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Deletion of Dentin Matrix Protein-1 Leads to a Partial Failure of Maturation of Predentin into Dentin, Hypomineralization, and Expanded Cavities of Pulp and Root Canal during Postnatal Tooth Development*

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The dentin matrix protein-1 (DMP-1) gene is identified in odontoblasts during both embryonic and postnatal development. *In vitro* study suggests that this noncollagen acidic phosphoprotein plays a role in mineralization. However, deletion of the *Dmp-1* gene has little effect on tooth development during embryogenesis. To address the role of DMP-1 in tooth during postnatal development, we analyzed changes of dentinogenesis in *Dmp-1* null mice from 3 days after birth to 1 year. Here we show that *Dmp-1* null mice postnatally develop a profound tooth phenotype characterized by a partial failure of maturation of predentin into dentin, enlarged pulp chambers, increased width of predentin zone with reduced dentin wall, and hypomineralization. The tooth phenotype of these mice is strikingly similar to that in dentin sialophosphoprotein (*Dspp*) null mice and shares some features of the human disease dentinogenesis imperfecta III. We have also demonstrated that DSPP levels are reduced in *Dmp-1* null mice, suggesting that DSPP is probably regulated by DMP-1 during dentinogenesis. Finally, we show the absence or delayed development of the third molar in *Dmp-1* null mice, which is probably secondary to defects in *Dmp-1* null bone. Taken together, these studies suggest that DMP-1 is essential for later dentinogenesis during postnatal development.

Dentin is a mineralized tissue that closely resembles bone in composition and mechanism of formation. The mechanisms for mineralization are largely unclear, although two hypotheses are proposed to explain initiating mineralization: matrix vesicles in mantle dentin and collagen-phosphophoryn complexes in circumpulpal dentin (1). The *in vitro* studies also suggest that phosphorylated extracellular matrix (ECM)[1] proteins localized within collagen gap zones can bind calcium and phosphate ions in an appropriate conformation to nucleate the formation of apatite crystals (2, 3). One of the noncollagenous proteins that appears to play an important role in dentin ECM formation and mineralization is dentin matrix protein-1 (DMP-1).

DMP-1, an acidic phosphorylated extracellular matrix protein (4), is expressed in odontoblasts that secrete matrix proteins to form dentin. Using protein chemistry approaches, some progress has been made in identifying the normally processed forms of DMP-1 in mineralized tissues (5). Although full-length DMP-1 has been cloned and sequenced, the corresponding intact protein has not been isolated from mineralized tissues. However, two proteolytic fragments, a 37-kDa N-terminal fragment and a 57-kDa C-terminal fragment, have been isolated from bone and dentin extracts (5). Recent studies suggest that DMP-1 can be cleaved by bone morphogenetic protein-1/tolloid-like proteinases (6).

In vitro studies suggest that overexpression of *Dmp-1* induces differentiation of mesenchymal cells to odontoblast-like cells and enhances mineralization (7) and that DMP-1 can bind to Ca^{2+} and initiate mineral deposition *in vitro* (8). However, effects of recombinant DMP-1 on *in vitro* mineralization are controversial and depend on the phosphorylation status of DMP-1.[2] In addition, ectopic application of the recombinant DMP-1 showed no apparent effect on mineralization (9). To determine the *in vivo* role of DMP-1 in dentinogenesis and mineralization, we have cloned and sequenced the *Dmp-1* gene (GenBank™ accession number AJ242625) and have generated *Dmp-1* null mice, in which exon 6 of *Dmp-1* was replaced by a *lacZ* reporter gene and a neo-cassette (10). The expression of *Dmp-1*, reflected by X-gal staining, is not only identified in odontoblasts but also in pulpal cells, precursor cells of odontoblasts during embryonic development. However, there is no apparent early tooth phenotype observed in the *Dmp-1* null embryos and newborns, suggesting that DMP-1 may be redundant or nonessential for early dentinogenesis and mineralization (10).

The purpose of this study was to determine whether DMP-1 is required for dentinogenesis and mineralization during postnatal development by characterization of the *Dmp-1 lacZ* knock-in mice from day 3 to 1 year old. Here we show that *Dmp-1* null mice postnatally develop a profound tooth phenotype characterized by

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[1] The abbreviations used are: ECM, extracellular matrix; DMP, dentin matrix protein; X-gal, 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside; RT, reverse transcriptase; micro-CT, micro-computed tomography.

[2] Tartaix, P. H., Doulaverakis, M., George, A., Fisher, L. W., Butler, W. T., Qin, C., Salih, E., Tan, M., Fujimoto, Y., Spevak, L., and Boskey, A. L. (February 9, 2004) *J. Biol. Chem.* 10.1074/jbc.M314114200.

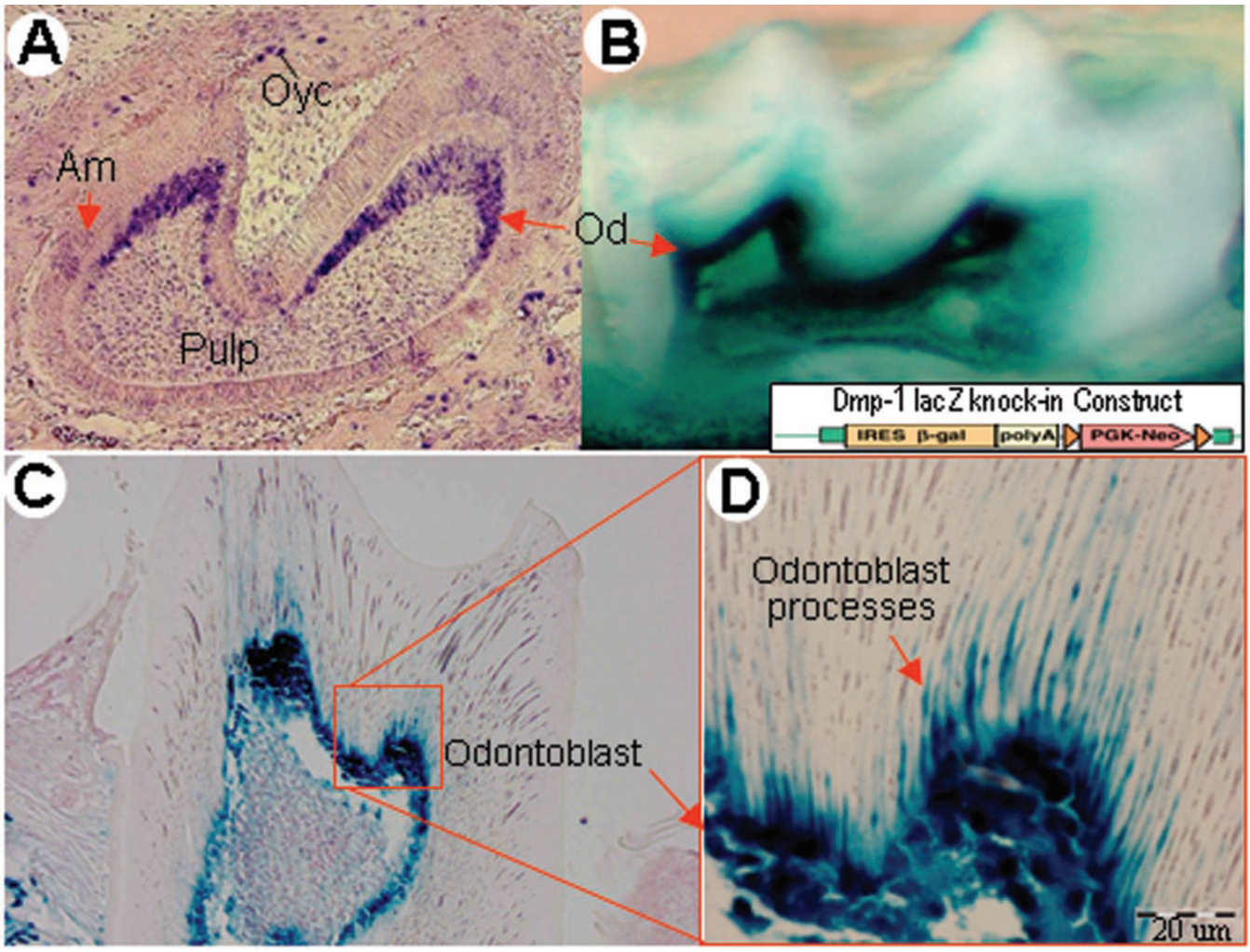

FIG. 1. **Dmp-1 signal is expressed in odontoblasts during development.** *In situ* hybridization was performed on decalcified paraffin sections from 17-day postcoitum embryos using probes for *Dmp-1* (*A*). A *Dmp-1* mRNA signal (*purple*) is detected in the odontoblast (*Od*) layer and osteocytes (*Oyc*). A much lower signal is identified in pulp cells and ameloblasts (*Am*). With whole mount X-gal staining of 5-week first molar (*B*) and sectioning (*C*), *Dmp-1-lacZ* signal (*blue*) is mainly observed in the odontoblast layer and odontoblast cell processes (*D*). Note that exon 6 of *Dmp-1* is replaced by a *lacZ* reporter; thus, expression of *lacZ* reflects endogenous *Dmp-1* activity (10).

enlarged pulp chambers, increased width of predentin zone with reduced dentin thickness, and hypomineralization. The tooth phenotype of these mice is strikingly similar to dentin sialophosphoprotein (*Dspp*) null mice (11) and shares some features of the human dentin disease dentinogenesis imperfecta III. In addition, we demonstrate that DSPP is reduced in *Dmp-1* null mice, suggesting that DSPP could be directly or indirectly controlled by DMP-1 during dentinogenesis.

EXPERIMENTAL PROCEDURES

Generation of Dmp-1 Null Mice—Mice deficient in *Dmp-1* were generated by gene targeting in embryonic stem cells as described previously (10). To obtain *Dmp-1*-deficient teeth, heterozygous *Dmp-1* null mice were interbred to generate homozygotes in the C57BL/6 background (>95%) or CD-1 outbred background in the expected Mendelian ratio. Males and females were both fertile. All experiments were performed using a protocol approved by the Institutional Animal Care and Use Committees of University of Missouri-Kansas City and NIEHS, National Institutes of Health, laboratory animal facilities.

Tail PCR Genotyping—Genotyping of *Dmp-1* null mice was determined by PCR analysis of genomic DNA extracted from tail with primers p01 (5′-CTTGACTTCAGGCAAATAGTGACC-3′) and p02 (5′-GCGGAATTCGATAGCTTGGCTG-3′) to detect the targeted allele (280 bp) and primers p01 (5′-CTTGACTTCAGGCAAATAGTGACC-3′) and 5′-CTGTTCCTCACTCTCACTGTGC-3′ to detect the wild-type allele (410 bp).

β-Galactosidase (lacZ) Expression Assay—β-Galactosidase staining was assessed from 5-week-old heterozygous jaw using the method described previously (12). Briefly, mandibles were fixed by immersion in ice-cold 4% paraformaldehyde for 30 min, and then washed three times with phosphate-buffered saline for 5 min each. The specimens were then stained overnight in freshly prepared X-gal solution (1 mg/ml) at 32 °C, followed by refixation, decalcification, paraffin-embedding, sectioning, and counterstaining.

In Situ Hybridization—The digoxigenin-labeled *Dmp-1* cRNA probe from exon 6 was prepared by using an RNA Labeling Kit (Roche Applied Science). Preparation of a 0.6-kb mouse *Dmp-1* RNA probe and *in situ* hybridization on paraffin sections were carried out essentially as described previously (13). Digoxigenin-labeled *Dmp-1* probe was detected in an enzyme-linked immunoassay with a specific anti-digoxigenin-AP antibody conjugate and the color substrates nitro blue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate according to the manufacturer's instructions (14).

Tooth Isolation and High Resolution Radiography—For better imaging of the teeth on radiographs, fresh mandibles dissected from wild-type and *Dmp-1* null mice were incubated in lysis buffer (2× SSC, 0.2% SDS, 10 mM EDTA, 10 mg/ml Proteinase K) for 1–2 days, depending on the age of the mouse. After muscles surrounding teeth were digested, the mandibles were washed in 1× phosphate-buffered saline, and molars were extracted using a dissection scope. Mandibles and isolated first molars were then examined on a Faxitron model MX-20 Specimen Radiography System with a digital camera attached (Faxitron x-ray Corp., Buffalo Grove, IL).

Micro-computed Tomography (Micro-CT)—Three-dimensional images of 12-month molars from wild-type and *Dmp-1* null mice were scanned with a compact fan beam-type tomograph, also referred to as micro-CT (Micro-CT 40; Scanco Medical AG, Bassersdorf, Switzerland).

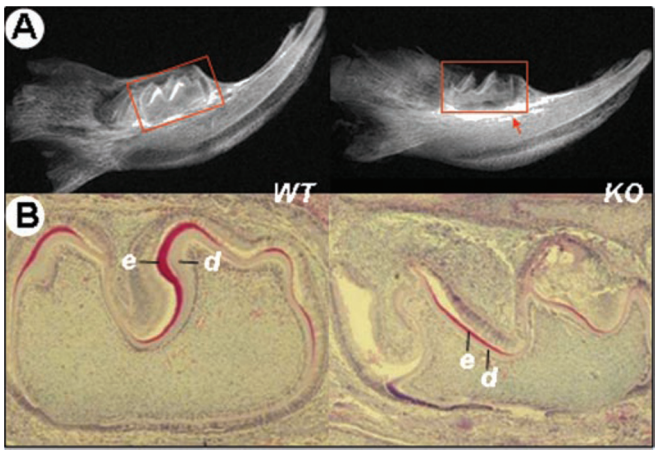

FIG. 2. **Deletion of *Dmp-1* leads to an early defect in tooth morphology and mineralization.** *A*, representative radiographs of jaws show a lower mineral content in the *Dmp-1* null teeth (*KO*, *right panel*) compared with the wild-type control (*WT*, *left panel*), although mineral in surrounding bone appears increased in the *Dmp-1* null pup (*arrow*). *B*, hematoxylin/eosin (*H&E*) stainings of first molar indicate an overall reduction in layers of dentin (*d*) and enamel (*e*) in the *Dmp-1* null tooth compared with the control.

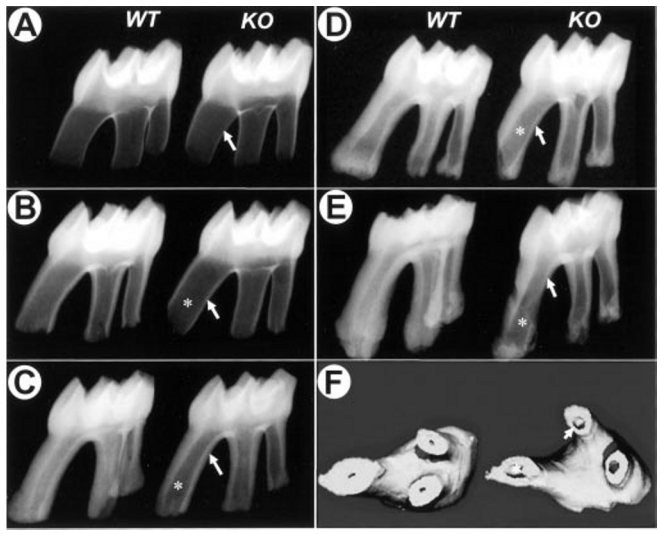

FIG. 3. **Decreased dentin thickness and increased cavities of pulp and root canals in *Dmp-1* null mice.** Representative radiographs (*A*, 3 weeks; *B*, 1 month; *C*, 3 months; *D*, 5 months; *E*, 12 months) and micro-CT (*F*) of upper first molar from wild-type control (*WT*) and *Dmp-1* null (*KO*) mice show a overall reduction of mineralization and ECM thickness of teeth. At 3 weeks, the mutant molar starts to show a reduction in root dentin thickness and mineral density, compared with the control molar (*A*, *arrows*). As the age increases, the reduction in *Dmp-1* null dentin thickness is more dramatic and accompanied with enlarged pulp chambers and root canals in *Dmp-1* null mice (*stars*).

Images were reconstructed with EVS Beam software using a global threshold at 1400 Hounsfield units.

Environmental Scanning Electron Microscopy and Backscattered Scanning Electron Microscopy—Mandibles from control and *Dmp-1* null mice were dissected and fixed in 2% paraformaldehyde and 2.5% glutaraldehyde in 0.1 M cacodylate buffer solution (pH 7.4) at room temperature for 4 h and then transferred to 0.1 M cacodylate buffer solution. After dehydration in ascending concentrations of ethanol, the first molars were fractured under a dissecting microscope and mounted on aluminum stubs with the fractured surface upward facing, sputter-coated with Au-Pd, and examined with field emission environmental scanning electron microscopy (Philips XL30; FEI Co.).

Preparation of Histological Sections—Mandibles from control and *Dmp-1* null mice were dissected and fixed in 4% paraformaldehyde at 4 °C overnight, followed by demineralization in 10% EDTA solution (Sigma) over 3 weeks and then dehydrated, embedded in paraffin, and sectioned at 6-μm thickness.

Immunohistochemistry—Immunostaining of DSPP (LF-153) and biglycan (LF-159) (15) (both polyclonal antibodies were kindly provided by Dr. Larry Fisher from NIDCR, National Institutes of Health) was performed on paraffin sections. After deparaffinization and rehydration, the sections were immersed in 3% H_2O_2 to quench endogenous peroxidase and further digested with 1 mg/ml trypsin for 30 min at 37 °C. Sections were then blocked with 1% bovine serum albumin containing 1% goat serum at room temperature for 2 h. The rabbit anti-mouse DSPP or biglycan polyclonal antibody was added at a dilution of 1:1000 or 1:2000, respectively, in phosphate-buffered saline, and the sections were incubated overnight at 4 °C. After washing, sections were coated with biotinylated anti-rabbit antibody (Vector Laboratories, Burlingame, CA) at a dilution of 1:200 and then incubated at room temperature for 60 min. The sections were washed again and incubated with the ABC reagent (Vector Laboratories) at room temperature for 60 min. The 3,3′-diaminobenzidine substrate was used to visualize immunoreaction sites. Sections were counterstained with hematoxylin and mounted on glass slides. Negative controls were obtained by substituting the primary antibody with rabbit serum.

RNA Extraction and Reverse Transcription-Polymerase Chain Reaction (RT-PCR)—Molars from 1-week-old wild-type and *Dmp-1* null mice were collected under the dissecting microscope. RNA was extracted by using TRIZOL reagent (Invitrogen), and 30 cycles of RT-PCR were performed using SuperScript™ II Reverse Transcriptase (Invitrogen) according to the manufacturer's protocols. The oligonucleotide primers used in the study are the following: *Dmp-1* forward (5′-CAGCCGTTCTGAGGAAGACAGTG-3′) and reverse (5′-CCTGCCCCTGGTTCTCCTTCTGA-3′); *Dspp* forward (5′-GGCATAATCAAAACACCGCTGC-3′) and reverse (5′-GGGGAAATAGGGAAATGACAAAGG-3′); and *Gapdh* forward (5′-ATGGAGAAGGCTGGGGCTC-3′) and reverse (5′-ACGGATACATTGGGGGTAG-3′).

RESULTS

Expression Pattern of Dmp-1 in Odontoblast Layer during Development—To set the foundation for studies of DMP-1 function in dentinogenesis, the expression patterns of *Dmp-1* in embryonic teeth were examined. With i*n situ* hybridization, a *Dmp-1* signal was observed in the odontoblast layer at E17.5 (Fig. 1*A*). In addition, a low level of *Dmp-1* was noted in pulpal precursor cells of odontoblasts, as well as a transient *Dmp-1* expression in the preameloblasts (Fig. 1*A*).

Next we investigated whether *Dmp-1* is expressed postnatally in teeth using the *Dmp-1 lacZ* knock-in mice where a *lacZ* neo-cassette was used to replace exon 6 of the *Dmp-1* gene (10). The advantage of this approach is that *lacZ* expression reflects endogenous *Dmp-1* activity, since this reporter gene is under the control of the endogenous *Dmp-1* promoter (10). The homozygous mice carrying two copies of this transgene will be null for the *Dmp-1* gene, which was used for studies of tooth phenotype in mice that lack the *Dmp-1* gene (see below). The first molar of 5-week-old *Dmp-1-lacZ* heterozygous mouse was selected for tracking *Dmp-1* expression. A strong *Dmp-1 lacZ* signal is detected in both odontoblast layer and odontoblast processes (Fig. 1, *B–D*).

Dmp-1 Null Pups Display an Early Defect in Both Dentin and Enamel—Previously, we showed that *Dmp-1* null embryos or newborns appeared grossly normal and could not be distinguished from control littermates by radiographs and histology (10). To determine the role of DMP-1 in postnatal tooth development, we examined the mineralization and morphogenesis of the *Dmp-1* null teeth from birth to 1 year of age. To follow changes over time noninvasively, x-ray analysis and micro-CT were used. No differences were observed between wild-type and heterozygous mice, suggesting that loss of a single copy of the *Dmp-1* gene or gain of a truncated DMP-1 protein has no effect on tooth development.

However, qualitative differences between 3-day-old *Dmp-1* null pups and their littermates were documented by radiographs and hematoxylin/eosin staining (Fig. 2). Mineral con-

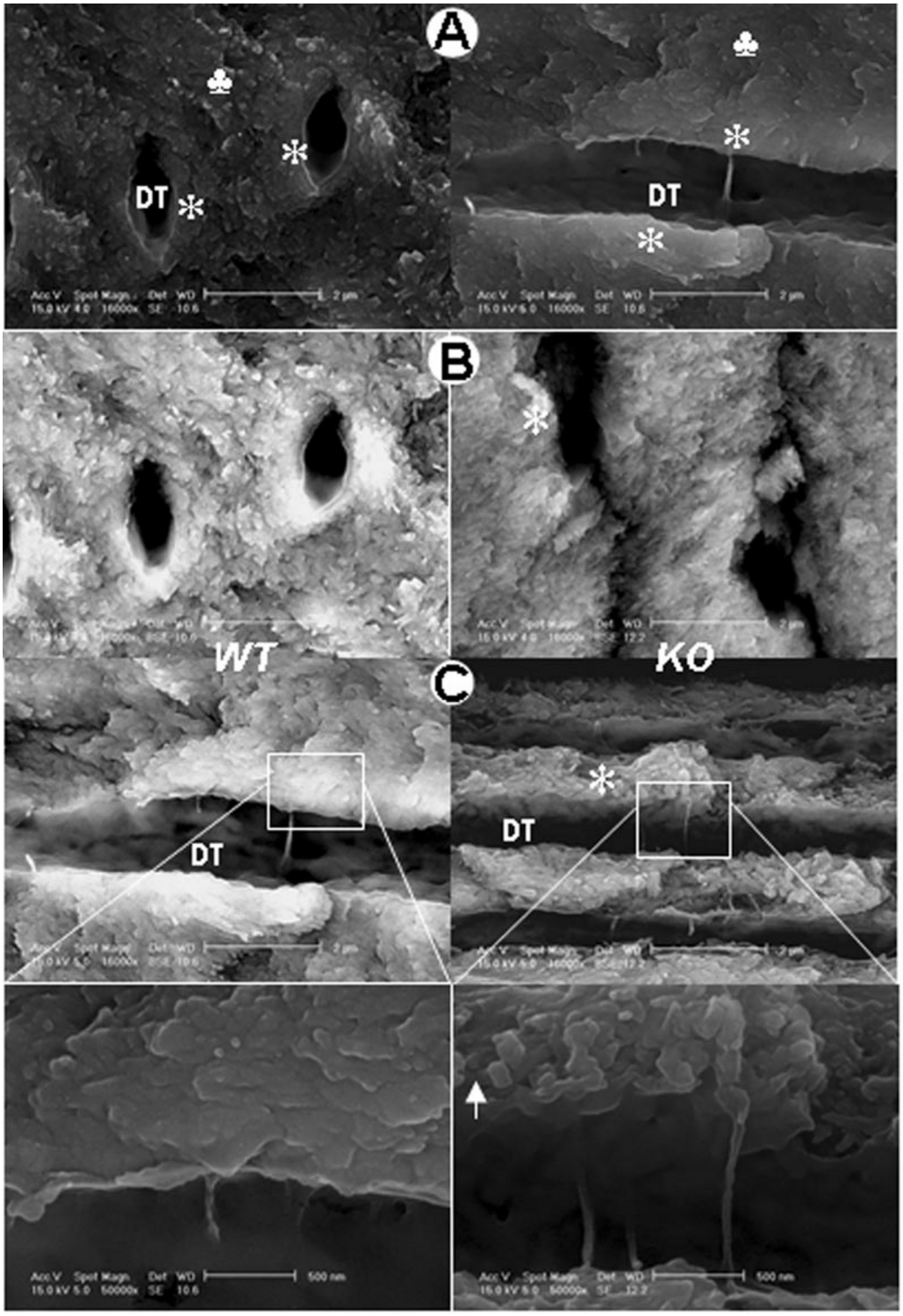

FIG. 4. **Decreased mineralization is associated with a dramatic alteration of dentin matrix structure in *Dmp-1* null mice.** Scanning electron microscopy (*SE*) images of cross-sections (*A*, *left*) and longitudinal sections (*A*, *right*) from fractured 3-month wild-type first molar (*A* and *B*) shows the normal structure of dentin: dentin tubule (*DT*), peritubular dentin (*), and intertubular dentin (♣). Representative backscattered scanning electron microscopy images in both cross-section (*B*) and sagittal section (*C*) reveal a dramatic decrease in mineralization and a striking change in dentin matrix structure in 3-month-old *Dmp-1* null (*KO*, *stars*) compared with the wild-type (*WT*) mice. A further enlarged scanning electron microscopy image of a wall of the KO peritubular area shows that matrix is poorly organized with increased spaces (*arrow*), suggesting a critical role of DMP-1 in control of dentin matrix morphology.

tents in *Dmp-1* null teeth are decreased (Fig. 2*A*). The layers of predentin (newly formed unmineralized dentin matrix), dentin (mature mineralized dentin matrix), and enamel (the hardest of all mineralized tissues, formed by ameloblasts) were reduced in *Dmp-1* null pups (Fig. 2*B*). In contrast, the mineral content in surrounding alveolar bone appears increased in the *Dmp-1* null pup when compared with controls (Fig. 2*B*).

Impaired Mineralization and Dentin Structure in Dmp-1 Null Mice during Postnatal Dentinogenesis—To address the *in vivo* roles of DMP-1 in mineralization of dentinogenesis, the first molars from the wild-type and *Dmp-1* null mice were removed from the maxilla for radiograph examinations from 3

weeks to 12 months in age (Fig. 3). Overall, there is no apparent change in tooth shape and size between the wild-type and the *Dmp-1* null mice, suggesting that DMP-1 has no effect on tooth pattern formation. The earliest change in mineralization was revealed by a reduction in thickness of the wall of tooth roots (Fig. 3*A*). By 1 month of age, the *Dmp-1* null mice showed an increase in both the pulp chamber and the root canal (Fig. 3*B*). A similar change was also documented in *Dmp-1* null molars at the ages of 3, 5, and 12 months (Fig. 3, *C–E*) and further confirmed by the micro-CT data (Fig. 3*F*).

Next, we analyzed changes in mineralization and dentin structure using scanning electron microscopy and backscattered scanning electron microscopy that allows differentiated visualization of mineralized tissues. Normal dentin is characterized by the presence of numerous dentin tubules surrounded by a ring of hypermineralized peritubular dentin (Fig. 4, *A* and *left panels* of *B* and *C*). In contrast, little mineral was seen in the *Dmp-1* null dentin (Fig. 4, *B* and *C*, *right panels*), suggesting that DMP-1 is required for mineralization during postnatal dentinogenesis. In addition, the dentin peritubular ultrastructure is poorly organized with an increased space. The normal smooth surface of the peritubular wall is replaced by a coarse and irregular dentin surface in *Dmp-1* null mice (Fig. 4, *B–D*, *right panels*). It is not clear that the impaired matrix structure is the consequence or the cause of a defect in mineralization during dentinogenesis.

Impaired Maturation of Predentin into Dentin in Dmp-1 Null Mice—During dentinogenesis, the first formed odontoblastic ECM is called predentin (a nonmineralized tissue), where mineral is later deposited and matured to dentin, a mature mineralized tissue. To better understand why increased pulp chambers and reduced dentin walls occurred in *Dmp-1* null mice (Fig. 3), we did a series of histological analysis in both molar and incisors at different ages (Fig. 5). Hematoxylin/eosin-stained sections from 1-month first molars (Fig. 5*A*), 12-month first molars (Fig. 5*B*), and 3-month incisors (Fig. 5*C*) clearly show that the predentin zone is expanded, whereas the dentin zone is decreased in the *Dmp-1* null mice compared with the controls. This may explain, in part, the increased volume of the pulp chamber and the root canal, as well as the reduced thickness of the dentin walls observed by radiographs (Fig. 3). In addition, we have also examined the enamel layer in *Dmp-1* null mice, which is thinner compared with that in the control mice (Fig. 5*C*). Note that a low level of *Dmp-1* is expressed by preameloblasts (Fig. 1*A*) that secret enamel ECM.

The Level of DSPP, a Critical Molecule in Dentin, Is Reduced in Dmp-1 Null Mice—In addition to DMP-1, there is another important noncollagenous protein in dentin, DSPP (16, 17). Both DMP-1 and DSPP are members of the SIBLING (small integrin-binding ligand *N*-linked glycoprotein) family of acidic, phosphorylated glycoproteins (18, 19). They share a similar genomic structure and are located in the same region on the chromosome. Their amino acid properties are very similar, and both need to be processed further into N-terminal and C-terminal fragments (5, 6). Particularly, *Dspp* null mice display a strikingly similar phenotype in teeth as observed in our *Dmp-1* null mice: reduced thickness of dentin, increased thickness of predentin, enlarged pulp chamber, and hypomineralization. Thus, we investigated whether there was a potential linkage between DMP-1 and DSPP during postnatal dentinogenesis. First, we examined DSPP protein level in *Dmp-1* null first molars from 1 month to 12 months in age with a polyclonal antibody against the mouse DSPP. In the wild-type molar, a strong DSPP signal is detected in both the dentin and the odontoblast (Fig. 6, *A–D*, *upper panels*), whereas a reduction of DSPP signal is observed in *Dmp-1* null molar (Fig. 6, *A–D*, *lower panels*). Next, we asked whether this change was caused by a reduction of DSPP expression or an increase in degradation of DSPP in mice lacking *Dmp-1*. With the RT-PCR method at 30 cycles, we measured the *Dspp* mRNA level in 1-week-old *Dmp-1* null teeth compared with that in the control. As shown in Fig. 6*E*, *Dspp* transcripts are reduced in *Dmp-1* null teeth, suggesting a reduction of *Dspp* expression. This is further confirmed by real time RT-PCR data, which showed a 25% reduction of *Dspp* in *Dmp-1* null molars compared with the control. We therefore propose that DSPP can be regulated directly or indirectly by *Dmp-1* and that some of dentinogenesis defects in *Dmp-1* null mice are probably caused by a reduction of DSPP (Fig. 6*F*).

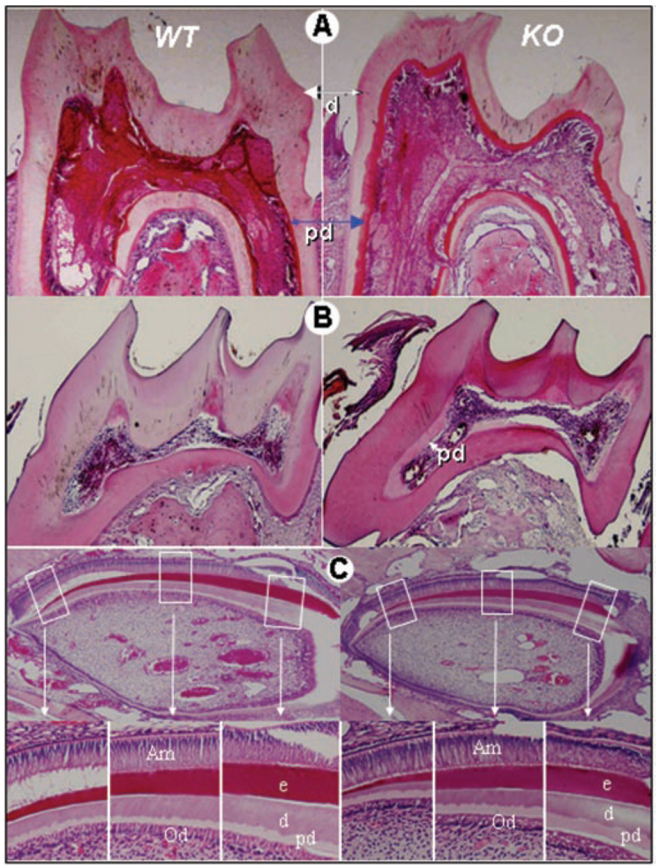

FIG. 5. **Deletion of *Dmp-1* leads to defects in maturation of predentin to dentin.** Predentin (*pd*), the newly formed nonmineralized matrix, matures into a mineralized dentin (*d*) during development. Representative hematoxylin/eosin-stained sections of molars (*A*, 1 month, lower first molar; *B*, 12 months, upper first molar) and incisors (*C*, 3 months) show an extended predentin (*red layer* in newly formed matrix; *light pink layer* in adult mice) and a reduced dentin in *Dmp-1* null mice (*KO*) compared with the wild-type (*WT*) mice, suggesting that DMP-1 is required for maturation of predentin into dentin. Note that the enamel layer is thinner in the *Dmp-1* null mice, indicating that DMP-1 may play a role in normal amelogenesis (*C*). *Am*, ameloblasts; *Od*, odontoblasts; *e*, enamel.

An Increased Biglycan May Be Associated with the Dentin Defects in Dmp-1 Null Mice—Biglycan, one of the small leucine-rich proteoglycan family members, is secreted by odontoblasts. A high concentration of biglycan inhibits the growth and proliferation of mineral crystals *in vitro* (20). Interestingly, biglycan null mice display growth retardation and decreased bone mass (21), whereas ectopic ossification is observed in biglycan and fibromodulin double null mice (22). To address whether there is a change in biglycan expression in *Dmp-1* null mice, a polyclonal antibody to mouse biglycan was used for immunostaining of the first molars from 3-week, 3-month, and 12-month-old mice (Fig. 7, *WT*, *upper panels*; *KO*, *lower panels*).

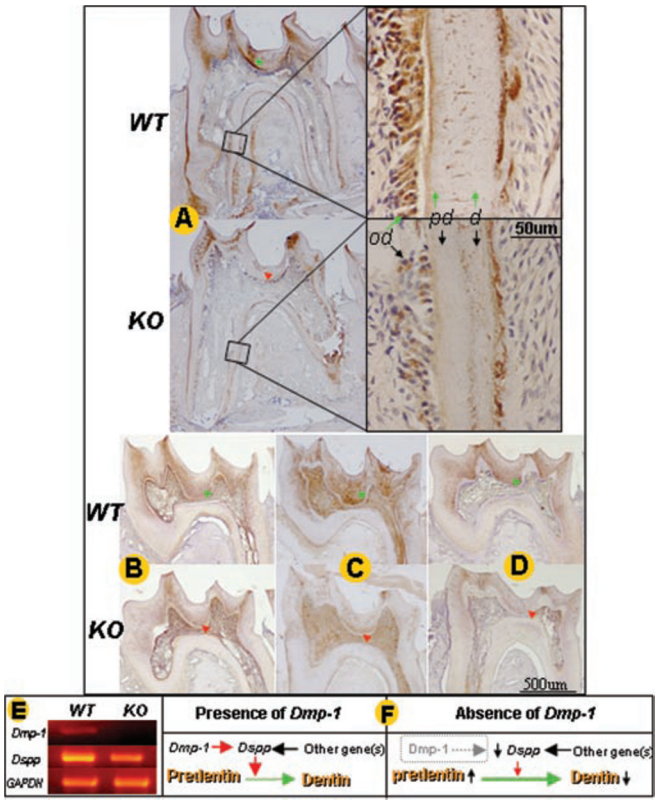

FIG. 6. **Dentin sialophosphoprotein (DSPP) is reduced in *Dmp-1* null mice.** To address whether DSPP is a targeted molecule of DMP-1, an antibody to mouse DSPP was used for immunostaining of the first molars (*A*, 1 month; *B*, 3 months; *C*, 5 months; *D*, 12 months). DSPP protein expression is shown by the *brown/yellow color*, and a general reduction of staining in *Dmp-1* null first molar (*arrowheads*) suggests that DSPP protein level is lower in the *Dmp-1* null first molar (*lower panels*). High magnification of a 1-month first molar (*A*, *inset*) shows that DSPP protein level in odontoblasts (*od*) is also reduced in the mutant molar. This change is associated with increased thickness of predentin (*pd*) and reduced thickness of dentin (*d*) in *Dmp-1* null molar. RT-PCR was performed using the first molar RNA for detection of *Dspp* change at 30 cycles. *Dspp* mRNA is also decreased in the *Dmp-1* null molar (*E*). Based on these changes, a working hypothesis is proposed (*F*). *WT*, wild type.

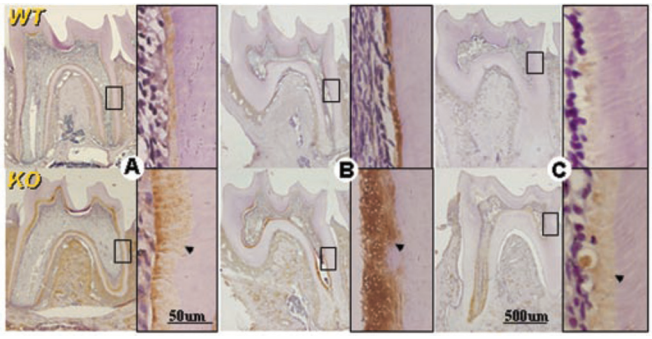

FIG. 7. **Biglycan protein level is increased in *Dmp-1* null predentin.** Imbalance of biglycan distribution greatly affects mineralization status. To find out whether biglycan level is altered in *Dmp-1* null mice, a polyclonal antibody to mouse biglycan was used for immunostaining of the first molars from 3-week (*A*), 3-month (*B*), and 12-month (*C*) mice (wild type (*WT*) in *upper panel*; knockout (*KO*) in *lower panel*). Biglycan protein expression is shown in *brown*, and an increase of biglycan is documented in *Dmp-1* null predentin.

A dramatic increase of biglycan in the predentin of *Dmp-1* null mice was observed in the expanded predentin, which has been reported in *Dspp* null mice (11). However, there was no change in biglycan mRNA level (data not shown), suggesting that there is no increase in biglycan production in *Dmp-1* null mice.

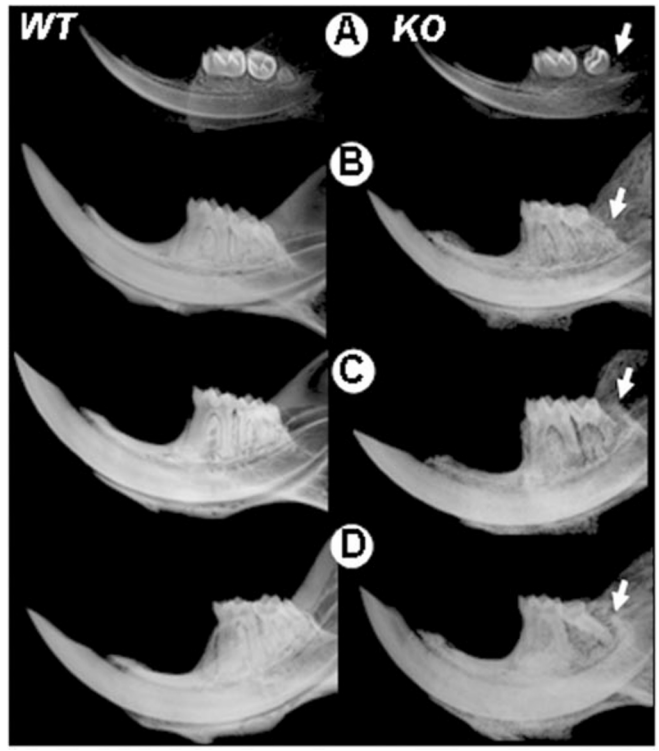

FIG. 8. **The third molar is either missing or retarded in *Dmp-1* null mice.** Representative radiographs of mandibles from 2 weeks (*A*), 3 months (*B*), 5 months (*C*), and 12 months (*D*) show that the third molar is absent or delayed in development in *Dmp-1* null mice (*right panel*, *arrows*).

The Third Molar Is Retarded in Dmp-1 Null Mice—Dmp-1 null phenotype appears postnatally (see above), and formation of the third molar is mainly during the first 2 weeks after birth. We therefore examined a potential effect of the absence of *Dmp-1* on development of the third molar from 2 weeks to 1 year in age. As shown in Fig. 8 (*right panels*, *arrows*), the third molar is either absent or delayed in development in *Dmp-1* null mice. Based on analysis over 200 *Dmp-1* null mice with C57/B6 background, ~10% of *Dmp-1* null mice display this defect. In contrast, the other defects described above exhibit a 100% penetrance. The detailed mechanism is unknown and is currently under investigation.

DISCUSSION

During dentinogenesis, odontoblasts secrete unmineralized, collagen-rich extracellular matrices termed predentin. As a precursor of dentin (a bone-like mineralized tissue), predentin lies between the mineralization front and the odontoblast layer. Later, the predentin is transformed to the mineralized tissue when apatite crystals are deposited within and around collagen fibrils (1, 23, 24). This process requires mechanisms that control the site and rate of apatite formation. In other words, the rate of formation of the unmineralized precursor layer should be the same as that of mineralization. Imbalances of these dynamic processes would lead to pathological conditions such as expansion of the predentin layer and reduction in the dentin layer as observed in some in some human dentin diseases (25). Although the mechanisms for controlling this process are largely unknown, noncollagenous proteins in dentin have been proposed to be critical for this process during dentinogenesis (23). In this study, we provide strong *in vivo* evidence to support DMP-1 roles in late stages of dentinogenesis with a *Dmp-1 lacZ* knock-in null animal model as described below.

First, *Dmp-1* is continuously expressed in dentin after birth

(Fig. 1, *B* and *C*), and *Dmp-1* null pups display defects starting with hypomineralization and reduction of dentin thickness at day 3 after birth (Fig. 2). Second, enlarged pulp chambers, expanded root canals, and decreased thickness in the dentin walls exist throughout the *Dmp-1* null mouse life span, although data are shown only up to 1 year (Figs. 3 and 5), suggesting that a defect (instead of a simple delaying) exists in later stages of dentinogenesis. Third, a defect in maturation of predentin to dentin, as reflected by an expanded predentin zone and reduced thickness of dentin, is documented in both incisors and molars in the mutants (Figs. 5–7). Fourth, the dentin ultrastructure is rough and disorganized, with little mineral deposited in mutant peritubular areas (Fig. 4), suggesting that DMP-1 is required for both the organic and inorganic components in dentin. All of the above phenotype is 100% penetrance, suggesting that none of the existing genes can compensate for the role of DMP-1 in later stages of dentinogenesis and mineralization. Paradoxically, there is no apparent phenotype observed in *Dmp-1* null embryos or newborns, although *Dmp-1* is active in the odontoblastic layer during this developmental stage (10). It is of note that mineralization in dentin, where DMP-1 plays a critical role, occurs mainly after birth. This difference of mineralization in dentin before and after birth may explain partially for defects observed in later stages of dentinogenesis.

To determine and identify what other molecules are involved in dentinogenesis could be perturbed in the *Dmp-1* null mice, we have screened over 10 factors and matrix proteins (bone morphogenic proteins 2 and 4, PTHrP, Cbfa1, type 1 collagen, alkaline phosphatase, bone sialoprotein, osteopontin, DSPP, and biglycan) by both RT-PCR and immunostaining. Of this group, there is little change seen in *Dmp-1* null mice (data not shown) except for a reduction in DSPP (Fig. 6) and the accumulation of biglycan in dentin matrix (Fig. 7). The significance of these findings is described as below.

DSPP, like DMP-1, is a member of the SIBLING family and highly expressed in odontoblasts during dentinogenesis (26). Both genes are located on the same chromosome location and share similar biochemical and genomic DNA features (17–19). *In vitro* studies have shown that dentin phosphoprotein, a cleaved product of DSPP, is actively involved in mineralization *in vitro* (27, 28). Mutations in the human *DSPP* gene are associated with the disease dentinogenesis imperfecta (29, 30). Finally, both *Dmp-1* null mice (Figs. 2–8) and *Dspp* null mice (11) display a very similar defect in later stages of dentinogenesis, suggesting a strong connection between these two genes. Here we show that DSPP is reduced in *Dmp-1* null mice at both the mRNA and protein levels (Fig. 7). This finding is in agreement with an *in vitro* study by Narayanan *et al.* (7), in which overexpression of *Dmp-1* induced *Dspp* expression. These authors also reported DMP-1 in the nucleus (31). Taken together, DSPP is probably one of the targeted molecules by DMP-1, and changes in DSPP may explain, in part, some of the defects observed in *Dmp-1* null mice (see Fig. 6*E* for support of our hypothesis).

However, *Dspp* null mice did not show tooth phenotype until 2–3 months of age (11). Furthermore, *Dspp* null mice display a high incidence of pulp exposures, a feature not found in *Dmp-1* null teeth. This suggests that the absence of DMP-1 itself or changes of other factors due to loss of DMP-1 may play a critical role in the development of defects in *Dmp-1* null dentinogenesis. Changes in the biglycan level in *Dmp-1* null predentin matrix are probably one example. As stated earlier, maintenance of normal biglycan levels in mineralized tissues is critical, since a high concentration of biglycan inhibits the growth and proliferation of mineral crystals *in vitro* (20). Deletion of biglycan leads to growth retardation and bone mass reduction (21) or to ectopic ossification when deleted with fibromodulin together (22). In *Dspp* null mice, both biglycan mRNA (as shown by RT-PCR) and protein (as shown by immunostaining) levels were increased (11). Similarly, Sreenath *et al.* (11) demonstrated the increased production and accumulation of decorin, another small leucine-rich proteoglycan secreted by odontoblast, in *Dspp* null mice. In contrast, there is no alteration in the level of biglycan mRNA (data not shown) despite the increase of biglycan protein in *Dmp-1* null predentin (Fig. 7). This suggests that the presence of DMP-1 is required for removing of biglycan in predentin matrix either directly or indirectly. In addition, there is no change in either mRNA or protein levels of decorin in *Dmp-1* null mice (data not shown), indicating that DMP-1 and DSPP may have distinct roles in dentinogenesis.

One of the most surprising findings in this study is that the third molar is either missing or retarded in some of *Dmp-1* null mice (Fig. 8). Tooth agenesis is a common inherited condition and has been linked in some cases to mutations in Msx-1 or Pax-9 (32–36). Deletion of Msx-1 or Pax-9 leads to tooth agenesis in mice (37, 38), suggesting that these early transcription factors are required for tooth formation and patterning. In contrast, *Dmp-1* is mainly active in the odontoblasts, a terminated cell, and deletion of *Dmp-1* has no effect on general tooth shape and size (Figs. 3 and 8). Additionally, tooth agenesis only accounts for ~10% of *Dmp-1* null mice in which severe alveolar bone defects coexist, suggesting that this defect is probably secondary to that of the bone defects.

In summary, by characterizing *Dmp-1* null mice, our studies clearly demonstrated that DMP-1 is required for mineralization and maturation of predentin into dentin during later stages of dentinogenesis. The *Dmp-1* tooth phenotype is identical, and 100% penetrance in both inbred C57/B6 background and outbred CD-1 background (data not shown). This indicates that tooth defects observed in *Dmp-1* null mice are most likely independent of genetic background. In this study, we have also excluded a potential effect of the neo-cassette on the *Dmp-1* null tooth phenotype, since the tooth phenotype is identical in *Dmp-1* null mice after removal of the floxed neo-cassette by breeding with CMV-Cre transgenic mice (data not shown). The above defects could not be rescued by a high calcium diet (data not shown), suggesting that the effect of DMP-1 on mineralization is local. Last, our initial mechanism studies show that DSPP can be directly or indirectly controlled by DMP-1. However, reduction of DSPP can only explain in part the defects displayed in *Dmp-1* null mice.

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