extra teeth showed upregulation of *Lrp6* expression and Wnt activity in the diastema at E14.5 (Figure 4C, 4C', and 4G; Ohazama *et al.*, 2008).

Expression of Dkks in the diastema

Dickkopfs (Dkks) are secreted proteins that typically antagonize Wnt/ β -catenin signaling (Bafico *et al.*, 2001; Nusse, 2001; Niehrs, 2006). The Dkk family comprises four evolutionarily conserved gene members (*Dkk1–Dkk4*). In the diastema, *Dkk2* and *Dkk3* were expressed in mesenchyme, whereas *Dkk4* showed no expression (Figure 5C–5E'). *Dkk1* was expressed in presumptive maxillary bone adjacent to the diastema (Figure 5A

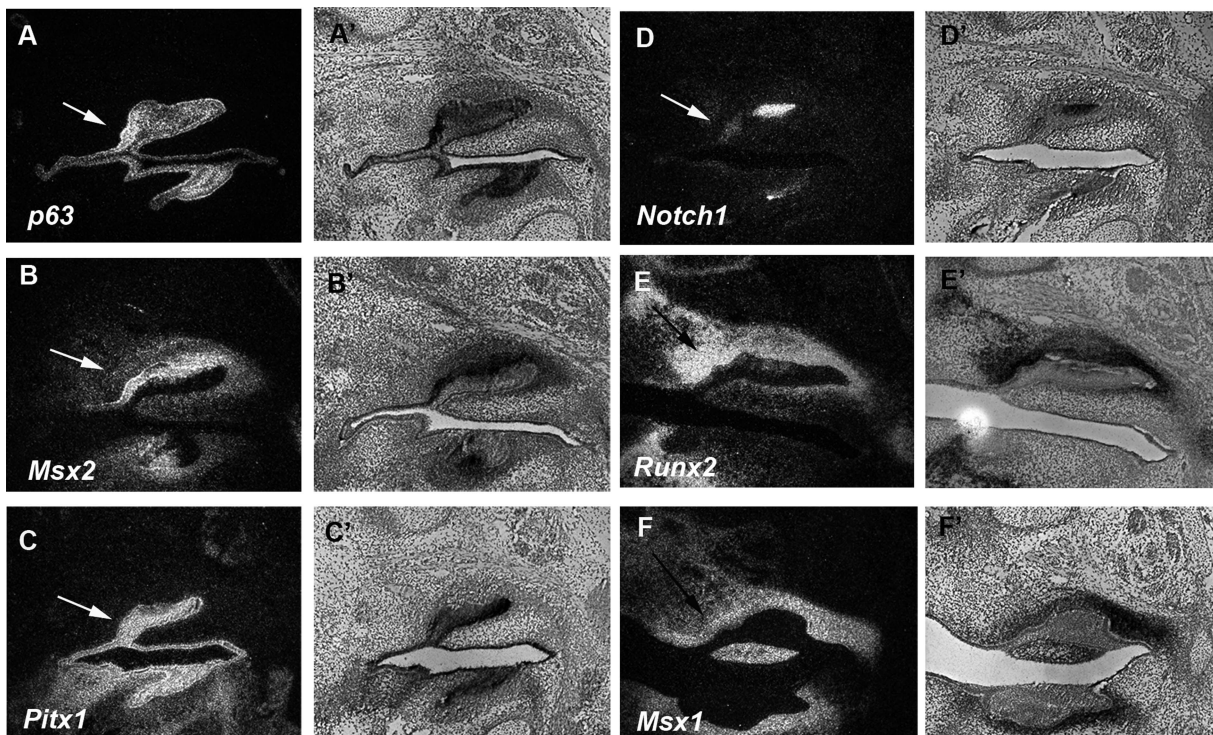


Figure 2 Tooth-related genes in the diastema. Dark field (A, B, C, D, E, and F) and light field (A', B', C', D', E', and F') images of radioactive *in situ* hybridization on sagittal sections in wild-type mice at E14.5. The expression of *p63* (A and A'), *Msx2* (B and B'), *Pitx1* (C and C'), and *Notch1* (D and D') were found in upper diastema epithelium. *Runx2* (E and E') and *Msx1* (F and F') expression were found in upper diastema mesenchyme. Both black and white arrows indicate rudimentary tooth bud.

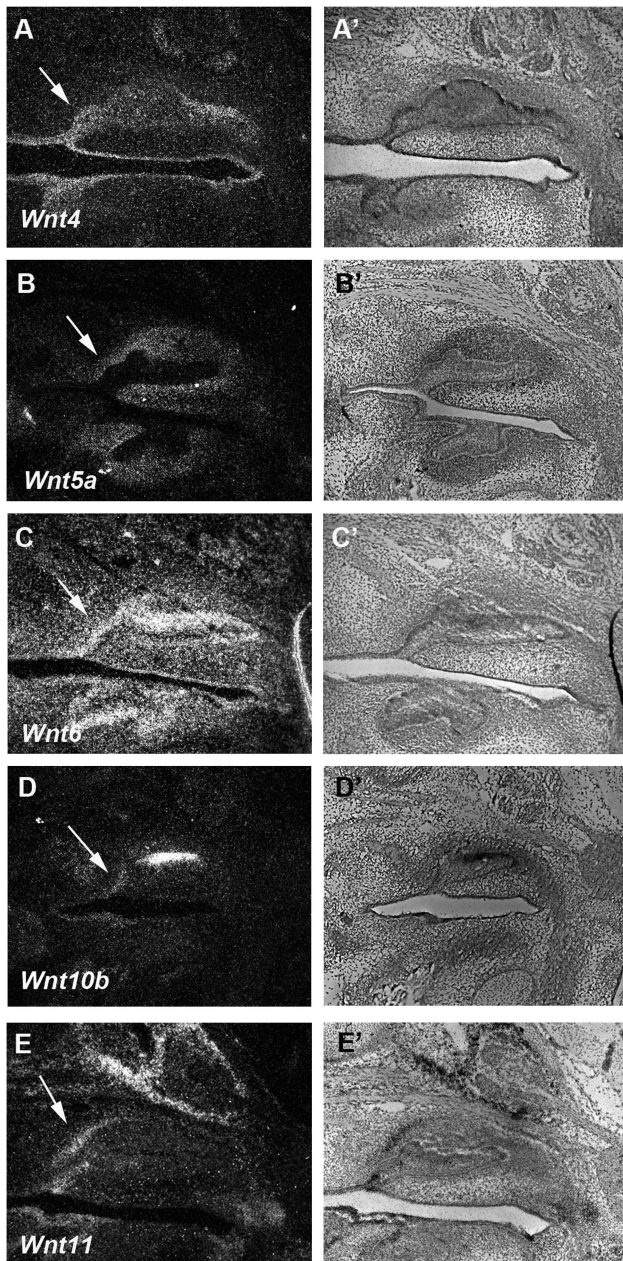


Figure 3 Wnt ligands in the diastema. Dark field (A, B, C, D, and E) and light field (A', B', C', D', and E') images of radioactive *in situ* hybridization on sagittal sections in wild-type mice at E14.5. Weak expression of *Wnt4* and *Wnt10b* (A, A', D, and D') was detected in diastema epithelium. *Wnt5a* expression (B and B') was observed in diastema mesenchyme. *Wnt6* and *Wnt11* (C, C', E, and E') were expressed in both diastema epithelium and mesenchyme. Arrows indicate rudimentary tooth bud.

and 5A'). No obvious differences in *Dkk1* expression could be detected in *Lrp4* mutant diastema (Figure 5B and 5B').

Wnt signaling-related molecules including ligands, co-receptors, and inhibitors are thus expressed in the diastema and upregulation of Wnt activity is observed in *Lrp4* mutant diastema.

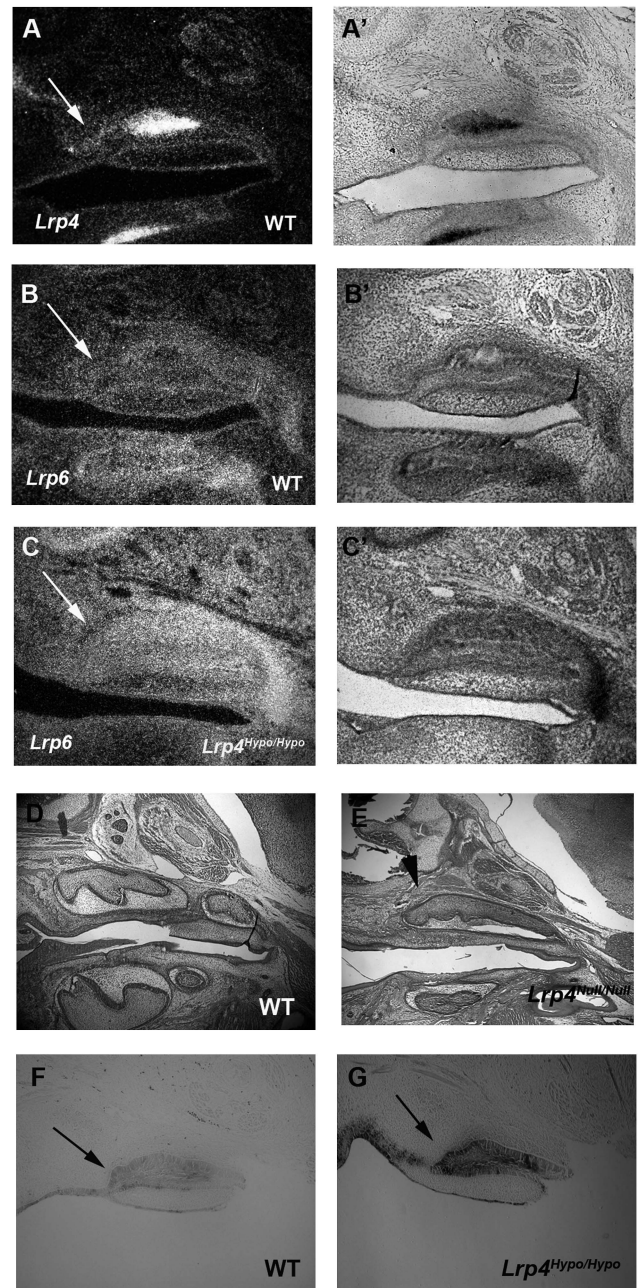


Figure 4 Wnt signaling in the diastema. Dark field (A, B, and C) and light field (A', B', and C') images of radioactive *in situ* hybridization on sagittal sections in wild-type mice at E14.5. Weak expression of *Lrp4* (A and A') and *Lrp6* (B and B') was observed in both upper diastema epithelium and mesenchyme. Upregulation of *Lrp6* expression was detected in *Lrp4*^{Hypo/Hypo} mutants (C and C'). Supernumerary tooth in the diastema was found in *Lrp4*^{Null/Null} mutants at E18.5 (arrowhead in E). The first and the second molars were present in wild-type at E18.5 (D). Upregulation of β -gal activity in the diastema was shown in *Lrp4*^{Hypo/Hypo}; *BAT-gal* mice at E14.5 (arrow in G) compared with β -gal activity in the diastema of wild-type at E14.5 (arrow in F). Both black and white arrows indicate rudimentary tooth bud.

Discussion

Canonical Wnt signaling comprises a complex molecular network. In the absence of Wnt ligands, β -catenin is phosphorylated by a destruction complex that contains the

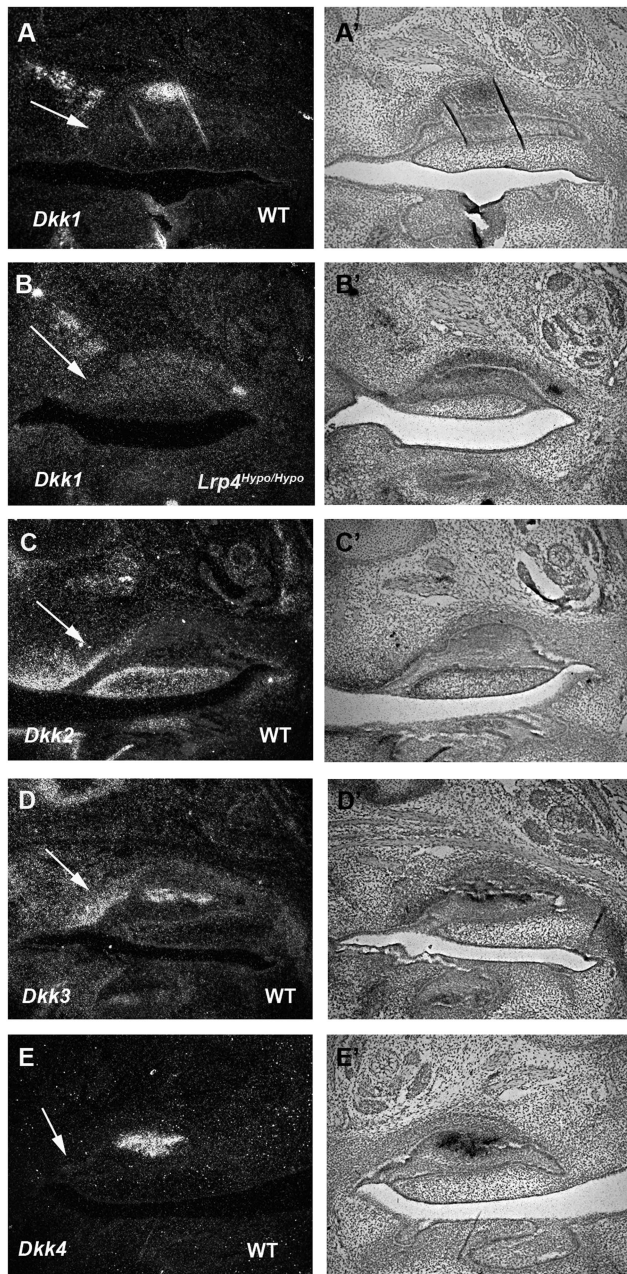


Figure 5 Dkk expression in the diastema. Dark field (A, B, C, D, and E) and light field (A', B', C', D', and E') images of radioactive *in situ* hybridization on sagittal sections in wild-type mice at E14.5. The expression of *Dkk1* (A and A') was found in presumptive maxillary bone adjacent to the diastema in wild-type. Normal expression of *Dkk1* (B and B') was detected in *Lrp4*^{Hypo/Hypo} mutant. *Dkk2* (C and C') and *Dkk3* (D and D') were expressed in upper diastema mesenchyme. *Dkk4* expression (E and E') could not be detected in the diastema. Arrows indicate rudimentary tooth bud.

scaffolding protein Axin and glycogen synthase kinase 3 (Gsk3). In the presence of the ligands, the binding of Wnt ligands to Frizzled/Lrp5/6 recruits a complex formed by Dishevelled-Axin-Gsk3 β to Frizzled. Gsk3 β then phosphorylates Lrp6 that allows Axin binding to Lrp6.

β -catenin becomes stabilized, accumulates in the cytoplasm, and translocates to the nucleus where it mediates the transcription of Wnt target genes (Logan and Nusse, 2004; Nusse, 2005; Clevers, 2006). Lrp4 and Lrp6 belong to the low-density lipoprotein receptor family that is a group of evolutionarily conserved transmembrane receptors found in mammals and other organisms. Recent findings have shown that these receptors function in a broad spectrum of diverse biological processes, including lipid and vitamin homeostasis, brain development, synaptic plasticity, and signal transduction (Herz and Bock, 2002; Nykjaer and Willnow, 2002). Although there is structural similarity between Lrp4 and Lrp5/6, the cytoplasmic domain of Lrp4 lacks the Axin-binding motifs that are present in Lrp5 and Lrp6. Therefore, Lrp4 does not transmit canonical Wnt signaling, whereas Lrp5 and Lrp6 are essential molecules in the canonical Wnt signaling cascade (Pinson *et al.*, 2000; Tamai *et al.*, 2000; Wehrli *et al.*, 2000). Overlapping expression of *Lrp4* and *Lrp6* in the diastema suggests that the presence of Lrp4 results in reduction of ligand binding to Frizzled/Lrp5/6, which leads to attenuation of Wnt signaling in the diastema.

Wise (Ectodin, Sostdc1, and USAG-1) is a secreted Wnt modulator that is expressed in the developing diastema (Itasaki *et al.*, 2003; Kassai *et al.*, 2005; Ohazama *et al.*, 2008). Although Wise and Wnt ligands share the same binding domain of Lrp6, Wise does not activate Wnt signalling (Itasaki *et al.*, 2003). The presence of Wise therefore results in the reduction of ligands binding to Frizzled/Lrp5/6, which leads to attenuation of Wnt activity in the diastema. *Wise* mutant mice show extra teeth in the diastema, although upregulation of Wnt signaling has not been reported in the *Wise* mutant diastema (Kassai *et al.*, 2005; Murashima-Suginami *et al.*, 2008). Dkks are secreted molecules that inhibit Wnt signaling. *Dkk1* inhibits Wnt signaling by a different mechanism to Wise since *Dkk1* interacts with a different domain from Wise-binding region on Lrp6 (Itasaki *et al.*, 2003). *Dkk1* was expressed in the diastema, suggesting that Wnt signaling can be reduced by both secreted molecules, *Dkk1* and Wise in the diastema. *Dkk2* and *Dkk3* expression was also observed in the diastema, although *Dkk2* and *Dkk3* can either activate or inhibit Wnt pathway depending on the tissue context, the level of Lrp5/6, and the presence of Dkk receptor Kremlin2 (Brott and Sokol, 2002; Li *et al.*, 2002; Mao and Niehrs, 2003; Hoang *et al.*, 2004; Hackam, 2005; Yue *et al.*, 2008). It is conceivable that these molecules are involved in switching on/off Wnt signaling to induce and regress the vestigial epithelial buds in the diastema.

Supernumerary tooth formation in the diastema has been found in mice with mutations of *Lrp4*, *Sprouty2/4*, *Wise*, *Polaris*, and *Gas1* (Zhang *et al.*, 2003; Kassai *et al.*, 2005; Klein *et al.*, 2006; Murashima-Suginami *et al.*, 2008; Ohazama *et al.*, 2008, 2009). It has been shown that upregulation of Shh and Fgf signaling causes extra tooth

formation in *Polaris/Gas1* and *Sprouty2/4* mutants, respectively (Zhang *et al.*, 2003; Klein *et al.*, 2006; Ohazama *et al.*, 2009). Upregulation of Wnt signaling was observed in *Lrp4* mutant mice and ectopic teeth in the diastema were also observed in mice misexpressing *ectodysplasin A1 (EdaA1)* and *EdaA1* receptor (*Edar*; Grüneberg, 1966; Mustonen *et al.*, 2003; Pispá *et al.*, 2003; Tucker *et al.*, 2004; Peterková *et al.*, 2005). It has however not been shown which signaling pathways are involved in *EdaA1/Edar* for tooth formation in the diastema. *EdaA1* and *Edar* belong to the TNF ligand family and TNF receptor family, respectively, which are responsible for X-linked hypohidrotic ectodermal dysplasia in humans (Kere *et al.*, 1996). Spontaneous mutation of the *EdaA1* in mice shows the presence of supernumerary teeth in the diastema and over-expression of *EdaA1* or *Edar* also induces extra tooth formation in the diastema (Grüneberg, 1966; Mustonen *et al.*, 2003; Pispá *et al.*, 2003; Tucker *et al.*, 2004; Peterková *et al.*, 2005). These results suggest that fine tuning of *EdaA1/Edar* is necessary to inhibit tooth formation in the wild-type diastema. It is known that there is a cross talk between *EdaA1/Edar* and Wnt/ β -Catenin in hair follicles, suggesting the possibility that alteration of *EdaA1/Edar* induces Wnt signaling activity in the diastema (Zhang *et al.*, 2009).

Signaling pathways such as Wnt, Fgf, Bmp, and Shh play critical roles in development. Secreted or membrane bound antagonists play a role in regulating the activity of these pathways by controlling the extracellular concentration of these ligands. We found that Wnt ligands, *Dkks*, *Lrp4*, and *Lrp6*, are expressed in the diastema, suggesting that Wnt signaling is involved in the repression of diastema tooth formation, modulated by these molecules.

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