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Noggin inhibition of mouse dentinogenesis

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Abstract

Objectives: The Bone Morphogenetic Proteins (BMPs) direct tooth development and still express in the adult tooth. We hypothesized that inhibition of BMP function would therefore disrupt dentinogenesis by differentiated odontoblasts.

Methods: We generated mice overexpressing the BMP-inhibitory protein Noggin in differentiated odontoblasts and osteocytes under control of a *Dmp1* promoter-driven cre transgene. We compared the dentin phenotype in these mice with that in WT littermates and in mice with a *Smad4* odontoblast/osteocyte knockout mediated by the same cre and therefore lacking all BMP and Tgfß signaling in the same tissues.

Results: Three-month-old first molars from both Noggin-expressing and Smad4-deleted mice showed decreased dentin volume with enlarged pulp cavities, and both displayed less organized and mineralized dentinal tubules compared to WT. The Smad4-ablated phenotype was more severe. While dentin sialophosphopritein (DSPP) and bone sialoprotein (BSP) were decreased in the dentin of both lines, dentin matrix protein 1 (DMP1) was sharply increased in Noggin-expressing teeth.

Conclusions: The phenotypes we observed in Noggin-overexpressing and Smad4-conditional knockout teeth resemble the phenotype of Dentinogenesis Imperfecta (DGI) type III. Our results show that BMPs regulate post-natal dentinogenesis and that BMP-inhibitory proteins like Noggin play a role in that regulation. The increased severity of the Smad4 phenotype indicates that Tgfß ligands, in addition to BMPs, play a crucial role in post-developmental dentinogenesis.

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Conflict of interest

All of the authors declare no conflict of interest.

Ethical statement

All animal procedures were approved by the Institutional Animal Care and Use Committee (IACUC) of the Texas A&M University College of Dentistry.

Keywords

bone morphogenetic protein; transforming growth factor beta; dentin; dentinogenesis imperfecta; tooth

1. Introduction

Mammalian tooth development begins with an invagination of the ectoderm into the underlying cranial neural crest-derived mesenchyme. A series of reciprocal signals between these two layers shapes the tooth through the bud, cap, and bell stages, with the ectodermal tissue eventually forming enamel while the mesenchyme gives rise to dentin [1]. Prominent among these reciprocal signals are the Bone Morphogenetic Proteins (BMPs), members of the Transforming Growth Factor ß (Tgfß) superfamily of secreted molecules [2-4]. BMPs 2 and 4, in particular, play central roles in initial tooth patterning events [5, 6]. Their initial secretion by the ectoderm initiates mesenchymal progression toward odontogenesis. Subsequent BMP4 expression by the mesenchyme then acts back on the ectoderm to induce amelogenesis.

BMPs 2 and 4 continue to be expressed in the enamel and dentin layers and both play roles in cell differentiation in these tissues. Conditional deletion of *Bmp2* in mouse ameloblasts using an *Osx*-cre causes reduced and hypomineralized enamel [7], while deletion with a 3.6*Col1a1*-cre leads to marked reduction of dentin in both root and crown due to deficient odontoblast differentiation [8]. Deletion of *Bmp4* with the 3.6*Col1a1*-cre also reduces odontoblast differentiation marker expression leading to reduced dentin [9]. We recently reported that both BMP 2 and 4 continue to regulate dentin formation by mature odontoblasts after their differentiation [10].

BMPs bind to BMP type I receptors (BMPRIs) or activin-like kinase (ALK) 2, 3, or 6. This complex then binds to the BMP type II receptor (BMPRII), a serine/threonine kinase that phosphorylates the type I receptor. The activated type I receptor phosphorylates a set of Smad proteins called receptor Smads (R-Smad1, 5, and 8) that bind to Smad4, a co-Smad used by all BMP signaling pathways. The assembled Smad complex accumulates in the nucleus where it mediates BMP-dependent gene transcription [11, 12]. As Smad4 is a common co-Smad for BMP and Tgfß receptors, its deletion has more severe consequences for development than the loss of one or the other alone [13].

In addition to the canonical, Smad-mediated signaling pathway, BMP receptors can also signal through a non-canonical pathway involving activation of p38 and ERK MAP kinases [14, 15]. Although this pathway is important for BMP function during tooth formation [15], we do not yet know the extent to which it contributes to BMP-mediated dentin formation in the mature tooth.

A number of secreted binding proteins, including Noggin, Follistatin, Ectodin, and Chordin, regulate BMP activity *in vivo* by preventing their binding to BMP receptor complexes [16-19]. Noggin, in particular, is known to inhibit BMP 2, 4, and 7 signaling during early gastrulation [20, 21]. Ectopic expression of Noggin during early tooth development blocks

BMP signals and arrests tooth formation at the lamina/early bud stage, while its application later at e12 can convert presumptive incisors to a molar fate by disrupting transcription downstream of BMP receptors [22, 23]. Because of its high affinity for all three of these BMPs, controlled transgenic Noggin overexpression is a feasible way to suppress their activity without the practical complications of creating a triple conditional knockout in one mouse.

We recently created a double conditional knockout of *Bmp2* and *4* under the control of a *Dmp1*-cre [10]. These double-knockout mice displayed reduced and disorganized dentin and enlarged pulp cavities compared to wild type (WT). The *Dmp1*-cre is expressed by terminally differentiated odontoblasts [24] so these phenotypes demonstrated that BMPs 2 and 4 regulate dentinogenesis in mature cells. In this study, we used a Noggin-overexpressing allele under control of the same *Dmp1*-cre to explore the effect of suppressing BMP activity beyond only that of 2 and 4. We further compared the dentin phenotypes in these mice with a *Dmp1*-cre conditional knockout of *Smad4* which ablated all BMP and Tgfß signaling in differentiated odontoblasts.

2. Materials and methods

2.1. Animals

Smad4 conditional knockout, *Dmp1*-Cre, *pMes-Nog,* and *Smad4*$^{fl/fl}$ mice have been described previously [24-26]. *Smad4*$^{fl/fl}$ mice or *pMes-Nog* mice were mated with *Dmp1-Cre* transgenic mice to generate the *Smad4* conditional-knockout mice and mice overexpressing Noggin, respectively. Genotypes were confirmed by polymerase chain reaction (PCR) using primers specified in Table 1. Age- and gender-matched WT littermates were used as controls. All animal procedures were approved by the Institutional Animal Care and Use Committee (IACUC) of the Texas A&M University College of Dentistry.

2.2. Radiography and Microcomputed Tomography (μCT)

Mandibles from three-month-old mice were fixed for 48 hours in 4 % formaldehyde, stored in 70 % ethanol at 4 °C, and analyzed with a Faxitron MX-20 (Faxitron X-ray Corp., Buffalo Grove, IL). Three-month-old gender-matched mandibles were scanned in 7.0 μm slice increments using a μCT35 imaging system (Scanco Medical, Basserdorf, Switzerland) to assess the overall shape and structure and in 3.5 μm increments for quantification. (n = 4 each for each group).

2.3. Tissue Preparation and Histology Evaluation

Teeth from three-month-old mice were demineralized in 14 % EDTA (pH 7.4) at 4 °C for 2 weeks, processed for paraffin embedding, and sectioned at 5 μm for hematoxylin and eosin (H&E) or Picrosirius Red to staining [27]. Anti-DSP-2C12.3 monoclonal antibody was used at 2 μg/mL. Anti-DMP1 monoclonal antibody against the C-terminal region of DMP1 was used at 5 μg/mL. Anti-BSP monoclonal antibody 10D9.2 was used at 5 μg/mL. All IHC experiments used the Mouse on Mouse and DAB (3,3′-diaminobenzidine) kits with Methyl Green counterstain (Vector Laboratories, Burlingame, CA).

2.4. In Situ Hybridization

In situ hybridization was performed on sections of six-week-old molars as previously described [28]. RNA probes were labeled with the DIG DNA Labeling and Detection Kit from Roche (Indianapolis, IN) and detected by an enzyme-linked immunoassay as per manufacturer's instructions, with Nuclear Fast Red counterstain (Vector Laboratories, Burlingame, CA).

2.5. Statistical Analysis

Student's t-test was used to validate the differences between two groups. $P < 0.05$ was considered statistically significant. Data were presented as mean ± SEM.

3. Results

To explore BMP signaling in postnatal dentinogenesis we employed a transgenic mouse line expressing cre under control of the *Dmp1* gene. Cre from this allele is expressed at low levels in teeth before e18.5 and at high levels in odontoblasts and pulp odontoblast precursors after birth, as well as in osteocytes [24]. We crossed this line with the *pMes-Nog* allele in which Noggin expression is unleashed in cre-expressing cells to create odontoblast Noggin-overexpressing mice (*Dmp1*-Cre;*pMes-Nog*, referred to as Nog-O here). We also crossed the *Dmp1*-cre into a *Smad4* loxP allele to generate an odontoblast-selective *Smad4* knockout line (*Dmp1*-Cre;$Smad4^{fl/fl}$ referred here as Smad4cKO). We examined the dentin in these mice at three months of age so as to examine the role of BMP inhibition on postnatal formation of primary and secondary dentin.

3.1. X-ray and micro-CT analysis of noggin-overexpressing teeth

Hemi-mandibles from three-month-old WT, Nog-O, and Smad4cKO mice were examined by x-ray. First molars from Nog-O mice appeared normal in size and shape compared to the ones from WT littermates (Fig. 1a,b) but displayed a reduction in root dentin thickness, especially near the furcation, resulting in an enlarged pulp cavity compared to controls (red arrow in 1b). Smad4cKO first molars displayed a much more severe phenotype than Nog-O teeth (Fig. 1c). They appeared much smaller, with much less dentin and a correspondingly enlarged pulp cavity. The enamel layer, however, did not appear to be affected. In addition to the tooth phenotype, Smad4cKO mandibles had reduced alveolar bone density surrounding the roots. This is likely the result of *Dmp1*-cre expression causing *Smad4* knockout in the osteocytes. The incisors in Nog-O and Smad4cKO showed no phenotype on x-ray and thus, were not analyzed further.

Quantitative μ-CT analysis of first molars supported the x-ray observations. Sagittal sections through the middle portion of the first molar revealed an increase in the pulp chamber width of molars in Nog-O mice (red bracket in Fig. 2b) compared to WT (Fig. 2a). Coronal section through the mesial root of the first molar of these mice also showed similar changes (Fig. 2d,e). Coronal dentin appeared thinner in Nog-O mice (Fig. 2e) than in WT mice (Fig. 2d). Crown and root dentin were even more dramatically reduced in the molars of Smad4cKO mice with a correspondingly enlarged pulp cavity (Fig. 2c,f). Some mineralized nodules were also apparent in coronal pulp of Smad4cKO mice (Fig. 2f, red arrow) indicating pulp

calcifications. We generated heat maps of the dentin thicknesses in each group using the μ-CT data to visually observe differences in density distribution throughout the first molars. High density areas are shown in red in the images while the least dense regions appear green. Dentin was thickest in the buccal portion of WT molar crowns with thickness decreasing towards the root apices (Fig. 2g). Molars of Nog-O mice showed an irregular distribution with some denser areas close to the root apex (Fig. 2h). Dentin was thin throughout the Smad4cKO molars as indicated by the absence of red areas (Fig. 2i). Thus, not only the dentin amount but also the distribution of dentin mineral density was affected by BMP inhibition in these mice.

Three dimensional volumetric measurements of first molars were done using the μ-CT data on four mice from each group. These measurements are shown in Table 2 and graphed in Figure 3. Enamel was excluded from the analyses by thresholding. Total Nog-O tooth volumes were relatively unchanged but average dentin volume was reduced to 88 % of WT (Fig. 3a). Thus, the dentin volume as a fraction of total tooth volume fell 8 % (Fig. 3b). The loss of dentin created an increase in pulp cavity size. Pulp volume in Nog-O mice was 20 % higher than in the controls (Fig. 3a). These changes created a reduction in the apparent density, which is defined as the density when considering all tissues and spaces in the tooth (Fig. 3c). The material density of the dentin, however, was not significantly changed by the Noggin overexpression. This is in spite of the loss of the higher density areas in the heat map (revealed as red spots in Figure 2) and suggests more of a density redistribution throughout the tooth rather than simply an overall loss.

Total tooth and dentin volumes in Smad4cKO first molars were reduced to 79 % and 62 % of WT, respectively. The loss of dentin created a substantially increased pulp volume, 41 % greater than in WT and 21 % greater than in Nog-O mice (Fig. 3a). The enlarged pulp cavity in Smad4cKO molars contributed to an overall reduced apparent density compared to WT molars. Smad4cKO dentin material density was also reduced (Fig. 3c).

3.2. Histological changes in Nog-O and Smad4cKO molars

Hematoxylin and eosin staining showed well-ordered dentinal tubules in sections of Nog-O first molars similar to the ones in the controls. There was, however, a substantial increase in the width of the unmineralized predentin layer suggesting a delay in dentin maturation caused by BMP inhibition (Fig. 4a,b, red arrow in b). Smad4cKO sections exhibited an even more dramatic decrease in mineralization throughout the proximal dentin adjacent to the pulp (Fig. 4c). Their dentinal tubules were disorganized and far less stained, as opposed to the regularly oriented tubules in the dentin of WT mice. Interestingly, despite the overall reduction in dentin mineralization, a discrete unmineralized predentin layer was absent in the Smad4KO as if the natural progression of dentin maturation was suppressed throughout.

We examined collagen fiber structure using Picrosirius Red (PR) to differentiate between thinner reticular fibers and thicker fibers [27]. WT dentin appeared evenly stained with bright red color under transmitted light microscopy (Fig. 4d). When viewed under polarized light, the thick collagen fibers ranged from red to orange in WT mice (Fig. 4g). The Nog-O and Smad4cKO collagen fibers had irregular distributions with patches of thin fibers (light pink) interspersed between the areas of thick fibers (Fig. 4e,f). Under polarized light, these

thin reticular fibers appeared green to yellow indicating that both Nog-O (Fig. 4h) and Smad4cKO mice (Fig. 4i) had fewer thick fibers than WT mice. Collagen plays a central role in the nucleation of the mineralized matrix in teeth and has been shown to bind to several proteins involved in the matrix formation [29]. Thus, irregular collagen fibril formation and orientation may contribute to the mineralization defects in Nog-O and Smad4cKO dentin.

3.3. Expression of dentin ECM proteins is altered by Noggin overexpression or Smad4 deletion

To understand the mechanism underlying the phenotypic changes observed in these mice, we performed immunohistochemical staining for dentin matrix proteins. Dentin sialophosphoprotein (DSPP), the most abundant non-collagenous protein in dentin, was drastically reduced in Nog-O and Smad4cKO mice compared to WT (Fig. 5a-c). Bone sialoprotein (BSP) was also reduced in Nog-O and Smad4cKO dentin. Surprisingly, expression of DMP1 protein was dramatically increased in Nog-O dentin compared to WT, even though it was decreased and more diffusely distributed throughout the tubules of Smad4cKO dentin.

We confirmed the DMP1 immunohistochemistry results with *in situ* hybridization which revealed a marked increase in *Dmp1* mRNA in Nog-O mice (Fig. 6b) and decreased mRNA in Smad4cKO mice (Fig. 6c) compared to WT. We also investigated whether the changes we observed in predentin of Nog-O teeth and dentin structure in SmadcKO teeth might be associated to changes in collagen gene expression. *Col1a1* mRNA signal appeared somewhat reduced in the odontoblast layer of Nog-O teeth as indicated by less punctate staining in Fig. 6e compared to WT in 6d, suggesting that their widened layer of unmineralized predentin was not caused by accelerated collagen production. In contrast, odontoblast *Col1a1* signal in Smad4cKO mice was more intense than in WT (Fig. 6d,f). Overall, *Smad4* knockout in odontoblasts not only produced a more severe dentin phenotype than Noggin overexpression but also a different pattern of matrix protein expression. These *in situ* data argue against deficient type I collagen production being a cause of the observed dentin defects in Smad4cKO teeth.

4. Discussion

We generated mice expressing the BMP-antagonist Noggin under control of a *Dmp1*-cre transgene. The transgene is expressed primarily in terminally differentiated odontoblasts [24]. Thus, we were able to examine the effect of suppressing BMP activity on dentin formation in the postnatal and mature tooth. Nog-O mice displayed reduced dentin and increased pulp volume with a thickened pre-dentin layer, similar to what we previously described in a dentin-specific knockout of *Bmp2* and *4* using the same *Dmp1*-cre allele [10]. The Nog-O phenotype appears marginally less severe; however, this may be due to a slightly less than complete suppression of BMPs 2 and 4 by the Noggin protein as compared to their complete genetic knockout. Nevertheless, a comparison of data suggests that the role of BMPs in dentin formation is mediated entirely by BMPs 2 and 4 and not by other additional members of the BMP family subject to Noggin inhibition, such as BMP7 [30, 31].

Consistent with this interpretation, deletion of BMP7, though it is expressed in dentin, shows no dentin defects. It should be noted that there are other BMP family members that are not susceptible to Noggin suppression [20], but to our knowledge, roles for these in dentin formation have not been described.

Molars from *Smad4 Dmp1*-cre conditional knockout (Smad4cKO) mice had a far more severe dentin phenotype than those from Nog-O mice. Rather than having a discrete but thickened predentin layer as in Nog-O mice, Smad4cKO teeth showed an expanded region of poorly organized dentinal tubules radiating out from the pulp with thinner collagen fibers than in the WT. Smad4 mediates intracellular signaling from BMPs and other Tgfß superfamily receptors. Thus, the more severe phenotype in Smad4cKO mice is consistent with roles for other Tgfß-related ligands beyond merely BMPs 2 and 4.

While there are BMPs not susceptible to Noggin inhibition (i.e: BMPs 3, 13, and 14) that could theoretically be nullified by *Smad4* knock out in odontoblasts, there are no known roles for these BMPs in dentin [20]. However, Tgfß signaling has been reported to be required for odontoblast differentiation. Oka and coworkers showed that molars of mice in which *Tgfß2* had been deleted by a neural crest-specific *Wnt1*-cre, from which dentin is derived, presented with reduced dentin and disrupted dentinal tubules in a kidney transplantation model [32]. We do not know the relative extent to which Tgfß2 and BMPs 2 and 4 contribute to dentin formation in the adult tooth, but our data are consistent with a role for Tgfß2 in addition to these two Noggin-susceptible BMPs.

We also observed a dramatic loss of alveolar bone density in Smad4cKO mice compared to WT (Fig. 1c). Tgfß and BMP signaling are known to be important for bone formation [33, 34]. The *Dmp1*-cre allele is expressed in osteocytes, which regulate bone remodeling [35], and so the reduced bone density in these mice is most likely a cell-autonomous phenotype attributable to recombination of the *Smad4* allele in these cells.

The dentin defects we observed in both Nog-O and Smad4cKO mice resemble the phenotypes of *Dspp* and *Dmp1* knockout mice, both of which display features of human dentinogenesis imperfecta (DGI) type III, namely thinner dentin, a widened predentin layer, and enlarged pulp chamber [36, 37]. Surprisingly, while DMP1 was reduced as expected in Smad4cKO molars, it was dramatically upregulated in Nog-O teeth. This upregulation was also observed at the mRNA level, suggesting increased transcription of the *Dmp1* gene or possibly increased message halflife. We cannot explain the mechanism behind the difference in DMP1 expression between these two mouse models with the data currently available. However, the consequence of this finding for our understanding of the dentin formation mechanism is interesting. The previously published DGI-like phenotypes of *Dspp* and *Dmp1* knockouts indicate that both proteins are crucial for proper dentin formation, but the Nog-O phenotype reported here shows that increased *Dmp1* expression cannot rescue the dentin phenotype in the context of BMP inhibition. Earlier studies from our research group indicated that DSPP is a downstream effector of DMP1, as transgenic overexpression of DSPP was able to rescue the tooth and bone defects of DMP1-deficient mice [28, 38]. Yet, the increased expression of DMP1 in the Nog-O mice did not cause an increase in endogenous DSPP production. Further, the DSPP mutant mouse model of DGI shows

decreased levels of matrix DSPP and elevated odontoblast *Dmp1* mRNA levels similar to what we observe in the Nog-O mice suggesting that altered DSPP somehow affects *Dmp1* gene expression [36]. Thus, the relation between these two genes is more complex than a linear upstream-downstream regulation.

Collagen mRNA was increased in the Smad4cKO odontoblast layer, and we observed this phenomenon in *Bmp2/4* double knockout mice [10]. We do not know whether the increased levels of message in these mice means that more collagen protein is being produced or exported by Smad4cKO odontoblasts. If more collagen matrix is being exported, then the dentin defects we observe are a result of an inability to properly assemble and mineralize the matrix in spite of abundant collagen. However, it is also possible that increased amounts of collagen are being translated by the cells but not properly exported thereby causing a backup of the secretory machinery and a potentially damaging unfolded protein response. Our current data cannot distinguish between these or other possible mechanisms, and ongoing studies will need to address this question.

Our previous studies of *Bmp* 2 and 4 *Dmp1*-cre double-knockout mice were not able to address whether these proteins mediate their effects on dentin formation through the canonical Smad pathway, the non-canonical pathway, or both. The current study does not directly address nor completely resolve this issue. Resolution of the relative contributions of these two pathways awaits the generation of knockout models specific to these individual pathways.

5. Conclusion

Our data demonstrate that dentin formation in postnatal molars depends on Smad-based signaling downstream of BMPs 2 and 4 and likely Tgfßs. Disruption of this signaling models human DGI type III. Therefore, these pathways may present viable targets for treatment of the human disease.

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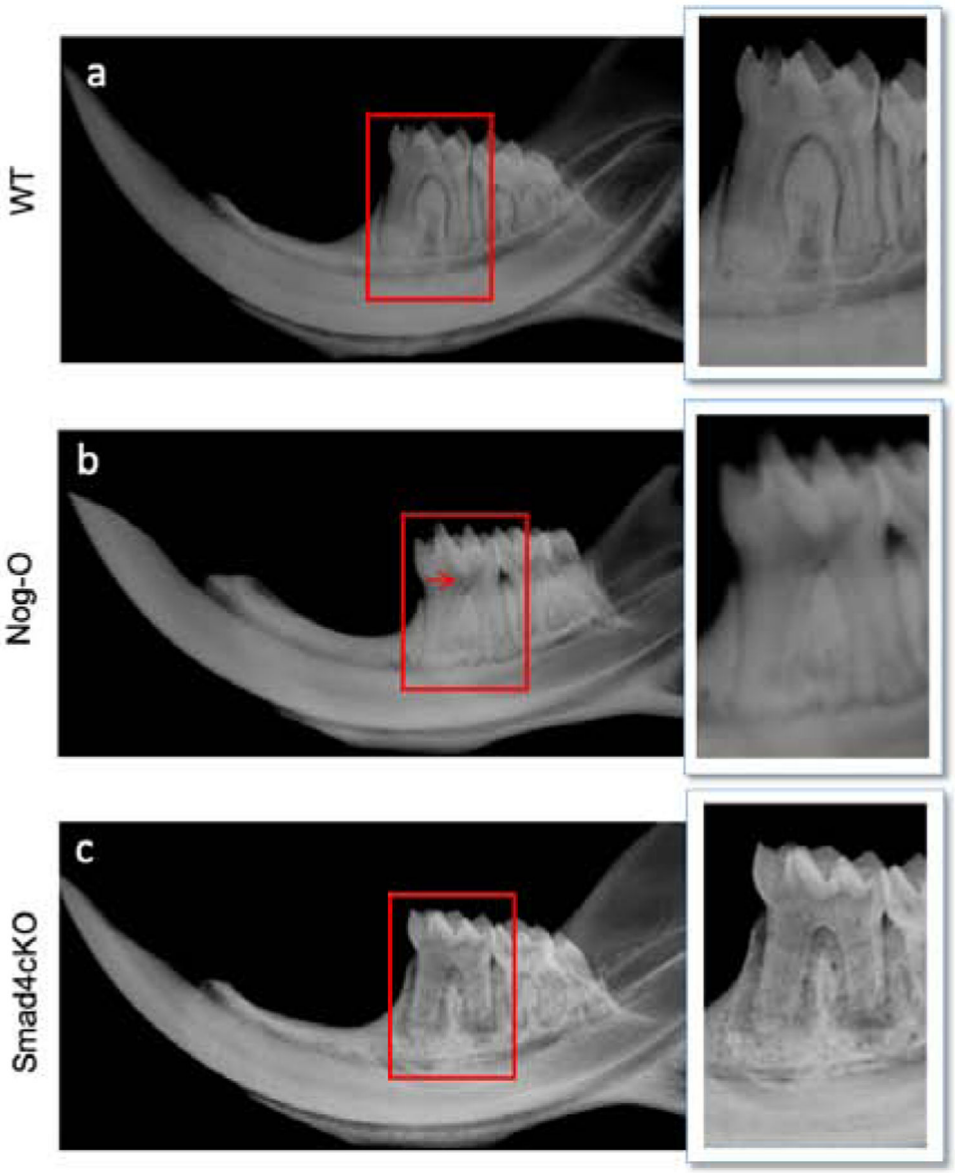

Figure 1. Plain x-ray radiography of Noggin-overexpressing and Smad 4 knockout teeth.

(a) Two dimensional x-rays of hemi-mandibles from 3-month old WT mice show normal appearance of the dentin, alveolar bone, and periodontal ligament space. (b) *Dmp1*-Cre;*pMes-Nog* (Nog-O) first molars show a wider pulp cavity (red arrow). (c) *Dmp1*-Cre;*Smad4*$^{fl/fl}$ (Smad4cKO) molars displayed increased radiolucency of the bone around the first molar, a greatly enlarged pulp cavity, and thin dentin in both root and crown. The insets in each panel show the first molars at higher magnification.

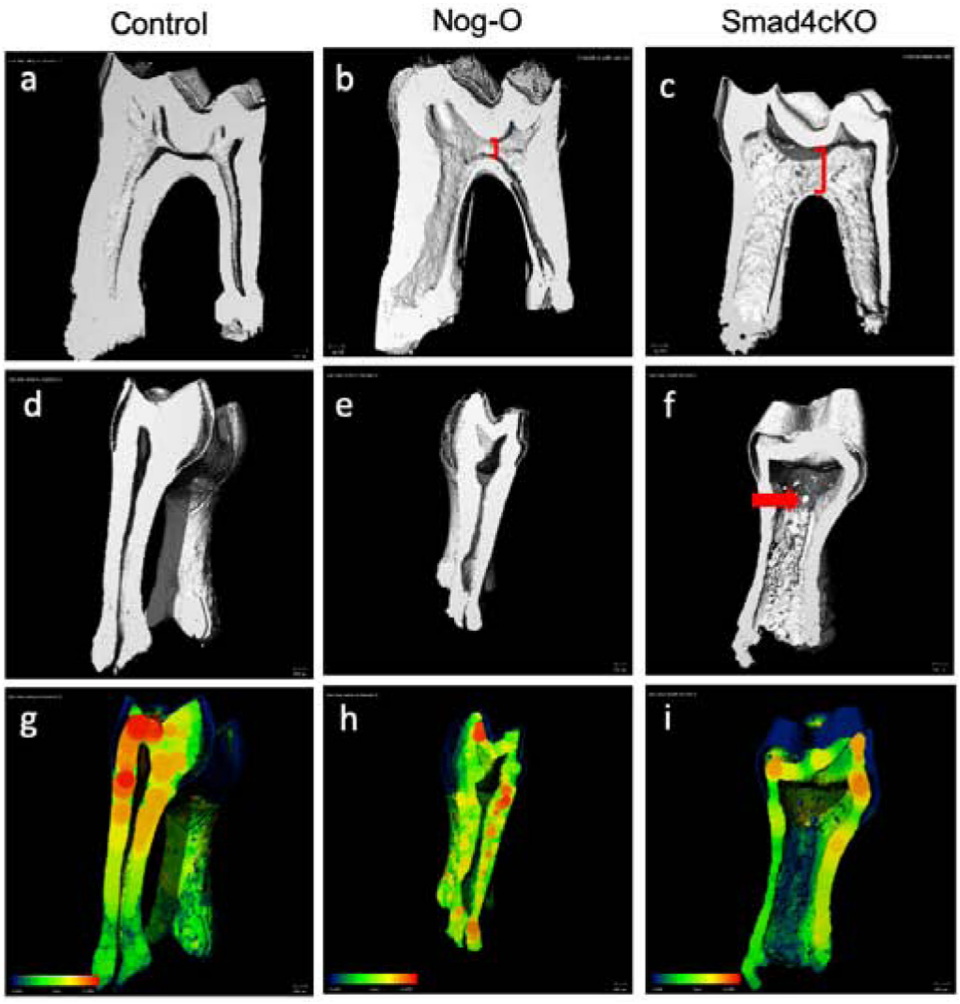

Figure 2. Micro computed tomography imaging of Nog-O and Smad4cKO first molars.

Three dimensional micro-CT images of first molars of 3-month-old mice showed a larger pulp chamber in Nog-O mice (b,e, red bracket in b) and visibly thinner dentin in the crown compared to WT (a,d). Smad4cKO molars (c,f) showed a dramatically thinned dentin layer with correspondingly enlarged pulp chamber (bracket in c). Smad4cKO pulp chambers also contained calcified nodules (f, red arrow). The density heat map shows areas of greater density in red and least dense in green (g-i). WT molars had areas of the highest density in the crown (g). Nog-O molars had fewer regions in red and less total red area (h). Smad4cKO showed no areas in red indicating reduced dentin density in the overall structure (i). Scale bar = 200 μm.

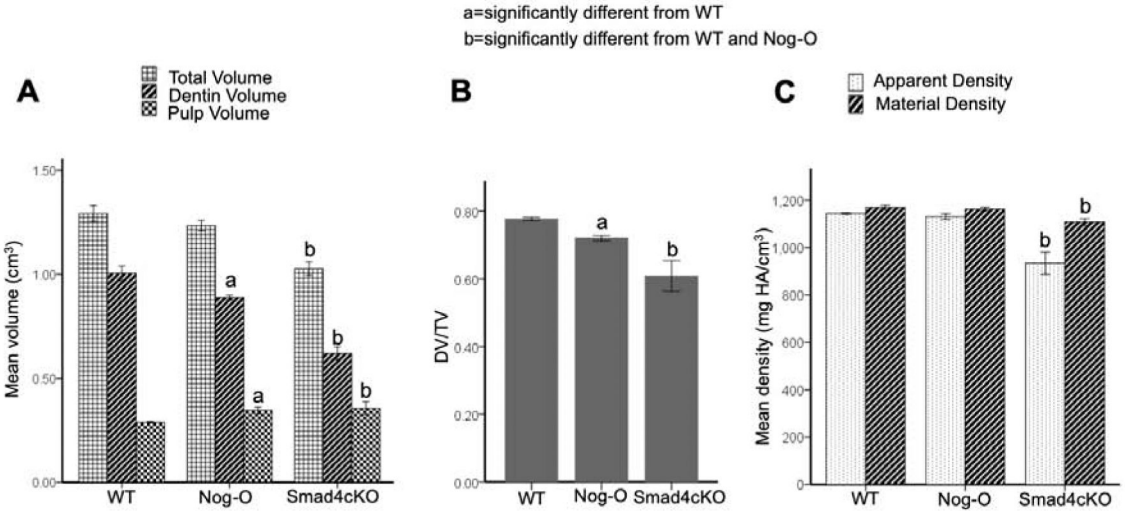

Figure 3. Quantification of volume and density in Nog-O and Smad4cKO molars.

(A) Total tooth volume in WT and Nog-O molars was similar. Both Nog-O and Smad4cKO had significantly less dentin volume than WT and correspondingly increased pulp volumes. (B) Dentin volume as a fraction of total volume (DV/TV) was significantly reduced in both Nog-O and Smad4cKO molars. (C) The apparent density of Nog-O teeth (density calculated throughout the tooth, including pulp space and dentin) was similar in WT and in Nog-O teeth, but was significantly reduced in Smad4cKO teeth. The material density (density of the dentin alone) was not significantly lower in Nog-O and Smad4cKO teeth despite the absence of regions of maximal density in these teeth (red spots on the heat map in Figure 2). Error bars are ± SEM. “a” and “b” denote $p \leq 0.05$ for comparison with WT and for comparison with WT and Nog-O, respectively. n=4 for each group.

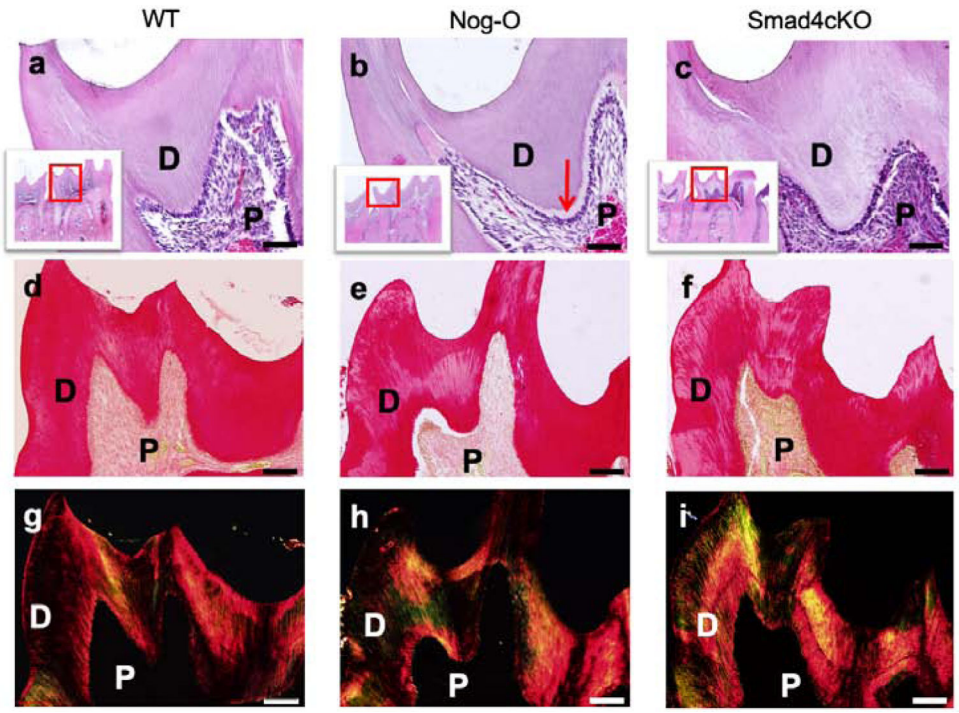

Figure 4. Histological evaluation of first molar dentin.

(a-c) H&E staining and high magnification view of the distal cusp of the first molar showed altered dentin ultrastructure. Nog-O teeth had thinner dentin and a widened predentin zone (b, red arrow) compared to WT teeth (a). There was severe disorganization of the dentin tubules in Smad4cKO mice with more irregular dentin in the area towards the pulp (c). Picrosirius Red (PR) staining in WT dentin showed uniform staining indicating well-organized collagen fibrils under transmitted (d) and polarized light (g). Nog-O mice showed uneven PR staining (e) with more green stain under polarized light (h) indicating the presence of thinner collagen fibrils in that area. Smad4cKO teeth showed expanded areas of light pink PR staining under transmitted (f) that appeared yellow and green under polarized light (i), reflecting the presence of widespread disorganization of thin collagen fibrils. Scale bar =100 μm. D, dentin; P, pulp cavity. Red squares in insets show the higher magnification images in relation to the surrounding tooth.

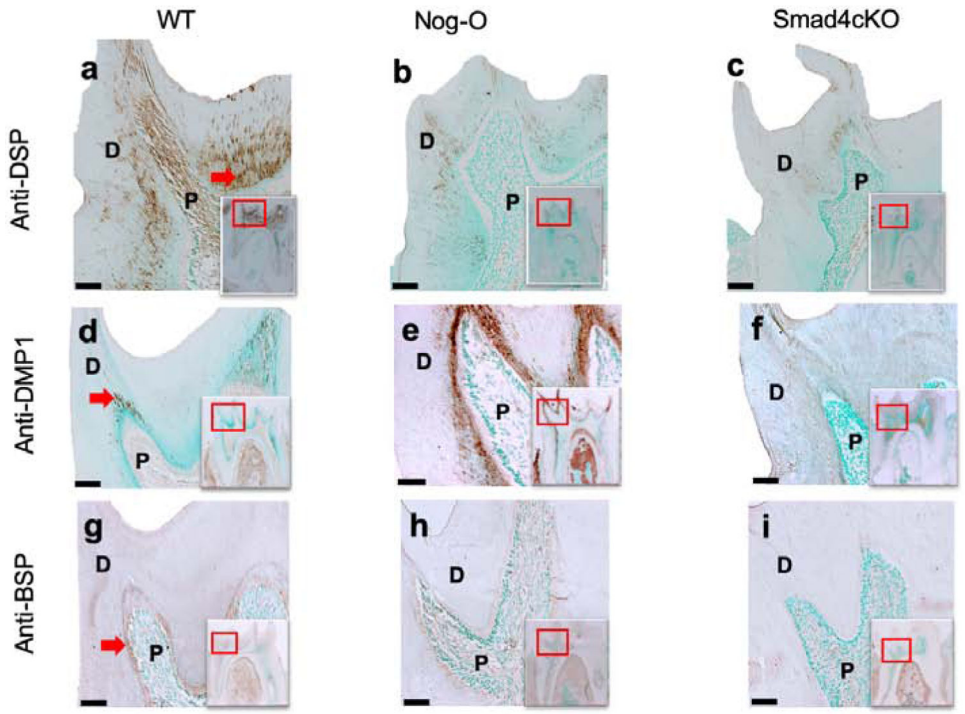

Figure 5. Expression of late odontoblast markers in Nog-O and Smad4cKO molars.

Immunohistochemical staining showed normal expression of DSP, DMP1, and BSP in WT teeth (red arrows in a, d, and g, respectively). Nog-O teeth had reduced DSP (b) and BSP (h) but a marked increase in DMP1 in the odontoblast layer (e). Smad4cKO teeth had a substantial reduction in all three matrix proteins compared to WT mice (c,f,i). D, dentin; P, pulp cavity. Red squares in insets indicate magnified area in the context of the surrounding tooth. Scale bar = 50 μm.

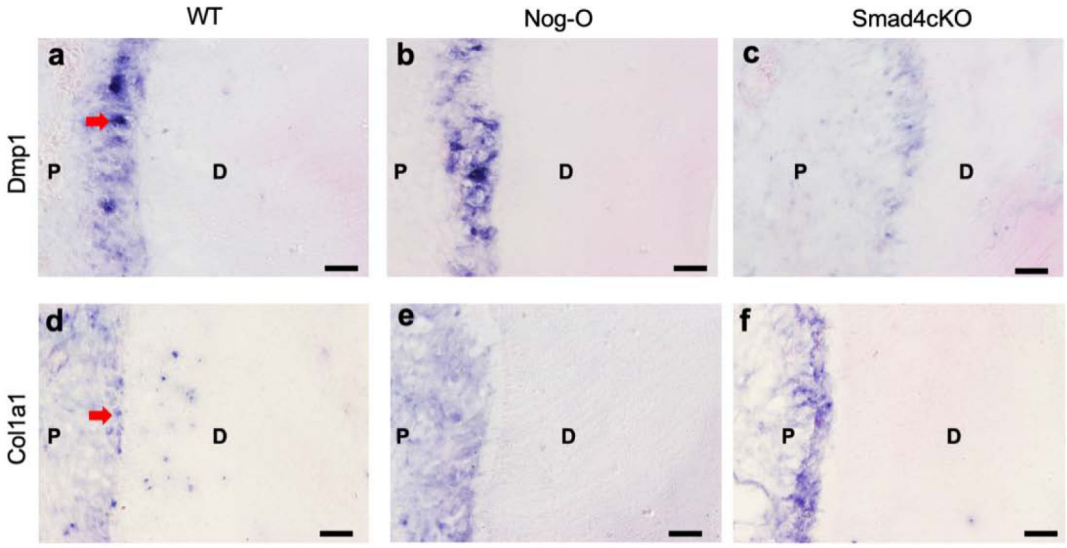

Figure 6. *In-situ* hybridization of Dmp1 and Col1a1 mRNAs in odontoblast layers.

Dmp1 mRNA was increased in Nog-O odontoblasts (b) compared to WT odontoblasts (a) while Smad4cKO odontoblasts produced much less Dmp1 message (c). Col1a1 message was not appreciably changed in the Nog-O odontoblast layer (e) compared to WT (d), but Smad4cKO odontoblasts showed increased Col1a1 mRNA (f). Scale bar = 20 μm. D, dentin; P, pulp cavity.

Table 1.

Primer sequences used for genotyping

Mouse line	Primer sequences
Dmp1-Cre	Forward: 5′- CCCGCAGAACCTGAAGATG -3′ Reverse: 5′- GACCCGGCAAAACAGGTAG -3′
pMes-Nog	Forward: 5′- CCCCCTGAACCTGAAACATA -3′ Reverse: 5′- GGCGGATGTGTAGATAGTGCT -3′
Smad4$^{fl/fl}$	Forward: 5′- TAAGAGCCACAGGGTCAAGC -3′ Reverse: 5′- TTCCAGGAAAAACAGGGCTA -3′

Table 2.

Quantification of micro-CT data

	WT	Nog-O	Smad4cKO
	Mean ± SEM	**Mean ± SEM**	**Mean ± SEM**
Total Volume (cm^3)	1.29±0.039	1.23±0.025	1.02±0.032
Dentin Volume (cm^3)	1.00±0.036	0.88±0.011	0.62±0.031
Pulp Volume (cm^3)	0.29±0.003	0.35±0.016	0.41±0.057
DV/TV	0.77±0.005	0.72±0.008	0.60±0.045
Apparent Density (mg/cm^3)	1144.05±3.24	1130.95±12.41	933.40±44.94
Material Density (mg/cm^3)	1169.21±9.88	1162.74±7.66	1107.97±14.44