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Dentin sialoprotein and dentin phosphoprotein have distinct roles in dentin mineralization

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Abstract

Dentin sialophosphoprotein (DSPP), a major non-collagenous matrix protein of odontoblasts, is proteolytically cleaved into dentin sialoprotein (DSP) and dentin phosphoprotein (DPP). Our previous studies revealed that DSPP null mice display a phenotype similar to human autosomal dominant dentinogenesis imperfecta, in which teeth have widened predentin and irregular dentin mineralization resulting in sporadic unmineralized areas in dentin and frequent pulp exposure. Earlier in vitro studies suggested that DPP, but not DSP, plays a significant role in initiation and maturation of dentin mineralization. However, the precise in vivo roles of DSP and DPP are far from clear. Here we report the generation of DPPcKO mice, in which only DSP is expressed in a DSPP null background, resulting in a conditional DPP knockout. DPPcKO teeth show a partial rescue of the DSPP null phenotype with the restored predentin width, an absence of irregular unmineralized areas in dentin, and less frequent pulp exposure. Micro-computed tomography (micro-CT) analysis of DPPcKO molars further confirmed this partial rescue with a significant recovery in the dentin volume, but not in the dentin mineral density. These results indicate distinct roles of DSP and DPP in dentin mineralization, with DSP regulating initiation of dentin mineralization, and DPP being involved in the maturation of mineralized dentin.

Keywords

dentin sialophosphoprotein; biomineralization; dentin volume; dentin mineral density; chondroitin sulfate proteoglycan

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1. Introduction

Mineralization of dentin is a complex process regulated by the collagenous matrix which is largely comprised of type I collagen, many non-collagenous proteins (NCPs), and minerals. Dentin sialophosphoprotein (DSPP) is one of the key NCPs involved in tooth development and mineralization (Butler and Ritchie, 1995). It is highly expressed in odontoblasts and transiently expressed in ameloblasts (D'Souza et al., 1997; Bègue-Kirn et al., 1998; MacDougall et al., 1998). However, a low level of DSPP expression has also been reported in bones, kidneys, lungs, salivary glands, and sweat glands (Xiao et al., 2001; Qin et al., 2002, 2003a; Ogbureke and Fisher, 2004, 2005, 2007; Alvares et al., 2006; Verdelis et al., 2008). Many heterogeneous mutations in the human DSPP gene have been linked to the two most common hereditary diseases affecting dentin, dentinogenesis imperfecta (DGI), and dentin dysplasia (DD) (Hart and Hart, 2007; Kim and Simmer, 2007; McKnight et al., 2008). Patients with DGI and DD typically have amber-brown, opalescent teeth, and their tooth enamel is often broken off, exposing the underlying dentin, which can lead to pulp exposure and accelerated tooth attrition. We previously generated the *Dspp*^{-/-} mice to delineate the precise functions of DSPP in dentinogenesis (Sreenath et al., 2003a). These *Dspp*^{-/-} mice show tooth defects similar to those seen in patients suffering from DGI and DD, with widened predentin, irregular mineralization front, and hypomineralization resulting in frequent pulp exposure. The *Dspp*^{-/-} phenotype confirmed the important roles of DSPP in dentin mineralization. Interestingly, the *Dspp*^{+/-} mice did not show any obvious abnormalities, suggesting that haploinsufficiency does not cause any obvious phenotype in these mice.

So far, a full-length DSPP protein has never been isolated or identified in dentin. DSPP is cleaved into dentin sialoprotein (DSP) and dentin phosphoprotein (DPP), also known as phosphophoryn (MacDougall et al., 1997). Interestingly, a third polypeptide, dentin glycoprotein (DGP), is cleaved out from the C-terminal end of porcine DSP by matrix metalloproteinases such as MMP-2 and MMP-20 (Yamakoshi et al., 2005a). DSP and DPP are the most abundant NCPs in dentin ECM (Yamakoshi et al., 2008), and some of the earlier studies suggest that DPP is more abundant than DSP in the mineralized dentin (Butler et al., 1981; Butler, 1998). DSP, the amino-terminal part of DSPP, is a sialic acid-rich, glycosylated protein. It is a member of the SIBLING (Small Integrin-Binding Ligand N-linked Glycoproteins) family, which also includes bone sialoprotein (BSP), dentin matrix protein-1 (DMP-1), osteopontin (OPN), and matrix extracellular phosphoglycoprotein (MEPE). It has been reported that both porcine and bovine DSP are chondroitin sulfate-type proteoglycans (Yamakoshi et al., 2005b; Sugars et al., 2006). DPP contains repeat sequences of serine-serine-aspartic acid (in humans over 200 tandem copies and in mice about 100 copies) and most of them are phosphorylated, contributing to their acidic nature (Jonsson and Fredriksson, 1978; McKnight et al., 2008).

DPP is present mostly in the mineralized matrices of primary dentin (Weinstock and Leblond, 1973; Jontell and Linde, 1983; MacDougall et al., 1985; Rahima et al., 1988), suggesting a possible role in dentin mineralization. DPP has a strong affinity for Ca²⁺ and it significantly promotes the growth of hydroxyapatite crystals when bound to collagen fibrils in vitro (Zanetti et al., 1981; Milan et al., 2006). The phosphate moieties in DPP are believed to be important for the role of DPP in dentin mineralization (He et al., 2005; Milan et al., 2006). In contrast, DSP has been reported to have limited or no effect on in vitro formation and growth of hydroxyapatite crystals (Boskey et al., 2000). Due to the fact that DSP and DPP are produced by the cleavage of DSPP, the *Dspp*^{-/-} phenotype does not shed much light on individual roles of DSP or DPP in dentin mineralization. Therefore, in order to investigate the in vivo roles of DSP and DPP as an individual matrix protein in dentin mineralization, we generated and characterized DPPcKO mice in whose odontoblasts express DSP in a *Dspp*^{-/-} background.

2. Results

2.1. Generation of DSP transgenic mice in the *Dspp*^{-/-} background (DPPcKO mice)

We engineered a transgenic construct whose DSP expression is driven by the DSPP promoter (Fig. 1a). The coding sequence for DSP spanned from ATG to the stop codon inserted after ³⁷⁰Ser (³⁷⁰Ser was first hypothesized as the C-terminal of DSP (Butler et al., 1992; Ritchie et al., 1994, 1998; Butler, 1998)). Using this construct we generated 2 TgDSP lines, one with a high level of DSP expression (line B) and the other with a low level of DSP expression (line D). Interestingly, histological and microradiographic analysis revealed that neither of the TgDSP mouse lines had any obvious phenotype as compared to wild-type mice (data not shown). These mouse lines were crossed with *Dspp*^{-/-} mice to obtain *TgDSP/Dspp*^{-/-} (DPPcKO) mice. In order to analyze whether *Dsp* was expressed sufficiently in DPPcKO molars, we first analyzed the mRNA expression level of *Dsp* and *Dpp* portion in *Dspp*^{+/-}, *Dspp*^{-/-}, and DPPcKO-B molars by quantitative real-time PCR (Fig. 1b). *Dsp* expression level was approximately 8-fold higher in DPPcKO-B molars than in *Dspp*^{+/-} molars. As expected, no expression was observed in *Dspp*^{-/-} molars, while *Dpp* portion of *Dspp* mRNA was detectable only in *Dspp*^{+/-} mice. Next, we determined the amount of DSP protein deposited into molar dentin by ELISA (Fig. 1c). *Dspp*^{+/-} and DPPcKO-B molars contained identical amounts of DSP protein.

Immunohistochemical analysis of 16-day-old mice revealed that the DSP expression pattern in DPPcKO-B molars was similar to the endogenous DSP expression pattern seen in *Dspp*^{+/-} mice (Figure 1d and f). In both *Dspp*^{+/-} and DPPcKO-B mice, DSP expression was mainly restricted to odontoblasts and dentin, and a slight expression was observed in predentin and tooth pulp. No DSP staining was detected in *Dspp*^{-/-} molars (Fig. 1e). On the other hand, immunostaining for DPP revealed positive staining in *Dspp*^{+/-} odontoblasts and dentin (Fig. 1g), but none in DPPcKO-B (Fig. 1i) or in *Dspp*^{-/-} mice (Fig. 1h). Next, we performed immunostaining on tooth sections from 3-month-old mice, because by this age the teeth are fully grown and their eruption is complete. In DPPcKO-B and *Dspp*^{+/-} molars, a strong DSP staining was detected in the primary dentin, especially along the dentinal tubules, odontoblast layer, and also in the odontoblast processes, but less in the predentin. The DSP expression level in DPPcKO-B molars was similar to that in *Dspp*^{+/-} molars (Fig. 1 j-l). Similar expression levels and patterns were also observed in the molars of the 2-, 5-, and 9-month-old transgenic mice (data not shown).

2.2. Restored width of predentin and absence of sporadic unmineralized areas in DPPcKO dentin

In order to characterize the role of DSP in dentin mineralization, we examined molars of the DPPcKO-B mice that did not show any pulpal exposure. During dentinogenesis, odontoblasts first secrete collagens and some NCPs to form predentin, and then they secrete some NCPs directly into the mineralization front (the border between predentin and dentin). It is believed that these secreted NCPs participate in conversion of predentin into dentin. Similar to the wild-type molars, *Dspp*^{+/-} molars showed a normal, narrow predentin and smooth mineralization front along the odontoblasts layer (Fig. 2a and d). *Dspp*^{-/-} molars, on the other hand, had a wider predentin, an irregular mineralization front, and randomly distributed unmineralized areas in the circumpulpal dentin (Fig. 2b and e). Interestingly, the predentin width of DPPcKO-B molars was much narrower than that of the *Dspp*^{-/-} molars. Unlike the *Dspp*^{-/-} dentin, DPPcKO-B dentin had no unmineralized areas (Fig. 2c and f). However, the width of predentin still appeared to be wider than that of *Dspp*^{+/-} molars (Fig. 2 d and f).

2.3. Partial rescue of dentin thickness in DPPcKO mice

To further investigate the precise role of DSP in conversion of unmineralized predentin to mineralized dentin, we conducted radiographic and Scanning Electron Microscopy (SEM) analyses of the first molars of 3-month-old *Dspp*^{+/-}, *Dspp*^{-/-}, and DPPcKO-B mice. Radiographic analysis of the upper first molars indicated a normal thickness of dentin in the molar roots of *Dspp*^{+/-} mice (Fig. 3a). However, *Dspp*^{-/-} molar roots had reduced dentin thickness, accompanied by widened root canals (Fig. 3b). In contrast, the thickness of the molar root dentin in DPPcKO-B mice appeared wider than that of *Dspp*^{-/-} mice (Fig. 3c). Interestingly, both the crown and roots of the DPPcKO-B mice appeared more translucent than those of *Dspp*^{+/-} mice. SEM analysis of the first lower molars of the *Dspp*^{-/-} mice showed that the dentin was thin, resulting from a widened pulpal area (the total volume of predentin and pulpal cavity) and an irregularly mineralized front (Fig. 3e). However, DPPcKO-B mice displayed much wider dentin, as compared to *Dspp*^{-/-} mice (Fig. 3f). This was accompanied by a thinner predentin and a smooth mineralization front in DPPcKO-B molars as shown in Fig. 2. Notably, *Dspp*^{-/-} molars showed more severe defects in the pulp region of the horn area (Fig. 3h). Hypomineralized dentin seemed to be much wider in the *Dspp*^{-/-} molars than in DPPcKO-B molars (Fig. 3i).

2.4. DSP restores the dentin volume but not the dentin mineral density of *Dspp*^{-/-} molars

We previously reported the lack of DSPP didn't affect the mRNA expression levels of type I collagen, the major organic component of predentin and dentin (Sreenath et al., 2003a). This observation indicated that the lack of either DSP or DPP did not alter the expression level of type I collagen, resulting in the total volume of predentin and dentin remaining unchanged. On the other hand, based on the results shown in Fig. 2, DSP expression in DPPcKO mice may have promoted the conversion of predentin into dentin, probably resulting in an increase in the dentin volume and mineralization. Therefore, we examined the dentin volume density and the dentin mineral density by micro-CT analysis. We performed this analysis on the lower first molars of the 3-month-old male mice that did not show any pulpal exposure. As shown in Figure 4a and b, the dentin volume density and the dentin mineral density were significantly reduced in *Dspp*^{-/-} mice, as compared to those of *Dspp*^{+/-} mice. Conversely, the dentin volume density in DPPcKO-B mice was similar to that of *Dspp*^{+/-} mice. However, the dentin mineral density of DPPcKO-B mice was significantly lower than that of the *Dspp*^{+/-} control mice. Additionally, it is worth noting that enamel volume density and enamel mineral density remained unaltered in the *Dspp*^{+/-}, *Dspp*^{-/-}, and DPPcKO-B mice (Fig. 4c and d). Taken together, these findings indicate that DSP expression in the dentin of DPPcKO-B mice increased dentin volume density but not dentin mineral density.

2.5. Reduced frequency of pulp exposure in DPPcKO molars

One of the key characteristics of the *Dspp*^{-/-} tooth phenotype was the frequent pulp exposure due to severe tooth attrition (Sreenath et al., 2003a). To determine the effects of DSP expression on the frequency of pulp exposure, we analyzed the upper first molars of 4-month-old *Dspp*^{+/-}, *Dspp*^{-/-}, and DPPcKO mice, since pulp exposure is more common in first molars at this age. We could easily distinguish molars that had pulp exposure by staining their sections with hematoxylin and eosin stain (Fig. 5 b-e). In the molars, with pulp-exposure, we observed pulpal exposure mostly in distal and central cusps, which resulted in pulpal degeneration associated with bacterial infection. None of the *Dspp*^{+/-} mice had molars with pulpal exposure, whereas 41.5% of the *Dspp*^{-/-} mice had teeth which showed pulpal exposure. However, less than 20% of DPPcKO mice displayed pulpal exposure in their teeth (Fig. 5a).

2.6 DSP is the chondroitin/dermatan sulfate proteoglycan in mouse molars

Proteoglycans in hard tissues such as bones and teeth are believed to be essential factors required to induce and maintain mineralization. Recent reports indicate that DSP exists as a chondroitin sulfate-attached proteoglycan in porcine and bovine dentin (Yamakoshi et al., 2005b; Sugars et al., 2006). To determine whether DSP is also present as a chondroitin sulfate-attached proteoglycan in the molars of *Dspp*^{+/-}, *Dspp*^{-/-}, and DPPcKO-B mice, molar dentin proteins (aliquots of the proteins used for DSP analysis shown in Fig. 1c) were analyzed by Western blotting. This analysis revealed that both endogenous and transgenic DSP appeared as a smear (Fig. 6a, lane 1 and 5, open bracket). The smear on the blot ranged from 90 kDa to more than 210 kDa in *Dspp*^{+/-} dentin, whereas it ranged from 90 kDa to less than 210 kDa in DPPcKO-B dentin. Chondroitinase ABC treatment of both protein preparations resulted in a distinct band of ~111 kDa (Fig. 6a, lane 2 and 6, asterisk). This indicates that the smears contained chondroitin/dermatan sulfate-type proteoglycans, and DSP was mainly secreted into dentin as a proteoglycan. Additionally, we also found bands under the ~111 kDa band in *Dspp*^{+/-} and DPPcKO-B dentin after chondroitinase ABC treatment. These extra bands seemed to be DSP fragments, or possibly DGP, because this antibody was made against exon 4 of mouse DSPP. No staining was detected in *Dspp*^{-/-} dentin either with or without chondroitinase ABC treatment (Fig. 6a lane 3 and 4).

3. Discussion

In order to delineate specific roles of DSP and DPP in dentin development and mineralization, we utilized an authentic mouse DSPP promoter to express DSP in *Dspp*^{-/-} background. This odontoblast-specific expression of DSP partially ameliorates dentin defects of *Dspp*^{-/-} mice. Interestingly, DSP expression alone restored dentin volume, but not dentin mineral density, and also reduced the frequency of pulp exposure.

We previously reported genomic cloning and characterization of the mouse DSPP gene, including its entire promoter (Feng et al., 1998). Subsequently, we validated the mouse DSPP promoter for its ability to drive odontoblast-specific expression in the developing molars of the LacZ transgenic mouse lines (Sreenath et al., 1999). Moreover, we utilized the same promoter to express Cre recombinase (Sreenath et al., 2003b) and TGF- β 1 in odontoblasts (Thyagarajan et al., 2001). In these mouse models, the DSPP promoter-driven expressions of the transgenes in the developing molars were similar to endogenous patterns of spatiotemporal expression of the DSPP gene. As shown in Fig. 1I, immunostaining of the molar section from the DPPcKO mouse confirms that the DSP expression profile also matches the endogenous profile seen in the controls (Fig. 1J). Moreover, DSP mRNA level in DPPcKO molar dentin was higher than in *Dspp*^{+/-} dentin. However, the DSP protein level in dentin was apparently similar in the *Dspp*^{+/-} and DPPcKO molars (Fig. 1b and c), suggesting that there may be mechanism(s) at the translational or post-translational level that regulates amount of DSP deposited into dentin of these mice. This idea is consistent with the fact that over-expression of DSP in wild-type mice showed no obvious phenotypes (data not shown). These results and previous findings mentioned above validate our approach to express DSP using the DSPP promoter in *Dspp*^{-/-} background. SEM and micro-CT data showed that DSP expression in these mice mainly reversed the deficiency in the dentin volume but not in the dentin mineral density observed in *Dspp*^{-/-} mice (Fig. 3, 4). The difference in the dentin volume deficiency identified by micro-CT data reflected the existence of some sporadic unmineralized areas in *Dspp*^{-/-} dentin but not in DPPcKO dentin. Some of the sporadic unmineralized areas were much larger than the diameter of calcospherites (calcifying globules in or near the mineralization front), indicating that the initiation of mineralization was inhibited in *Dspp*^{-/-} dentin but was largely restored in DPPcKO dentin (Fig. 2). On the other hand, the dentin mineral density was significantly lower in DPPcKO mice as well as *Dspp*^{-/-} mice, suggesting that the

process of mineral maturation, such as the assembly of hierarchically ordered crystal structures, requires DPP. Therefore, the reduced frequency of pulp exposure in DPPcKO mice was due to the rescue of dentin volume (Fig. 5). Since the predentin width of DPPcKO mice was slightly wider than in *Dspp*^{+/-} mice, DPP itself, or a combination of DSP and DPP, may accelerate predentin-dentin conversion.

As previously reported, DSP in rat dentin extract migrated either as a single major band (100 kDa) which had nominal effects on formation and growth of hydroxyapatite crystals in vitro (Boskey et al., 2000), or as a high molecular weight DSP (HMW-DSP) (200 kDa~), whose function still remains unknown (Qin et al., 2003b). Moreover, Qin and co-worker also reported that HMW-DSP showed more heterogeneity in glycosylation of the extremely large amounts of carbohydrates, such as N-linked carbohydrates, sialic acid, and O-linked oligosaccharides (Qin et al., 2003b). As shown in Fig. 6, most of the DSP in *Dspp*^{+/-} and DPPcKO mice was secreted into dentin as HMW-DSP containing chondroitin sulfate chain (Fig. 6a), though the size of HMW-DSP in DPPcKO mice seems to be smaller than in *Dspp*^{+/-} mice. This suggests that DSP synthesis as a full length DSPP is critical for induction of complete posttranslational modification in odontoblasts. However, DSP in DPPcKO molars could significantly restore the chondroitin sulfate level in dentin, indicating the HMW-DSP containing chondroitin sulfate chain is responsible for the predentin-dentin conversion (data not shown).

In predentin, proteoglycans such as biglycan and decorin organize type I collagen into a more fibrillar form near the mineralization front in order to induce the proper crystal formation along the collagen fibrils and inside the fibrils (Goldberg et al., 1995; Embery et al., 2001). Interestingly, these proteoglycans are also known to be secreted into the mineralization front directly from odontoblasts as well as the phosphoproteins (Weinstock and Leblond, 1973; Jontell and Linde, 1983; MacDougall et al., 1985; Rahima et al., 1988; Lormée et al., 1996; Embery et al., 2001). They interact with collagen fibrils through their core proteins, and their chondroitin sulfate chain pierces into the collagen fibrils as a needle-like structure to capture Ca²⁺ ions and bond to hydroxyapatite. This leads to nucleation of hydroxyapatite crystals around and inside the collagen fibrils (Goldberg et al., 1995; Dechichi et al., 2007). Moreover, as compared to predentinal proteoglycans, dentinal proteoglycans, which are largely proteoglycans containing chondroitin sulfate (Waddington et al., 2003), have 19 times the binding affinity for hydroxyapatite (Milan et al., 2004). Thus, dentinal DSP might facilitate initiation of hydroxyapatite formation along or inside the collagen fibril, leading to conversion of the predentin to dentin at the mineralization front.

In summary, our studies with DPPcKO mice revealed that DSP and DPP have distinct roles in dentin mineralization. In DPPcKO mice, predentin is narrower, with almost the same width as in *Dspp*^{+/-}, and the mineralization front is much smoother than in *Dspp*^{-/-} mice. However, dentin is still hypo-mineralized, suggesting that DSP is more involved in regulation of dentin mineralization's initiation, whereas DPP is more involved in maturation of dentin mineralization. Future investigations of the DSP and DPP and their roles in the deposition of other NCPs in dentin matrix, may provide better insight into their precise roles in dentinogenesis.

4. Experimental procedures

4.1. Plasmid construction

As depicted in Fig. 1a, the genomic fragment spanning 2.5 kb of the upstream *Dspp* 5' promoter region, and a coding region from exon 1 to the middle of intron 4, were cloned by digestion of the previously described DSPP genomic clone with *Xba*I (Feng et al., 1998). We previously reported that the genomic fragment spanning the DSPP regulatory element from 2.5 kb upstream of the DSPP promoter region to exon 2 was sufficient for the expression of the

transgene mimicking the endogenous expression pattern of the DSPP gene (Sreenath et al., 1999,2003b). The *Xba*I-digested fragment was ligated with the PCR product that included the rest of intron 4 and a part of exon 5 (from the DSP peptide to residue ³⁷⁰Ser), followed by an introduced stop codon. This entire fragment was cloned into a pIRES-DsRed vector (Clontech, Mountain View, CA). The 13.5-kb fragment containing the entire transgene was confirmed by DNA sequences analysis.

4.2. Generation of DSP transgenic mice in a DSPP null background (DPPcKO mice)

TgDSP mice were generated by injecting the 13.5-kb transgenic fragment described above into zygotes of FVBN mice to generate TgDSP lines, which were characterized for their DSP expression levels. Two of these transgenic lines were crossed with *Dspp*^{-/-} mice (Sreenath et al., 2003a). Founder mice with genotype TgDSP (+) and *Dspp*^{+/-} were identified by PCR analysis. These mice were crossed with *Dspp*^{-/-} mice, and their progeny with the genotype of TgDSP (+) *Dspp*^{-/-} (DPPcKO) were identified by PCR. Because *Dspp*^{+/-} mice had a normal tooth phenotype, *Dspp*^{+/-} littermates were used as controls. Studies were performed in compliance with the National Institutes of Health (NIH) guidelines on the care and use of laboratory and experimental animals. All experimental procedures were approved by the Animal Care and Use Committee of the National Institute of Dental and Craniofacial Research.

4.3. PCR genotyping

Genotyping was performed by PCR analysis of genomic DNA extracted from tail snips using the following primers: the forward primer (GATAGCAATGGACACCAAGGA GTG), and the reverse primer (ACTTCCGGTTAGATTCGTCGCTG) for determination of *Dspp*^{+/-} or *Dspp*^{-/-} mice. In addition, the following primers were used: the forward primer (GTTTCATGCGCTTCAAGGTGCGCATGGAG) and the reverse primer (GACTTGAACCTCCACCAGGTAGTGGCCGC) for identification of TgDSP mice.

4.4. RNA extraction and Real time-PCR

Mice were euthanized by CO₂ inhalation, and the molars from P20 mice were crushed carefully by using mortar and pestle, and then total RNA was isolated by Trizol reagent kit (Invitrogen) and real-time quantitative PCR reactions were prepared with iQ SYBR Green supermix and carried out in a PTC-200 thermal cycler with Chromo4 Real-Time PCR Detection System (Bio-rad). *Gapdh* was used as an internal reference control. Primer pairs for target genes were as follows: *Dsp* portion, sense (5'-CTTCCCAAATGGACACAATG-3') and antisense (5'-ATGCTTCTGACTGGCTGATG-3'); *Dpp* portion, (5'-AAAGGAATAGCCCAAAGCAA-3') and antisense (5'-CCATCACTATTGCTGCTGCT-3'); *Gapdh* sense (5'-AATGTGTCCGTCGTGGATCTGA-3') and antisense (5'-GATGCCTGCTTACCACCTTCT-3').

4.5. Extraction of tooth protein and detection of DSP

Mice were euthanized by CO₂ inhalation, and the molars (lower first, second, and upper first) were extracted and tooth proteins were isolated by procedures described earlier (Fisher et al., 1983). Extracted teeth were crushed by using mortar and pestle, and incubated with 4 mol guanidine HCl in the presence of Complete mini EDTA-free protease inhibitor (Roche, Alameda, CA) for 24 h at RT in order to remove proteins not incorporated into the mineralized matrix. The residue was then demineralized with 4 mol guanidine HCl, 0.5 mol EDTA plus protease inhibitors for 2 days at RT. The solution containing proteins incorporated into the mineralized matrix was dialyzed against 4 mol guanidine for 1 day, then against distilled water for another 2 days. Dialyzed solution was lyophilized and dissolved into distilled water containing protease inhibitor. Protein samples were treated for 24 h at 37 °C with or without

0.1 unit of protease-free chondroitinase ABC (Seikagaku America, St. Petersburg, FL) and then reduced with DTT and loaded onto Novex 4–20% Tris-glycine gels (Invitrogen), electrophoresed, and transferred onto a PVDF membrane (Invitrogen). The membrane was blocked with 3% non-fat dry milk in PBS solution and then incubated with anti-DSP antibody (LF-153)(Ogbureke and Fisher, 2004) (1:2000)(this antibody is kind gifts from Dr. Larry Fisher, NIDCR, NIH, Bethesda MD). The immunoreactive proteins were identified using Supersignal chemiluminescent substrate (Pierce, Rockford, IL).

4.6. Enzyme-linked immunoabsorbent assay (ELISA)

Ninety-six-well, flat-bottom microtiter plates (Immunolon 2HB, Thermo Fisher Scientific, Waltham, MA) were coated with protein extracted from teeth in triplicate in a serial dilution in PBS (Dulbecco's Phosphate Buffered Saline, containing calcium and magnesium) overnight at 4 °C. Plates were washed 3 times with 0.1% Tween20- PBS and then blocked with 3% bovine serum albumin (BSA) in PBS for 5 h at RT. Plates were washed 5 times with 0.1% Tween20- PBS and incubated with anti-DSP antibody (1:3000) for 90 min at RT. After washing, the wells were incubated with goat anti-rabbit IgG antibodies conjugated with HRP in PBS (1:3000) for 1 h at RT. They were washed and detected with 3, 3', 5, 5'- Tetramethyl Benzidine substrate solution (TMB, Pierce) for 30 min at RT. After addition of 2 N HCl to stop the colorimetric reaction, optical density was measured at 450 nm using a microtiter plate reader.

4.7. Immunohistochemistry

Immunostaining for DSP and DPP was performed on 5-µm thick paraffin sections. Skulls from postnatal 16 (P16), or 3-month-old mice were dissected and fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS) for 24 hr at 4 °C. The skulls were then decalcified with 0.1 mol EDTA in PBS for 3 weeks at 37 °C, and dehydrated through a graded ethanol series, placed into xylene, and then embedded in paraffin. For staining, the sections were dewaxed and treated with Peroxidase I (Biocare Medical Concord, CA) for 5 min at RT to inactivate endogenous peroxidase. Next, sections were blocked with Rodent Block M (Biocare Medical) for 30 min at RT, and then incubated with rabbit polyclonal anti-DSP (1:2000) for 2h at RT or anti-DPP (Alvares et al., 2006) (1:1000) (a kind gift from Dr Keith Alvares, Northwestern University Medical School, Chicago, IL) overnight at 4 °C. The immune complexes were formed using Rodent HRP-Polymer (Biocare Medical) for 30 min at RT, and the peroxidase reaction in the immune complexes was visualized by a chromogen substrate 3,3'-Diaminobenzidine reaction, according to the manufacturer's instructions (Sigma). The sections were counterstained with hematoxylin and then mounted.

4.8. Microradiography

Three-month-old mice were euthanized by CO₂ inhalation, and the upper first molars were separated and radiographed using a Faxitron MX20 Specimen Radiography System (Faxitron X-ray Corp., Wheeling, IL) for 120 seconds at 15 kV (Sreenath et al., 2003a). The images were captured on X-OMAT TL film (Eastman Kodak, Rochester, NY).

4.9. Scanning electron microscope analysis

For scanning electron microscopy (SEM) analysis, mandibles were dissected from 3-month-old male mice and fixed in 2% glutaraldehyde and 2% paraformaldehyde in 0.1 mol cacodylate buffer, pH 7.2. These mandibles were later rinsed in 2% glutaraldehyde and 2% paraformaldehyde in 0.1 mol cacodylate buffer, pH 7.2, and 2% osmium tetra-oxide, then incubated at room temperature for 24 h. Three successive transverse sections of the lower first molars were obtained using a saw with a diamond disk under cold-water conditions (Accutom-Streuer, Copenhagen, Denmark). The sections were further dehydrated in a graded ethanol

series, carefully dried, glued to aluminum stubs, sputter-coated with gold-palladium, and examined using an electron microscope (JSM 35C; Jeol, Peabody, MA) operating at 20 kV.

4.10. Micro-CT analysis

The dissected mandibles were analyzed by a high-resolution micro-tomographic imaging system (μ CT 40, Scanco Medical AG, Brüttisellen, Switzerland) equipped with a 5- μ m focal spot X-ray tube as a source. A two-dimensional CCD, coupled with a thin scintillator as a detector, permitted acquisition of 42 tomographic images in parallel. The mandibles were oriented in the micro-CT scanner in such a way that the incisor was oriented perpendicular to the rotation axis of the scanner. The X-ray tube was operated at 70 kVp and 114 μ A, with an integration time set to 300 ms. Scans were performed at an isotropic, nominal resolution of 10 μ m (high-resolution mode).

The whole mandibles were scanned, which resulted in an average scan height of 3.5 mm and a measurement time of approximately 2 hours. A constrained 3D Gaussian filter ($\sigma = 1.2$, support of one voxel) was used to partly suppress the noise in the volumes. Then the dentin and enamel phases were segmented from bone by a global thresholding procedure (Rüeggsegger et al., 1996), with threshold values set between 34% to 58% of the maximum grayscale value for dentin, and a threshold value of 58% for enamel. Furthermore, special algorithms based on morphological operators were used to remove partial volume effects.

Volume density and mineral density were then separately determined for the enamel and dentin phases of the first molar. Dentin or enamel volume density was calculated as the ratio of the percent volume occupied by the mineralized space, divided by total dentin or enamel volume, respectively, using a direct and 3D approach (Hildebrand et al., 1999). Mineral density values were calculated for each phase separately from the X-ray absorption levels, based on a linear calibration equation determined by a hydroxyapatite (HA) phantom, which was provided by the micro-CT manufacturer (Nazarian et al., 2008).

4.11. Histological analysis and measurement of the frequency of pulp exposure

Four-month-old mouse skulls were dissected and 5- μ m sections were prepared as described above. Sections containing upper first molars were stained with hematoxylin (Sigma) and eosin (Sigma) to determine whether pulp was exposed or not.

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Fig. 1. Generation of DPPcKO mice

a, A diagrammatic representation of the transgenic DSP construct showing the genomic DSP sequence (5.7-kb regulatory element and DSP domain) followed by a stop codon, IRES-DsRed, and SV 40 ploy(A). Exons are colored green, stop codon is blue, BMP-1 cleavage site is gray, IRES-DsRed is red, and SV40 poly(A) is yellow. **b**, Relative mRNA expression of *Dsp* and *Dpp* analyzed by real-time PCR using specific primer pairs. mRNA expression of *Dsp* in DPPcKO-B molars is eight folds higher than in *Dspp*^{+/-} molars. *Dsp* is not detectable in *Dspp*^{-/-} molars. *Dpp* is only detectable in *Dspp*^{+/-} molars. *Dsp* and *Dpp* expression level was normalized using *Gapdh* expression level. **c**, DSP protein levels in dentin of *Dspp*^{+/-}, *Dspp*^{-/-}, and DPPcKO-B molars. EDTA fraction of dentin proteins, isolated from molars of

P20 mice ($n > 40$), were coated on the individual wells of 96-well plates at different concentrations. DSP was determined using anti-DSP antibody. *Dspp*^{+/-} and DPPcKO-B dentins contain almost same level of DSP where as *Dspp*^{-/-} dentin does not contain any. Each column represents the mean $\pm$ SD. **d-l**, Expression pattern of DSP and DPP in *Dspp*^{+/-}, *Dspp*^{-/-}, and DPPcKO-B molars. DSP expression (d, e, f) in P16 mice shows endogenous DSP (d) and transgenic DSP (f) expression in odontoblasts, dentin, and pulp, but not in *Dspp*^{-/-} (e) molars. DPP expression (g, h, i) in P16 mice shows only endogenous DPP expression (g) in *Dspp*^{+/-} molars but none in *Dspp*^{-/-} (h) or DPPcKO-B (i) molars. Expression of DSP (j-l) in lower first molars of 3-month-old mice shows endogenous DSP (j) and transgenic DSP (l) equally deposited in the primary dentin. No positive staining is observed in *Dspp*^{-/-} molars (k). d, dentin; pd, predentin; od, odontoblast; p, pulp. Bars in d-i = 25 μ m. j-l = 200 μ m.

Fig. 2. Restored width of predentin and absence of sporadic unmineralized areas in dentin of DPPcKO mice

H&E staining of the upper first molars of *Dspp*^{+/-} mice (a and d), *Dspp*^{-/-} mice (b and e) with widened predentin and sporadic unmineralized areas in irregular dentin, and DPPcKO-B mice (c and f) with the restored predentin width (still slightly wider than *Dspp*^{+/-} mice) and absence of the sporadic unmineralized areas. The boarder between predentin and dentin is indicated by line. The unmineralized area is shown by an asterisk, and the dentin-enamel junction is shown by an arrowhead. d, dentin; pd, predentin; p, pulp. Bars in a–c = 200 μm. d–f = 50 μm.

Fig. 3. Partial rescue of dentin defects in DPPcKO mice

a–c, Radiographic analysis of the upper first molars from 3-month-old mice shows normal thickness of molar root in *Dspp*^{+/-} (a, arrow), thinner root in *Dspp*^{-/-} (b, arrow), and an apparently wider root in DPPcKO-B mice (c, arrow) than that of *Dspp*^{-/-} mice. Total translucency of DPPcKO-B mice in both crown and root appeared to be higher than that of *Dspp*^{+/-} mice. **d–i,** SEM analysis of the lower first molars from 3-month-old mice shows dense dentin and a smooth mineralization front in *Dspp*^{+/-} teeth (d), narrow thickness of dentin and an irregular mineralization front (arrowhead) in *Dspp*^{-/-} teeth (e), and a much smoother mineralization front in DPPcKO-B (f) teeth. High magnification of the pulp horn area shows severe defects in *Dspp*^{-/-} (h), mild defects in DPPcKO-B (i), and no defects in *Dspp*^{+/-} (g)

mice. d, dentin; p, pulp; ph, pulp horn; e, enamel; ab, alveolar bone. Bars in d–f = 200 μ m.
Bars in g–i = 50 μ m.

Fig. 4. Transgenic DSP expression rescues the dentin volume density but not the dentin mineral density seen in *Dspp*^{-/-} mice. Micro-CT analysis was carried out on the lower first molar for all 3 groups of mice

Panel **a** shows dentin volume density, and panel **c** shows enamel volume density, which was calculated as the volumetric ratio of the percent volume occupied by the mineralized space divided by total dentin or enamel volume, respectively. Panel **b** shows dentin mineral density, and panel **d** shows enamel mineral density, meaning the amount of hydroxyapatite per unit volume in mg. Dentin volume density but not dentin mineral density is restored in DPPcKO-B dentin (a and b). No significant difference is observed in enamel volume density and enamel mineral density (c and d). Each column represents the mean and standard deviation (SD). Statistical analysis is performed using ANOVA and pos hoc comparison techniques for Turkey HSD test; *n*=3, **P* < 0.05, N.S., not significant.

Fig. 5. Reduced frequency of pulp exposure in DPPcKO molars

a, 41.5% of *Dspp*^{-/-} (41 molars), 19.4% of DPPcKO-B (36 molars), 18.9% of DPPcKO-D (37 molars), and none of *Dspp*^{+/-} (26 molars) upper first molars had pulp-exposure and degeneration. **b–e**, H&E staining of the upper first molars from 4-month-old mice showing the occurrence of pulp exposure and degeneration. Representative images of intact teeth show healthy pulp tissues (**b** and **d**; *Dspp*^{+/-} tooth). Image of pulp-exposed teeth show communication between the oral area and pulp; therefore, pulp is degenerated by oral bacteria invasion (**c** and **e**; *Dspp*^{-/-} tooth). The total volume of predentin and dentin in pulp-exposed teeth is always lesser than that of intact teeth because of the absence of ECM secretion after odontoblasts are necrotized by oral bacteria infection. High magnification of the distal cusp area of (**b**) and (**c**) is shown in (**d**) and (**e**), respectively. d, dentin; p, pulp. Statistical analysis: all samples are classified into pulp-exposure teeth or intact teeth, and then the frequency of each DPPcKO mice was compared with that of *Dspp*^{+/-} mice, respectively, by unpaired Student's t-test; **P* < 0.05

Fig. 6. Identification of mouse DSP as a proteoglycan containing chondroitin sulfate in dentin
a, Immunoblots of the same proteins used in Fig.1c show both endogenous (lane 1) and transgene (lane 5) DSP as a smear. After chondroitinase ABC (Ch ABC) treatment, the bands are distinct (lane 2 and 6), indicating that DSP in *Dspp*^{+/+} and DPPcKO-B dentin deposit as a chondroitin/dermatan sulfate proteoglycans. There is an absence of positive bands in *Dspp*^{-/-} samples with or without chondroitinase treatment (lane 3 and 4). The smeared band shown with the open bracket is for the DSP proteins without Ch ABC treatment. An asterisk indicates main band with Ch ABC treatment.