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*

Received for publication, January 16, 2004, and in revised form, February 13, 2004
Published, JBC Papers in Press, February 13, 2004, DOI 10.1074/jbc.M400490200

Ling Ye‡, Mary MacDougall§, Shubin Zhang‡, Yixia Xie‡, Jianghong Zhang‡, Zubing Li‡, Yongbo Lu‡, Yuji Mishina‡, and Jian Q. Feng‡‡

From the ‡Department of Oral Biology, School of Dentistry, University of Missouri-Kansas City, Kansas City, Missouri 64108, the §Department of Pediatric Dentistry, University of Texas Health Science Center at San Antonio, San Antonio, Texas 78284, and ¶Laboratory of Reproductive and Developmental Toxicology, NIEHS, National Institutes of Health, Research Triangle Park, North Carolina 27709

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 (Dsp) 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 DSP 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)§ 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. 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\textsuperscript{TM} 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 pulp 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

---

§ This work was partially supported by NIDCR, National Institutes of Health, Grants DE04055 and DE13430 (to J. Q. F.), DE13221 (to J. Q. F. and M. M.), and DE09875 (to M. M.). The costs of publication of this article were defrayed in part by the payment of page charges. This paper is available on line at http://www.jbc.org

‡ Tartaiex, 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.
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′-CTTGACTTCAGCCTATGCAAGC-3′) and p02 (5′-GGCTTAGTCTGTTGGCTG-3′) to detect the targeted allele (280 bp) and primers p01 (5′-CTTGACTTCAGCCTATGCAAGC-3′) and 5′-CTGTTCCTCACTCTAGCCTG-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 immunosassay 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. 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).
Roles of DMP-1 in Postnatal Dentinogenesis

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 60 min. The sections were washed again and incubated at 37°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% H2O2 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 on tooth development.

**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’-CAGCGCTTCT-GAGGAAGACAGTG-3’) and reverse (5’-CCTGCCCCCTTGCTCCT-TCTGA-3’); Dspp forward (5’-GGCATATACTAAACACCCGCTGC-3’) and reverse (5’-GGGGAAATAGGAAATGACAAGG-3’); and Gapdh forward (5’-ATGGGAAAGCTGGGCTGCT-3’) and reverse (5’-ACGG-ATACATTGGGGTACG-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 in situ hybridization, a Dmp-1 signal was observed in the odontoblast layer at E17.5 (Fig. LA). 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. LA).

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-1lacZ heterozygous mouse was selected for tracking Dmp-1 expression. A strong Dmp-1 lacZ signal is detected in both odontoblast layer and odontoblast processes (Figs. 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 by radiographs or 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-
tents in Dmp-1 null teeth are decreased (Fig. 2A). 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. 2B). In contrast, the mineral content in surrounding alveolar bone appears increased in the Dmp-1 null pup when compared with controls (Fig. 2B).

**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.

**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
first, we examined DSPP protein level in between DMP-1 and DSPP during postnatal dentinogenesis. thus, we investigated whether there was a potential linkage predentin, enlarged pulp chamber, and hypomineralization. null mice: reduced thickness of dentin, increased thickness of strikingly similar phenotype in teeth as observed in our null mice: reduced thickness of dentin, increased thickness of null mice showed an increase in both the pulp chamber and the root canal (Fig. 3B). 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. 3F).

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, A–B, D–E). 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 incisor at different ages (Fig. 5). Hematoxylin/eosin-stained sections from 1-month first molars (Fig. 5A), 12-month first molars (Fig. 5B), and 3-month incisors (Fig. 5C) 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. 5C). Note that a low level of Dmp-1 is expressed by preameloblasts (Fig. 1A) 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. 6E, 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. 6F).

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, W, upper panels; KO, lower panels).

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-month, 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.
A dramatic increase of biglycan in the predentin of \textit{Dmp-1} null mice was observed in the expanded predentin, which has been reported in \textit{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 \textit{Dmp-1} null mice.

**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 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 \textit{in vivo} evidence to support DMP-1 roles in late stages of dentinogenesis with a \textit{Dmp-1 lacZ} knock-in null animal model as described below.

First, \textit{Dmp-1} is continuously expressed in dentin after birth.
Roles of DMP-1 in Postnatal Dentinogenesis

(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 morphogenetic proteins 2 and 4, PTTHrP, Cbfal, type I 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 phosphophoroprotein, 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 Dsp 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 Dsp 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. 6E for support of our hypothesis).

However, Dsp null mice did not show tooth phenotype until 2–3 months of age (11). Furthermore, Dsp 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 Dsp 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 odontoblasts, in Dmp-1 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 MxS-1 or Pax-9 (32–36). Deletion of MxS-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 defect 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.

Acknowledgments—We thank David Anderson for providing CMV-Cre mice and Tetsuo Kunieda and Takeo W. Tsutsui for initial collection of animal samples. We thank Drs. James Simmer and Charles Cobb for useful discussion and critical comments on the manuscript. We also thank Dr. Larry Fisher for providing polyclonal antibodies against biglycan and DSPP.

REFERENCES
1. Garant, P. R. (2003) in Oral Cells and Tissues (Dickson, A., ed) pp. 25–52, Quintessence Publishing, Chicago, IL
2. Hunter, G. K., Haukschka, P. V., Poole, A. R., Rosenberg, L. C., and Goldberg, H. A. (1986) Biochem. J. 237, 59–64
3. Glimecher, M. J. (1989) Anul. Rec. 254, 159–153
4. George, A., Sabaay, B., Simonian, P. A., and Veis, A. (1993) J. Biol. Chem. 268, 12624–12630
5. Gue, C. Bruno, J. C., Cook, R. G., Orkiszewski, R. S., Malone, J. P., Veis, A., and Butler, W. T. (2003) J. Biol. Chem. 278, 34700–34708
6. Stieglitz, B. M., Ayala, M., Narayanan, K., George, A., and Greenspan, D. S. (2004) J. Biol. Chem. 279, 9850–9858
7. Narayanan, K., Srinivas, R., Ramachandran, A., Hao, J., Quinn, B., and George, A. (2001) Proc. Natl. Acad. Sci. U. S. A. 98, 4516–4521
8. He, G., Dahl, T., Veis, A., and George, A. (2003) in Nut. Mater. 2, 552–558
9. Hassan, A. H., Evans, C. A., Zaki, A. M., and George, A. (2003) Connect. Tissue Res. 44, 30–41
10. Feng, J. Q., Huang, H., Lu, Y., Ye, L., Xie, Y., Tsutsui, T. W., Kunieda, T.,

Downloaded from http://www.jbc.org/ by guest on July 26, 2018
11. Sreenath, T., Thyagarajan, T., Hall, B., Longenecker, G., D’Souza, R., Heng, S., Wright, J. T., MacDougall, M., Sauk, J., and Kulkarni, A. B. (2003) J. Biol. Chem. 278, 24874–24880
12. Zhang, J., Tan, X., Contag, C. H., Lu, Y., Guo, D., Harris, S. E., and Feng, J. Q. (2002) Biochem. Biophys. Res. Commun. 293, 1412–1419
13. Feng, J. Q., Zhang, J., Dallas, S. L., Lu, Y., Chen, S. Tan, X., Owen, M., Harris, S. E., and MacDougall, M. (2002) J. Bone Miner. Res. 17, 1822–1831
14. Thompson, D. L., Sabbagh, Y., Tenenhouse, H. S., Drezner, M. K., Salisbury, J. L., Grande, J. P., Poeschla, E. M., and Kumar, R. (2002) J. Bone Miner. Res. 17, 311–320
15. Fisher, L. W., Stubbs, J. T., III, and Young, M. F. (1995) Acta Orthop. Scand. Suppl. 266, 61–65
16. MacDougall, M., Simmons, D., Luan, X., Nydegger, J., Feng, J., and Gu, T. T. (1997) J. Biol. Chem. 272, 835–842
17. Feng, J. Q., Luan, X., Wallace, J., Jeng, D., Ohshima, T., Kulkarni, A. B., D’Souza, R. N., Kozak, C. A., and MacDougall, M. (1996) J. Biol. Chem. 273, 9457–9464
18. Fisher, L. W., Torchia, D. A., Fohr, B., Young, M. F., and Fredarko, N. S. (2001) Biochem. Biophys. Res. Commun. 280, 460–465
19. Fedarko, N. S., Fohr, B., Robey, P. G., Young, M. F., and Fisher, L. W. (2000) J. Biol. Chem. 275, 16666–16672
20. Fredarko, N. S., Fohr, B., Robey, P. G., Young, M. F., and Fisher, L. W. (2000) J. Biol. Chem. 275, 16666–16672
21. Ameye, L., Aria, D., Jepsen, K., Oldberg, A., Xu, T., and Young, M. F. (2002) FASEB J. 16, 673–680
22. Butler, W. T., and Ritchie, H. (1995) Int. J. Dev. Biol. 39, 169–179
23. Ten Cate, A. (1998) Oral Histology: Development, Structure, and Function, pp. 128–149, Mosby, St. Louis, MO
24. Levin, L. S., Leaf, S. H., Jelmini, R. J., Rose, J. J., and Rosenbaum, K. N. (1983) Oral Surg. 56, 267–274
25. D’Souza, R. N., Cavender, A., Sunavala, G., Alvarez, J., Ohshima, T., Kulkarni, A. B., and MacDougall, M. (1997) J. Bone Miner. Res. 12, 2040–2049
26. Linde, A., Lussi, A., and Crenshaw, M. A. (1989) Calcif. Tissue Int. 44, 266–269
27. Zhang, X., Zhao, J., Li, C., Gao, S., Qiu, C., Liu, P., Wu, G., Qiang, B., Lo, W. H., and Shen, Y. (2001) Nat. Genet. 27, 151–152
28. Xiao, S., Yu, C., Chou, X., Yuan, W., Wang, Y., Bu, L., Fu, G., Qian, M., Yang, J., Shi, Y., Hu, L., Han, B., Wang, Z., Huang, W., Liu, J., Chen, Z., Zhao, G., and Kong, X. (2001) Nat. Genet. 27, 261–264
29. Narayanan, K., Ramachandran, A., Hao, J., He, G., Park, K. W., Cho, M., and George, A. (2003) J. Biol. Chem. 278, 17500–17508
30. Vastardis, H., Karimzad, N., Guthuz, S. W., Seidman, J. G., and Seidman, C. E. (1998) Nat. Genet. 13, 417–421
31. Stockton, D. W., Das, P., Goldenberg, M., D’Souza, R. N., and Patel, P. I. (2000) Nat. Genet. 24, 18–19
32. van den Boogaard, M. J., Dorland, M., Beemer, F. A., and van Amstel, H. K. (2000) Nat. Genet. 24, 342–343
33. van der Bogaard, M. J., Dorland, M., Beemer, F. A., and van Amstel, H. K. (2000) Nat. Genet. 24, 342–343
34. Piazza-Bowers, S. A., Guo, D. C., Cavender, A., Xue, L., Evans, B., King, T., Milewicz, D., and D’Souza, R. N. (2002) J. Dent. Res. 81, 129–133
35. Stokken, D. W., Das, P., Goldenberg, M., D’Souza, R. N., and Patel, P. I. (2000) Nat. Genet. 24, 18–19
36. Vieira, A. R. (2000) J. Dent. Res. 82, 162–165
37. Satokata, I., and Maas, R. (1994) Nat. Genet. 6, 348–356
38. Peters, H., Neubuser, A., Kratuchwil, K., and Balling, R. (1998) Genes Dev. 12, 2735–2747
