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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;Smad4$^{fl/fl}$* mice. Despite the lack of dentin, enamel formation appeared unaffected in *Osr2-IresCre;Smad4$^{fl/fl}$* 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;Smad4$^{fl/fl}$* 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; Ruoslahti 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;Tgfbr2$^{fl/fl}$* mice and of BMP signaling in *K14-Nog* or *OC-Cre;Smad4$^{fl/fl}$* 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 (ACVRL1), ALK2 (ACVR1), ALK3 (BMPR1A), ALK6 (BMPR1B)] phosphorylate

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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., 2008).

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;Smad4$^{fl/fl}$*). 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;Smad4$^{fl/fl}$* 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;Smad4$^{fl/fl}$* 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;Smad4$^{fl/+}$* male mice were crossed with *Smad4$^{fl/fl}$* female mice to generate *Osr2-IresCre;Smad4$^{fl/fl}$* alleles. *Osr2-IresCre;Smad4$^{fl/fl}$;TOPGAL* embryos were produced by crossing *Osr2-IresCre;Smad4$^{fl/+}$;TOPGAL* and *Smad4$^{fl/fl}$* 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., 2006).

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 prior to X-gal staining for *lacZ* expression. Detection of β-galactosidase (β-gal) activity in tissue sections was as previously described (Chai et al., 2000).

Whole molars (after 19 days kidney capsule transplantation) were dissected from the mandible and stained for β-gal activity according to standard procedures, as previously described (Chai et al., 2000). The molars were embedded in paraffin after decalcification and dehydration. Sections were cut at 8 μm and counterstained with Nuclear Fast Red.

Lower first molar organ culture

The lower first molars were microdissected from control and *Osr2-IresCre;Smad4$^{fl/fl}$* 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;Smad4$^{fl/fl}$* 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 (*Ambn*), Margarita Zeichner-David [University of Southern California (USC), USA]; amelogenin (*Amelx*), Malcolm Snead (USC, USA); *Bsp* (*Ibsp* – Mouse Genome Informatics), Tomoyo Sasaki (USC, USA); *Dspp*, Irma Thesleff (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.

Von Kossa staining

To detect calcium salts, Von Kossa staining was performed by immersing the sections in 5% silver nitrate solution (Merck), exposing the samples under a 100 W light bulb for 2.5 hours, stopping the reaction with 5% sodium thiosulfate (Sigma-Aldrich), and staining the sections with paragon-epoxy solution [0.73 g Toluidine Blue (Sigma-Aldrich) and 0.135 g basic fuchsin (Sigma-Aldrich) in 30% ethanol] for 2.5 minutes on a hot plate.

Quantitative (q) PCR analysis

RNA was isolated from dental papilla in vivo and dental mesenchymal cells in vitro using RNeasy Mini Kits (Qiagen). The QuantiTect Reverse Transcription Kit (Qiagen) was used for cDNA synthesis. qPCR was carried out on the iCycler (Bio-Rad) with gene-specific primers and SYBR Green. Values were normalized to β-actin using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001).

ELISA analysis

Total protein was obtained from dental mesenchymal cells in vitro using M-PER Mammalian Protein Extraction Reagent (Thermo Scientific). ELISAs for total β-catenin (Assay Designs) were carried out following the manufacturer's instructions.

Dental mesenchymal cell culture

Newborn dental papilla of lower first molars was mechanically dissected from *Smad4$^{fl/fl}$* mice and cultured in the alpha modification of Eagle's Medium (GIBCO/Invitrogen) supplemented with 20% fetal bovine serum. The culture medium was changed twice a week. Confluent cultures were collected by trypLE Express (GIBCO/Invitrogen) and subcultured under the same conditions.

Transfection of cells with adenovirus

Adherent primary dental mesenchymal cells (as mentioned above) were transfected with adenovirus (control, *Smad4$^{fl/fl}$* + Ad-CMV-eGFP; mutant, *Smad4$^{fl/fl}$* + Ad-CMV-Cre-eGFP; Vector Development Lab, Baylor College of Medicine) at 5000 virus particles/cell with GeneJammer as previously described (Fouletier-Dilling et al., 2005). Based on the eGFP expression, we harvested cells after 12, 24 or 36 hours transfection, respectively.

RESULTS

Early tooth development is unaffected in *Osr2-IresCre;Smad4$^{fl/fl}$* 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;Smad4$^{fl/fl}$* embryos. In contrast to *Wnt1-Cre*

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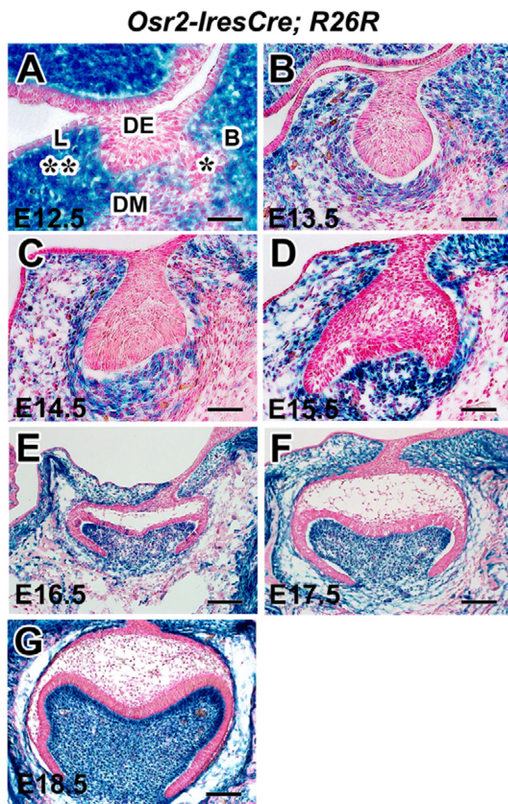

Fig. 1. *Osr2-IresCre* expression pattern during molar development. (**A-G**) Cre-mediated activation of *lacZ* expression assayed by X-gal staining (blue) in frontal sections of the lower first molar of E12.5-E18.5 *Osr2-IresCre;R26R* mouse embryos at the lamina (A), bud (B), cap (C,D) and bell (E-G) 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.

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;Smad4$^{fl/fl}$* 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;Smad4$^{fl/fl}$* 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;Smad4$^{fl/fl}$* 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;Smad4$^{fl/fl}$* (Fig. 2J) mice at E13.5. To confirm our in vivo data, we analyzed the expression level of *Msx1* using adenoviral 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;Smad4$^{fl/fl}$* 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;Smad4$^{fl/fl}$* 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;Smad4$^{fl/fl}$* mice (Fig. 3B′). To examine the status of odontoblast functional differentiation in *Osr2-IresCre;Smad4$^{fl/fl}$* 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;Smad4$^{fl/fl}$* mice, however, *Dspp* was undetectable (Fig. 3F). In addition, expression of *Amelx*, an ameloblast differentiation marker, was also undetectable in *Osr2-IresCre;Smad4$^{fl/fl}$* 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;Smad4$^{fl/fl}$* tooth germ were negative for SMAD4 (Fig. 3N).

Osr2-IresCre;Smad4$^{fl/fl}$ 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;Smad4$^{fl/fl}$* 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;Smad4$^{fl/fl}$* mice (Fig. 3D,D′,J,J′), although both were detectable in control samples (Fig. 3C,C′,I,I′). Our data indicate that loss of *Smad4* in the dental mesenchyme affects the terminal differentiation of odontoblasts.

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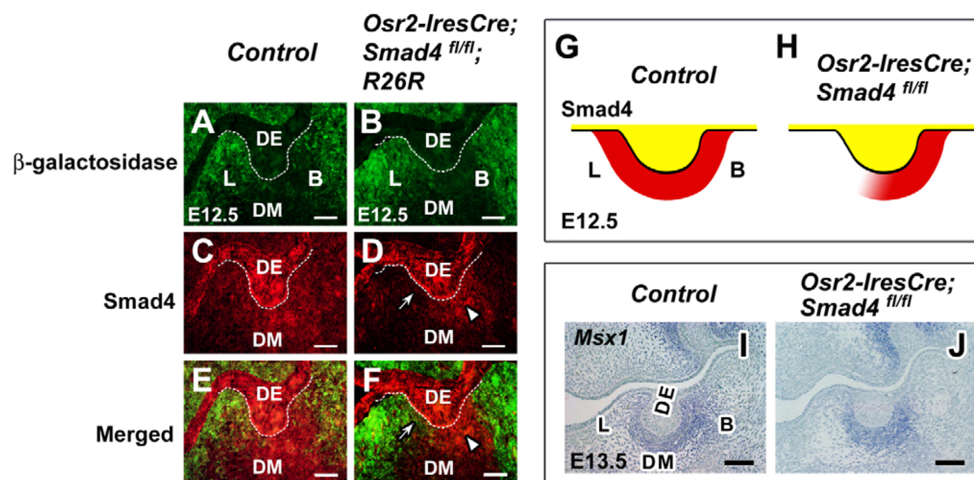

Fig. 2. Residual *Smad4* in the buccal mesenchyme of *Osr2-IresCre;Smad4$^{fl/fl}$* mice mediates BMP signaling to induce *Msx1* expression. (**A-F**) Immunofluorescence of β-gal (green in A,B,E,F) and SMAD4 (red in C-F) in control (*Osr2-IresCre;Smad4$^{fl/+}$;R26R*) and *Osr2-IresCre;Smad4$^{fl/fl}$;R26R* tooth germs at E12.5. Arrowheads and arrows point to the remaining SMAD4 expression in the buccal mesenchyme and to the absence of SMAD4 expression in the lingual mesenchyme, respectively, of *Osr2-IresCre;Smad4$^{fl/fl}$;R26R* mice. Broken lines indicate the basement membrane. (**G**,**H**) Schematic of SMAD4 expression in the dental epithelium (yellow) and mesenchyme (red) of control (G) and *Osr2-IresCre;Smad4$^{fl/fl}$* (H) mice at E12.5. (**I**,**J**) In situ hybridization for *Msx1* in control (I) and *Osr2-IresCre;Smad4$^{fl/fl}$* (J) tooth germs at E13.5. B, buccal; L, lingual; DE, dental epithelium; DM, dental mesenchyme. Scale bars: 50 μm in A-F; 100 μm in I,J.

Interestingly, after 1 week of culture, the ameloblasts in *Osr2-IresCre;Smad4$^{fl/fl}$* 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;Smad4$^{fl/fl}$* 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;Smad4$^{fl/fl}$* mice into kidney capsules to explore the cell fate of the dental mesenchymal cells. After 19 days cultivation under the kidney capsule, mineralized teeth were well formed with normal cusps in control samples (Fig. 4A). By contrast, *Osr2-IresCre;Smad4$^{fl/fl}$* teeth appeared translucent (Fig. 4B). Using histological analysis, we found that bone-like structures, with trapped cells in the lacunae, were formed ectopically in the dentin region of *Osr2-IresCre;Smad4$^{fl/fl}$* teeth (Fig. 4D). After further analysis by SEM, we found that dentin tubules were clearly visible in control mice (Fig. 4E) but that bone lacunae and trabeculae-like structures were detectable in the dentin region of *Osr2-IresCre;Smad4$^{fl/fl}$* mice (Fig. 4F). We hypothesized that loss of *Smad4* in dental mesenchymal cells might have changed their fate from odontoblasts into osteoblasts, resulting in ectopic bone formation. To test this hypothesis, we carried out in situ hybridization for *Dspp* and *Bsp*, which are differentiation markers for odontoblasts and osteoblasts, respectively. In control mice, *Dspp* expression was detectable in the odontoblast layer and in some ameloblasts (Fig. 4H,H′), and *Bsp* was only expressed in the alveolar bone around the molars (Fig. 4I,I′). By contrast, *Dspp* was undetectable in the dentin region of the lower first molars in *Osr2-IresCre;Smad4$^{fl/fl}$* mice (Fig. 4L,L′), although it was detectable in some ameloblasts of the lower second molar (Fig. 4L″). We observed strong expression of *Bsp* in the dentin region and in the alveolar bone around the molars in *Osr2-IresCre;Smad4$^{fl/fl}$* mice (Fig. 4M-M″). In addition, ectopic bone-like structure in the dentin region of *Osr2-IresCre;Smad4$^{fl/fl}$* mice was well mineralized, as assessed by Von Kossa staining (see Fig. S4 in the supplementary material).

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;Smad4$^{fl/fl}$* mice and after 19 days kidney capsule transplantation (P=1.48×10^{-09} and P=1.6×10^{-09}, 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;Smad4$^{fl/fl}$* mice and after 19 days kidney capsule transplantation, respectively (Fig. 4O). Conversely, *Bsp* expression was increased 3-fold in *Osr2-IresCre;Smad4$^{fl/fl}$* 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;Smad4$^{fl/fl}$* mice.

By contrast, ameloblasts in *Osr2-IresCre;Smad4$^{fl/fl}$* 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

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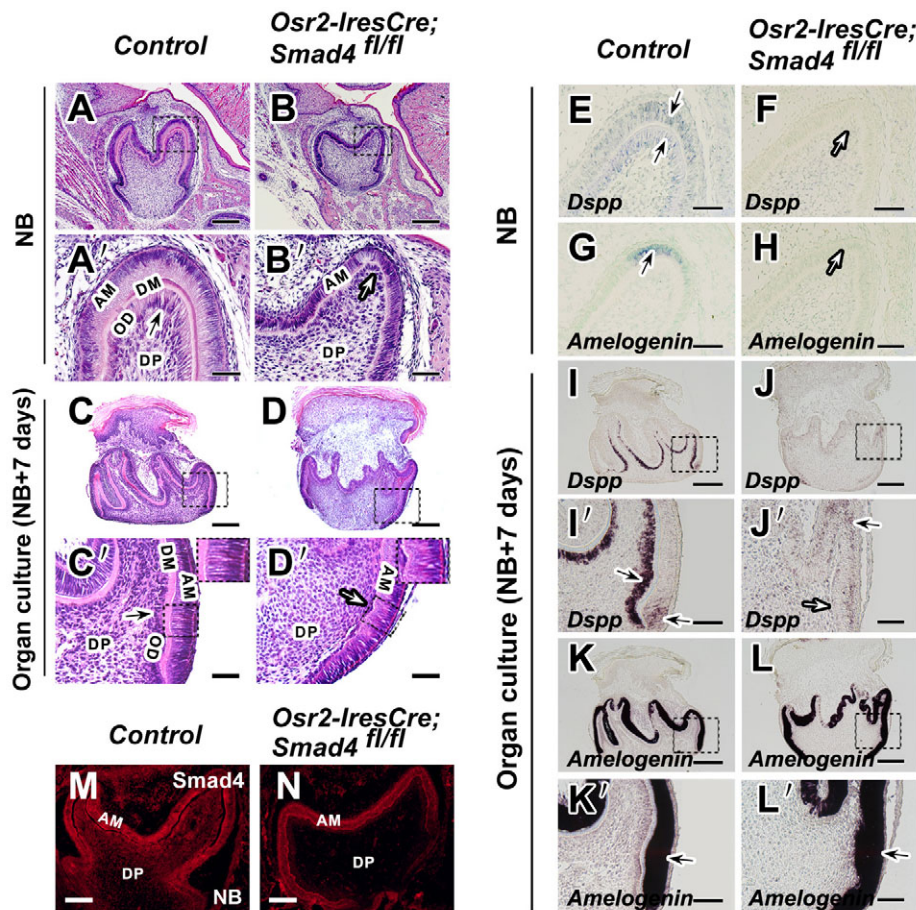

Fig. 3. Odontoblast and ameloblast differentiation in *Osr2-IresCre;Smad4*$^{fl/fl}$ mice. (**A-D′**) H&E staining of molar tooth germs of newborn (NB) control (A,A′) and *Osr2-IresCre;Smad4*$^{fl/fl}$ (B,B′) mice and after 7 days organ culture (C-D′). Boxed areas in frontal sections (above) are shown magnified beneath. The arrows indicate polarized odontoblasts in control tooth germ (black) and unpolarized odontoblasts in *Osr2-IresCre;Smad4*$^{fl/fl}$ tooth germ (white). Boxed areas in C′ and D′ are magnified (right top corner) to show polarized ameloblasts in control (C′) and *Osr2-IresCre;Smad4*$^{fl/fl}$ (D′) tooth germ. (**E-L′**) In situ hybridization for *Dspp* and amelogenin (*Amelx*) in newborn control (E,G) and *Osr2-IresCre;Smad4*$^{fl/fl}$ (F,H) mice and after 7 days organ culture (I-L′). Boxed areas (I-L) are shown magnified beneath. Black arrows indicate *Dspp* expression in odontoblasts and ameloblasts and *Amelx* expression in ameloblasts. White arrows indicate lack of expression of *Dspp* and *Amelx* in *Osr2-IresCre;Smad4*$^{fl/fl}$ mice. (**M**,**N**) Immunofluorescence of SMAD4 (red) in newborn control (M) and *Osr2-IresCre;Smad4*$^{fl/fl}$ (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′.

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* more than doubled after 12 hours of infection (Fig. 5B). *Bsp*, a late osteogenic marker, was upregulated by more than 2-fold after 24 hours of infection (Fig. 5B). Osteocalcin (*Bglap* – Mouse Genome Informatics) expression was also increased in *Smad4*$^{fl/fl}$ 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 in vitro downregulates genes that are involved in dentinogenesis and upregulates genes that are involved in osteogenesis, consistent with our in vivo data.

Upregulation of canonical WNT signaling in *Osr2-IresCre;Smad4*$^{fl/fl}$ 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., 2010; Ohazama et al., 2008). We hypothesized that ablation of *Smad4* in the dental mesenchyme upregulated the WNT signaling pathway, resulting in ectopic bone formation in teeth. We first investigated canonical WNT signaling using *TOPGAL* WNT reporter mice. WNT signaling, as assessed by X-gal staining, was increased in *Osr2-IresCre;Smad4*$^{fl/fl}$*;TOPGAL* teeth after 19 days kidney capsule transplantation (Fig. 6A-D). The percentage of β-gal-positive cells was almost 4-fold higher in *Osr2-IresCre;Smad4*$^{fl/fl}$*;TOPGAL* samples than in the control, a statistically significant increase (Fig. 6E). Although the endogenous expression of canonical WNT signaling is detectable in odontoblasts during postnatal tooth development using WNT reporter mice (Lohi et al., 2010), we found that the intensity of X-gal staining was dramatically increased in teeth from *Osr2-IresCre;Smad4*$^{fl/fl}$*;TOPGAL* mice compared with the control (Fig. 6A-E).

In addition, we analyzed the expression of *Axin2*, a canonical WNT target gene, in teeth from *Osr2-IresCre;Smad4*$^{fl/fl}$ mice using qPCR. *Axin2* expression was significantly increased in *Osr2-IresCre;Smad4*$^{fl/fl}$ 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 (Fig. 6G). mRNA and protein levels of β-catenin, a central component of the canonical WNT cascade, were also dramatically increased after loss of *Smad4* via adenoviral Cre infection in vitro for 12 and 24 hours, respectively (Fig. 6H,I). 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;Smad4*$^{fl/fl}$*;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.

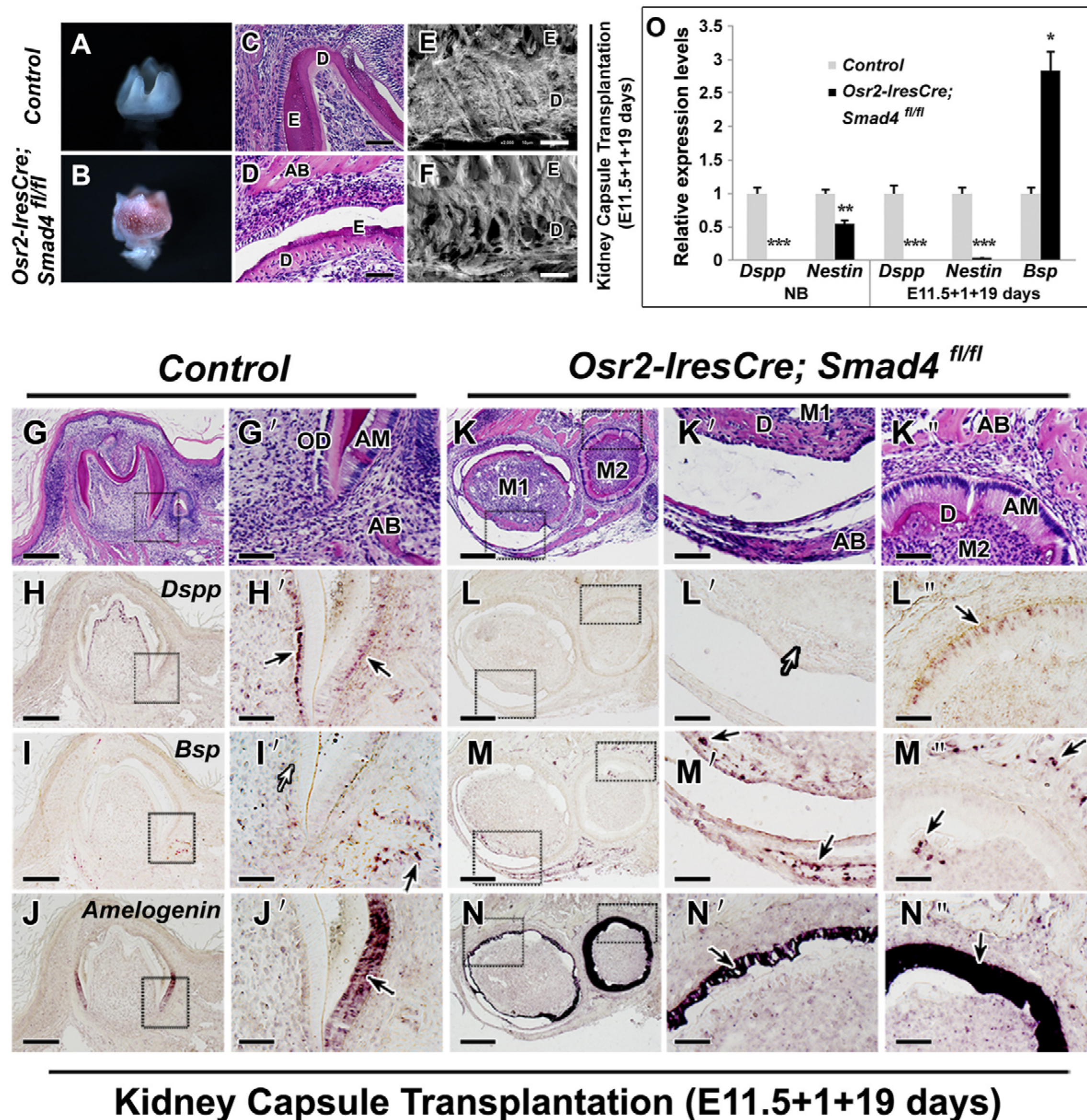

Fig. 4. Ectopic bone formation in *Osr2-IresCre;Smad4$^{fl/fl}$* mice. (**A-F**) Macroscopic views (A,B), H&E staining (C,D) and SEM analysis (E,F) of E11.5 control and *Osr2-IresCre;Smad4$^{fl/fl}$* molar tooth germs after 19 days kidney capsule transplantation. (**G-N″**) In situ hybridization analysis of odontoblast, osteoblast and ameloblast differentiation markers in E11.5 control (G-J′) and *Osr2-IresCre;Smad4$^{fl/fl}$* (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;Smad4$^{fl/fl}$* 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′-N′,K″-N″; 10 μm in E,F.

Dkk1 and *Sfrp1* are downregulated in teeth of *Osr2-IresCre;Smad4$^{fl/fl}$* mice

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;Smad4$^{fl/fl}$* 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;Smad4$^{fl/fl}$* 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

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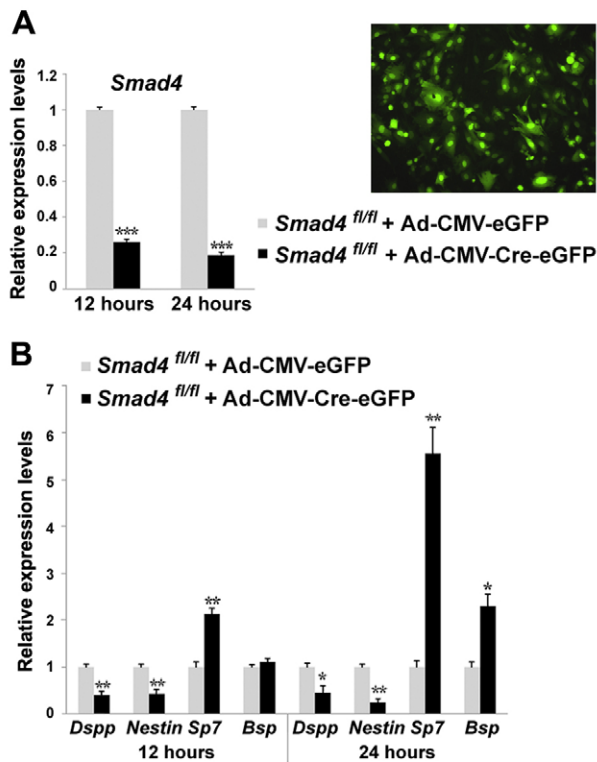

Fig. 5. Adenovirus-mediated Cre deletion of *Smad4* in vitro downregulates genes involved in dentinogenesis and upregulates genes involved in osteogenesis. (**A**,**B**) qPCR analysis of *Smad4* (A) and genes involved in dentinogenesis (*Dspp* and *Nes*) and osteogenesis (*Sp7* and *Bsp*) (B) in *Smad4*$^{fl/fl}$ dental mesenchymal cells after adenoviral Cre infection (Ad-CMV-Cre-eGFP) for 12 and 24 hours in vitro. Image in A illustrates the infection efficiency of Ad-CMV-Cre-eGFP (green). Values are expressed relative to adenoviral eGFP infection (Ad-CMV-eGFP). *, P<0.05; **, P<0.01; ***, P<0.001. Error bars indicate s.d.

newborn *Osr2-IresCre;Smad4*$^{fl/fl}$ teeth (see Fig. S7 in the supplementary material). Immunostaining of teeth confirmed that expression of DKK1 and SFRP1 was reduced in newborn *Osr2-IresCre;Smad4*$^{fl/fl}$ mice. In control mice, we detected DKK1 expression in preodontoblasts, odontoblasts and dental papilla (Fig. 7C,C′). In *Osr2-IresCre;Smad4*$^{fl/fl}$ 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;Smad4*$^{fl/fl}$ 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;Smad4*$^{fl/fl}$ dental mesenchyme using an exogenous WNT pathway inhibitor

To test whether upregulated canonical WNT signaling in *Osr2-IresCre;Smad4*$^{fl/fl}$ 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 *Smad4*$^{fl/fl}$ 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;Smad4*$^{fl/fl}$ 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;Smad4*$^{fl/fl}$ 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;Smad4*$^{fl/fl}$ 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.

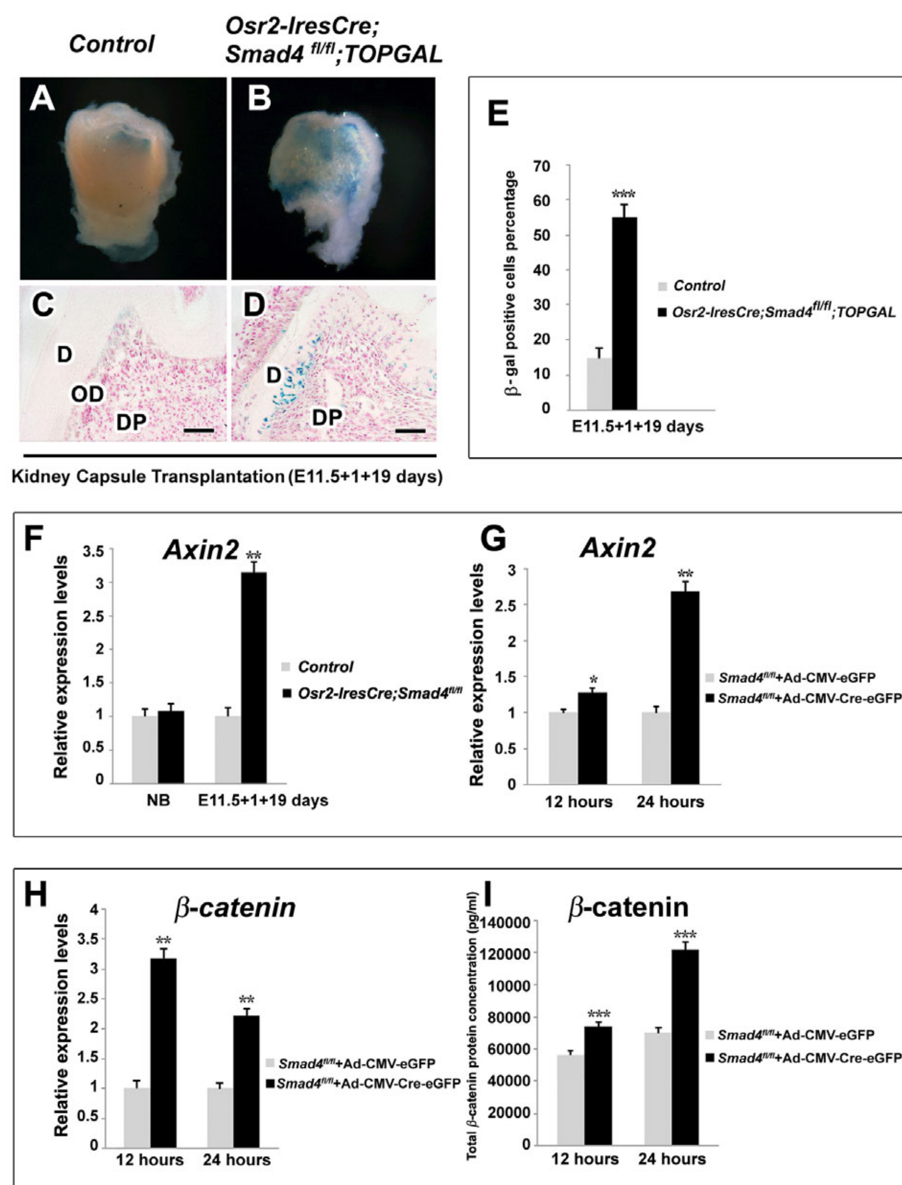

Fig. 6. Upregulation of canonical WNT signaling in *Osr2-IresCre;Smad4$^{fl/fl}$* mice. (**A**,**B**) Macroscopic view of tooth germs from *TOPGAL* mice cultured for 19 days under kidney capsules to assess canonical WNT signaling using X-gal staining. (**C**,**D**) Histological analysis of tooth germs cultured for 19 days under kidney capsules from *TOPGAL* mice. D, dentin; DP, dental pulp; OD, odontoblast. Scale bars: 50 μm. (**E**) Percentage of β-gal-positive cells in molars cultured for 19 days after kidney capsule transplantation from control and *Osr2-IresCre;Smad4$^{fl/fl}$;TOPGAL* mice. Cells were counted in 30 fields of molars for each genotype (*n*=3). Total cell number was obtained by counting Nuclear Fast Red-positive nuclei in the control (*Smad4$^{fl/fl}$;TOPGAL*) odontoblast layer and *Osr2-IresCre;Smad4$^{fl/fl}$;TOPGAL* dentin region. ***, $P<0.001$. (**F**,**G**) qPCR for *Axin2* in newborn (NB) control and *Osr2-IresCre;Smad4$^{fl/fl}$* dental papilla and after 19 days kidney capsule transplantation (F), and in *Smad4$^{fl/fl}$* dental mesenchymal cells after adenoviral Cre infection (Ad-CMV-Cre-eGFP) for 12 and 24 hours in vitro (G). Values are expressed relative to control and adenoviral eGFP infection (Ad-CMV-eGFP), respectively. *, $P<0.05$; **, $P<0.01$. (**H**,**I**) qPCR (H) and ELISA (I) analysis of β-catenin in *Smad4$^{fl/fl}$* dental mesenchymal cells after adenoviral Cre infection (Ad-CMV-Cre-eGFP) for 12 and 24 hours in vitro. qPCR values are expressed relative to adenoviral eGFP infection (Ad-CMV-eGFP). **, $P<0.01$; ***, $P<0.001$. Error bars indicate s.d.

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;Smad4$^{fl/fl}$* 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;Bmpr1a$^{fl/fl}$* 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. Accordingly, deletion of *Smad4*, the common mediator for the canonical TGFβ/BMP signaling pathway, causes morphological and functional defects in odontoblasts 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

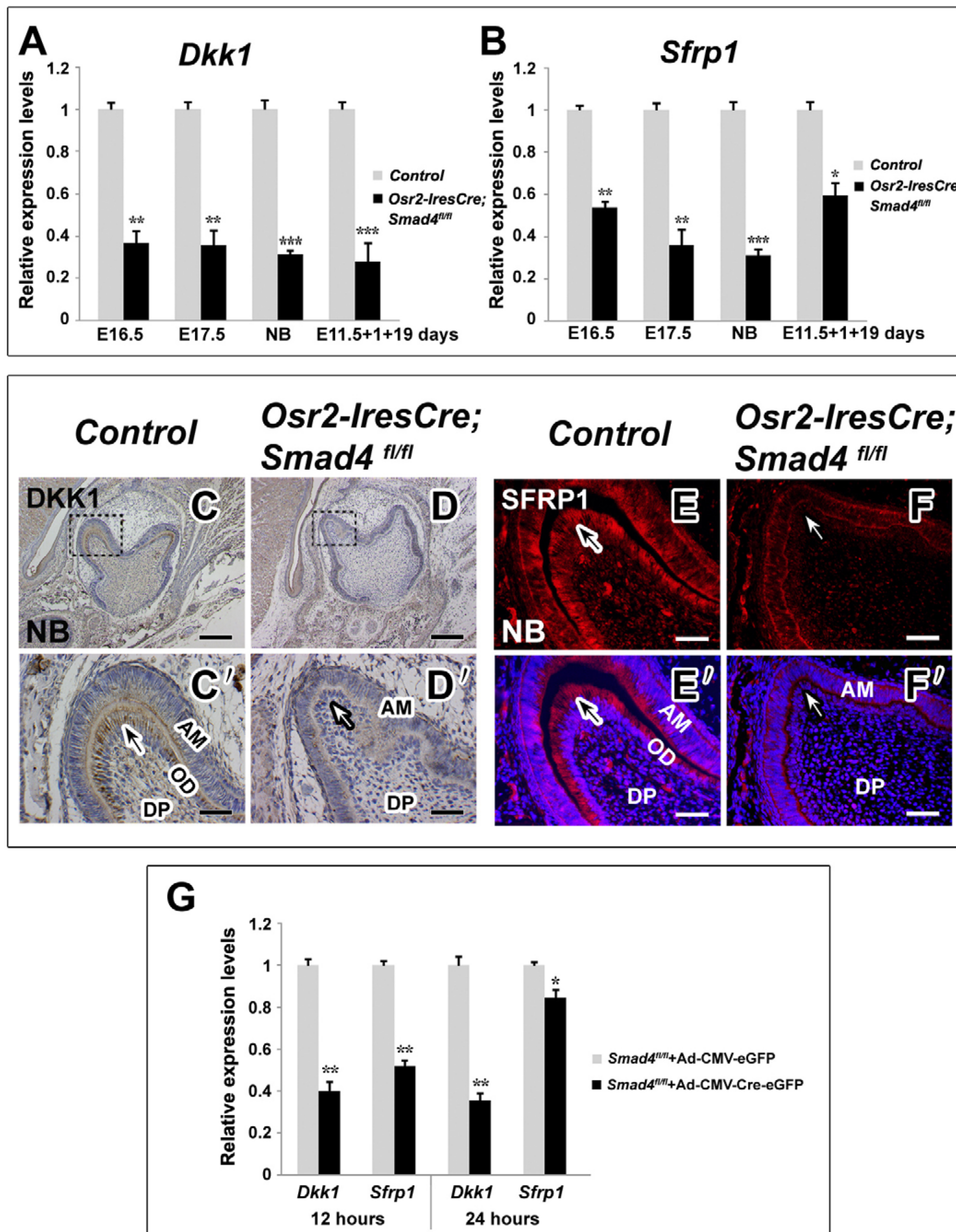

Fig. 7. Downregulation of *Dkk1* and *Sfrp1* in *Osr2-IresCre;Smad4$^{fl/fl}$* mice. (**A**,**B**) qPCR analysis of *Dkk1* (A) and *Sfrp1* (B) in dental papilla of E16.5, E17.5 and newborn (NB) control and *Osr2-IresCre;Smad4$^{fl/fl}$* mice and E11.5 control and *Osr2-IresCre;Smad4$^{fl/fl}$* molars after 19 days kidney capsule transplantation. Values are expressed relative to control. *, $P<0.05$; **, $P<0.01$; ***, $P<0.001$. (**C-D′**) Immunohistochemical staining of DKK1 (brown) counterstained with Hematoxylin (blue) using newborn molar tooth germs from control (C,C′) and *Osr2-IresCre;Smad4$^{fl/fl}$* (D,D′) mice. Boxed areas are shown magnified beneath. Black arrow indicates DKK1 expression in odontoblasts of control mice (C′), whereas the white arrow indicates the lack of detectable DKK1 expression in *Osr2-IresCre;Smad4$^{fl/fl}$* mice (D′). (**E-F′**) Immunofluorescence of SFRP1 (red) in newborn molar tooth germ from control (E,E′) and *Osr2-IresCre;Smad4$^{fl/fl}$* (F,F′) mice. Nuclei were stained with DAPI (blue; E′,F′). Black arrows indicate SFRP1 expression in odontoblasts of control mice (E,E′), whereas white arrows indicate lack of detectable SFRP1 expression in *Osr2-IresCre;Smad4$^{fl/fl}$* mice (F,F′). (**G**) qPCR of *Dkk1* and *Sfrp1* in *Smad4$^{fl/fl}$* dental mesenchymal cells after adenoviral Cre infection (Ad-CMV-Cre-eGFP) for 12 and 24 hours in vitro. Values are expressed relative to adenoviral eGFP infection (Ad-CMV-eGFP). *, $P<0.05$; **, $P<0.01$. Error bars indicate s.d. AM, ameloblast; DP, dental papilla; OD, odontoblast. Scale bars: 200 μm in C,D; 50 μm in E,F,C′-F′.

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;Smad4$^{fl/fl}$* 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;Smad4$^{fl/fl}$* 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).

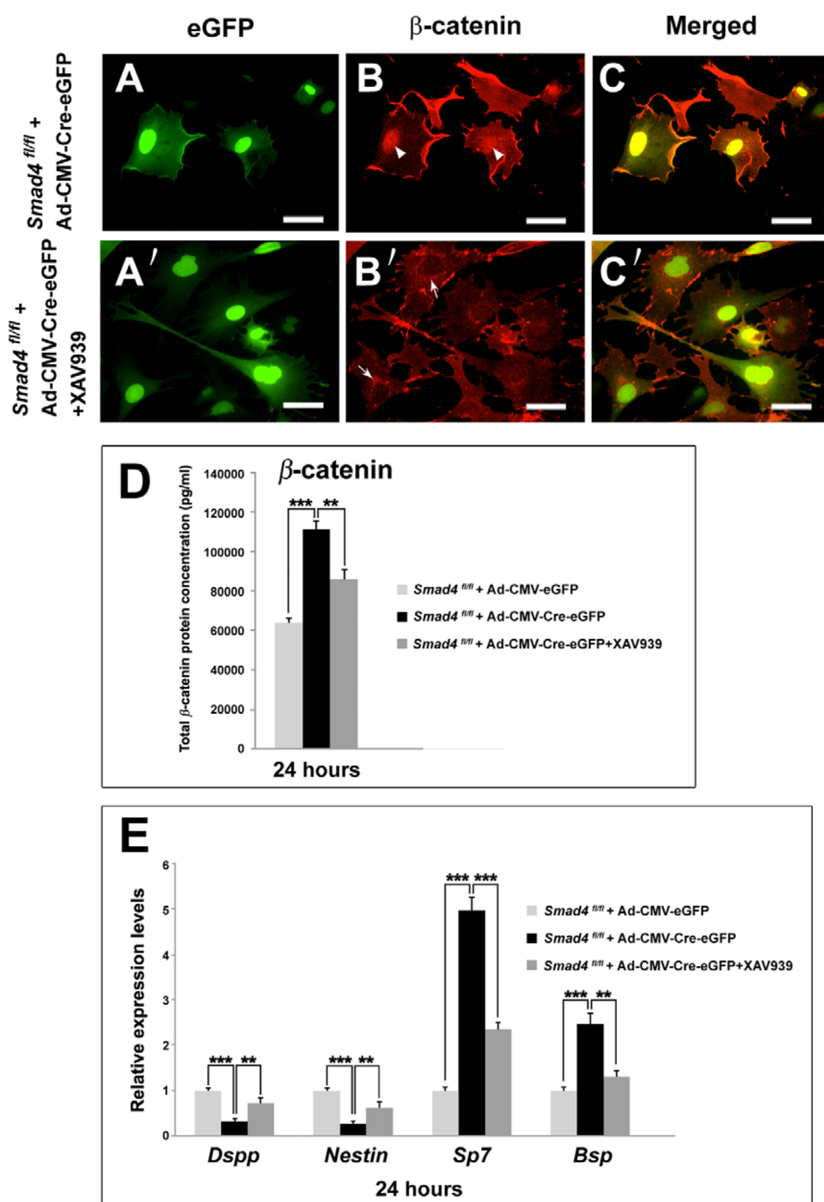

Fig. 8. Partial rescue of the cell fate change in *Osr2-IresCre;Smad4$^{fl/fl}$* dental mesenchyme using an exogenous WNT pathway inhibitor in vitro. (**A-C′**) Immunofluorescence of β-catenin (red) in *Smad4$^{fl/fl}$* dental mesenchymal cells after adenoviral Cre infection (Ad-CMV-Cre-eGFP) alone (A-C) and with XAV939 (10 μM) treatment (A′-C′) for 24 hours in vitro. Cells successfully infected with adenoviral Cre show eGFP fluorescence (green). Arrowheads and arrows point to β-catenin in the nuclei and cytoplasm, respectively. Scale bars: 50 μm. (**D**) ELISA analysis of β-catenin in *Smad4$^{fl/fl}$* dental mesenchymal cells after adenoviral Cre infection (Ad-CMV-Cre-eGFP) and XAV939 (10 μM) treatment for 24 hours in vitro. **, $P<0.01$; ***, $P<0.001$. (**E**) qPCR analysis of genes involved in dentinogenesis (*Dspp* and *Nes*) and osteogenesis (*Sp7* and *Bsp*) in *Smad4$^{fl/fl}$* dental mesenchymal cells after adenoviral Cre infection (Ad-CMV-Cre-eGFP) and XAV939 (10 μM) treatment for 24 hours in vitro. Values are expressed relative to adenoviral eGFP infection (Ad-CMV-eGFP). **, $P<0.01$; ***, $P<0.001$. Error bars indicate s.d.

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 adenoviral 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; Miquelajauregui 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

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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 *Osr2-IresCre;Smad4$^{fl/fl}$* 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 BMPR1A 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 (Kléber 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.

Supplementary material
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References
About, I., Laurent-Maquin, D., Lendahl, U. and Mitsiadis, T. A. (2000). Nestin expression in embryonic and adult human teeth under normal and pathological conditions. *Am. J. Pathol.* **157**, 287-295.
Adaimy, L., Chouery, E., Mégarbané, H., Mroueh, S., Delague, V., Nicolas, E., Belguith, H., de Mazancourt, P. and Mégarbané, A. (2007). Mutation in WNT10A is associated with an autosomal recessive ectodermal dysplasia: the odonto-onycho-dermal dysplasia. *Am. J. Hum. Genet.* **81**, 821-828.
Ahn, Y., Sanderson, B. W., Klein, O. D. and Krumlauf, R. (2010). Inhibition of Wnt signaling by Wise (Sostdc1) and negative feedback from Shh controls tooth number and patterning. *Development* **137**, 3221-3231.
Bain, G., Müller, T., Wang, X. and Papkoff, J. (2003). Activated beta-catenin induces osteoblast differentiation of C3H10T1/2 cells and participates in BMP2 mediated signal transduction. *Biochem. Biophys. Res. Commun.* **301**, 84-91.
Balemans, W., Patel, N., Ebeling, M., Van Hul, E., Wuyts, W., Lacza, C., Dioszegi, M., Dikkers, F. G., Hildering, P., Willems, P. J. et al. (2002). Identification of a 52 kb deletion downstream of the SOST gene in patients with van Buchem disease. *J. Med. Genet.* **39**, 91-97.
Barrow, J. R., Thomas, K. R., Boussadia-Zahui, O., Moore, R., Kemler, R., Capecchi, M. R. and McMahon, A. P. (2003). Ectodermal Wnt3/β-catenin signaling is required for the establishment and maintenance of the apical ectodermal ridge. *Genes Dev.* **17**, 394-409.
Bègue-Kirn, C., Smith, A., Ruch, J., Wozney, J., Purchio, A., Hartmann, D. and Lesot, H. (1992). Effects of dentin proteins, transforming growth factor beta 1 (TGF beta 1) and bone morphogenetic protein 2 (BMP2) on the differentiation of odontoblast in vitro. *Int. J. Dev. Biol.* **36**, 491-503.
Bègue-Kirn, C., Smith, A., Loriot, M., Kupferle, C., Ruch, J. and Lesot, H. (1994). Comparative analysis of TGF beta s, BMPs, IGF1, msxs, fibronectin, osteonectin and bone sialoprotein gene expression during normal and in vitro-induced odontoblast differentiation. *Int. J. Dev. Biol.* **38**, 405-420.
Bennett, C. N., Longo, K. A., Wright, W. S., Suva, L. J., Lane, T. F., Hankenson, K. D. and MacDougald, O. A. (2005). Regulation of osteoblastogenesis and bone mass by Wnt10b. *Proc. Natl. Acad. Sci. USA* **102**, 3324-3329.
Bodine, P. V. N., Zhao, W., Kharode, Y. P., Bex, F. J., Lambert, A.-J., Goad, M. B., Gaur, T., Stein, G. S., Lian, J. B. and Komm, B. S. (2004). The Wnt antagonist secreted frizzled-related protein-1 is a negative regulator of trabecular bone formation in adult mice. *Mol. Endocrinol.* **18**, 1222-1237.
Boyden, L. M., Mao, J., Belsky, J., Mitzner, L., Farhi, A., Mitnick, M. A., Wu, D., Insogna, K. and Lifton, R. P. (2002). High bone density due to a mutation in LDL-receptor-related protein 5. *N. Engl. J. Med.* **346**, 1513-1521.
Cam, Y., Neumann, M., Oliver, L., Raulais, D., Janet, T. and Ruch, J. (1992). Immunolocalization of acidic and basic fibroblast growth factors during mouse odontogenesis. *Int. J. Dev. Biol.* **36**, 381-389.
Chai, Y. and Slavkin, H. C. (2003). Prospects for tooth regeneration in the 21st century: a perspective. *Microsc. Res. Tech.* **60**, 469-479.
Chai, Y., Jiang, X., Ito, Y., Bringas, P., Han, J., Rowitch, D. H., Soriano, P., McMahon, A. P. and Sucov, H. M. (2000). Fate of the mammalian cranial neural crest during tooth and mandibular morphogenesis. *Development* **127**, 1671-1679.
Chen, J., Lan, Y., Baek, J.-A., Gao, Y. and Jiang, R. (2009). Wnt/beta-catenin signaling plays an essential role in activation of odontogenic mesenchyme during early tooth development. *Dev. Biol.* **334**, 174-185.
Chen, Y., Bei, M., Woo, I., Satokata, I. and Maas, R. (1996). Msx1 controls inductive signaling in mammalian tooth morphogenesis. *Development* **122**, 3035-3044.
Chen, Y., Whetstone, H. C., Youn, A., Nadesan, P., Chow, E. C. Y., Lin, A. C. and Alman, B. A. (2007). β-Catenin signaling pathway is crucial for bone morphogenetic protein 2 to induce new bone formation. *J. Biol. Chem.* **282**, 526-533.
Chung, I. H., Yamaza, T., Zhao, H., Choung, P. H., Shi, S. and Chai, Y. (2009). Stem cell property of postmigratory cranial neural crest cells and their utility in alveolar bone regeneration and tooth development. *Stem Cells* **27**, 866-877.
Danielian, P. S., Muccino, D., Rowitch, D. H., Michael, S. K. and McMahon, A. P. (1998). Modification of gene activity in mouse embryos in utero by a tamoxifen-inducible form of Cre recombinase. *Curr. Biol.* **8**, 1323-1326.
DasGupta, R. and Fuchs, E. (1999). Multiple roles for activated LEF/TCF transcription complexes during hair follicle development and differentiation. *Development* **126**, 4557-4568.
Dassule, H. R. and McMahon, A. P. (1998). Analysis of epithelial-mesenchymal interactions in the initial morphogenesis of the mammalian tooth. *Dev. Biol.* **202**, 215-227.
Dean, C. H., Miller, L.-A. D., Smith, A. N., Dufort, D., Lang, R. A. and Niswander, L. A. (2005). Canonical Wnt signaling negatively regulates branching morphogenesis of the lung and lacrimal gland. *Dev. Biol.* **286**, 270-286.
Donoghue, P. C. J., Sansom, I. J. and Downs, J. P. (2006). Early evolution of vertebrate skeletal tissues and cellular interactions, and the canalization of skeletal development. *J. Exp. Zool. B Mol. Dev. Evol.* **306B**, 278-294.
Ellies, D. L., Church, V., Francis-West, P. and Lumsden, A. (2000). The WNT antagonist cSFRP2 modulates programmed cell death in the developing hindbrain. *Development* **127**, 5285-5295.
Fjeld, K., Kettunen, P., Furmanek, T., Kvinnsland, I. H. and Luukko, K. (2005). Dynamic expression of Wnt signaling-related Dickkopf1, -2, and -3 mRNAs in the developing mouse tooth. *Dev. Dyn.* **233**, 161-166.

Fouletier-Dilling, C. M., Bosch, P., Davis, A. R., Shafer, J. A., Stice, S. L., Gugala, Z., Gannon, F. H. and Olmsted-Davis, E. A. (2005). Novel compound enables high-level adenovirus transduction in the absence of an adenovirus-specific receptor. *Hum. Gene Ther.* **16**, 1287-1297.

Gao, Y., Yang, G., Weng, T., Du, J., Wang, X., Zhou, J., Wang, S. and Yang, X. (2009). Disruption of Smad4 in odontoblasts causes multiple keratocystic odontogenic tumors and tooth malformation in mice. *Mol. Cell. Biol.* **29**, 5941-5951.

Gong, Y., Slee, R. B., Fukai, N., Rawadi, G., Roman-Roman, S., Reginato, A. M., Wang, H., Cundy, T., Glorieux, F. H., Lev, D. et al. (2001). LDL receptor-related protein 5 (LRP5) affects bone accrual and eye development. *Cell* **107**, 513-523.

Grotewold, L. and Ruther, U. (2002). The Wnt antagonist Dickkopf-1 is regulated by Bmp signaling and c-Jun and modulates programmed cell death. *EMBO J.* **21**, 966-975.

Guo, X., Day, T. F., Jiang, X., Garrett-Beal, L., Topol, L. and Yang, Y. (2004). Wnt/β-catenin signaling is sufficient and necessary for synovial joint formation. *Genes Dev.* **18**, 2404-2417.

He, W.-X., Niu, Z.-Y., Zhao, S.-L., Jin, W.-L., Gao, J. and Smith, A. J. (2004). TGF-[beta] activated Smad signalling leads to a Smad3-mediated down-regulation of DSPP in an odontoblast cell line. *Arch. Oral Biol.* **49**, 911-918.

He, X. C., Zhang, J., Tong, W.-G., Tawfik, O., Ross, J., Scoville, D. H., Tian, Q., Zeng, X., He, X., Wiedemann, L. M. et al. (2004). BMP signaling inhibits intestinal stem cell self-renewal through suppression of Wnt-beta-catenin signaling. *Nat. Genet.* **36**, 1117-1121.

Holmen, S. L., Giambernardi, T. A., Zylstra, C. R., Buckner-Berghuis, B. D., Resau, J. H., Hess, J. F., Glatt, V., Bouxsein, M. L., Ai, M., Warman, M. L. et al. (2004). Decreased BMD and limb deformities in mice carrying mutations in both Lrp5 and Lrp6. *J. Bone Miner. Res.* **19**, 2033-2040.

Huang, S.-M. A., Mishina, Y. M., Liu, S., Cheung, A., Stegmeier, F., Michaud, G. A., Charlat, O., Wiellette, E., Zhang, Y., Wiessner, S. et al. (2009). Tankyrase inhibition stabilizes axin and antagonizes Wnt signalling. *Nature* **461**, 614-620.

Huelsken, J., Vogel, R., Erdmann, B., Cotsarelis, G. and Birchmeier, W. (2001). beta-Catenin controls hair follicle morphogenesis and stem cell differentiation in the skin. *Cell* **105**, 533-545.

Jernvall, J. and Thesleff, I. (2000). Reiterative signaling and patterning during mammalian tooth morphogenesis. *Mech. Dev.* **92**, 19-29.

Kamiya, N., Ye, L., Kobayashi, T., Mochida, Y., Yamauchi, M., Kronenberg, H. M., Feng, J. Q. and Mishina, Y. (2008). BMP signaling negatively regulates bone mass through sclerostin by inhibiting the canonical Wnt pathway. *Development* **135**, 3801-3811.

Kamiya, N., Kobayashi, T., Mochida, Y., Yu, P. B., Yamauchi, M., Kronenberg, H. M. and Mishina, Y. (2010). Wnt inhibitors Dkk1 and Sost are downstream targets of BMP signaling through the type IA receptor (BMPRIA) in osteoblasts. *J. Bone Miner. Res.* **25**, 200-210.

Karcher-Djuricic, V., Staubli, A., Meyer, J. and Ruch, J. (1985). Acellular dental matrices promote functional differentiation of ameloblasts. *Differentiation* **29**, 169-175.

Kato, M., Patel, M. S., Levasseur, R., Lobov, I., Chang, B. H.-J., Glass, D. A., Hartmann, C., Li, L., Hwang, T.-H., Brayton, C. F. et al. (2002). Cbfa1-independent decrease in osteoblast proliferation, osteopenia, and persistent embryonic eye vascularization in mice deficient in Lrp5, a Wnt coreceptor. *J. Cell Biol.* **157**, 303-314.

Kawano, Y. and Kypta, R. (2003). Secreted antagonists of the Wnt signalling pathway. *J. Cell Sci.* **116**, 2627-2634.

Kléber, M., Lee, H.-Y., Wurdak, H., Buchstaller, J., Riccomagno, M. M., Ittner, L. M., Suter, U., Epstein, D. J. and Sommer, L. (2005). Neural crest stem cell maintenance by combinatorial Wnt and BMP signaling. *J. Cell Biol.* **169**, 309-320.

Ko, S. O., Chung, I. H., Xu, X., Oka, S., Zhao, H., Cho, E. S., Deng, C. and Chai, Y. (2007). Smad4 is required to regulate the fate of cranial neural crest cells. *Dev. Biol.* **312**, 435-447.

Kollar, E. and Baird, G. (1970). Tissue interactions in embryonic mouse tooth germs. *J. Embryol. Exp. Morphol.* **24**, 173-186.

Kratochwil, K., Galceran, J., Tontsch, S., 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.

Lan, Y., Wang, Q., Ovitt, C. E. and Jiang, R. (2007). A unique mouse strain expressing Cre recombinase for tissue-specific analysis of gene function in palate and kidney development. *Genesis* **45**, 618-624.

Leimeister, C., Bach, A. and Gessler, M. (1998). Developmental expression patterns of mouse sFRP genes encoding members of the secreted frizzled related protein family. *Mech. Dev.* **75**, 29-42.

Li, J., Sarosi, I., Cattley, R. C., Pretorius, J., Asuncion, F., Grisanti, M., Morony, S., Adamu, S., Geng, Z., Qiu, W. et al. (2006). Dkk1-mediated inhibition of Wnt signaling in bone results in osteopenia. *Bone* **39**, 754-766.

Little, R. D., Folz, C., Manning, S. P., Swain, P. M., Zhao, S.-C., Eustace, B., Lappe, M. M., Spitzer, L., Zweier, S., Braunschweiger, K. et al. (2002). A mutation in the LDL receptor-related protein 5 gene results in the autosomal dominant high-bone-mass trait. *Am. J. Hum. Genet.* **70**, 11-19.

Livak, K. J. and Schmittgen, T. D. (2001). Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) method. *Methods* **25**, 402-408.

Lohi, M., Tucker, A. S. and Sharpe, P. T. (2010). Expression of Axin2 indicates a role for canonical Wnt signaling in development of the crown and root during pre- and postnatal tooth development. *Dev. Dyn.* **239**, 160-167.

Loots, G. G., Kneissel, M., Keller, H., Baptist, M., Chang, J., Collette, N. M., Ovcharenko, D., Plajzer-Frick, I. and Rubin, E. M. (2005). Genomic deletion of a long-range bone enhancer misregulates sclerostin in Van Buchem disease. *Genome Res.* **15**, 928-935.

MacDonald, B. T., Joiner, D. M., Oyserman, S. M., Sharma, P., Goldstein, S. A., He, X. and Hauschka, P. V. (2007). Bone mass is inversely proportional to Dkk1 levels in mice. *Bone* **41**, 331-339.

Mani, A., Radhakrishnan, J., Wang, H., Mani, A., Mani, M.-A., Nelson-Williams, C., Carew, K. S., Mane, S., Najmabadi, H., Wu, D. et al. (2007). LRP6 mutation in a family with early coronary disease and metabolic risk factors. *Science* **315**, 1278-1282.

Massague, J. (2000). How cells read TGF-beta signals. *Nat. Rev. Mol. Cell Biol.* **1**, 169-178.

Massagué, J. and Gomis, R. R. (2006). The logic of TGFbeta signaling. *FEBS Lett.* **580**, 2811-2820.

Mina, M. and Kollar, E. (1987). The induction of odontogenesis in non-dental mesenchyme combined with early murine mandibular arch epithelium. *Arch. Oral Biol.* **32**, 123-127.

Miquelajauregui, A., Van de Putte, T., Polyakov, A., Nityanandam, A., Boppana, S., Seuntjens, E., Karabinos, A., Higashi, Y., Huylebroeck, D. and Tarabykin, V. (2007). Smad-interacting protein-1 (Zfhx1b) acts upstream of Wnt signaling in the mouse hippocampus and controls its formation. *Proc. Natl. Acad. Sci. USA* **104**, 12919-12924.

Mukhopadhyay, M., Shtrom, S., Rodriguez-Esteban, C., Chen, L., Tsukui, T., Gomer, L., Dorward, D. W., Glinka, A., Grinberg, A., Huang, S.-P. et al. (2001). Dickkopf1 is required for embryonic head induction and limb morphogenesis in the mouse. *Dev. Cell* **1**, 423-434.

Nakashima, M. (1994). Induction of dentin formation on canine amputated pulp by recombinant human bone morphogenetic proteins (BMP)-2 and -4. *J. Dent. Res.* **73**, 1515-1522.

Obara, N., Suzuki, Y. and Takeda, M. (2006). Gene expression of β-catenin is up-regulated in inner dental epithelium and enamel knots during molar tooth morphogenesis in the mouse. *Cell Tissue Res.* **325**, 197-201.

Ohazama, A., Johnson, E. B., Ota, M. S., Choi, H. J., Porntaveetus, T., Oommen, S., Itoh, N., Eto, K., Gritli-Linde, A., Herz, J. et al. (2008). Lrp4 modulates extracellular integration of cell signaling pathways in development. *PLoS ONE* **3**, e4092.

Oka, S., Oka, K., Xu, X., Sasaki, T., Bringas, P., Jr and Chai, Y. (2007). Cell autonomous requirement for TGF-beta signaling during odontoblast differentiation and dentin matrix formation. *Mech. Dev.* **124**, 409-415.

Oshima, T., Abe, M., Asano, J., Hara, T., Kitazoe, K., Sekimoto, E., Tanaka, Y., Shibata, H., Hashimoto, T., Ozaki, S. et al. (2005). Myeloma cells suppress bone formation by secreting a soluble Wnt inhibitor, sFRP-2. *Blood* **106**, 3160-3165.

Plikus, M. V., Zeichner-David, M., Mayer, J.-A., Reyna, J., Bringas, P., Thewissen, J. G. M., Snead, M. L., Chai, Y. and Chuong, C.-M. (2005). Morphoregulation of teeth: modulating the number, size, shape and differentiation by tuning Bmp activity. *Evol. Dev.* **7**, 440-457.

Rawadi, G., Vayssière, B., Dunn, F., Baron, R. and Roman-Roman, S. (2003). BMP-2 controls alkaline phosphatase expression and osteoblast mineralization by a Wnt autocrine loop. *J. Bone Miner. Res.* **18**, 1842-1853.

Ruch, J. (1987). Determinisms of odontogenesis. *Revis. Biol. Celular.* **14**, 1-99.

Ruch, J. (1990). Patterned distribution of differentiating dental cells: facts and hypotheses. *J. Biol. Buccale* **18**, 91-98.

Ruch, J., Karcher-Djuricic, V. and Gerber, R. (1973). Determinants of morphogenesis and cytodifferentiations of dental anloges in mice. *J. Biol. Buccale* **1**, 45-56.

Ruch, J., Lesot, H. and Bègue-Kirn, C. (1995). Odontoblast differentiation. *Int. J. Dev. Biol.* **39**, 51-68.

Ruoslahti, E. and Yamaguchi, Y. (1991). Proteoglycans as modulators of growth factor activities. *Cell* **64**, 867-869.

Rutherford, R. B., Spångberg, L., Tucker, M., Rueger, D. and Charette, M. (1994). The time-course of the induction of reparative dentine formation in monkeys by recombinant human osteogenic protein-1. *Arch. Oral Biol.* **39**, 833-838.

Sarkar, L. and Sharpe, P. T. (1999). Expression of Wnt signalling pathway genes during tooth development. *Mech. Dev.* **85**, 197-200.

Shi, Y. and Massagué, J. (2003). Mechanisms of TGF-beta signaling from cell membrane to the nucleus. *Cell* **113**, 685-700.

Siegel, P. M. and Massague, J. (2003). Cytostatic and apoptotic actions of TGF-beta in homeostasis and cancer. *Nat. Rev. Cancer* **3**, 807-820.

Sire, J. Y. (1994). Light and TEM study of nonregenerated and experimentally regenerated scales of Lepisosteus oculatus (Holostei) with particular attention to ganoine formation. *Anat. Rec.* **240**, 189-207.

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Sloan, A. J., Rutherford, R. B. and Smith, A. J. (2000). Stimulation of the rat dentine-pulp complex by bone morphogenetic protein-7 in vitro. *Arch. Oral Biol.* **45**, 173-177.
Soriano, P. (1999). Generalized lacZ expression with the ROSA26 Cre reporter strain. *Nat. Genet.* **21**, 70-71.
Suomalainen, M. and Thesleff, I. (2010). Patterns of Wnt pathway activity in the mouse incisor indicate absence of Wnt/beta-catenin signaling in the epithelial stem cells. *Dev. Dyn.* **239**, 364-372.
Takada, I., Mihara, M., Suzawa, M., Ohtake, F., Kobayashi, S., Igarashi, M., Youn, M.-Y., Takeyama, K.-i., Nakamura, T., Mezaki, Y. et al. (2007). A histone lysine methyltransferase activated by non-canonical Wnt signalling suppresses PPAR-gamma transactivation. *Nat. Cell Biol.* **9**, 1273-1285.
Terling, C., Rass, A., Mitsiadis, T., Fried, K., Lendahl, U. and Wroblewski, J. (1995). Expression of the intermediate filament nestin during rodent tooth development. *Int. J. Dev. Biol.* **39**, 947-956.
Thesleff, I. and Vaahtokari, A. (1992). The role of growth factors in determination and differentiation of the odontoblastic cell lineage. *Proc. Finn. Dent. Soc.* **88**, 357-368.
Thesleff, I., Keranen, S. and Jernvall, J. (2001). Enamel knots as signaling centers linking tooth morphogenesis and odontoblast differentiation. *Adv. Dent. Res.* **15**, 14-18.
Unda, F.-J., Martín, A., Hilario, E., Bègue-Kirn, C., Ruch, J.-V. and Aréchaga, J. (2000). Dissection of the odontoblast differentiation process in vitro by a combination of FGF1, FGF2, and TGFbeta1. *Dev. Dyn.* **218**, 480-489.
Unterbrink, A., O'Sullivan, M., Chen, S. and MacDougall, M. (2002). TGF beta-1 downregulates DMP-1 and DSPP in odontoblasts. *Connect. Tissue Res.* **43**, 354-358.
Winkler, D. G., Sutherland, M. S. K., Ojala, E., Turcott, E., Geoghegan, J. C., Shpektor, D., Skonier, J. E., Yu, C. and Latham, J. A. (2005). Sclerostin inhibition of Wnt-3a-induced C3H10T1/2 cell differentiation is indirect and mediated by bone morphogenetic proteins. *J. Biol. Chem.* **280**, 2498-2502.
Xu, X., Bringas, P., Jr, Soriano, P. and Chai, Y. (2005). PDGFR-alpha signaling is critical for tooth cusp and palate morphogenesis. *Dev. Dyn.* **232**, 75-84.
Xu, X., Han, J., Ito, Y., Bringas, J. P., Urata, M. M. and Chai, Y. (2006). Cell autonomous requirement for Tgfbr2 in the disappearance of medial edge epithelium during palatal fusion. *Dev. Biol.* **297**, 238-248.
Xu, X., Han, J., Ito, Y., Bringas, P., Jr, Deng, C. and Chai, Y. (2008). Ectodermal smad4 and p38 MAPK are functionally redundant in mediating TGF-beta/BMP signaling during tooth and palate development. *Dev. Cell* **15**, 322-329.
Yamazaki, H., Tsuneto, M., Yoshino, M., Yamamura, K.-I. and Hayashi, S.-I. (2007). Potential of dental mesenchymal cells in developing teeth. *Stem Cells* **25**, 78-87.
Yang, X., Li, C., Herrera, P.-L. and Deng, C.-X. (2002). Generation of Smad4/Dpc4 conditional knockout mice. *Genesis* **32**, 80-81.
Zhang, J., He, X. C., Tong, W.-G., Johnson, T., Wiedemann, L. M., Mishina, Y., Feng, J. Q. and Li, L. (2006). Bone morphogenetic protein signaling inhibits hair follicle anagen induction by restricting epithelial stem/progenitor cell activation and expansion. *Stem Cells* **24**, 2826-2839.