SMAD4-mediated WNT signaling controls the fate of cranial neural crest cells during tooth morphogenesis

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SUMMARY

TGFβ/BMP signaling regulates the fate of multipotential cranial neural crest (CNC) cells during tooth and jawbone formation as these cells differentiate into odontoblasts and osteoblasts, respectively. The functional significance of SMAD4, the common mediator of TGFβ/BMP signaling, in regulating the fate of CNC cells remains unclear. In this study, we investigated the mechanism of SMAD4 in regulating the fate of CNC-derived dental mesenchymal cells through tissue-specific inactivation of Smad4. Ablation of Smad4 results in defects in odontoblast differentiation and dentin formation. Moreover, ectopic bone-like structures replaced normal dentin in the teeth of Osr2-IresCre;Smad4fl/fl mice. Despite the lack of dentin, enamel formation appeared unaffected in Osr2-IresCre;Smad4fl/fl mice, challenging the paradigm that the initiation of enamel development depends on normal dentin formation. At the molecular level, loss of Smad4 results in downregulation of the WNT pathway inhibitors Dkk1 and Sfrp1 and in the upregulation of canonical WNT signaling, including increased β-catenin activity. More importantly, inhibition of the upregulated canonical WNT pathway in Osr2-IresCre;Smad4fl/fl dental mesenchyme in vitro partially rescued the CNC cell fate change. Taken together, our study demonstrates that SMAD4 plays a crucial role in regulating the interplay between TGFβ/BMP and WNT signaling to ensure the proper CNC cell fate decision during organogenesis.

KEY WORDS: TGFβ/BMP, SMAD4, Canonical WNT signaling, Odontoblast, Bone formation, WNT inhibitor, Mouse

INTRODUCTION

During vertebrate animal development, the cell fate of multipotential neural crest cells is controlled by the context-dependent integration of extrinsic and intrinsic signals that drive their differentiation. Networks of synergistic and antagonistic signals are likely to regulate the development of neural crest derivatives to produce correct cell numbers at the proper time and location. Neural crest-derived odontogenic mesenchymal cells contain multipotential stem cells and can differentiate into dentin-secreting odontoblasts as well as chondrocyte-like and osteoblast-like cells (Chai et al., 2000; Chung et al., 2009; Yamazaki et al., 2007). However, the crucial cues in the signaling network that regulate dental mesenchymal cell fate remain largely unknown.

During dentinogenesis, cranial neural crest (CNC)-derived odontoblast differentiation plays a crucial role in the secretion of predentin and dentin following terminal differentiation (Chai et al., 2000; Ruch, 1990). Odontoblast terminal differentiation is controlled by the inner enamel epithelium and is also dependent on matrix-mediated interactions (Cam et al., 1992; Ruch et al., 1995; Ruoslathi and Yamaguchi, 1991; Thesleff et al., 2001). Analysis of the expression patterns of growth factors during odontogenesis suggests that members of the transforming growth factor β (TGFβ) superfamily, IGFs, WNTs and FGFs contribute to odontoblast terminal differentiation (Bègue-Kirn et al., 1994; Cam et al., 1992; Fjeld et al., 2005; Lohi et al., 2010; Suomalainen and Thesleff, 2010; Thesleff and Vaahtokari, 1992). Within the TGFβ superfamily, TGFβ1, TGFβ2, TGFβ3, BMP2, BMP4, BMP7 and follistatin are expressed in the inner enamel epithelium, dental papilla and in polarizing and functional odontoblasts. Exogenous TGFβ1, BMP2, BMP4 and BMP7 can induce odontoblast differentiation and dentin formation in dental papilla cells in vitro (Bègue-Kirn et al., 1992; Nakashima, 1994; Rutherford et al., 1994; Sloan et al., 2000; Unda et al., 2000). In addition, exogenous TGFβ1 regulates DSPP and DMP1 expression in odontoblast cell lines (He et al., 2004; Unterbrink et al., 2002). Moreover, inhibition of TGFβ signaling in Wnt1-Cre;Tgbr2fl/fl mice and of BMP signaling in K14-Nog or OC-Cre;Smad4fl/fl mice results in abnormal dentin formation (Oka et al., 2007; Plikus et al., 2005; Gao et al., 2009). These data indicate that TGFβ/BMP signaling is involved in regulating dentinogenesis.

The TGFβ superfamily of cytokines comprises TGFβs, BMPs, activins and related proteins. TGFβ/BMP signaling plays an important role in regulating a broad spectrum of processes, including cell proliferation, differentiation, apoptosis, migration and extracellular matrix remodeling (Chai and Slavkin, 2003; Massague, 2000; Siegel and Massague, 2003). The canonical TGFβ/BMP signaling pathway involves binding of the ligand to initiate the assembly of a heteromeric complex of type II and type I receptors. The activated type I receptor phosphorylates SMAD proteins in the cytoplasm. The type I receptors for TGFβ, activin, nodal and myostatin [ALK4 (ACVR1B), ALK5 (TGFBR1), ALK7 (ACVR1C)] phosphorylate SMAD2 and SMAD3, whereas the BMP and AMH type I receptors [ALK1 (ACVR1L), ALK2 (ACVR1), ALK3 (BMPR1A), ALK6 (BMPR1B)] phosphorylate...
SMAD1, SMAD5 and SMAD8 (Massague and Gomis, 2006). These receptor-activated SMADs (R-SMADs) dissociate from the type I receptor and then oligomerize with a common partner, SMAD4. Activated SMAD complexes move into the nucleus, where they regulate the transcription of target genes (Shi and Massague, 2003). A recent study shows that SMAD4-independent signaling pathways are also important during craniofacial development (Xu et al., 2018).

SMAD4 plays a central role in regulating TGFβ/BMP signaling during organogenesis. However, the role of SMAD4 in regulating CNC cell fate determination remains unclear. In this study, we generated mutant mice in which Smad4 is specifically inactivated in the CNC-derived dental mesenchymal cells (Osr2-IresCre;Smad4fl/fl). We found that ablation of Smad4 in the dental mesenchyme results in a defect in odontoblast differentiation. Instead of dentin formation, ectopic bone-like structures form in Osr2-IresCre;Smad4fl/fl mice via a mechanism that involves upregulation of the canonical WNT signaling pathway. Despite the lack of dentin, enamel formation appears to be normal and therefore independent of dentinogenesis in Osr2-IresCre;Smad4fl/fl mice.

**MATERIALS AND METHODS**

**Generation of transgenic mice**

The Osr2-IresCre transgenic line (Lan et al., 2007), ROSA26 conditional reporter (R26R) transgene (Soriano, 1999), conditional Smad4 (Dpc4) allele (Yang et al., 2002) and TOPGAL transgenic allele (DasGupta and Fuchs, 1999) have been described previously. Mating Osr2-IresCre with R26R mice generated Osr2-IresCre;R26R embryos. Osr2-IresCre;Smad4fl/+ male mice were crossed with Smad4fl/+ female mice to generate Osr2-IresCre;Smad4fl/+ alleles. Osr2-IresCre;Smad4fl/+;TOPGAL embryos were produced by crossing Osr2-IresCre;Smad4fl/+;TOPGAL and Smad4fl/fl mice.

**Histological analysis and scanning electron microscopy (SEM)**

For histological analysis, samples were fixed in 4% paraformaldehyde and processed into paraffin-embedded serial sections using routine procedures. For general morphology, deparaffinized sections were stained with Hematoxylin and Eosin (H&E) using standard procedures. For SEM, samples were processed and viewed according to standard procedures as previously described (Xu et al., 2018).

**X-gal staining and detection of β-galactosidase activity**

Samples at various stages of embryonic development were fixed in 0.2% glutaraldehyde, passed through a sucrose series, embedded in O.C.T. Compound (Tissue-Tek) and sectioned on a cryostat at 10 μm thickness. Sections were cut at 8 μm and viewed according to standard procedures as previously described (Xu et al., 2018).

**Lower first molar organ culture**

The lower first molars were microdissected from control and Osr2-IresCre;Smad4fl/+ mutant mice at the newborn stage and cultured in BGJB culture medium (Gibco/Invitrogen) supplemented with 10% ascorbic acid and 1% penicillin and streptomycin. Tissues were harvested after 7 days in culture.

**Kidney capsule transplantation**

Kidney capsule transplantation was carried out as previously described (Xu et al., 2005). The first branchial arches were dissected from embryonic day (E) 11.5 control and Osr2-IresCre;Smad4fl/+ embryos and cultured for 1 day during genotyping. The explants were then grafted under kidney capsules. The grafting products were harvested after 19 days.

In situ hybridization

In situ hybridizations were performed following standard procedures (Xu et al., 2005). Digoxigenin-labeled antisense probes were generated from mouse cDNA clones that were kindly provided by several laboratories: ameloblastin (Anbhn), Margarita Zeichner-David [University of Southern California (USC), USA]; amelogenin (Amelx), Malcolm Sneed (USC, USA); Bsp ((Bsp – Mouse Genome Informatics), Tomoyo Sasaki (USC, USA); Dspp, Irma Thelessef [University of Helsinki, Finland].

**Immunostaining**

Immunostaining was performed using primary antibodies against SMAD4 (Abcam), β-catenin (BD Transduction Laboratories), β-gal (Abcam), DKK1 (R&D) and SFRP1 (Santa Cruz). Alexa Fluor 568 (Molecular Probes), DyLight 488 (Jackson ImmunoResearch) and the HistoStain SP Kit (Invitrogen) were used for detection. Sections were counterstained with Hematoxylin and DAPI.

**RESULTS**

Early tooth development is unaffected in Osr2-IresCre;Smad4fl/+ mice

To test our hypothesis that SMAD4-mediated TGFβ/BMP signaling is crucial for CNC cell fate determination during tooth morphogenesis, we generated Smad4 conditional knockout mice. Specific ablation of Smad4 in neural crest cells using the Wnt1-Cre recombination system leads to early mortality by E11.5, probably owing to heart development failure (Ko et al., 2007). To circumvent this early lethality, we crossed the Smad4 conditional allele (Yang et al., 2002) with Osr2-IresCre (Lan et al., 2007) to generate Osr2-IresCre;Smad4fl/+ embryos. In contrast to Wnt1-Cre
transgenic mice, which express Cre in the premigratory neural crest cells by E8.5 (Chai et al., 2000; Danielian et al., 1998). Cre activity is not detectable in the craniofacial region in Osr2-IresCre mice until E10.5 (Lan et al., 2007). The Osr2-IresCre transgene directed Cre activity in the dental mesenchyme throughout tooth development in Osr2-IresCre;R26R mice (Fig. 1A-G).

Surprisingly, tooth development progressed without obvious defects in Osr2-IresCre;Smad4fl/fl mice until the late bell stage (E18.5) (see Fig. S1 in the supplementary material). By contrast, our previous studies found that tooth development in Wnt1-Cre;Smad4fl/fl embryos is arrested at the dental lamina stage, suggesting that Smad4 is absolutely required in the CNC-derived dental mesenchyme for tooth development to advance into the bud stage (Ko et al., 2007). To understand this discrepancy, we re-examined Osr2-IresCre expression using X-gal staining from the bud to bell stages. As tooth buds developed from E12.5 to E14.5, Osr2-IresCre was expressed in a gradient in the developing tooth mesenchyme, with higher expression towards the lingual side and lower expression immediately buccal to the tooth buds (Fig. 1A-C). We also analyzed Osr2-IresCre expression using immunofluorescence of β-gal at E12.5 and found the same gradient of expression in the dental mesenchyme (Fig. 2A,B).

The initial tooth-generating potential resides within the dental epithelium, which is capable of inducing non-tooth-forming CNC-derived ectomesenchyme to develop into teeth (Jernvall and Thesleff, 2000; Mina and Kollar, 1987). Later, this tooth-forming potential shifts to the dental mesenchyme, coinciding with a shift in BMP signaling from the epithelium to the mesenchyme, where BMP signaling induces the expression of Msx1 (Chen et al., 1996). We hypothesized that the gradient of Osr2-IresCre expression in the dental mesenchyme results in a gradient of Smad4 deletion. Smad4 expression might persist in the buccal region of the tooth bud, which would mediate BMP signaling in the mesenchyme and allow teeth to develop to the bud stage. We found that Smad4 is expressed in the dental epithelium and mesenchyme in control mice at E12.5 (Fig. 2C,E,G). By contrast, Smad4 expression was not detectable in the lingual mesenchyme of the tooth bud in Osr2-IresCre;Smad4fl/fl mice at E12.5, although it was detectable in the buccal region (Fig. 2D,F,H). We also assayed the expression of Msx1 in the dental mesenchyme by in situ hybridization. Msx1 expression was clearly detectable in both control (Fig. 2I) and Osr2-IresCre;Smad4fl/fl (Fig. 2J) mice at E13.5. To confirm our in vivo data, we analyzed the expression level of Msx1 using adeno viral Cre infection to inactivate Smad4 in primary mesenchymal cells within the lower molar region at E13.5. Although Smad4 expression was reduced by ~80% after 24 hours of infection, the expression level of Msx1 was similar to that of the control (see Fig. S2 in the supplementary material). Our data indicate that Smad4 remaining in the buccal mesenchyme of tooth buds in Osr2-IresCre;Smad4fl/fl mice may be sufficient to mediate BMP signaling in the dental mesenchyme to induce Msx1 expression, resulting in normal tooth development during the early stages of embryogenesis.

**Cre-mediated inactivation of Smad4 in the dental mesenchyme affects odontoblast differentiation**

At the newborn stage, we found that odontoblasts of Osr2-IresCre;Smad4fl/fl mice failed to undergo polarized growth and formed a layer of non-polarized cuboidal cells with centrally located nuclei (Fig. 3B,B'). Moreover, dentin matrix was absent in Osr2-IresCre;Smad4fl/fl mice (Fig. 3B'). To examine the status of odontoblast functional differentiation in Osr2-IresCre;Smad4fl/fl mice, we assayed the expression of Dspp by in situ hybridization. In control mice, we detected Dspp expression in the odontoblast layer and in some ameloblasts at the newborn stage (Fig. 3E). In Osr2-IresCre;Smad4fl/fl mice, however, Dspp was undetectable (Fig. 3F). In addition, expression of Amelx, an ameloblast differentiation marker, was also undetectable in Osr2-IresCre;Smad4fl/fl samples as compared with the control (Fig. 3G,H). To confirm the successful inactivation of Smad4, we performed immunostaining using a Smad4 antibody and found that dental mesenchymal cells in newborn Osr2-IresCre;Smad4fl/fl tooth germ were negative for Smad4 (Fig. 3N).

Osr2-IresCre;Smad4fl/fl mice die within a day of birth, precluding an examination of tooth development at later stages. To determine whether the abnormal odontoblast differentiation in Osr2-IresCre;Smad4fl/fl tooth germ is due to delayed development, we cultured newborn tooth germ for 7 days ex vivo. After 1 week of culture, we still failed to find polarization and Dspp expression in odontoblasts from Osr2-IresCre;Smad4fl/fl mice (Fig. 3D,D',J'), although both were detectable in control samples (Fig. 3C,C',J'). Our data indicate that loss of Smad4 in the dental mesenchyme affects the terminal differentiation of odontoblasts.

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Fig. 1. Osr2-IresCre expression pattern during molar development. (A-G) Cre-mediated activation of lacZ expression assayed by X-gal staining (blue) in frontal sections of the lower first molar of E12.5-E18.5 Osr2-IresCre;R26R mouse embryos at the lamina (A), bud (B), cap (C,D) and bell (E-G) stage. X-gal staining is detectable in dental mesenchymal cells, but not in the dental epithelium. Note that Osr2-IresCre is expressed in a gradient in the developing tooth mesenchyme, with higher expression (**) lingual and lower expression (*) buccal to the tooth buds. B, buccal; L, lingual; DE, dental epithelium; DM, dental mesenchyme. Scale bars: 50 μm in A-D; 100 μm in E-G.
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Interestingly, after 1 week of culture, the ameloblasts in Osr2-IresCre;Smad4fl/fl mice were polarized and exhibited strong expression of Amelx and Ambn, comparable to that of control (Fig. 3C–D′,K–L′; see Fig. S3 in the supplementary material), suggesting that ameloblast differentiation shows slightly delayed development in the absence of Smad4. Strikingly, however, our data indicate that ameloblast differentiation is independent of functional odontoblast differentiation.

Ablation of Smad4 in the dental mesenchyme results in ectopic bone formation in the dentin region

Lack of dentin formation in Osr2-IresCre;Smad4fl/fl mice suggests that there is a possible change in cell fate. To test this hypothesis, we transplanted explants containing the lower first molars from E11.5 control and Osr2-IresCre;Smad4fl/fl mice into kidney capsules to explore the cell fate of the dental mesenchymal cells. After 19 days cultivation under the kidney capsule, mineralized capsules to explore the cell fate of the dental mesenchymal cells. Scale bars: 50 μm in A–F; 100 μm in I–J.

To confirm the changes in expression level, we quantified odontoblast and osteoblast differentiation-related genes using qPCR. Expression of Dspp was dramatically reduced in molars from newborn Osr2-IresCre;Smad4fl/fl mice and after 19 days kidney capsule transplantation (P = 1.48×10⁻⁹ and P = 1.6×10⁻⁶, respectively) (Fig. 4O). Previous studies have shown that nestin (Nes), a member of the intermediate filament family, is expressed in fully differentiated odontoblasts (About et al., 2000; Terling et al., 1995). We found that Nes expression was decreased by 50% and 97% in molars from newborn Osr2-IresCre;Smad4fl/fl mice and after 19 days kidney capsule transplantation, respectively (Fig. 4O). Conversely, Bsp expression was increased 3-fold in Osr2-IresCre;Smad4fl/fl versus control samples after 19 days kidney capsule transplantation (Fig. 4O). Taken together, our data indicate that loss of Smad4 in the dental mesenchyme results in ectopic osteoblast differentiation and bone formation in Osr2-IresCre;Smad4fl/fl mice.

By contrast, ameloblasts in Osr2-IresCre;Smad4fl/fl mice underwent normal differentiation, as confirmed by expression of Amelx (Fig. 4N–N′), and normal enamel formation adjacent to the ectopic bone (Fig. 4D,F). These observations further demonstrate that ameloblast differentiation and enamel formation are independent of functional odontoblast differentiation and dentin formation.

To confirm our in vivo data, we analyzed the changes in the expression level of dentinogenesis and osteogenesis genes using adenoviral Cre infection to inactivate Smad4 in primary dental mesenchymal cells. Smad4 expression was reduced by more than...
UPREGULATION OF CANONICAL WNT SIGNALING IN OSR2-IRESCRE;SMAD4FL/FL TEETH

WNT signaling regulates osteoblast differentiation and bone formation (Bennett et al., 2005; Takada et al., 2007). Previous studies have shown that BMP and WNT signaling may regulate each other during bone formation (Bain et al., 2003; Chen et al., 2007; Kamiya et al., 2010; Kamiya et al., 2008; Rawadi et al., 2003; Winkler et al., 2005) and tooth development (Ahn et al., 2007; Kamiya et al., 2010; Kamiya et al., 2008; Rawadi et al., 2003). Thus, ablation of Smad4 in primary dental mesenchymal cells in vitro downregulates genes that are involved in dentinogenesis and upregulates genes that are involved in osteogenesis, consistent with our in vivo data.

70% after 12 hours of infection and by 80% after 24 hours of infection, as assessed by qPCR (Fig. 5A). The levels of the odontoblast differentiation markers Dspp and Nes were reduced by 60% after 12 hours of infection (Fig. 5B), whereas expression of the osteoblast differentiation marker Sp7 was upregulated by more than 2-fold after 24 hours of infection (Fig. 5B). Osteocalcin (Bglap – Mouse Genome Informatics) expression was also increased in Smad4fl/flu primary dental mesenchymal cells after 36 hours of infection (see Fig. S5 in the supplementary material). Thus, ablation of Smad4 in primary dental mesenchymal cells results in the upregulation of the canonical WNT target gene, in teeth from newborn control (M) and Osr2-IresCre;Smad4fl/flu (N) molar tooth germs. AM, ameloblast; DM, dentin matrix; DP, dental papilla; OD, odontoblast. Scale bars: 200 μm in A-D, I-L; 100 μm in M,N; 50 μm in A’–D’,E–H’,I–L’.

UPREGULATION OF CANONICAL WNT SIGNALING IN OSR2-IRESCRE;SMAD4FL/FL TEETH

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In addition, we analyzed the expression of Axin2, a canonical WNT target gene, in teeth from Osr2-IresCre;Smad4fl/flu mice using qPCR. Axin2 expression was significantly increased in Osr2-IresCre;Smad4fl/flu molars after 19 days kidney capsule transplantation (Fig. 6F). Similarly, Axin2 expression was upregulated after Smad4 was inactivated in primary dental mesenchymal cells in vitro via adenoviral Cre infection in vitro for 12 and 24 hours, respectively (Fig. 6H,1). In addition, we also analyzed canonical WNT signaling in other Osr2-IresCre expression regions, such as the olfactory, periocular and tongue mesenchyme, using Osr2-IresCre;Smad4fl/flu;TOPGAL mice and did not find upregulated or ectopic canonical WNT signaling (see Fig. S6 in the supplementary material), which suggests that SMAD4-WNT interaction is tissue-specific during dentinogenesis. Taken together, our data indicate that loss of Smad4 in the dental mesenchyme results in the upregulation of the canonical WNT signaling pathway.
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The extracellular antagonists of the WNT signaling pathway can be divided into two functional classes: the SFRP class and the Dickkopf (DKK) class. In theory, the SFRP class will inhibit both canonical and non-canonical WNT pathways, whereas the DKK class specifically inhibits the canonical WNT pathway (Kawano and Kypta, 2003). We hypothesized that deletion of Smad4 in the dental mesenchyme upregulated the canonical WNT signaling pathway via suppression of WNT antagonist expression. Using qPCR, we assayed the expression of Dkk1, Dkk2, Sfrp1 and Sfrp2, which are WNT antagonists expressed during tooth development (Fjeld et al., 2005; Leimeister et al., 1998). In teeth of Osr2-IresCre;Smad4fl/fl mice, Dkk1 expression was dramatically reduced at E16.5, E17.5 and the newborn stage, and was downregulated by more than 70% after 19 days kidney capsule transplantation (Fig. 7A). Expression of Sfrp1 in Osr2-IresCre;Smad4fl/fl teeth was also significantly decreased at E16.5, E17.5, the newborn stage and after 19 days kidney capsule transplantation (Fig. 7B). By contrast, expression of Dkk2 and Sfrp2 was unchanged in E16.5 and

Fig. 4. Ectopic bone formation in Osr2-IresCre;Smad4fl/fl mice. (A-F) Macrosopic views (A,B), H&E staining (C,D) and SEM analysis (E,F) of E11.5 control and Osr2-IresCre;Smad4fl/fl molar tooth germs after 19 days kidney capsule transplantation. (G-N) In situ hybridization analysis of odontoblast, osteoblast and ameloblast differentiation markers in E11.5 control (G-J) and Osr2-IresCre;Smad4fl/fl (K-N) molar tooth germs after 19 days kidney capsule transplantation. Boxed areas are shown magnified to the right. Black arrows indicate expression of Dspp, Bsp or Amelx, whereas white arrows indicate lack of detectable expression. (O) qPCR for differentiation markers expressed by odontoblasts (Dspp and Nes) and osteoblasts (Bsp) using dental papilla from newborn (NB) control and Osr2-IresCre;Smad4fl/fl mice and E11.5 samples after 19 days kidney capsule transplantation. Values are expressed relative to control. *, P<0.05; **, P<0.01; ***, P<0.001. Error bars indicate s.d. AB, alveolar bone; AM, ameloblast; D, dentin; E, enamel; M1, lower first molar; M2, lower second molar; OD, odontoblast. Scale bars: 200 μm in G-N; 50 μm in C,D,G-H11032-N/H11032; 10 μm in E,F.

Dkk1 and Sfrp1 are downregulated in teeth of Osr2-IresCre;Smad4fl/fl mice

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newborn Osr2-IresCre;Smad4fl/fl teeth (see Fig. S7 in the supplementary material). Immunostaining of teeth confirmed that expression of DKK1 and SFRP1 was reduced in newborn Osr2-IresCre;Smad4fl/fl mice. In control mice, we detected DKK1 expression in preodontoblasts, odontoblasts and dental papilla (Fig. 7C,C′), In Osr2-IresCre;Smad4fl/fl mice, DKK1 was not detectable in the dental mesenchyme (Fig. 7D,D′). SFRP1 was strongly expressed in the odontoblast and ameloblast layers in control mice (Fig. 7E,E′). By contrast, SFRP1 expression was not detectable in the dental mesenchyme of Osr2-IresCre;Smad4fl/fl mice, although it was detectable in the ameloblast layer (Fig. 7F,F′). Similarly, expression of Dkk1 and Sfrp1 was downregulated when Smad4 was deleted via adenoviral Cre infection in vitro (Fig. 7G). These results suggest that Dkk1 and Sfrp1 are downstream effectors of Smad4, and that Smad4 deficiency in the dental mesenchyme might have increased canonical WNT signaling via the suppression of Dkk1 and Sfrp1 activity.

Partial rescue of the cell fate change in Osr2-IresCre;Smad4fl/fl dental mesenchyme using an exogenous WNT pathway inhibitor

To test whether upregulated canonical WNT signaling in Osr2-IresCre;Smad4fl/fl teeth causes the cell fate change in dental mesenchymal cells and the ectopic bone formation, we utilized a small molecule, XAV939, which selectively inhibits β-catenin-mediated transcription (Huang et al., 2009), to suppress the upregulated canonical WNT pathway. After addition of XAV939 (10 μM) to primary newborn Smad4fl/fl dental mesenchymal cell culture medium along with adenoviral Cre infection for 24 hours, translocation of β-catenin into the nuclei was affected, as judged by immunofluorescence (Fig. 8A–C′). By contrast, β-catenin translocation into the nuclei was clearly detectable after inactivation of Smad4 using adenoviral Cre infection alone for 24 hours (Fig. 8A–C). The β-catenin protein level was also dramatically decreased after XAV939 treatment for 24 hours, as assayed by ELISA (Fig. 8D). Our data indicate that XVA939 treatment effectively inhibits the upregulation of the WNT pathway in dental mesenchymal cells after inactivation of Smad4 via adenoviral Cre infection.

Next, we analyzed by qPCR the changes in the expression level of dentinogenesis and osteogenesis genes after XVA939 treatment. The levels of the osteoblast differentiation markers Sp7 and Bsp were reduced by ~50% after XVA939 treatment for 24 hours as compared with adenoviral Cre infection alone (Fig. 8E), whereas expression of the odontoblast differentiation markers Dspp and Nes was obviously increased after 24 hours treatment (Fig. 8E). Although XVA939 treatment reversed the changes in dentinogenesis and osteogenesis gene expression caused by inactivation of Smad4 via adenoviral Cre infection, the expression level of these genes was not completely restored to the control level (adenovirus-mediated eGFP infection only). These results suggest that inhibition of the upregulated canonical WNT pathway in Osr2-IresCre;Smad4fl/fl dental mesenchyme partially rescues the CNC cell fate change.

DISCUSSION

Despite the well-documented requirements for TGFβ/BMP signaling pathways during tooth development, we have found that loss of Smad4 has an unexpected impact on dentin formation: ectopic bone-like structures replace dentin in Osr2-IresCre;Smad4fl/fl mice. More importantly, our results indicate that SMAD4-WNT interaction plays an important role in regulating CNC cell fate determination. Our results provide a link between two important families of signaling molecules and a cellular mechanism that might explain how growth factor signaling pathways work together to exert a specific regulatory function during dentinogenesis. Furthermore, enamel formation appears to be independent of dentinogenesis. This discovery redefines the paradigm that enamel development depends on proper dentinogenesis and has significant implications for our understanding of the regulatory mechanisms of tooth development and regeneration.

Smad4 is required for odontoblast differentiation

SMAD4, the common intracellular mediator for the canonical TGFβ/BMP signaling pathway, plays a crucial role in regulating early tooth development (Ko et al., 2007). However, our study found that inactivation of Smad4 in the dental mesenchyme using the Osr2-IresCre line does not affect early tooth development. One explanation is that Smad4 remaining in the buccal mesenchyme of Osr2-IresCre;Smad4fl/fl tooth buds owing to the expression gradient of Osr2-IresCre might be sufficient to mediate BMP signaling and allow teeth to develop to the bud stage. Alternatively, it is possible that the induction of Msx1 expression in the dental mesenchyme by BMP signaling might not be mediated by the SMAD-dependent pathway.
TGFβ/BMP signaling has been shown to function during odontoblast differentiation and dentin formation. In order to investigate the functional significance of SMAD4 signaling in regulating dentinogenesis, we generated mice that specifically lack Smad4 expression in dental mesenchymal cells. We found that in Osr2-IresCre;Smad4fl/fl mice, CNC-derived dental mesenchyme differentiation is arrested at the late bell stage and secretory stage, with no detectable expression of Dspp. In Wnt1-Cre;Tgfbr2 fl/fl mice, odontoblast differentiation is only delayed and Dspp expression is eventually detectable (Oka et al., 2007). We also observed normal cell polarization in odontoblasts of newborn Osr2-IresCre;Bmpr1afl/fl mice, in which BMP signaling is blocked in the dental mesenchyme (see Fig. S8 in the supplementary material).

Thus, we hypothesize that the TGFβ and BMP signaling pathways work together in a fine balance to regulate odontoblast differentiation during dentinogenesis. Alternatively, it is conceivable that other members of the TGFβ signaling family might have a unique function in regulating dentinogenesis in a SMAD4-dependent manner.

**Ameloblast differentiation is independent of odontoblast differentiation**

Preameloblasts are derived from precursor cells in the inner enamel epithelium of the enamel organ. Upon differentiation, the epithelial preameloblasts exit the cell cycle and polarize, with a reorganization of cellular components. Previous studies have shown that reciprocal epithelial-mesenchymal interactions regulate ameloblast differentiation. Tissue recombination studies using dental and non-dental tissues have shown that ameloblast cytodifferentiation requires functional odontoblasts (Kollar and Baird, 1970; Ruch et al., 1973), and that acellular dentin matrices can also promote ameloblast cytodifferentiation (Karcher-Djuricic et al., 1985). When preodontoblasts differentiate into functional odontoblasts and start to secrete dentin matrix, the basement membrane breaks up and degrades, allowing direct interaction between preameloblasts and...
predentin-dentin (Ruch, 1987). Strikingly, we found that ameloblasts begin to polarize and show strong expression of Amelx and Ambn in the absence of odontoblast differentiation and dentin matrix formation in Osr2-IresCre;Smad4f/f mice. Our data suggest that ameloblast differentiation might be independent of functional odontoblast differentiation. A possible explanation is that, although there is no bona fide dentin in the tooth of Osr2-IresCre;Smad4f/f mice, there is bone-like tissue. This mineralized tissue might be sufficient to support ameloblast differentiation. The presence of dentin is not a prerequisite for enamel formation. Although our finding is somewhat surprising, an examination of enamel throughout evolution lends support to our discovery. Holostean fish, a member of a group of primitive bony fishes, can form ganoine that has been identified as true enamel on bony scales (Donoghue et al., 2006; Sire, 1994), consistent with our conclusion that the presence of ectopic bone in the dentin-forming site substitutes for dentin to induce ameloblast differentiation. From a regenerative medicine perspective, our study might provide useful future strategies for enamel regeneration.

**Dkk1 and Sfrp1 are downstream targets of Smad4**

Many WNT family members and WNT pathway mediators are expressed during tooth development, and the indispensable role of WNT signaling in tooth morphogenesis has been demonstrated in mouse and human studies (Adaimy et al., 2007; Chen et al., 2009; Dassule and McMahon, 1998; Kratochwil et al., 2002; Obara et al., 2006; Sarkar and Sharpe, 1999). Previous reports have shown that WNT ligands (Wnt5, Wnt6 and Wnt10a) are expressed both in the dental mesenchyme and in odontoblasts (Suomalainen and Thesleff, 2010). WNT/β-catenin activity is detectable in the mesenchyme and odontoblasts in BATGAL and TOPGAL reporter mice (Suomalainen and Thesleff, 2010).
Consistent with the expression patterns of WNT ligands and mediators, we report here that DKK1 and SFRP1, which are inhibitors of the WNT pathway, are also expressed in the dental mesenchyme and odontoblasts. Thus, WNT signaling self-regulation might play a role during odontoblast differentiation and dentinogenesis.

In our study, we found that expression of DKK1 and SFRP1 was dramatically downregulated at the mRNA and protein levels following the loss of Smad4 in vivo. These results, which were replicated in cell culture with adeno viral Cre-mediated inactivation of Smad4, suggest that both Dkk1 and Sfrp1 are downstream targets of Smad4. Previous studies support this notion because inhibition of BMP signaling with dorsomorphin, an inhibitor of SMAD-dependent BMP signaling, suppresses the expression of Dkk1 in osteoblasts (Kamiya et al., 2010). Moreover, BMP2 and BMP4 induce Dkk1 expression during limb development in mouse and chicken (Grotewold and Ruther, 2002; Mukhopadhyay et al., 2001). In addition, other studies suggest possible cross-talk between SFRPs and BMP signaling (Ellies et al., 2000; Miquelajuregui et al., 2007; Oshima et al., 2005). However, the present study provides the first in vivo evidence that BMP/TGFβ relies on SMAD4 to regulate Dkk1 and Sfrp1 expression during dentinogenesis.

**SMAD4-mediated BMP/TGFβ and WNT interaction and CNC cell fate determination**

There is a growing body of evidence to suggest that the TGFβ/BMP and WNT signaling pathways regulate one another synergistically or antagonistically (Ahn et al., 2010; Barrow et al., 2003; Guo et al., 2004; He et al., 2004; Huelsken et al., 2001; Ohazama et al., 2008), but the possible interplay of the WNT/β-catenin and TGFβ/BMP signaling pathways for CNC cell fate determination has not been demonstrated during tooth development in vivo. In this study, we found that ablation of Smad4 in dental mesenchyme upregulates the canonical WNT signaling pathway through β-catenin in vivo and in vitro. Our findings are consistent with other studies that have shown an inhibitory effect of BMP signaling on WNT signaling during organogenesis of tissues such as bone (Kamiya et al., 2010; Kamiya et al., 2008), joint (Guo et
al., 2004), lung (Dean et al., 2005), hair (Zhang et al., 2006) and intestine (He et al., 2004). Collectively, our data indicate that loss of Smad4 signaling upregulates WNT signaling, which is likely to be via inhibition of Dkk1 and Sfrp1 expression.

WNT signaling regulates osteoblast differentiation and bone formation (Bennett et al., 2005; Takada et al., 2007). Studies of mouse and human mutations related to the WNT pathway have shown defects in osteogenesis and bone mass (Balemans et al., 2002; Bennett et al., 2005; Bodine et al., 2004; Boyden et al., 2002; Gong et al., 2001; Holmen et al., 2004; Kato et al., 2002; Li et al., 2006; Little et al., 2002; Loots et al., 2005; Mani et al., 2007; Takada et al., 2007). Both DKK1 and SFRP1 are expressed in bone and play crucial roles during bone formation (Bodine et al., 2004; MacDonald et al., 2007). Deletion of Dkk1 or Sfrp1 preferentially activates WNT signaling in osteoblasts, leading to enhanced bone formation in vivo (Bodine et al., 2004; MacDonald et al., 2007). Intriguingly, we found that disrupting Smad4 in the dental mesenchyme causes ectopic bone-like structure formation in the dentin region and enhances the WNT pathway. More importantly, suppression of the upregulated canonical WNT pathway in Oster2-IresCre;Smad4flox/flox dental mesenchyme partially rescues the CNC cell fate change. We suggest that these two phenotypes are linked, and this relationship would be consistent with previous studies in which loss of BMPRIA signaling upregulates WNT signaling by inhibiting Dkk1 and Sost expression in bone and increases bone mass (Kamiya et al., 2010; Kamiya et al., 2008).

The temporal and spatial combination of signals determines neural crest cell fates. Previous studies have demonstrated that the combinatorial activity of the BMP and WNT signaling pathways promotes sensory neuron fate during early neural crest cell development (Klíber et al., 2005). After migration, neural crest-derived dental mesenchymal cells still possess the potential to differentiate into dentin-secreting odontoblasts as well as chondrocyte-like and osteoblast-like cells during craniofacial development (Chai et al., 2000; Chung et al., 2009; Yamazaki et al., 2007). Here, we have demonstrated that SMAD4 is indispensable for odontoblast differentiation during tooth development and that loss of Smad4 in the dental mesenchyme results in ectopic osteoblast differentiation and bone formation via WNT pathway upregulation. Thus, the interplay between the TGFβ/BMP and WNT signaling pathways also functions to ensure proper cell fate determination during postmigratory neural crest cell development and organogenesis. From a clinical perspective, our study might help to provide etiological clues of heritable dentin disorders and suggest novel therapeutically useful strategies and candidates for future investigation.

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Competing interests statement

The authors declare no competing financial interests.

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Belguith, H., de Mazancourt, P. and Mégarbané, A. (2007). Mutation in Dkk1 promotes sensory neuron fate during early neural crest cell fates. Previous studies have demonstrated that the temporal and spatial combination of signals determines neural crest cell fates. Previous studies have demonstrated that the combinatorial activity of the BMP and WNT signaling pathways promotes sensory neuron fate during early neural crest cell development (Klíber et al., 2005). After migration, neural crest-derived dental mesenchymal cells still possess the potential to differentiate into dentin-secreting odontoblasts as well as chondrocyte-like and osteoblast-like cells during craniofacial development (Chai et al., 2000; Chung et al., 2009; Yamazaki et al., 2007). Here, we have demonstrated that SMAD4 is indispensable for odontoblast differentiation during tooth development and that loss of Smad4 in the dental mesenchyme results in ectopic osteoblast differentiation and bone formation via WNT pathway upregulation. Thus, the interplay between the TGFβ/BMP and WNT signaling pathways also functions to ensure proper cell fate determination during postmigratory neural crest cell development and organogenesis. From a clinical perspective, our study might help to provide etiological clues of heritable dentin disorders and suggest novel therapeutically useful strategies and candidates for future investigation.


