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The Role of Bone Morphogenetic Proteins 2 and 4 in Mouse Dentinogenesis

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Abstract

Objective—The bone morphogenetic proteins (BMPs) play crucial roles in tooth development. However, several BMPs retain expression in the dentin of the fully patterned and differentiated tooth. We hypothesized that BMP signaling therefore plays a role in the function of the differentiated odontoblast, the job of which is to lay down and mineralize the dentin matrix.

Design—We generated mice deficient in *Bmp2* and *4* using a dentin matrix protein 1 (*Dmp1*) promoter-driven cre recombinase that was expressed in differentiated odontoblasts.

Results—The first and second molars of these *Bmp2* and *Bmp4* double conditional knockout (DcKO) mice displayed reduced dentin and enlarged pulp chambers compared to cre-negative littermate controls. DcKO mouse dentin in first molars was characterized by small, disorganized dentinal fibers, a wider predentin layer, and reduced expression of dentin sialophosphoprotein (DSPP), dentin matrix protein 1 (DMP1), and bone sialoprotein (BSP). DcKO mouse odontoblasts demonstrated increased type I collagen mRNA production, indicating that the loss of BMP signaling altered the rate of collagen gene expression in these cells. *Bmp2* and *Bmp4* single *Dmp1*-cre knockout mice displayed no discernable dentin phenotype.

Conclusions—These data demonstrate that BMP signaling in differentiated odontoblasts is necessary for proper dentin production in mature teeth.

Keywords

Dentin; Bone Morphogenetic Protein 2; Bone Morphogenetic protein 4; Odontoblast

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Ethical Approval

Animal use was approved by the Institutional Animal Care and Use Committee of the Texas A&M University. No other ethical approval was sought.

CONFLICT OF INTEREST

The authors declare no conflict of interest

INTRODUCTION

The mammalian tooth begins as an invagination of embryonic ectoderm into the adjacent mesenchyme. Reciprocal signals between these layers guide their development through the bud, cap, and bell stages to form the mature tissues of the tooth (Tummers & Thesleff, 2009). Crucial among these signals are the bone morphogenetic protein (BMP) members of the transforming growth factor beta (TGF β) superfamily. BMP 2 and BMP4 secretion by the early lamina stage ectoderm induces mesenchymal expression of the Msx1 and 2 homeodomain transcription factors that are essential for progression to the bud stage (Neubuser, Peters, Balling, & Martin, 1997; Nie, Luukko, & Kettunen, 2006). Furthermore, BMP4 secretion specifies the identity of the presumptive tooth as an incisor in the embryonic mandible, and inhibition of BMP signaling by appropriately-timed application of Noggin protein changes tooth fate from incisor to molar (Tucker, Matthews, & Sharpe, 1998). Beginning with the bud stage, BMP4 expression appears in the mesenchyme as well, and inactivation of the BMP receptor *Bmpr1a* in epithelial or mesenchymal layers arrests development between the bud and cap stages, highlighting the essential role of BMP signaling in early tooth progression (Åberg, Wozney, & Thesleff, 1997; Vainio, Karavanova, Jowett, & Thesleff, 1993).

BMP expression continues past the cap stage to tooth maturity, and several studies of cell- and stage-specific BMP deletions in mice have demonstrated their importance in dentin and enamel formation after initial tooth patterning. Feng, et al. reported that conditional cre/lox-mediated deletion of *Bmp2* in ameloblasts using an osterix-cre leaves thin and hypomineralized enamel (Feng et al., 2011). Yang and colleagues then demonstrated that *Bmp2* ablation in differentiating odontoblasts and osteoblasts using the 3.6*Colla1*-cre results in severe decreases in root and crown dentin (Yang et al., 2012). This cre causes recombination of the *Bmp2* loxP allele after specification to the odontoblast lineage, but before terminal differentiation, and results in deficient expression of osterix, collagen, and dentin sialophosphoprotein (DSPP). *Bmp4* knockout in dentin and bone using the same 3.6*Colla1*cre also leads to reduced dentin and enlarged pulp chambers caused by reduced expression of key odontoblast differentiation transcription factors (Gluhak-Heinrich et al., 2010). Together, these data demonstrate that BMPs direct cell differentiation and gene expression in the fully patterned tooth.

Dentinogenesis is an ongoing process accomplished by differentiated odontoblasts once tooth patterning is complete. Odontoblasts must form the primary dentin of the tooth crown, and then generate secondary dentin throughout life and tertiary dentin in response to injury. BMPs 2, 4, and 7 continue to be expressed in dentin after birth (Butler, Mikulski, Urist, Bridges, & Uyeno, 1977; Nakashima, Nagasawa, Yamada, & Reddi, 1994), and we hypothesized that BMP signaling plays an ongoing role in dentin production by the differentiated odontoblast. Although BMP7 has distribution and expression patterns similar to those of BMP2 and 4, its ablation in mice appears to have little consequence for tooth development, possibly because of functional redundancy with other BMP members or related growth factors (Dudley & Robertson, 1997; Helder et al., 1998). We therefore designed the present study to define the function of BMP2 and 4 in differentiated odontoblasts *in vivo*. We generated a mouse model in which the *Bmp2* and 4 alleles were

deleted in the presence of cre recombinase under the control of the *Dmp1* promoter (*Dmp1-Cre*) (Lu et al., 2007). The striking dentin phenotype seen in these double conditional knockout mice demonstrates that BMP signaling plays a pivotal role in dentin synthesis and maturation.

MATERIALS AND METHODS

Generation of *Dmp1-cre;Bmp2^{flf};Bmp4^{flf}* (double conditional knockout, DcKO) mice

The generation of transgenic mice expressing cre recombinase under the control of a 9.6-kb *Dmp1* promoter + 4 kb of intron 1 fragment (*Dmp1-Cre*) was described previously (Lu et al., 2007). Mice harboring conditional alleles of the *Bmp2* or *Bmp4* gene (*Bmp2*-floxed or *Bmp4*-floxed mice) were also described previously and were purchased from The Jackson Laboratory (Bar Harbor, ME, USA) (Liu et al., 2004; Ma & Martin, 2005). To study the inactivation of *Bmp2* and *4* in differentiated odontoblasts, these three lines were mated to generate *Dmp1-cre;Bmp2^{flf};Bmp4^{flf}* experimental group animals (referred to as double conditional knockout, DcKO mice) and *Bmp2^{flf};Bmp4^{flf}* littermate controls. Tail biopsies were analyzed by polymerase chain reaction (PCR) with primers recommended by The Jackson Laboratory. All animal procedures were approved by the Institutional Animal Care and Use Committee (IACUC) of the Texas A&M University College of Dentistry.

Quantitative real time polymerase chain reaction (qPCR)

Total RNA was extracted from the molars of three-week-old DcKO and control mice, treated with DNase I (Promega, Madison, WI), and purified with the RNeasy Mini Kit (Qiagen, Inc., Valencia, CA). RNA (1 µg/ml per sample) was transcribed into cDNA with SuperScript III reverse transcriptase (Invitrogen, San Diego, CA). qPCR reactions were performed using the Brilliant SYBR Green QPCR Master Mix (Applied Biosystems; Foster City, CA) and the CFX-96 Real-Time PCR Detection System (Bio-Rad; Hercules, CA). Primers for relative (qPCR) of *Bmp2* and *Bmp4* were those used previously by Wang, et al. (Wang et al., 2010). The housekeeping gene, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), was used as the internal control. The $\Delta\Delta C_t$ method was used to calculate gene expression levels normalized to GAPDH value. Reactions were performed in triplicate on samples from three separate experiments and expressed as a relative fold change in gene expression compared to the control.

Plain X-ray Radiography and Microcomputed Tomography (µCT)

Mandibles from one-month and three-month-old mice from both groups were dissected, fixed for 48 hours in 4% formaldehyde and stored in 70% ethanol at 4°C. The mandibles were then analyzed with a Faxitron MX-20 specimen radiography system (Faxitron X-ray Corp., Buffalo Grove, IL). For the µ-CT analyses, 3-months old gender matched mandibles were scanned using a µCT35 imaging system (Scanco Medical, Basserdorf, Switzerland). The whole mandible was scanned in 7.0 µm slice increments at medium resolution to assess the overall shape and structure. Following this evaluation, a high-resolution scan of the molar region in 3.5 µm slice increments was made for quantification purposes. (n = 4 each for wild type control and DcKO groups). For quantification, we used the Scanco MicroCT evaluation software. The tooth bearing area of the mandibles were scanned in a vertical

position with long axis of the mandible perpendicular to the floor, which would generate the cross sections of the teeth in each slice. Contour lines were drawn around the first and second molars to define the region of interest. Once the entire molar was defined, the slices were submitted for analyses to generate the total volume (TV), dentin volume (DV), pulp volume (PV), DV/TV and PV/TV ratios and the apparent and mean densities.

Tissue Preparation and Histology Evaluation

Under anesthesia, the control and DcKO mice at postnatal one and three months were perfused from the ascending aorta with 4% formaldehyde in 0.1 M phosphate-buffered saline. The mandibles were dissected and further soaked in the same fixative for 48 hours, followed by demineralization in 14% EDTA (pH 7.4) at 4°C for 2 weeks. The tissues were processed for paraffin embedding, and serial 5 µm sections were prepared. The sections were stained with hematoxylin and eosin (H&E) for histological analyses. Picrosirius Red staining (Junqueira, Bignolas, & Brentani, 1979) was performed to assess the morphology and organization of the collagen fibrils.

For the immunohistochemistry analyses, anti-DSP-2C12.3 monoclonal antibody was used at a concentration of 2.05 µg/ml. Anti-DMP1 monoclonal antibody that recognizes the C-terminal region of DMP1 was used at a concentration of 4.7 µg/ml. Anti-BSP monoclonal antibody 10D9.2 was used at a concentration of 4.5 µg/ml.

All the IHC experiments were carried out using the mouse on mouse kit for monoclonal antibodies (Vector Laboratories, Burlingame, CA). The 3, 3'-diaminobenzidine (DAB) kit (Vector Laboratories) was used for color development according to the manufacturer's instructions. Methyl Green was used as the counterstain.

In Situ Hybridization

In situ hybridization was performed as described previously to assess *Col1a1* mRNA levels in the molars of six-week-old mice for each group (Jani et al., 2016). The RNA probes were labeled with digoxigenin using a RNA labeling kit (Roche, Indianapolis, IN) and were detected by an enzyme-linked immunoassay with a specific anti-digoxigenin-alkaline phosphatase antibody conjugate and alkaline phosphatase substrate (Roche), following the manufacturer's instructions. Nuclear fast red was used for counterstaining.

Statistical Analysis

Statistical evaluations of the data were conducted by independent Student's t-test to validate the differences between two groups. $P < 0.05$ was considered statistically significant. The data were presented as mean $\pm$ SD.

RESULTS

Reduced BMP2 and 4 expression in Dmp1-cre double knockout mice

We employed *Dmp1*-cre transgenic mice in which cre activity correlates with endogenous DMP1 expression in osteocytes and odontoblasts. Cre is expressed in these mice at low levels in teeth before embryonic day 18.5 and then at high levels in odontoblasts after birth

and is also found in odontoblastic precursor cells in the pulp (Lu et al., 2007). To ablate *Bmp2* and *4* selectively in odontoblasts, we therefore generated mice that were homozygous for *Bmp2* and *Bmp4* loxP alleles and carried the *Dmp1*-driven cre transgene. Molars from mice containing the cre (double conditional knockouts, DcKO) were analyzed for expression of *Bmp2* and *Bmp4* messages to assess the degree of gene ablation compared to their cre-negative (control) littermates. Quantitative real time PCR showed that *Bmp2* expression was reduced by $77\pm 0.012\%$ in DcKO molars (Fig. 1a) compared to controls (Fig. 1a) control mice. Similarly, the expression of *Bmp4* was reduced by $68\pm 0.023\%$ in DcKO mice (Fig. 1b) compared to control mice (Fig. 1b). Although knockout of these messages was incomplete, they represented substantial reductions such that we reasoned they would affect BMP signaling and result in a phenotype. Furthermore, because the RNA for these experiments was isolated from whole teeth, it included mRNA from other cells, which would not be affected by *Dmp1*-cre-mediated recombination, thus possibly resulting in an underestimation of the BMP reduction in dentin.

Reduced dentin in DcKO mice

We used plain X-ray radiography and micro-computed tomography (μ -CT) to characterize the dentin of one and three month old DcKO mice. X-Ray radiography showed a drastic decrease in the dentin thickness of both first and second molars in DcKO mice (Fig. 2b) compared to control mice (Fig. 2a) at one month of age. The pulp cavity was enlarged, and roots were shorter with wider apices. At three months, the reduction in DcKO dentin was less severe than at one month, but was still substantial (Fig. 2d).

Three-dimensional μ -CT imaging of the molars in three-month-old mice confirmed the dentin and pulp cavity changes seen in the radiographs. Compared to the control mice (Fig. 2e and f) the first and second molars of DcKO mice (Fig. 2g and h respectively) showed reduced overall dentin thickness and enlarged pulp chambers with the second molar showing a more severe reduction than the first. Density pattern evaluation of the first and second molars (Figs. 2i to l) also revealed specific areas of dentin loss in each DcKO molar. First molars of control mice showed maximum density on the mesial half of the crown and root (Fig. 2i, red color), while in DcKO first molars, that density was mostly lost (Fig. 2k). Maximum dentin density in control second molars occurred on the occlusal surface (Fig. 2j, red color), whereas second molars of DcKO mice (Fig. 2l) lacked these dense areas completely. Interestingly, some areas of the roots in DcKO molars had slight increases in density over their control counterparts.

Quantification of the μ -CT data showed that the total volumes of DcKO first and second molars were not significantly different from those of control mice, indicating normal tooth patterning (Fig. 3a, d). This was predicted in the *Dmp1* mediated knockout model in which *Bmp* alleles are not ablated until the final stage of odontoblast differentiation. Within the teeth, however, the dentin volumes of first (Fig. 3a) and second (Fig. 3d) molars from DcKO mice were only 83 and 80 percent, respectively, of those from their control brethren. These changes caused a significant reduction in the dentin volume to total volume ratio (DV/TV), an index of mineral density across the entire tooth. The DV/TV ratio was reduced by 8% in DcKO first molars (Fig. 3b) and 17% in DcKO second molars (Fig. 3e) compared to

controls. Pulp cavity volumes in DcKO mice were also 10 and 50 percent greater than in controls for the first and second molars, respectively (Fig 3a,d). When these pulp volumes were normalized to total volume, the PV/TV ratio for first molars was increased by 13% over controls (Fig. 3b) and for second molars by 52% (Fig. 3e). There was a concomitant significant reduction for both molars in apparent density, which is the density of all tissues and spaces measured across the entire volume of the tooth (Fig. 3c,f). This illustrates that the loss of dentin volume and increase in pulp volume caused a significant increase in the over all density of the tooth taken as a whole. However, a reduction in material density, which measures the density of the solid tissue only without the pulp cavity, was significant in the second molars of DcKO mice and not in the first (Fig. 3c,f). This suggests that, although the amount of dentin in the first molar was reduced by *Bmp* knockout, the density of that dentin was not compromised to the extent as it was in the second molar. These data, collectively, support the X-ray and μ -CT imaging analyses in which both molars were affected by *Bmp2* and *Bmp4* deletion, but the second molar more severely so.

Increased predentin and impaired fibril organization in DcKO mice

We employed histological stains of tooth sections to further characterize the structural nature of the dentin deficiencies caused by the double deletion of *Bmp2* and *4*. Hematoxylin and Eosin (H&E) stain of three-month-old first molars showed that DcKO teeth had a much wider predentin layer than in controls, suggesting delayed mineralization of the dentin matrix produced by odontoblasts (Fig. 4a,b). The dentin fibrils in DcKO molars also appeared more disorganized than in control teeth. To assess the quality of collagen fibers in the dentin of these mice, we stained the molars with Picosirius red and examined them under polarized light. Larger collagen fibers prepared this way appear bright red or orange, as we observed in the control teeth (Fig. 4c). The DcKO dentin, however, displayed a larger proportion of yellow and green fibers, indicative of smaller, more disorganized collagen fibrils (Fig. 4d). Thus, not only did the odontoblasts of DcKO mice produce less dentin than their control counterparts, but that dentin also mineralized more slowly and was less organized.

Reduced odontoblast marker expression in DcKO odontoblasts

Our histological and μ -CT analyses pointed to impaired function of DcKO odontoblasts. We therefore examined the expression of dentin matrix proteins as a measure of odontoblast function using immunohistochemistry. Dentin sialoprotein (DSP) expression, normally seen in dentin and pulp, was drastically reduced in DcKO dentin (Fig. 5a,b). Similarly, the DMP1 found in the dentinal tubules of control mice, was mostly absent in DcKO molars (Fig. 5c,d). BSP was expressed in the newly formed dentin layer of control teeth, but was completely absent in DcKO teeth (Fig. 5e,f).

In addition to non-collagenous odontoblast markers that affect the maturation and mineralization of dentin matrix, we examined whether DcKO odontoblasts were deficient in their ability to produce collagen matrix, as this would explain the observed reduction of dentin in these teeth. We used *in situ* hybridization with a riboprobe for *Col1a1* mRNA to assess newly expressed collagen from odontoblasts in mature teeth. Surprisingly, DcKO odontoblasts residing at the new dentin/pre-dentin layer expressed substantially more *Col1a1*

message than control odontoblasts (Fig. 5g,h). This suggests that the thicker pre-dentin layer observed in DcKO molars may be due to increased secretion of matrix as well as to delayed mineralization of that matrix.

Discussion

Multiple studies have demonstrated the significance of BMP signaling during the early stages of tooth development (Bei & Maas, 1998; Gluhak-Heinrich et al., 2010; Jia et al., 2013; Nakashima et al., 1994; Rakian et al., 2013; Tucker et al., 1998; Vainio et al., 1993; Yang et al., 2012). However, the role of BMP signaling in dentinogenesis after the tooth is patterned is largely unknown. To investigate that role, we deleted both *Bmp2* and *Bmp4* from mice using a *Dmp1*-cre. Because this transgene faithfully recapitulates the expression pattern of *Dmp1*, it caused gene ablation within the tooth primarily in terminally differentiated odontoblasts, although there is some cre expression in the pulp, which could result in recombination and knockout in some pre-odontoblasts in this space (Lu et al., 2007). The resulting double knockout (DcKO) mice had reduced dentin thickness compared to wild type controls, with disorganized dentinal fibers and enlarged pulp chambers. The teeth of these mice also showed increased pre-dentin layer thickness and reduced expression of dentin ECM proteins DMP1, DSPP and BSP. Overall then, these data paint a picture of mutant odontoblasts that are fully differentiated, but functionally deficient in their ability to produce mature dentin matrix.

The dentin defects we observed in our BMP DcKO mice are similar to those seen in *Dspp*-null mice and *Dmp1*-null mice, and mirrors the phenotype of human dentinogenesis imperfecta (DGI), which is characterized by thinner dentin, enlarged pulp chamber and widened predentin (Lu et al., 2007; Sreenath et al., 2003; Xiao et al., 2001). Thus, while it appears from mouse models that lack of *Dspp* and *Dmp1* directly causes DGI, it is possible that defective BMP signaling in mature odontoblasts may create deficiencies in these proteins and thus also result in DGI.

Interestingly, while we observed reduced dentin matrix marker proteins in DcKO teeth, *Colla1* mRNA expression was increased. We do not know at present whether this means that the mutant odontoblasts secrete more collagen or if the immature collagen fibrils remain stuck inside the cells. Thus, we do not know the extent to which the increased predentin layer is caused by accelerated export of collagen, delayed mineralization of the secreted matrix, or both. Collagen transport and secretion requires the packaging of procollagen into specific vesicles at the endoplasmic reticulum (ER) (Stephens, 2012). If collagen production is increased but the cells' export is somehow not up to the task, ER stress could result in an unfolded protein response (UPR) and inability of odontoblasts to secrete the non-collagenous matrix proteins that mature the dentin matrix. Indeed, membrane trafficking defects have been linked to human disease (De Matteis & Luini, 2011). Characterizing the collagen secretion mechanism in DcKO odontoblasts may therefore be necessary to understand the role of BMP signaling in dentinogenesis.

We also generated single knockout mice that were deficient in either *Bmp2* or *Bmp4* using the *Dmp1* cre. These mice did not have any significant dentin defects visible on x-ray (Fig.

S1). This contrasts with *Bmp2* or *Bmp4* single knockout during early tooth development, when inactivation of either causes severe dentin and enamel defects in mice. Thus, while *Bmp2* and *Bmp4* play unique roles in tooth development, our data indicate that they are redundant in mature dentin. This is perhaps not surprising given that both are expressed in the same cells and share the same serine/threonine kinase receptor complex (Lavery, Swain, Falb, & Alaoui-Ismaili, 2008; Mueller & Nickel, 2012). It therefore follows that opportunities for single mutations that cause disease in dentinogenesis are more plentiful downstream of the BMP receptor than mutation of each ligand simultaneously. BMP2 and 4 bind to the type I/II receptor complex to cause binding of receptor-specific intracellular Smad proteins to the Co-Smad protein Smad4. These Smad heterodimers translocate to the nucleus to activate transcription. Both BMPs can also activate the non-canonical Erk/Mek, p38/MAPK and JNK signaling pathways to regulate gene expression (Chen, Deng, & Li, 2012). Our future genetic dissection of these pathways in odontoblasts will reveal which are crucial for dentin formation and shed light on the causes of dysfunctional dentinogenesis in humans.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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HIGHLIGHTS

- Adult dentin-specific BMP2/4 double knockout mice have reduced, disorganized dentin
- Double mutant odontoblasts have deficient matrix protein expression
- BMP2 and 4 are important for dentin synthesis by differentiated odontoblasts

Fig. 1. Conditional deletion of *Bmp2* and *Bmp4*

Bmp2 and *Bmp4* mRNA levels in the molars of 3-weeks-old DcKO and control mice.

Control mRNA level is set as 1. (a) The *Bmp2* mRNA level in the DcKO mice (red bar) was 23 ± 0.012 % of control mice.

(b) the *Bmp4* mRNA level reduced to 32 ± 0.029 % of control.

All values are mean of three independent experiments $\pm$ SD.

Fig. 2. Changes in dentin and pulp volumes

Plain X-ray radiography showed that at both one-month and three-months age, the dentin layer in DcKO mice (b,d) had reduced thickness and pulp chambers were enlarged when compared to dentin in control mice (a,c). Three-dimensional imaging from Micro-CT confirmed that dentin layers were thinner in DcKO mice versus the controls (e–h) and that the densities of the mesial and occlusal portions of the first and second molars, respectively, in three-month-old DcKO mice were greatly reduced (i–l). Scale bar=100 μ m in e–l. Red=higher density.

Fig. 3. Quantification of dentin volume and density

(a) Micro-CT data analysis showed that the total volume (TV) of first molar did not change in the DcKO (1.29 ± 0.08 vs. 1.15 ± 0.11 cm³), while there was a significant decrease in the dentin volume (DV) in DcKO mice (1.00 ± 0.07 vs. 0.83 ± 0.09 cm³) and increase in pulp volume (0.28 ± 0.01 vs. 0.32 ± 0.02 cm³). (b) The ratio of dentin volume to total volume (DV/TV) of the first molar was reduced in the DcKO (0.78 ± 0.01 control vs. 0.72 ± 0.02 DcKO). Pulp volume to total volume (PV/TV) for first molars was increased (0.24 ± 0.01 vs. 0.27 ± 0.02). (c) There was a significant decrease in the apparent density (1144 ± 6 vs. 1049 ± 48 mg/cm³), but not the material density (1169 ± 20 vs. 1131 ± 27 mg/cm³) of dentin in the first molars of DcKO mice. (d) The dentin volume was drastically reduced in second molars of DcKO mice (0.54 ± 0.01 vs. 0.43 ± 0.02 cm³). (e) The dentin to total volume ratio was also reduced in second molars (0.75 ± 0.02 vs. 0.62 ± 0.02). PV/TV ratio was increased (0.25 ± 0.02 vs. 0.38 ± 0.01). (f) Apparent density (1137 ± 54 vs. 991 ± 17 mg/cm³) and material density (1261 ± 31 control vs. 1095 ± 44 mg/cm³) of second molars were reduced in DcKO mice compared to control mice. All values are mean $\pm$ SD. * denotes statistical significance to $p < 0.05$. $n=4$ for each group.

Fig. 4. Histological evaluation of dentin in first molars

H&E staining and high magnification view of the distal cusp of the first molar showed reduction in dentin thickness, with less densely packed dentinal fibers in DcKO mice (b) compared to control mice (a). The predentin thickness was wider in DcKO mice (b, red arrow). Picrosirius red staining and polarized light microscopy revealed the irregular and less dense collagen fibers in DcKO mice (d, white arrow) compared to control mice (c). P, pulp cavity, D, dentin. Scale bar: 100 μ m in e–l.

Fig. 5. Reduced expression of late odontoblast markers

Immunohistochemical staining showed a reduction in the expression of DSPP, DMP1 and BSP late odontoblast markers in DcKO mice (b, d, and f respectively) compared to control mice (red arrow in a, c, and e respectively). In-situ hybridization revealed increased *Col1a1* mRNA levels in DcKO mice (h) compared to the control mice (g). Scale bar, 100 μ m. Counter stain is methyl green.