Inhibition of Wnt signaling by Wise (Sostdc1) and negative feedback from Shh controls tooth number and patterning

Youngwook Ahn¹, Brian W. Sanderson¹, Ophir D. Klein² and Robb Krumlauf¹,3,*

SUMMARY
Mice carrying mutations in Wise (Sostdc1) display defects in many aspects of tooth development, including tooth number, size and cusp pattern. To understand the basis of these defects, we have investigated the pathways modulated by Wise in tooth development. We present evidence that, in tooth development, Wise suppresses survival of the diastema or incisor vestigial buds by serving as an inhibitor of Lrp5- and Lrp6-dependent Wnt signaling. Reducing the dosage of the Wnt co-receptor genes Lrp5 and Lrp6 rescues the Wise-null tooth phenotypes. Inactivation of Wise leads to elevated Wnt signaling and, as a consequence, vestigial tooth buds in the normally toothless diastema region display increased proliferation and continuous development to form supernumerary teeth. Conversely, gain-of-function studies show that ectopic Wise reduces Wnt signaling and tooth number. Our analyses demonstrate that the Fgf and Shh pathways are major downstream targets of Wise-regulated Wnt signaling. Furthermore, our experiments revealed that Shh acts as a negative-feedback regulator of Wnt signaling and thus determines the fate of the vestigial buds and later tooth patterning. These data provide insight into the mechanisms that control Wnt signaling in tooth development and into how crosstalk among signaling pathways controls tooth number and morphogenesis.

KEY WORDS: Wnt signaling, Shh, Fgf, Wnt antagonists, Feedback regulation, Tooth development, Mouse

INTRODUCTION
The extensive variation in tooth number and morphology in vertebrates raises important questions about how patterning of dentition is controlled during evolution and development. In mammals, teeth develop sequentially in an anterior-to-posterior direction. The initiation of tooth development is characterized by thickening of the oral ectoderm and subsequent condensation of neural-crest-derived mesenchyme around the invaginating epithelium to form tooth buds (Tucker and Sharpe, 2004). Signaling between the dental epithelium and mesenchyme modulates the survival and growth of tooth buds. This signaling is crucial for determining tooth number, as rudimentary or vestigial buds initially form in the toothless diastema region between the incisors and molars but degenerate without reaching the cap stage (Peterkova et al., 2006). At the beginning of the cap stage, a transient epithelial signaling center, called the enamel knot, is induced at the tip of the tooth bud and it regulates tooth growth and morphogenesis (Tucker and Sharpe, 2004).

Mutations in a number of genes encoding components of major signaling pathways have been shown to influence tooth number. This is consistent with the idea that crosstalk between several major signaling pathways, such as Fgf, Shh, Wnt and Bmp, regulates tissue interactions and modulates tooth formation (Tummers and Thesleff, 2009). Many of these pathways are reiteratively used at different stages of tooth development. In the case of Wnt signaling, tooth development is arrested at the early bud stage when Wnt signaling is inactivated, either by conditional knockout of β-catenin or by overexpression of the Wnt antagonist dickkopf1 (Dkk1) in the dental epithelium (Andl et al., 2002; Liu et al., 2008). Conversely, ectopic Wnt activation leads to supernumerary teeth as well as abnormal cusp patterning (Jarvinen et al., 2006; Wang et al., 2009). Therefore, tight control of Wnt signaling activity is essential for normal tooth development, yet it is unclear how this control is achieved and how Wnt signaling interacts with other signaling pathways during tooth development.

Wise (also known as Sostdc1, ectodin and USAG-1) was identified in a functional screen as a gene encoding a conserved secreted protein capable of modulating canonical Wnt signaling (Itasaki et al., 2003). In vitro assays revealed that Wise and the closely related Sost protein bind to the extracellular domain of the Wnt co-receptors Lrp5 and Lrp6 and inhibit Wnt signaling (Ellies and Krumlauf, 2006; Itasaki et al., 2003; Li et al., 2005; Lintern et al., 2009; Semenov et al., 2005). In vitro assays have also revealed that Wise can bind to the extracellular domain of the related Lrp4 receptor (Ohazama et al., 2008). Based on mutations of Lrp4 in humans and mice, it has been postulated that Lrp4 can modulate Wnt signaling mediated by Lrp5 and Lrp6 (Choi et al., 2009; Li et al., 2010; Ohazama et al., 2008; Weatherbee et al., 2006). In addition, Wise is phylogenetically related to several subgroups of Bmp antagonists within the cystine-knot superfamily, and Wise has been shown to bind to a subset of Bmps in vitro and to influence Bmp signaling (Laurikkala et al., 2003). Therefore, Wise has the potential to provide multiple regulatory inputs into Lrp5/6, Bmp- and Lrp4-dependent pathways.

Wise loss-of-function mutants display defects in many aspects of tooth development including tooth number, size and cusp pattern (Kassai et al., 2005; Yanagita et al., 2006). Loss of Wise can increase the sensitivity to excess Bmp in cultured teeth, suggesting that Wise might have a function as a Bmp antagonist in teeth (Kassai et al., 2005). Mice homozygous for a hypomorphic allele of Lrp4 displayed tooth defects similar to those of Wise-null mice,
suggesting that they might cooperate in regulating Wnt signaling (Ohazama et al., 2008). Furthermore, a recent study with cultured Wise-deficient incisors suggested that Wise from the dental mesenchyme can limit tooth formation via inhibition of both Bmp and Wnt signaling (Munne et al., 2009). As signaling pathways play diverse roles at multiple stages of tooth development it is important to understand the stage- and process-specific mechanisms through which Wise exerts its in vivo regulatory activity.

In this study, we have used Wise-null mutants and a transgenic gain-of-function system to investigate crosstalk between signaling pathways and the molecular and cellular mechanisms that regulate the development and ultimately the number of teeth. We present genetic evidence that Wise inhibits Wnt signaling dependent upon the Wnt co-receptors Lrp5 and Lrp6 to suppress survival of vestigial buds in the incisor and molar regions. We found that the Fgf and Shh pathways are major downstream targets of Wise-regulated Wnt signaling and that Shh acts as a negative-feedback regulator of Wnt signaling during the bud-to-cap transition. Our data provide insight into how signaling pathways interact with each other to regulate cellular processes which govern key aspects of tooth formation.

MATERIALS AND METHODS

Mouse strains
Lrp5, Lrp6, Top-Gal, Ctnnb1(ex3)fx, Shh<sup>GFP</sup>, Shh<sup>cre</sup>ERT, Shh<sup>-/-</sup>, Shh<sup>+</sup>, Pch1<sup>-/-</sup>, R26<sup>Top-Gal</sup>, Fgffr1, Fgf2, Fgf10, Bmpr1a and K14-Cre mice were described previously (Chiang et al., 1996; DasGupta and Fuchs, 1999; Chen et al., 2000; Harada et al., 1999; Harle et al., 2004; Kato et al., 2002; Milenkovic et al., 1999; Min et al., 1998; Mishina et al., 2002; Pinson et al., 2000; Soriano, 1999; Trokovic et al., 2003). All experiments involving mice were approved by the Institutional Animal Care and Use Committee of the Stowers Institute for Medical Research (Protocol #2008-0002).

Generation of transgenenic mice
To produce a Wise-LacZ BAC reporter, a LacZ-SV40polyA sequence was inserted in-frame into the first coding exon of Wise in a mouse BAC clone, RP23-166E23, which contains an 191 kb genomic region. BAC DNA was prepared using Qiagen Maxi-Prep Kit, linearized with PI-Scel and used for pronuclear injection into C57Bl6/JxCBA-F1 embryos. A 2.2 kb promoter of the human keratin 14 gene was amplified from a BAC clone (RP11-434D2) using the primers 5′-AAGACTTGG-GGCGGTGGGTTGGATG-3′ and 5′-GAAGCTTGAGCGAG-CTTGCGTGAGGTTGGGATG-3′ and subcloned into the pCMs-EGFP vector (Clontech) replacing the CMV promoter. The mouse Wise cDNA was inserted downstream of the promoter. The 3.5 kb insert was gel-purified and injected into either C57Bl6/JxCBA-F1 or Top-Gal one-cell embryos.

β-gal staining, in situ hybridization and BrdU analysis
For β-gal staining, embryonic jaws were fixed in either 0.1% paraformaldehyde with 0.2% glutaraldehyde or 4% paraformaldehyde (PFA) for one hour on ice. After washing in phosphate-buffered saline, samples were stained in X-gal for 6-20 hours at room temperature. Wholemount in situ hybridization was performed with jags fixed in 4% PFA according to standard protocols using DIG-labeled riboprobes. Cell proliferation was measured by injecting BrdU into pregnant females 2 hours before embryo harvest. Histological samples were paraffin-embedded, sectioned at 8 µm and stained with a rabbit caspase 3 antibody (Cell Signaling) or a mouse anti-BrdU antibody (Amersham). To induce Cre-recombination in Shh<sup>cre</sup>ERT embryos, Tamoxifen was injected into pregnant females at the dose of 3-6 mg/40 g body weight.

qPCR arrays
Embryonic day E13.5 tooth germs were dissected from 14 mandibles of Wise-null embryos and Wise heterozygous littermates. Total RNA was isolated and used for qPCR according to the manufacturer’s protocol (SABiosciences). Four replicas were run for each signaling pathway array and the statistical analysis was performed using the software provided by the manufacturer. To compare gene expression between Wise<sup>-/-</sup> and Wise<sup>-/-;Shh<sup>+/+</sup></sup> tooth germs at E13.5, total RNA was extracted from six mandibular tooth germs for each genotype and four replicas were run with a TaqMan Array (Applied Biosystems).

RESULTS

Tooth phenotypes in Wise-null mice
We have used Wise-null mutants, which exhibit a variety of tooth defects, to investigate the genetic mechanisms that regulate the number and shape of teeth. Our Wise-null mice were generated by knock-in of a selection cassette into the first exon of the gene. Homozygous mutants displayed all the tooth abnormalities previously reported by other groups, including supernumerary incisors and molars, fused molars and cusp defects (Fig. 1; see Fig. S1A-F in the supplementary material) (Kassai et al., 2005; Yanagita et al., 2006). The maxillary molar region of Wise-null mice displayed extensive fusion of anterior teeth with full penetration, resulting in two teeth in each jaw quadrant (Fig. 1B). In the mandibular molar region, there were two general phenotypes observed. In two-thirds of the animals, in place of the three molars (M1-M3) seen in control mice, four teeth (T1-T4) were observed in each jaw quadrant. In the remaining animals, we observed T1-T2 and/or T2-T3 fusions (Fig. 1B-H). We also observed a small extra tooth lateral to T2 on the lingual side and a varying number of small teeth in the T4 region (Fig. 1B). The frequency of the fusions and lateral teeth varied with strain backgrounds and was higher in C57BL6.

Genetic interactions between Wise and the Fgf and Bmp pathways
It is known that mutations in Spry2 or Spry4, which are Fgf antagonists, result in formation of a premolar-like tooth due to elevated Fgf signaling and survival of a diastema bud (Klein et al., 2006; Peterkova et al., 2009). As the supernumerary teeth in the Wise-null tooth phenotypes were rescued by reducing dosage of genes encoding Fgf receptors (Fgfr1 or Fgfr2) or ligands (Fgf10) (Klein et al., 2006), we attempted to rescue Wise-null tooth phenotypes through a similar strategy. However, the appearance of four molar teeth or supernumerary maxillary incisors in Wise-null mice was not affected in any of the compound mutant mice (see Fig. S1 in the supplementary material). Thus, modest reductions in Fgf signaling were not sufficient to compensate for the loss of Wise with respect to tooth number. As Wise has been implicated as a Bmp antagonist, we also attempted to rescue the Wise tooth phenotypes by reducing levels of Bmp signaling through removal of a copy of the Bmpr1a type I receptor gene. There were no significant changes in any of the phenotypes in Wise<sup>-/-;Bmpr1a<sup>-/-</sup></sup> mice (see Fig. S1J,N in the supplementary material).

Dosage-dependent rescue of Wise tooth phenotypes by Lrp5 and Lrp6
Wise has been implicated in modulation of the canonical Wnt signaling pathway through its ability to interact with the extracellular domain of the Lrp5 and Lrp6 co-receptors (Itasaki et al., 2003). We hypothesized that elevated Wnt signaling might account for abnormal tooth development in Wise-null mice. Therefore, we attempted to lower levels of Wnt signaling by reducing the dosage of the Lrp5 and Lrp6 co-receptor genes. Reduction in copies of Lrp5 and/or Lrp6 themselves did not result in tooth abnormalities (data not shown). However, in Wise-null
mice, we observed strong genetic interactions between Wise and Lrp5 and Lrp6, as evidenced by rescue of all incisor and molar phenotypes.

Removal of only a single copy of Lrp6 in Wise-null mice rescued both the supernumerary maxillary (90%) and mandibular (100%) incisor phenotypes (Fig. 1C,H; data not shown). Lrp6<sup>−/−</sup> mutants are embryonic lethal and hence we could not test this combination. Removing one copy of Lrp5 had no effect (Fig. 1D,H), but in Wise<sup>−/−</sup>;Lrp5<sup>−/−</sup> mice, supernumerary incisors were rescued in 22% of cases (Fig. 1E,H). When mice carried one or two Lrp5-null alleles in addition to an Lrp6<sup>−/−</sup> allele, the supernumerary incisor phenotypes were completely rescued (Fig. 1G,H).

With respect to the abnormalities in the mandibular molars of Wise-null mice, removing one copy of either Lrp5 or Lrp6 rescued some aspects of the phenotype, as evidenced by the absence of a lateral supernumerary molar in 98% of cases (Fig. 1C,D). Although molar fusions completely disappeared in Wise<sup>−−</sup>;Lrp5<sup>−/−</sup> mice, only T<sub>5</sub>-T<sub>3</sub> fusions were rescued in Wise<sup>−−</sup>;Lrp6<sup>−/−</sup> mice (Fig. 1H). Removing two of the four copies of the co-receptors resulted in a smaller T<sub>1</sub>, but four molars were still present (Fig. 1E,F). Finally, in Wise<sup>−−</sup>;Lrp5<sup>−/−</sup>;Lrp6<sup>−/−</sup> mice, the normal pattern of three molars in each jaw quadrant was restored in the majority of cases (5/6), including a fairly normal cusp pattern (Fig. 1G).

The maxillary molar region of Wise-null mice displayed two teeth in each jaw quadrant owing to extensive fusion of anterior teeth (Fig. 1B). Removing one copy of Lrp6 had no effect on this fusion phenotype (Fig. 1C,H). However, the fusion phenotype was impacted by dosage of Lrp5, as three teeth were observed in 25% of Wise<sup>−−</sup>;Lrp5<sup>−/−</sup> mice and three or four teeth were observed in 92% of Wise<sup>−−</sup>;Lrp5<sup>−/−</sup> mice (Fig. 1D,E,H). In the majority of animals (5/6), removing three of the four copies of the co-receptors (Wise<sup>−−</sup>;Lrp5<sup>−/−</sup>;Lrp6<sup>−/−</sup>) also restored the normal tooth number, size and cusp pattern in the maxilla (Fig. 1G).

The dosage-dependent rescue by decreases in Lrp5 and Lrp6 demonstrates that most, if not all, of the diverse tooth defects of Wise-null mice are mediated by Lrp5/6-dependent processes. Although these experiments show that the Wnt co-receptors have overlapping and additive roles in tooth development, they also illustrate that the individual Lrp5 and Lrp6 genes contribute differently to regional aspects of tooth development. To probe this issue, we examined the expression pattern of Lrp5 by in situ hybridization and of Lrp6 by detection of LacZ expression from the Lrp6-null allele. We found that both genes are broadly expressed in dental epithelium and mesenchyme (data not shown). Hence, differential patterns of expression do not appear to account for specific roles for each co-receptor in tooth development. With respect to Wnt ligands, expression analyses have showed that multiple Wnt ligands are differentially expressed in early tooth germs (Sarkar and Sharpe, 1999).

**Elevated Wnt signaling leads to continuous development of R2**

The dependence of the Wise phenotypes on Lrp5 and Lrp6 suggests that elevated Wnt signaling in the absence of Wise causes the tooth abnormalities both in incisors and molars. Previously, Munne et al. reported an additional epithelial Wnt activity in Wise-null incisors utilizing the Top-Gal transgenic line (DasGupta and Fuchs, 1999; Munne et al., 2009). To monitor Wnt signaling during molar development, we also used the Top-Gal reporter and observed dynamic changes in the relative levels, number and spatial distribution of sites of Top-Gal expression during early stages of tooth development (E12.5-E15.5) in Wise mutants (Fig. 2A-H). Therefore, we investigated how these early alterations in Wnt activity might account for tooth abnormalities observed in adult Wise-null mice.

In mouse tooth development, premolars do not form, contributing to a toothless diastema region between the incisors and molars (Fig. 3A). Tooth buds are initiated in the diastema but they regress (Peterkova et al., 2006; Viriot et al., 2000). Two diastema buds, called ‘MS’ and ‘R2’ for historical reasons, form in a progressive anterior-posterior (AP) manner (Fig. 3A). MS is the first to form (E12.5), followed by R2 in the adjacent posterior territory (E13.5). At E14.5, as MS and R2 continue to regress, the lack of markers for such transient structures has made it difficult to follow the fate of the diastema buds.
We generated parasagittal sections to precisely map the domains of reporter staining (Fig. 2A’-H’). Top-Gal expression was colocalized with the markers of the epithelial signaling center in early tooth buds as well as the enamel knot of the cap stage molars (Fig. 2; see Fig. S2 in the supplementary material). In control mice, the MS and R2 vestigial buds were marked by Top-Gal (Fig. 2; see Fig. S2 in the supplementary material). In control mice, the temporal changes in Wnt signaling together with alterations in the root pattern suggest that, in Wise mutants, R2 overcame developmental arrest and continued to grow, eventually forming T1. Correlated with these changes in R2, M1 displayed delayed development and subsequently gave rise to the second tooth (T2; Fig. 3A).

**Wise expression in tooth development**

To monitor Wise expression during tooth development, we generated transgenic mice harboring a Wise-LacZ BAC reporter construct. The Wise-LacZ expression mimicked endogenous gene expression in the tooth germ (Fig. 2K; see Fig. S3D,E in the supplementary material) (Laurikkala et al., 2003). At E12.5, Wise expression was dynamic and appeared in the epithelium immediately surrounding the signaling center and in the underlying mesenchyme of MS (see Fig. S2D in the supplementary material). At E14.5, the Wise-LacZ reporter was strongly expressed in the condensing mesenchymal cells adjacent to R2 (Fig. 2K,K’). Weaker expression was also detected in the outermost layer of mesenchymal cells in the M1 region (Fig. 2K’). Wise-LacZ reporter expression was strongly upregulated in the developing R2 region of Wise-null tooth germ (Fig. 2L,L’), suggesting the presence of a negative-feedback loop.

**Fig. 2. Elevated Wnt signaling and continuous development of the R2 vestigial bud.**

(A-H) Wholemount Top-Gal expression in tooth germs of E12.5-E15.5 mandibles. (A’-H’) Parasagittal sections (anterior to the left) of the tooth germs from panels A-H show Top-Gal expression in the epithelial signaling center of the vestigial buds MS and R2, and in M1. (I-J’) Tamoxifen (Tmx)-inducible mutation of β-catenin leads to ectopic Wnt activation in Shh-expressing cells of R2 and M1 and continued development of R2. Tmx was injected at E13.5. (K-L’) Wise-LacZ BAC reporter expression in E14.5 tooth germs. Parasagittal (K,L) and frontal (K’-L’) sections showing reporter expression in mesenchymal cells. The dotted lines indicate the boundary between the dental epithelium and mesenchyme.
Overexpression of Wise disrupts tooth development

We used gain-of-function to investigate the inhibitory potential of Wise on the Wnt pathway in tooth development. Transgenic mice overexpressing Wise in epidermal tissues using the human keratin 14 promoter (K14-Wise) displayed a variety of tooth abnormalities including reduced size, loss of M3 in the maxilla and cusp defects (Fig. 4A-E). To determine how Wnt signaling is affected by Wise overexpression, we also monitored Top-Gal expression in K14-Wise transgenic embryos. We found that tooth germs were growth-retarded and displayed reduced levels of Top-Gal expression (Fig. 4F-I). The data indicate that ectopic Wise can disrupt tooth development by inhibiting Wnt signaling.

Sustained proliferation and survival of the R2 bud in the Wise-null mice

The loss of Wise resulted in elevated epithelial Wnt activity, which promoted the continuous development of R2. To investigate the basis of altered R2 development, we examined rates of cell proliferation and cell death (Fig. 5A-H). BrdU incorporation assays showed that the non-proliferating epithelial signaling center of R2 was surrounded by proliferating epithelial and mesenchymal cells in both control and Wise-null embryos at E13.5 (Fig. 5A,B). No obvious differences were observed between control and Wise-null tooth germs at this stage. However, in Wise-null tooth germs at E14.5, unlike control mice, robust proliferation was detected in the underlying mesenchyme of R2 (Fig. 5C,D, asterisk). In addition, the non-proliferating epithelial signaling center of R2 was maintained only in the mutants. At this stage, a new epithelial signaling center (M1) emerged posterior to R2 in control mice, but evidence for initiation of M1 was not observed in the posterior region of Wise-null mice, providing further evidence for delayed development of M1 (Fig. 5C,D).

To examine the rate of cell death, cells undergoing apoptosis were labeled by immunostaining against activated caspase 3 (Shigemura et al., 2001). A small group of apoptotic cells marked the primary enamel knot from E14.5-E15.5, but no significant difference was observed between control and mutant tooth germs over these stages (Fig. 5E-H). This indicated that apoptosis was unlikely to play a crucial role in determining the fate of R2. This is in agreement with recent genetic studies that suggested apoptosis is largely dispensable for tooth development (Matalova et al., 2006; Setkova et al., 2007).

The fate and contribution of cells from MS and R2 to M1 has been difficult to determine because these structures are normally transient in nature and undergo degeneration. We first examined the expression of Shh, an established enamel knot marker (Hardcastle et al., 1998). In control animals, Shh was progressively activated in a transient manner in MS and R2 and, by E15.5, Shh expression was observed only in the M1 enamel knot of control mice (Fig. 5I). By contrast, as previously shown in Wise-null mice (Kassai et al., 2005), Shh expression was sustained in R2 and also present in M1 at E15.5 (Fig. 5J). To trace the fate of cells descended from the MS and R2 in control and Wise-null mice, we utilized a GFP-Cre knock-in allele of the Shh gene (Harfe et al., 2004). In combination with an R26R-floxstop-LacZ reporter line, descendants of cells that had expressed Shh at earlier stages were genetically marked. At E15.5, the putative MS and R2 regions anterior to M1 were occupied by the descendants of the Shh-expressing cells in control tooth germs (Fig. 5K).
we utilized the Tamoxifen-inducible ShhCreERT allele to mark cells at a specific stage. By comparing LacZ-positive cells after Tamoxifen injection at E12.5 and E13.5, we confirmed that descendants of MS contribute to the dental epithelial cells anterior to R2 at E15.5 (Fig. 5M-N’). Staining in both the MS and R2 regions was greatly expanded in Wise–null tooth germs (Fig. 5L,L’). This was consistent with continued expression of Shh in R2 at earlier stages and underscored the fact that MS was also altered in the mutant (see Figs S2, S3 in the supplementary material).

**Fgf and Shh pathways are major targets of Wise-regulated Wnt signaling**

Our analyses demonstrate that inactivation of Wise leads to increased signaling activity in R2 as early as E13.5, whereas the morphological differences only become apparent a day later. To examine the degree to which signaling pathways were misregulated in the R2 of Wise-null mice, tooth germs were dissected from E13.5 mandibles and expression analysis was performed using qPCR arrays designed for Wnt, Tgfβ and/or Bmp, hedgehog and growth factor pathways (SABiosciences). Differential expression was confirmed for some of the genes by wholemount in situ hybridization (see Fig. S3 in the supplementary material). Major changes were observed in components of the Fgf and Shh pathways with a large increase in several Fgf genes and Shh (Table 1), whereas some minor alterations were observed in Tgfβ and/or Bmp or other pathways (see Table S1 in the supplementary material).

**Genetic interaction between Wise and Shh**

The significant changes in gene expression observed for several components of the Shh signaling pathway and elevated Patch1-LacZ expression (see Fig. S3 in the supplementary material) in the Wise-null tooth buds prompted us to investigate genetic interactions between Wise and Shh. There were no detectable tooth phenotypes in the Shh+/GFPcre or Wise+/– heterozygous mice (Fig. 6A). Hence, we were surprised to discover that a supernumerary tooth anterior to M1 was generated in Wise+/–;Shh+/GFPcre mice with high penetrance (81%, n=42; Fig. 6B). The ShhGFPcre allele displayed a dynamic pattern of GFP expression in the diastema buds and molars that mimicked expression of the endogenous Shh gene (Fig. 6D-L). The tooth phenotype in double-heterozygous embryos correlated with changes in the temporal pattern of Shh as GFP expression was significantly elevated in R2 and restricted to a smaller domain of cells in M1 at E14.5 (Fig. 6H). At E15.5, double-heterozygous embryos continued to express GFP in R2 and expression in the M1 enamel knot appeared to be smaller and shifted posteriorly (Fig. 6K). These results indicate that, in the compound heterozygous mutants, R2 escaped developmental arrest to form a cap-stage tooth and initiation and/or growth of M1 was delayed. Two additional Shh-null alleles, ShhCreERT and Shhneo, also generated the same

Table 1. Differentially expressed genes in Wise-null tooth germs at E13.5 identified in qPCR arrays

<table>
<thead>
<tr>
<th>Gene</th>
<th>t-test (P-value)</th>
<th>Fold upregulation or downregulation (Mut/Het)</th>
</tr>
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<tr>
<td>Dkk1</td>
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<tr>
<td>Foxn1</td>
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<td>Left1</td>
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<td>Fgf4</td>
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<td>Dlx2</td>
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Genes with >1.4-fold change (P<0.05) are shown.
supernumerary tooth phenotype when combined with the Wise mutant allele (data not shown). A reduction in the dosage of Shh significantly increased the severity of Wise-null molar phenotypes (Fig. 6C,M).

These genetic interactions demonstrated that phenotypes in Wise mutant backgrounds were highly sensitive to the dosage of Shh and thus presumably to levels of Shh signaling. Therefore, we tested the effect of reducing Ptch1, which encodes a negative regulator of Shh signaling. Removal of one copy of Ptch1 significantly decreased the frequency of the supernumerary tooth phenotype in Wise+/−;Shh+/GFPcre mice (33%, n=18, P=0.0002; Fig. 6N). This suggests that reduced Shh signaling was the cause of the defect in Wise+/−;Shh+/GFPcre mice.

Shh negatively regulates Wnt signaling in tooth germs
The effects seen upon reduction of Shh might reflect changes in Wnt signaling. Therefore, we measured Top-Gal activity in tooth germs with varying dosages of Wise and Shh (Fig. 7A-H). There was no change in patterns of reporter expression in Wise+/− or Shh+/GFPcre mice compared with control animals. However, in the Wise+/−;Shh+/GFPcre tooth germs, Top-Gal expression was significantly elevated in R2 and delayed in M1 (Fig. 7D). Elevation of Wnt signaling was also observed in fungiform taste papillae (I,J). Furthermore, reducing the dosage of Lrp6 rescued the supernumerary tooth formation in the majority of Wise+/−;Shh+/−;Lrp6−/− mice (20/22; Fig. 6N). The elevated Wnt signaling and genetic rescue by Lrp6−/− in the compound mutants closely resemble those observed in Wise−/− mice, suggesting that the reduction in Shh signaling leads to sustained growth of R2 through an elevation in Wnt signaling. These genetic data point to a role for Shh as an antagonist of Wnt signaling and a suppressor of the bud-to-cap transition of R2.

In addition, enhanced tooth fusion in Wise−/−;Shh+/− mice implies that Shh is required for separation of teeth by antagonizing Wnt signaling as the fusion phenotype was highly sensitive to dosage of Lrp5. To investigate this idea, we have utilized a conditional allele of Shh (Shhfx) in combination with a K14-Cre driver to delete Shh in the dental epithelium. In the K14-Cre;Shhneo/fx mice, Top-Gal expression in the maxilla where a single extended domain of Top-Gal expression was associated with tooth fusion (Fig. 7M-P). To rule out the possibility that the elevated Wnt signaling is an indirect effect of disruption in earlier tooth development, Shh was...
temporally reduced in R2 and M1 buds using the ShhCreERT allele. Two days after Tamoxifen injection, Top-Gal was upregulated in the tooth buds, which appeared to be fused (Fig. 7Q,R). These data support the idea that Shh suppresses the survival of R2 and prevents fusion between neighboring teeth by antagonizing Wnt signaling. We also observed elevated levels of Top-Gal staining in taste papilae and hair follicles in these conditional Shh mutants (Fig. 7T,J; data not shown). Therefore, Shh might have a related role in modulating Wnt signaling in other tissue contexts (Iwatsuki et al., 2007).

To identify candidate genes that might participate in the antagonistic action of Shh on Wnt signaling, we utilized qPCR to examine the expression of 90 selected genes relevant to tooth development, including the differentially regulated genes in Wise-null tooth germs (Table 1). Besides Shh, Dkk1 was downregulated by about 50% in Wise+/−;Shh+/− tooth germs at E13.5 compared with Wise+/− tooth germs (Fig. 7S). Dkk1 was the only gene that showed more than 1.6-fold change (P<0.05), suggesting that it is an early downstream target of Shh (data now shown). This raises the possibility that simultaneous reduction of the two Wnt antagonists, Wise and Dkk1, might account for the elevation of Wnt signaling above a threshold level and lead to survival of R2 in Wise+/−;Shh+/− mice.

**DISCUSSION**

In this study, we have demonstrated that precise control of the level of Wnt signaling plays a crucial role in determining whether diastema buds survive and go on to develop into mature teeth. We have also shown that survival of a diastema bud results in delayed development of the adjacent posterior molar buds, suggesting that reduced inhibitory signals resulting from the regression of R2 might be a prerequisite for the timely initiation of M1. These tissue interactions between developing buds are consistent with a temporal model for tooth formation in which pre-existing tooth buds inhibit initiation of new posterior teeth and form a progressive inhibitory cascade affecting the timing of tooth formation (Kavanagh et al., 2007). We have shown that, in tooth development, Wise suppresses survival of the diastema and incisor vestigial buds by serving as an inhibitor of Lrp5- and Lrp6-dependent Wnt signaling. Our genetic and expression analyses reveal that the Fgf and Shh pathways are major downstream targets of the Wnt signaling regulated by Wise. Furthermore, through genetic interaction studies, we have discovered that Shh acts as a negative-feedback regulator of Wnt signaling during the bud-to-cap transition. Our data provide insight into the mechanisms that control the levels of Wnt signaling and crosstalk between the Wnt, Shh and Fgf pathways that regulate the timing and number of teeth.

**Wise as a Wnt antagonist**

In Wise mutants, our qPCR array data indicated that expression of Wnt pathway components was only moderately affected and the Wnt pathway activity as a whole was highly elevated, as detected by Top-Gal. Conversely, overexpression of Wise resulted in reduced Wnt activity in the dental epithelium and inhibition of tooth growth. The phenotypes of our K14-Wise mice (Fig. 4; data not shown) were also strikingly similar to those of K14-Dkk1 mice, with abnormal development of multiple tissues including hair follicles, mammary glands, taste buds and teeth (Andl et al., 2002; Chu et al., 2004; Liu et al., 2008). The upregulation of Wise-LacZ reporter expression in Wise-null tooth germs implies that Wise might be activated by signaling molecules from the enamel knot through a negative-feedback loop. This is consistent with the elevated Wise expression observed in tooth germs with constitutively active Wnt signaling (Jarvinen et al., 2006; Liu et al., 2008).

The genetic interaction data revealed that, in the absence of Wise, multiple aspects of abnormal tooth development are highly sensitive to the dosage of Lrp5 and Lrp6. Complete rescue of the Wise-null molar and incisor phenotypes was only observed in Lrp5+/−;Lrp6−/− mice, indicating that both genes contribute to Wnt signaling in tooth development. This supports a model whereby Wise acts as a Wnt antagonist through its direct interaction with these Wnt co-receptors. In the absence of inhibition by Wise, there is an elevation of Lrp5/6-dependent Wnt signaling, which induces tooth phenotypes. Decreasing copies of the Lrp5/6 co-receptors rescues the Wise-null phenotypes presumably through restoration of normal levels of Wnt activity. There were no obvious tooth abnormalities in Lrp5−/−;Lrp6−/− mice in the presence of Wise. This indicates that one copy of Lrp6 is sufficient to provide levels of Wnt signaling able to potentiate normal tooth development. This could be a consequence of feedback mechanisms that would compensate for reduced levels of the co-receptor (Fig. 8).

Lrp4 might provide an additional means through which Wise mediates antagonistic action on Wnt signaling. Lrp4 can antagonize canonical Wnt signaling when overexpressed in cultured cells (Li et al., 2010). Although in vitro Wise can bind to Lrp4 (Ohazama et al., 2008), it is unknown whether interactions with Wise can influence Lrp4 function. Developmental abnormalities associated with inactivation of Lrp4 were similar to Wnt loss-of-function phenotypes in certain tissues rather than phenotypes caused by excess Wnt signaling (Choi et al., 2009; Weatherbee et al., 2006). This suggests that the function of Lrp4 can be context-dependent. As mice homozygous for a hypomorphic allele of Lrp4 displayed tooth defects similar to those of Wise-null mice, it was proposed that Wise binds to Lrp4 to initiate intracellular events leading to inhibition of Wnt signaling (Ohazama et al., 2008). Overgrowth and fusion of molars in these Lrp4 mutant mice was associated with elevated Wnt signaling, as assayed by BAT-gal staining in bell-stage tooth germs (Ohazama et al., 2008), but the early changes in Wnt, Shh and other signaling activities have not been examined in Lrp4 mutant mice. Preliminary analyses of mice homozygous for a null allele of Lrp4 (Weatherbee et al., 2006) have shown that loss of Lrp4 does not phenocopy loss of Wise during R2 development. This raises the possibility that Lrp4 and Wise play distinct roles in the diastema buds and that multiple molecular mechanisms underlie tooth defects in these two mouse mutant models.

**Signaling network in diastema tooth development**

In the diastema region of the mouse, it has been proposed that phylogenetic memory of odontogenesis is manifested by the formation of vestigial tooth buds that undergo degeneration without reaching the cap stage. In mouse models with genetic disruptions of major signaling pathways, a supernumerary tooth forms in front of M1, indicating that crosstalk between many pathways is involved in controlling diastema tooth development. In this regard, our analyses with Wise mutants have shed light on the important role of Wnt signaling in these events.

Data with the Top-Gal reporter in control and Wise-null mice demonstrated that Wnt signaling is sequentially activated in epithelia of the two vestigial buds, MS and R2, and then subsequently in molar buds M1, M2 and M3. However, during normal development, Top-Gal expression is rapidly downregulated
in MS and R2 in contrast to the M1-M3 buds, coincident with the inability of the rudimentary buds to make the bud-to-cap transition. Reporter analysis showed that Wise is highly expressed in the mesenchymal cells that surround the arrested buds. The loss of Wise leads to elevated and sustained levels of Wnt signaling in the epithelium and continuous development of R2.

Together with the fate mapping data that shows the persistence of descendants of Shh-positive cells, the results from cell proliferation and cell death analyses suggest that the transition is controlled largely by proliferative signals from the epithelial signaling center to the surrounding mesenchyme. Fgf4 is a strong candidate for the signals, as it is an epithelial target of Wnt signaling and can rescue the developmental arrest of cultured Lef1 mutant tooth germs with its ability to activate mesenchymal Fgf3 expression (Kratochwil et al., 2002). Our quantitative expression analyses showed that Fgf4, as well as Fgf3, are highly upregulated in Wise-null tooth germs, further supporting the notion that Fgf signaling is a major downstream target of epithelial Wnt signaling.

There are similarities and differences in the roles for Wise in molar and incisor development. In both molars (Fig. 2) and incisors (Munne et al., 2009), Wnt signaling is significantly elevated, in agreement with the idea that Wise can inhibit the Wnt pathway. Furthermore, reducing the dosage of the Lrp co-receptors rescues supernumerary tooth phenotypes in molars and incisors (Fig. 1). With respect to cell death in R2 and incisors, there were a limited number of apoptotic cells at early stages (Fig. 5) (Munne et al., 2009). However, reduction in the number of apoptotic cells was reported in later stage incisors (Munne et al., 2009; Murashima-Suginami et al., 2007), suggesting that apoptosis might play a different role in incisors compared with molars.

**Interaction between Shh and Wnt signaling in diastema tooth development**

An important aspect of the regulatory network uncovered by our studies was the nature of the genetic interactions between Wise (Wnt signaling) and Shh in tooth development. In normal tooth development, there was a tight spatial and temporal correlation between Wnt activity and Shh expression in the epithelial signaling center of tooth buds. The elevated Wnt signaling in Wise-null mice led to increased Shh expression in the R2 bud. Ectopic activation of Wnt signaling in the dental epithelium has been shown to induce Shh expression in tooth buds (Jarvinen et al., 2006; Wang et al., 2009). Inactivation of Wnt signaling results in loss of Shh expression (Liu et al., 2008). These data indicate that the level of Shh expression in the developing tooth bud is dependent upon the relative level of Wnt signaling. Conversely, we have found that Shh modulates levels of Wnt signaling. In Wise+/−;Shh−/− mice, Wnt signaling was significantly elevated, leading to survival of R2 and supernumerary tooth formation. This suggests that Shh signaling normally participates in a negative-feedback loop that controls the level of Wnt signaling in R2 (Fig. 8).

A key aspect of this regulatory model is that maintaining a proper balance between Wnt and Shh signaling is more important than the absolute level of either signaling activity. For example, during normal development of R2, levels of Wnt signaling are relatively low and transient. Therefore, low levels of Shh are sufficient to repress Wnt activity and suppress the bud-to-cap transition of R2. Phenotypes arise when either of the pathways is disrupted and normal regulatory feedback modulations are unable to restore a proper balance at the appropriate time. Fig. 8 presents a model for the regulatory interactions between signaling components in R2 of wild-type and genetic mutant backgrounds.

This hypothesis was further supported by the observation that reducing the dosage of Ptch1 (elevating Shh signaling) or Lrp6 (reducing Wnt signaling) rescues the supernumerary tooth phenotype of Wise+/−;Shh−/− mice. We also observed elevated Wnt signaling in Shh-deficient tooth buds. This inhibitory role of Shh on Wnt signaling in the bud-to-cap transition is consistent with the recent finding that ectopic Shh activity in K14-Shh mice arrests tooth development at the bud stage (Cobourne et al., 2009). In addition to the early role in R2, Shh signaling is required for proper separation of teeth (Grillit-Linde et al., 2002; Ohazama et al., 2008), consistent with the exacerbated fusion in Wise−/−;Shh+/− mice. The elevated Wnt signaling in the Shh-deficient tooth germ suggests that Shh prevents tooth fusion by antagonizing Wnt signaling.

Recently, ectopic tooth formation was reported in mice deficient for polaris and Gas1 (Ohazama et al., 2009). Polaris is a component of primary cilia which is required for Shh signaling (Huangfu et al., 2003). Gas1 acts as a facilitator of Shh signaling in different developmental contexts (Allen et al., 2007; Martelli and Fan, 2007; Seppala et al., 2007). Our genetic data on interactions between Shh and Wnt signaling suggest that the supernumerary tooth in polaris and Gas1 mutant mice results from disruption of the Wnt-Shh feedback loop in R2, in which temporal reduction in Shh signaling results in elevated Wnt signaling and hence survival of R2.

In conclusion, our findings highlight how the processes of tooth development are highly sensitive to spatiotemporal changes in Wnt signaling activity. Even a small disruption in the signaling network, through loss or gain of Wnt antagonists (Wise) or feedback regulators such as Shh, is sufficient to change the fate of tooth buds, leading to abnormal tooth number and size. Changes in
expression of signaling modulators such as Wise might represent an important mechanism that underlies the evolutionary diversity in mammalian dentition.

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Competing interests statement

The authors declare no competing financial interests.

Supplementary material

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References


Kleinhans, W., Galceran, J., Jernvall, J., Roth, W. and Grosschedl, R. (2002). Fgf4, a direct target of LEF1 and Wnt signaling, can rescue the arrest of tooth organogenesis in Lef1(–/–) mice. Genes Dev. 16, 3173-3185.


