Role of NH2-terminal fragment of Dentin Sialophosphoprotein (DSPP) in dentinogenesis

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Abstract

DSPP is a large precursor protein that is proteolytically processed into three fragments: a NH2-terminal fragment [dentin sialoprotein (DSP), a proteoglycan form (DSP-PG)] and a COOH-terminal fragment [dentin phosphoprotein (DPP)]. *In vitro* studies indicate that DPP is a strong initiator and regulator of hydroxyapatite crystal formation and growth, but the role(s) of the NH2-terminal fragment of DSPP (i.e., DSP and DSP-PG) in dentinogenesis remain unclear. This study focuses on the function of the NH2-terminal fragment of DSPP in dentinogenesis. Here, transgenic mouse lines expressing the NH2-terminal fragment of DSPP driven by 3.6-kb type I collagen promoter (*Col 1a1*) were generated and crossbred with *Dspp* null mice to obtain mice that express the transgene but lack the endogenous *Dspp* (*Dspp* KO/DSP Tg). We found dentin from the *Dspp* KO/DSP Tg mice was much thinner; more poorly mineralized and remarkably disorganized compared to the *Dspp* KO mice. The fact that *Dspp* KO/DSP Tg mice exhibited more severe dentin defects than the *Dspp* null mice indicates that the NH2-terminal fragment of DSPP may inhibit dentin mineralization or may serve as an antagonist against the accelerating action of DPP and serve to prevent predentin from being mineralized too rapidly during dentionogenesis.

Genetic studies have shown that dentin sialophosphoprotein (DSPP) plays an essential role in dentinogenesis (1–3). For example, the dentin in *Dspp* knockout mice is hypomineralized and the predentin is widened, creating a phenotype similar to that of dentinogenesis imperfecta type III in humans (3). While studies have demonstrated the critical role of DSPP in dentin formation, the mechanism(s) by which DSPP function(s) in biomineralization remain largely unclear.

DSPP, first identified by cDNA cloning (4), is a large protein that undergoes proteolytic processing to form the NH2-terminal fragment known as dentin sialoprotein (DSP), proteoglycan form of the NH2-terminal fragment referred to as DSP-PG and a COOH-

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CONFLICTS OF INTEREST
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terminal fragment named dentin phosphoprotein (DPP) (5–7). The sequence of the NH_2-terminal fragment (DSP or DSP-PG) lies in the 5′-portion, while the DPP sequence is located in the 3′-region of the DSPP transcript (4, 8–10). Studies done in our laboratory and others established the presence of a proteoglycan form of DSP (known as DSP-PG) (5–7). DSP/DSP-PG and DPP are abundant in the dentin extracellular matrix (ECM) whereas the protein representing the entire sequence (DSPP) is scarcely present, which led to the belief that the processed fragments may be the functional forms of DSPP. Recent work in our laboratory has shown that blocking the proteolytic fragmentation of DSPP inactivates the function(s) of this molecule during dentinogenesis (11). This observation provides strong evidence that the proteolytic processing of DSPP is an activation event which releases the functional fragments that exert distinct molecular role(s) during dentin formation (11).

DPP, discovered in 1967, is the most abundant non collagenous protein (NCP) in the dentin ECM (12, 13). The unusual feature of DPP is the presence of large amounts of aspartic acid (Asp) and phosphoserine (Pse), mostly found in the repeating sequences of $(Asp-Pse-Pse)_n$ and $(Asp-Pse)_n$ (14,15). Several *in vitro* mineralization studies have indicated that DPP is an important initiator and modulator for the formation and growth of hydroxyapatite (HA) crystals (16–18). It is believed that the DPP secreted by the odontoblasts is transported to the mineralization front where it binds to collagen fibrils promoting the formation of initial HA. As this mineralization process proceeds, and more predentin is converted to dentin, these mineral crystals grow in an oriented fashion under the modulation of DPP and other non-collagenous proteins (NCPs) that bind to the growing HA faces (19, 20).

DSP, discovered in 1981, is much less abundant than DPP in dentin ECM (21). DSP [protein without the glycosaminoglycan (GAG) chains]does not have a significant effect on the *in vitro* mineral formation and growth (22). Since there is no *in vivo* data concerning the role of DSP in dentinogenesis, the functions of this fragment is presently undefined. The recently discovered proteoglycan form of DSP (DSP-PG) is a major component in the proteoglycan pool of the dentin extracellular matrix (ECM) and appears to be more abundant than DSP (5, 6). We believe that the NH_2 terminal fragment of DSPP (DSP/DSP-PG) must play a role in biomineralization, distinct from that of the COOH-terminal fragment of DSPP (DPP). DSP-PG from the NH_2-terminal sequence of DSPP was found in the dentin ECM at higher amounts than the core protein DSP, which led us to believe that DSP-PG may be the functional form of the NH_2-terminal fragment. We hypothesize that the role of DSP-PG may be to prevent the predentin from being mineralized too rapidly and to serve as an antagonist of DPP in dentinogenesis.

In order to examine the function of the NH_2-terminal fragment of DSPP in dentinogenesis, we expressed the NH_2-terminal fragment of DSPP in the *Dspp*-null background (referred as "*Dspp* KO/DSP Tg") to analyze the *in vivo* effects of this fragment in a gain of function experimental approach. In this study, we systematically characterized the dentin of *Dspp* KO/DSP Tg mice and compared it to the dentin from *Dspp*-null (*Dspp* knockout or *Dspp* KO) mice and wild type (WT) mice. We observed that the dentin of the *Dspp* KO/DSP Tg mice appeared much worse compared to the *Dspp*-null mice. The results from this investigation support our hypothesis that the NH_2-terminal fragment of DSPP has an inhibitory role in dentinogenesis.

MATERIALS AND METHODS

Generation of mutant mice

Transgenic mice expressing the NH_2-terminal fragment of DSPP in the wild type (WT) background were generated using techniques described in our previous reports (11, 23). Briefly, a pBCKS construct containing a 3.6-kb rat *Col 1a1* promoter was used to generate

the transgene (24). The NH$_2$-terminal fragment construct consisting of mouse DSPP signal peptide, three VSV epitopes, and the cDNA sequence encoding the NH$_2$-terminal portion of mouse DSPP (25) were inserted downstream of the 3.6-kb rat *Col 1a1* promoter in the pBCKS construct. A detailed description of the restriction enzymes and procedures used in generating the transgenic mice is given in our previous publication (23). We obtained eight independent founders expressing the NH$_2$-terminal portion of mouse DSPP in the WT (C57BL/6J) background. These mouse lines expressing different levels of the transgene were crossbred with *Dspp*-null (*Dspp*-KO) mice (strain name: B6; 129-Dspptm1Kul/Mmnc; MMRRC, UNC, Chapel Hill, NC). By this crossbreeding scheme, we generated the mice expressing the NH$_2$-terminal fragment of the DSPP transgene in the *Dspp*-KO background (i.e., without the endogenous *Dspp* gene). These mice were referred to as "*Dspp*-KO/DSP Tg" mice. We analyzed the effects of the transgene on dentin formation in *Dspp*-KO/DSP Tg mice derived from two of the eight transgenic mouse lines, line 4 (with a higher level of expression for the transgene) and line 7 (with a lower expression level of the transgene). In this report, we described in detail the data from the analyses of *Dspp*-deficient mice expressing the higher level of the transgene (line 4).

The polymerase chain reaction (PCR) primers for detecting the DSP transgene were: forward, 5′-CCAGTTAGTACCACTGGAAAGAGAC-3′; reverse, 5′ TCATGGTTGGTGCTATTCTTGATGC-3′; PCR amplification using this pair of primers would yield a 521 bp PCR product from the transgene and a 676 bp product for the endogenous *Dspp* gene when mouse genomic DNA was used template. The primers used to identify the endogenous *Dspp* alleles and null alleles (containing Lac Z gene) are described in our previous publication (11).

The animal protocols used in this study were approved by the Animal Welfare Committee of Texas A&M Health Science Center Baylor College of Dentistry (Dallas, TX). A multidisciplinary experimental approach was used to characterize the dentin of the three mouse genotypes: 1) *Dspp*-KO/DSP Tg mice, 2) *Dspp*-KO mice, and 3) WT (C57/BL6J) mice.

Expression levels of the DSP transgenes in teeth

Quantitative real-time PCR (qPCR) was performed to determine the DSP/DSP-PG mRNA expression level in the incisors of the eight founder mice in the wild type genetic background as previously described (11, 26). Briefly, total RNA was extracted from the mouse incisors with an RNeasy mini kit (Qiagen; Germantown, MD). The RNA (1 ug/per sample) was reverse-transcribed into cDNA using the QuantiTect Rev Transcription Kit (Qiagen; Germantown, MD). The DSP primers used for quantitative real-time PCR were: forward, 5′-AACTCTGTGGCTGTGCCTCT-3′ (in exon 3) and reverse, 5′-TATTGACTCGGAGCCATTCC-3′ (in exon 4). The real-time PCR reactions were performed using PCR conditions as we previously reported (26). The housekeeping gene, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), was used for standardization (26).

Extraction and isolation of noncollagenous proteins (NCPs) from mouse dentin and detection of DSPP-related proteins

For extraction of DSP/DSP-PG and other DSPP-related proteins from dentin, maxillary incisors from six 3-month-old WT and *Dspp*-KO/DSP Tg mice (12 incisors/group) were processed using the protocol described in our previous reports (26, 27). The extracts from the incisors were separated into 80 fractions (0.5 ml/fraction) by Q-Sepharose ion-exchange chromatography (Amersham Biosciences; Uppsala, Sweden) with a gradient ranging from 0.1 to 0.8 M NaCl in 6 M urea solution (28). The same amount of sample (60 ul) from every third fraction was treated with 3% β-mercaptoethanol (β-ME) to break the disulfide bonds of

the dimeric form of DSP and was then loaded onto 10% SDS-PAGE gels. First, Stain-All staining was performed to visualize the acidic non-collagenous proteins (NCPs) in these fractions. Next, Western immunoblotting was carried out for the fractions that might contain any DSPP-related proteins. For Western immunoblotting, we used an anti-DSP monoclonal antibody (anti-DSP-2G7.3) at a dilution of 1:2000 (5). The blots were incubated in alkaline phosphatase-conjugated anti-rabbit IgG (Sigma-Aldrich; St. Louis, MO) secondary antibody at a dilution of 1:5000. This step was followed by incubation in a chemiluminescent substrate CDP-star (Ambion, Austin, TX, USA) for 5 min and exposure to X-ray films. Purified wild-type rat dentin extract (0.3 µg) previously shown to contain DSP and DPP proteins were used as positive controls for the Stains-all staining and Western immunoblotting analyses (DSP control).

Flat X-ray radiography and micro-computed tomography (µ-CT)

The mandibles from the 3-month-old and 6-month-old mice (5 mice/group) were dissected from the WT, *Dspp*-null and *Dspp*-KO/DSP Tg mice and analyzed with the Faxitron MX-20 Specimen Radiography System (Faxitron X-ray, Buffalo Grove, IL, USA). For the µ-CT analyses, we performed triplicate analyses of 3-month-old mice from the WT, *Dspp*-null and *Dspp*-KO/DSP Tg mice (n = 3). The mandibles were scanned using µ-CT35 imaging system (Scanco Medical, Basserdorf, Switzerland), to assess their structure and morphology as we previously described (11, 29).

Histology and immunohistochemistry

For histology and immunohistochemistry (IHC) analyses, the 3- and 6-month-old mice from the three groups were perfused from the ascending aorta with 4% paraformaldehyde in 0.1 M phosphate buffer. The samples were fixed in 4% paraformaldehyde for 48 hr. The samples (mandibles) were then decalcified in 8% EDTA (pH 7.4) at 4°C for approximately 2 wk. The mandibular tissues were processed for paraffin embedding, and 5-µm serial sections were prepared. The sections were either stained with hematoxylin & eosin (H&E) or used for IHC analyses. The anti-DSP monoclonal antibody, referred to as "anti-DSP-2C12.3" was used at a dilution of 1:800 (30). The anti-biglycan antibody ("LF-159"; a gift from Dr. Larry Fisher of the Craniofacial and Skeletal Diseases Branch, National Institutes of Health, Bethesda, MD, USA) was used at a dilution of 1:1000. All the IHC experiments were carried out with the M.O.M. kit and DAB kit (Vector Laboratories, Burlingame, CA, USA) according to the manufacturer's instructions.

Resin-casted scanning electron microscopy (SEM)

Mandibles from 3-month-old WT, *Dspp*-null and *Dspp*-KO/DSP Tg mice (5 mice/group) were dissected and fixed in 2% paraformaldehyde with 2.5% glutaraldehyde in 0.1 M cacodylate buffer solution (pH 7.4) at room temperature. A detailed protocol used in processing and polishing the samples for resin-casted SEM has been described in a previous study (11). The dentin surface of all the three samples was acid-etched with 37% phosphoric acid for 2–10 s and washed with 5.25% sodium hypochlorite for 5 min. The samples were then coated with gold and palladium as described previously (31). A FEI/Philips XL30 Field emission environmental SEM (Philips, Hillsboro, OR, USA) was used to perform the SEM analyses with a digital record prepared for representative specimens.

RESULTS

Expression of the DSP transgenes in the teeth

The relative mRNA expression level of the NH_2-terminal fragment of DSPP in the incisors from the eight founder mice was determined by real-time PCR analyses (Fig. 1). The

forward and reverse primers used in these analyses were located in exons 3 and 4 respectively, corresponding to an amplicon within the NH_2-terminal region of the *Dspp* gene. For the purpose of calculations, the mRNA level of the WT sample (Fig. 1, line 1) was set as one. The mRNA expression in the incisors of the eight founder mice (Fig. 1, lines 2–9) was then calculated relative to that of the WT mouse incisor. Triplicate analyses were performed. Real-time analyses revealed that the founder mice of line 4 (Fig. 1) had the highest DSPP mRNA level, which was ~13 times that of the WT samples. The *Dspp*-KO/DSP Tg mice derived from this line of founder mice were systematically characterized and reported in detail in this report. It should be noted that although different expression levels for the transgene were observed among the different transgenic mouse lines (Fig. 1, lines 2–9), they all contained higher levels of DSPP mRNA when compared with the WT mice (Fig. 1, line 1). The transgenic mice expressing the NH_2-terminal fragment of DSPP in the WT background did not show any significant difference in their teeth or their bone when compared to the WT mice (data not shown).

Extraction and separation of NCPs and detection of DSPP-related proteins

Stains-All staining and Western immunoblotting were utilized to identify the DSPP-related proteins in the dentin extracts from *Dspp*-KO/DSP Tg and WT mice. All the chromatographic fractions from the dentin extracts that putatively contain DSPP-related products were analyzed by SDS-PAGE with Stains-All detection (Fig. 2). In the extracts from the WT incisors, protein bands matching the migration rate of DSP, DSPP (Fig. 2A, fractions 27 to 39), as well as DPP (Fig. 2A, fractions 45 to 57), were clearly detected by affinity to the Stains-All dye. The protein bands matching the migration rate of the full length DSPP appeared to be much weaker than that of DSP in the WT samples (Fig. 2A, fractions 27 to 39). However, only large amount of DSP (Fig. 2B, fractions 27 to 39) was observed in the *Dspp*-KO/DSP Tg mice. No protein bands matching DSPP or DPP were detected in the dentin extracts of the *Dspp*-KO/DSP Tg mice. In the Western immunoblotting analyses, DSP and DSPP were clearly detected in the dentin extracts from the WT (Fig. 3A), while only DSP (without DSPP) could be identified in the dentin extracts from the *Dspp*-KO/DSP Tg mice (Fig. 3B). DSP-PG was detected as strong smears in the late fractions (fractions 69–75) in the extracts from the WT and *Dspp*-KO/DSP Tg (Fig. 3C,D). The results from both the Stains-All and Western immunoblotting analyses proved that for the *Dspp*-KO/DSP Tg mice, only the NH_2-terminal fragment of DSPP (DSP/DSP-PG) was expressed (i.e., without DPP or full-length DSPP).

Anti-DSP immunostaining

Anti-DSP immunostaining showed positive reactivity in the odontoblasts, as well as the dentin matrix of the WT and *Dspp*-KO/DSP Tg mice (Fig. 3E,F). In the *Dspp*-KO/DSP Tg mice (Fig. 3F), the anti-DSP immunostaining appeared much stronger than that of the WT mice (Fig. 3D) due to the higher expression levels of the transgene. The anti-DSP immunohistochemistry results from the *Dspp*-KO/DSP Tg mice also showed that the NH_2-terminal fragment of DSPP derived from the transgene was secreted into the dentin ECM (Fig. 3F).

Flat X-ray radiography and micro-computed tomography (µ-CT)

Faxitron X-ray radiography (Fig. 4) revealed the dentin structure and morphology in the 3- and 6-month-old mice. The X-ray analyses showed enlarged pulp chambers with reduced dentin thickness in the *Dspp* KO mice compared to the WT mice, which is a characteristic feature of the KO mice (Figs. 4A,B,D,E). The expression of NH_2-terminal fragment of DSPP from the engineered transgene worsened the dentin defects observed in the *Dspp* KO/ DSP Tg mice (Figs. 4C,F). Samples from the *Dspp*-KO/DSP Tg mice showed extremely thin and radiolucent dentin with remarkably enlarged pulp chambers (Figs. 4C.F).

Similar findings were observed from the μ-CT (Fig. 5) analyses of 3-month old mice, in which the alveolar bone and dentin in the *Dspp*-KO/DSP Tg mice (Figs. 5C,F) appeared considerably more porous and thinner than those of the *Dspp* KO mice (Figs. 5B,E). The μ-CT analyses showed that the dentin and alveolar bone defects in the 6-month-old *Dspp*-KO/DSP Tg mice (data not shown) were similar those observed in flat X-ray radiographic images of mice at the same age.

Histological Analysis

In the histological analyses, the pulp chamber in the mandibular molars of the *Dspp* KO mice appeared significantly larger with widened predentin zones as compared to the WT mice at both ages (Figs. 6A,B,D,E). The defects of the predentin-dentin complex in the *Dspp*-KO⁄DSP Tg mice (Figs. 6C,F) appeared noticeably worse than those of the *Dspp*-KO mice (Figs. 6B,E) with the former mice demonstrating extremely thin dentin. The predentin zone in the *Dspp*-KO⁄DSP Tg mice had dramatically expanded to replace almost the entire predentin-dentin complex at both 3 and 6 months of age (Figs. 6C,F).

Resin-cast SEM

The resin-cast SEM analyses (Fig. 7) showed well organized and evenly distributed dentinal tubules running parallel to one another and perpendicular to the dentino-enamel junction (DEJ) in the WT sample (Fig. 7A). The *Dspp* KO mice (Fig. 7B) on the other hand, revealed disrupted and disorganized dentinal tubules. In the *Dspp*-KO/DSP Tg mice (Fig. 7C), only an extremely small number of dentinal tubules could be visualized. The few dentinal tubules that were discernable in the *Dspp*-KO/DSP Tg mice appeared irregular and collapsed at several places (Fig. 7C).

Anti-biglycan immunostaining

Since the proteoglycan biglycan is primarily localized in the predentin zone of the molars, we used anti-biglycan immunostaining (Fig. 8) to localize the predentin zone in the three groups of mice. A very narrow predentin zone was evident in the WT mice (Fig. 8A), whereas the predentin zone in the *Dspp* KO (Fig. 8B) appeared comparatively wider. The biglycan immununoreactivity in the *Dspp*-KO⁄DSP Tg mice (Fig. 8C) was much stronger and appeared over a wider area than that observed in the *Dspp* KO mice (Fig. 8B). In fact, the biglycan was distributed in almost the entire predentin-dentin complex, confirming the histological finding that nearly the whole predentin-dentin complex was not mineralized in the *Dspp*-KO⁄DSP Tg mice. In other words, the histology and biglycan immunostaining analyses showed that in the *Dspp*-KO⁄DSP Tg mice, the mineralized dentin was almost non-existent, composed almost entirely of the premature and unmineralized predentin. Due to this reason, we were unable to measure the thickness of dentin and the predentin in order to quantify the ratio between the two.

We analyzed the teeth of *Dspp*-KO/DSP Tg mice derived from two of the eight lines of the founder mice: line 4 (with a higher level of expression for the transgene) and line 7 (with a lower expression level of the transgene). While in both cases, expressing the NH_2-terminal fragment (at a higher or lower level) in the *Dspp* knockout background significantly worsened the *Dspp*-deficient dentin defects, the dentin abnormalities in the *Dspp*-deficient mice expressing the higher level of the transgene (line 4) were worse than in the mice expressing the lower expression level of the transgene (line 7, data not shown). These observations suggest that expression levels of the NH_2-terminal fragment of DSPP correlated with its inhibitory potential in dentinogenesis.

DISCUSSION

In the dentin ECM, large amounts of the processed NH_2-terminal (DSP-PG/DSP) and COOH-terminal (DPP) fragments are present, along with only trace amounts of the full-length DSPP (27). Several recent reports indicate that the cleavage of DSPP at the peptide bond corresponding to Gly^{451}-Asp^{452} in mouse DSPP is essential for releasing the NH_2-terminal and COOH-terminal fragments of this protein (32–34). More recently, we showed that blocking the cleavage of mouse DSPP at this site leads to the loss of its function in dentinogenesis (11). These recent findings support the belief that proteolytic processing of DSPP may be an activation process, which releases the active fragments, DSP-PG/DSP and DPP.

DSP-PG/DSP and DPP with dramatically different biochemistry features are likely to play different roles in dentinogenesis. Several excellent reports have demonstrated the role of DPP in nucleation and modulation of apatite crystal formation (16–18). However, very little is known about the role of the function of the NH_2-terminal fragment of DSPP (DSP-PG and/or DSP). It has recently been discovered that DSP-PG consists of two glycosaminoglycan (GAG) chains, which are attached to Ser^{242} and Ser^{254} in the mouse DSPP sequence and the GAG chains are made of chondroitin-4-sulfate (35). It is also worth noting that DSP-PG appears more abundant than DSP (5, 6). In this study, we generated the *Dspp*-KO/DSP Tg mice to investigate the function of the NH_2-terminal fragment of DSPP (DSP-PG and/or DSP). These mice did not contain DPP or the endogenous *Dspp* gene but instead only possessed DSP-PG and DSP derived from the transgene. The immunohistochemistry analyses with anti-DSP monoclonal antibody confirmed that the NH_2-terminal fragment was properly secreted in the dentin ECM. We thoroughly characterized the dentin of the *Dspp*-KO/DSP Tg mice in comparison with the *Dspp*-KO and WT mice. Our analyses revealed that the dentin of the *Dspp*-KO/DSP Tg mice was remarkably thinner than that of the *Dspp*-KO mice. The mineralized dentin layer that was present in the WT mice could not be seen in the *Dspp*-KO/DSP Tg mice, while instead, structures resembling unmineralized predentin were observed in this region of the tooth in the *Dspp*-KO/DSP Tg mice. The structure of the dentinal tubules in the *Dspp*-KO/DSP Tg mice was more severely disrupted when compared to the *Dspp*-KO mice. These findings indicate that the NH_2-terminal fragment of DSPP may exert an inhibitory role during dentinogenesis. We suggest that the NH_2-terminal fragment of DSPP may prevent the predentin from being mineralized too rapidly. Data from this study has helped to delineate one role for the NH_2-terminal fragment in tooth formation.

In these experiments, we also noted that the *Dspp*-KO/DSP Tg mice revealed more severe defects in the alveolar bone than the *Dspp*-KO mice; this finding is consistent with our recent observation that DSPP plays an essential role in maintaining the health of periodontal tissues including the alveolar bone and cementum (36).

In the extracellular matrix of dentin, the ratio of DPP to DSP is approximately 10:1. Even when the presence of DSP-PG is taken into account, the ratio of DPP to DSP is not close to 1:1. Therefore, there must be an intrinsic, yet unknown, mechanism to which the inequality in molar ratio between the NH_2-terminal and COOH-terminal fragments of DSPP is attributed. One possibility is that NH_2-terminal fragment with few or no phosphates might be degraded faster than the highly phosphorylated COOH-terminal fragment of DSPP. We envision that in the degradation process of the NH_2-terminal fragment of DSPP, the two GAG chains of DSP-PG are first removed and then, the initial degradation product of DSP-PG (i.e., DSP) is broken into small fragments by proteinases such as matrix metalloproteinase-20 (MMP-20) present in the dentin extracellular matrix. Clearly, more studies are needed to address these puzzling questions.

From the *in vivo* data generated in this study, we cannot conclude whether DSP or DSP-PG was responsible for the observed phenotypic changes (inhibitory effects on mineralization). DSP has been shown to have no significant effects on *in vitro* mineralization (37). Proteoglycans are present as an amorphous gel in the predentin region of the teeth and occupy spaces between the collagen fibrils acting as ground substances providing a medium for transport and diffusion (38). Chondroitin sulphate, of which the GAG chains of DSP-PG are made, shows a decreasing gradient from the pulp towards the mineralized front of dentin (39, 40); the gradient shifting in the distribution of proteoglycan chondroitin sulphate appears to be associated with the changes in the activities of matrix metalloproteinase-3 (MMP-3, i.e., stromelysin-1 or proteoglycanase) in the pulp-predentin-dentin complex (41). Several *in vitro* studies have shown that chondroitin sulfate in solution inhibits the formation and growth of hydroxyapatite crystals in collagen gels (42–45). Recent *in vitro* mineralization analyses showed that the proteoglycan form of the NH_2-terminal fragment of dentin matrix protein 1 (DMP1) inhibits the formation and growth of hydroxyapatite crystals (46). Based on the finding in this study that *Dspp* KO/DSP Tg mice had more severe dentin defects than the *Dspp* null mice, along with previous observation that DSP has no significant effects on *in vitro* mineralization (37) and the fact that chondroitin sulfate proteoglycans inhibit the formation and growth of hydroxyapatite crystals, we believe that DSP-PG was responsible for the observed inhibitory effects on dentinogenesis in the *Dspp* KO/DSP Tg mice. We hypothesize that during dentinogenesis, DSP-PG primarily present in predentin serves as an antagonist against the mineralization-accelerating action of DPP and thus this proteoglycan prevents predentin from being mineralized too rapidly. At the mineralization front where predentin is converted to dentin, DSP-PG is degraded and the loss of DSP-PG allows the mineralization process to proceed. As stated above, in the degradation process of DSP-PG, the two GAG chains are first removed and DSP-PG is converted to DSP, and then DSP is further broken into small fragments and/or amino acids by proteinases such as MMP-3 and MMP-20. While these purported roles of DSP-PG or DSP and their degradation processes in dentinogenesis are conjecture, they represent hypotheses that best fit all the data. Clearly, experiments are warranted to test the *in vitro* effects of DSP-PG on the formation and growth of hydroxyapatite crystals.

Additionally, in recent years, DSPP expression has also been seen in several non-mineralized tissues such salivary glands, cartilage, liver, kidney and brain (26, 47–49). Investigations to examine the roles of the cleaved fragment DSP and/or DSP-PG in these soft tissues, as well as in long bones, are also needed for a more complete understanding of DSPP in maintaining the structural integrity of these diverse tissues.

## Acknowledgments

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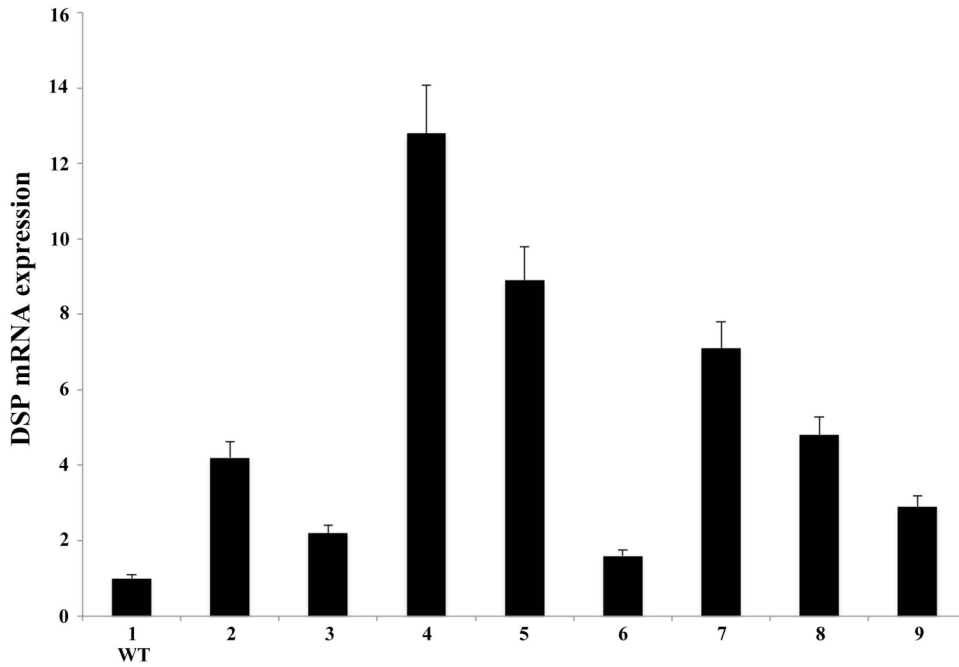
Figure 1. Levels of DSPP mRNA in the incisors of the transgenic founder mice
Real-time PCR analyses showed the different DSPP mRNA levels in the eight founder
transgenic mice expressing the NH_2-terminal fragment of DSPP transgene in the WT
background (lines 2–9). For calculation purposes, the WT sample (line 1) was set as 1 and
mRNA levels in the eight transgenic mice were calculated relative to that of the WT. The
results were from triplicate analyses (n = 3) for each group. The mRNA level of the
transgenic mice in line 4 was the highest (approximately 13 fold of that of the endogenouse
gene expression in the WT mice). This mouse line was used to crossbreed with *Dspp* KO
mice to generate the "*Dspp* KO/DSP Tg" mice that expressed the NH_2-terminal fragment of
DSPP without the endogenous *Dspp* gene.
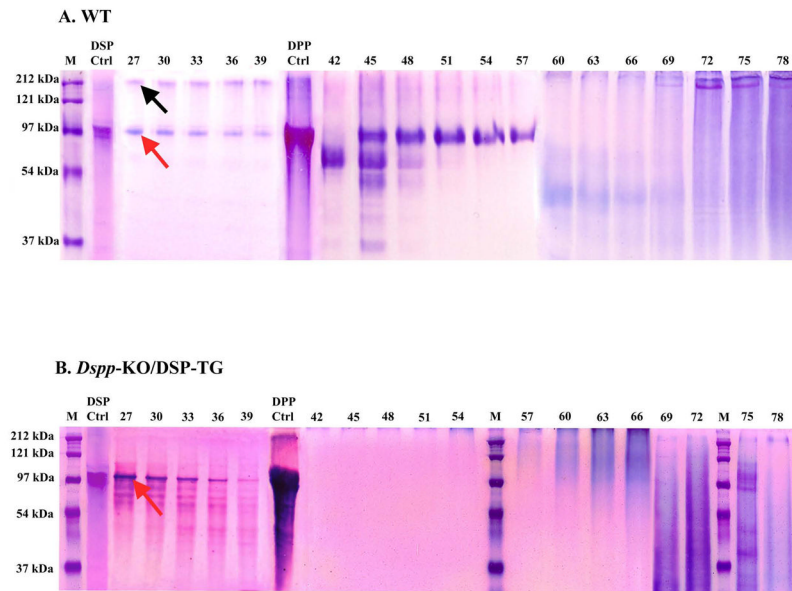
Figure 2. Stains-All staining of acidic proteins in the NCP extracts from mouse incisor dentin
The NCPs were extracted from the incisor dentin of six 3-month-old WT (A) and *Dspp* KO/DSP Tg mice (B). The extracted NCPs were separated into 80 fractions (0.5 ml/fraction) by a Q-Sepharose ion-exchange column. The numbers on the top of each gel image represent the fraction numbers. Equal amounts (60 μl) of every third fraction were loaded onto 10% SDS-PAGE gel. In the WT sample (A), protein bands corresponding to both DSP (~ 97 kDa, red arrow) and DSPP (~ 200 kDa, black arrow) were detected in fractions 27 to 39. The major blue bands in fractions 45 to 57 in the WT sample (A) were primarily composed of DPP. In the *Dspp* KO/DSP Tg mice (B), only the DSP protein bands (~ 97 kDa, red arrow) were observed in fractions 27 to 39. No DSPP or DPP proteins bands were detected. Some smaller protein bands in fractions 60–72 of the *Dspp* KO/DSP Tg samples (B) appeared stronger than in the wild type (A); this observation indicated that overexpressing the NH$_2$-terminal fragment of DSPP (DSP and DSP-PG) might have a direct or indirect effect on the expression levels of other acidic proteins and proteoglycans (e.g., biglycan). M, molecular weight standard; DSP, pure DSP isolated from rat dentin; DPP, pure DPP isolated from rat dentin.
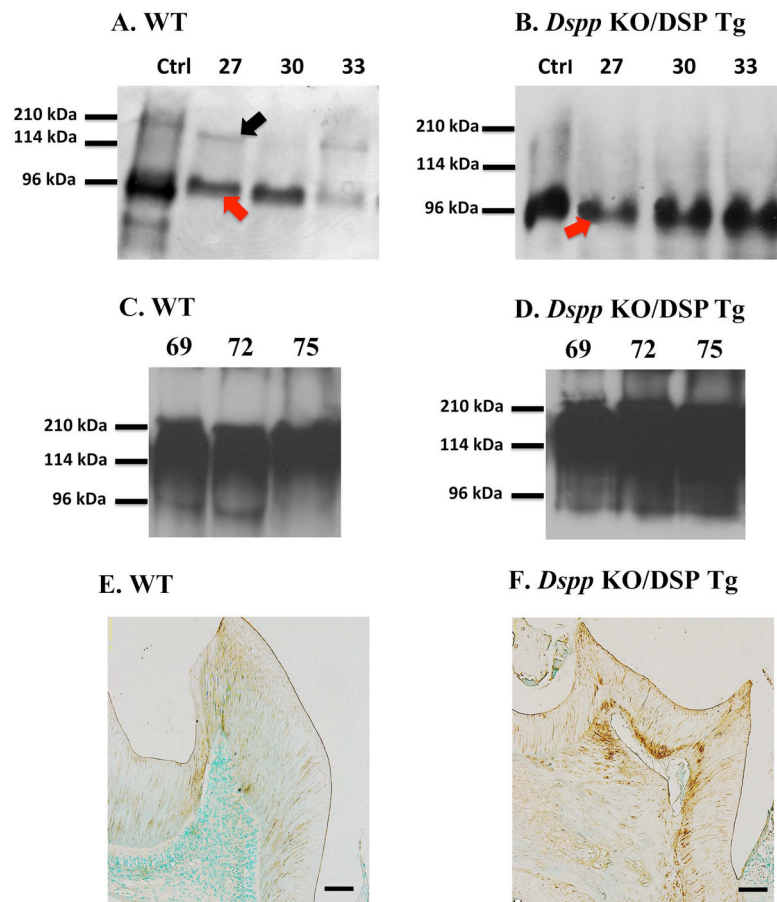
Figure 3. Western immunoblotting and anti-DSP immunohistochemistry to detect DSP in the mouse dentin

Western immunoblotting analyses (A to D) with monoclonal anti-DSP antibody were done to detect DSPP-related molecules in the dentin extracts of WT (A, C) and *Dspp* KO/DSP Tg mice (B, D). The purified rat dentin extract (0.3 μg) containing rat DSPP and DSP was used as a positive control (Ctrl). The Western immunoblotting results from the chromatographic fractions (27, 30, 33, 69, 72 and 75) that had strong signals for DSP/DSPP/DSP-PG in Stains-All analyses (Fig 2) were used for Western immunoblotting analyses. While both DSP (red arrow) and DSPP (black arrow) were detected in the dentin extracts from the WT mice (A), only DSP (red arrow) was detected in the *Dspp* KO/DSP Tg mice (B). In fractions 69–75, DSP-PG was detected as strong smears both in the WT and *Dspp*-KO/DSP Tg (C and D respectively); please note the abundance of DSP-PG in the *Dspp* KO/DSP Tg mice. The findings from both Stains-All and Western immunoblotting analyses showed that the *Dspp* KO/DSP Tg mice only contained the NH$_2$-terminal fragment of DSPP (DSP and DSP-PG), without any products from the endogenous *Dspp* gene.

For anti-DSP immunohistochemistry analyses (E and F), the samples from the 3-month-old WT (E) and *Dspp* KO/DSP Tg mice (F) were evaluated. Anti-DSP activity was observed in the matrix of the first mandibular molars in both WT (E) and *Dspp* KO/DSP Tg mice (F). The signal for the anti-DSP antibody in the dentin of the *Dspp* KO/DSP Tg mice (F) was relatively stronger than in the WT mice (E). Bar: 100 μm
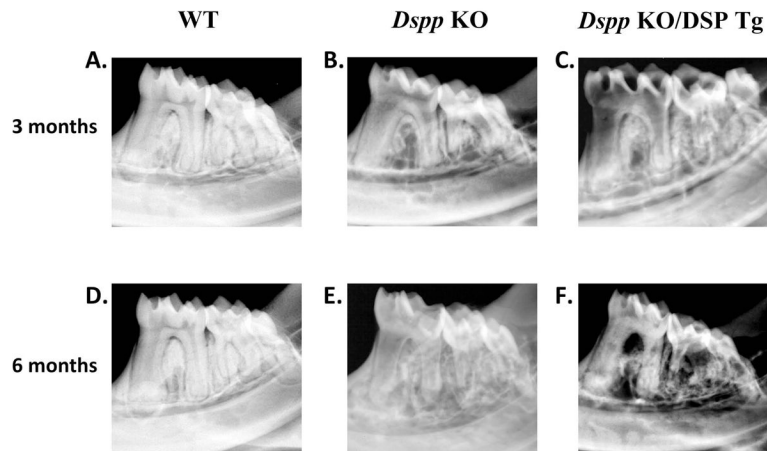
Figure 4. Flat X-ray radiography analyses of mandibles from 3- and 6-month-old mice
At 3 and 6 months of age, the WT mice (A and D) showed evenly distributed and well mineralized radio-opaque dentin with small pulp chamber. The mandibular molars in the *Dspp* KO mice (B and E) had an enlarged pulp chamber and thinner dentin compared to the WT mice. The tooth defects in the *Dspp* KO/DSP Tg mice (C and F) were worse than those of the *Dspp* KO mice, with much thinner, radiolucent dentin and a more significant loss of the alveolar bone.

WT   *Dspp* **KO**   *Dspp* **KO/DSP Tg**
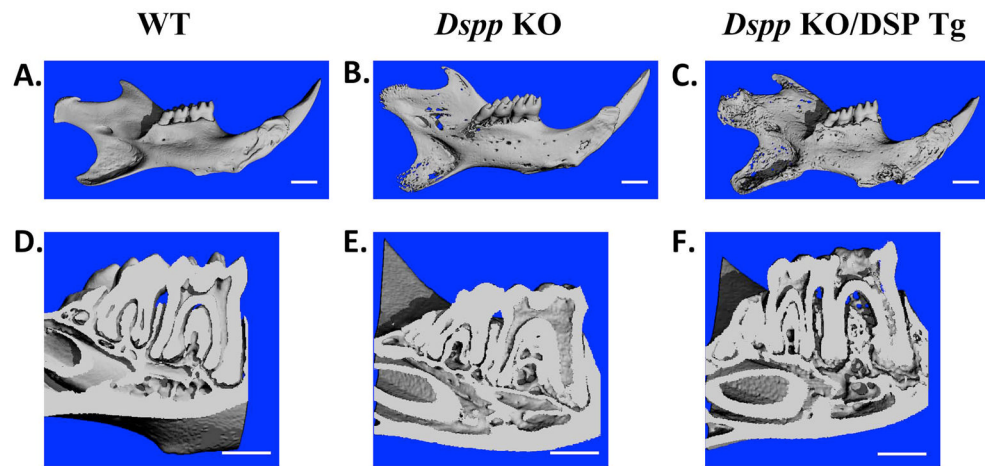
**Figure 5. μ-CT analyses of mandibles from 3-month-old mice**
WT (A and D), *Dspp* KO (B and E) and *Dspp* KO/DSP Tg mice (C and F) were evaluated for their overall morphology (A, B and C) and dentin phenotypes in the molar cross sections (D, E and F). The WT mice showed evenly distributed and well mineralized dentin (A and D). The mandible of the *Dspp* KO mice (B) had porosities when compared to the WT sample. Molar cross section of the *Dspp* KO mice (E) showed an enlarged pulp chamber and thinner dentin compared with the WT mice (D). The defects in the *Dspp* KO/DSP Tg mice (C and F) were even more pronounced with marked increase in porosities and reduction in dentin thickness compared to the *Dspp* KO mice. Bar: 1 mm
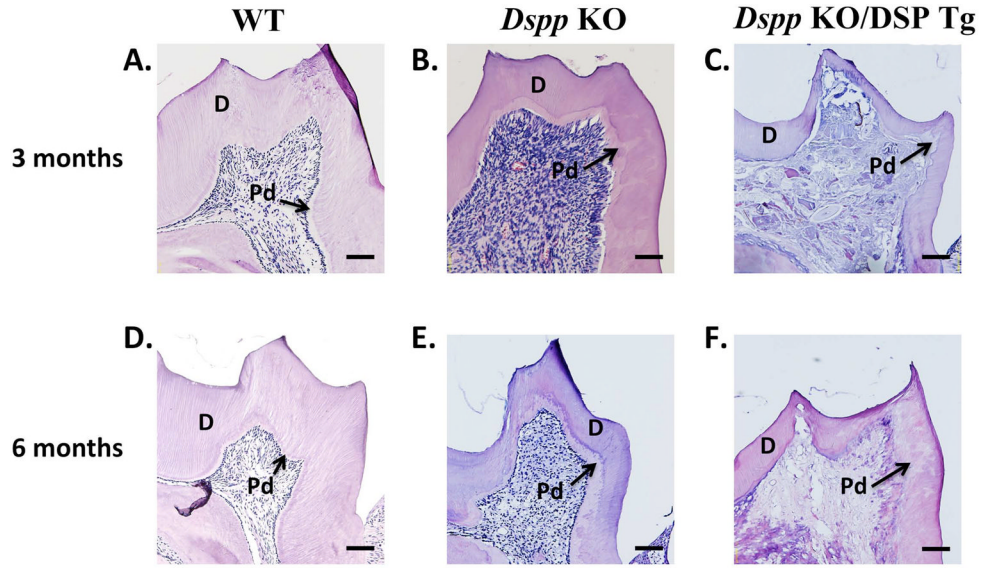
Figure 6. H&E staining in 3- and 6-month-old mice

At 3 and 6 months, the *Dspp* KO mice (B and E) had wider predentin (denoted as "Pd") and thinner dentin (denoted as "D") compared to the WT mice (A and D). The *Dspp* KO/DSP Tg mice (C and F) displayed more severe dentin abnormalities compared to those of the *Dspp* KO mice (B and E). The dentin of the *Dspp* KO/DSP Tg (C and F) mice was almost non-existent with almost the entire layer consisting of the unmineralized predentin structure. The pulp showed evidence of ectopic calcification (C and F), which appeared worsened at 6-months (F) than 3 months of age. This could possibly be due to chronic inflammation arising from pulp exposure. All the six sections showed in this figure were processed, sectioned, stained and visualized using the same settings and protocols. The differences in the staining intensity seen in the sections were probably due to the difference in the matrix composition (e.g., increased amounts of proteoglycans in the *Dspp* KO/DSP Tg and *Dspp* KO mice). Bar: 100 μm.

WT   *Dspp* KO   *Dspp KO/DSP Tg*
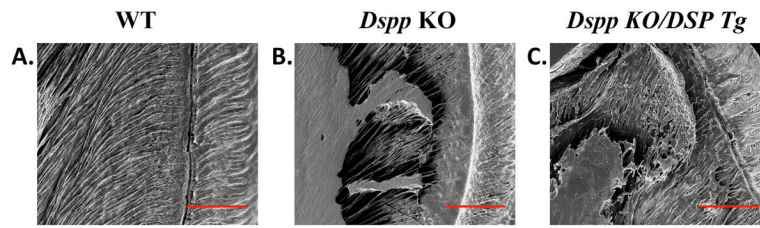
Figure 7. Resin-casted and acid-etched SEM analyses
In the resin infiltration and acid-etched SEM images of the mandibular first molar, the dentinal tubules in the WT mice (A) had a uniform distribution of dentinal tubules running to parallel to one another towards the DEJ. The dentinal tubules in the *Dspp* KO mice (B) appeared fewer in number when compared to the WT sample and were disorganized in their orientation. The dentinal tubules in the *Dspp* KO/DSP Tg mice (C) were completely collapsed, tangled and could not even be distinguished. Bar: 50 μm
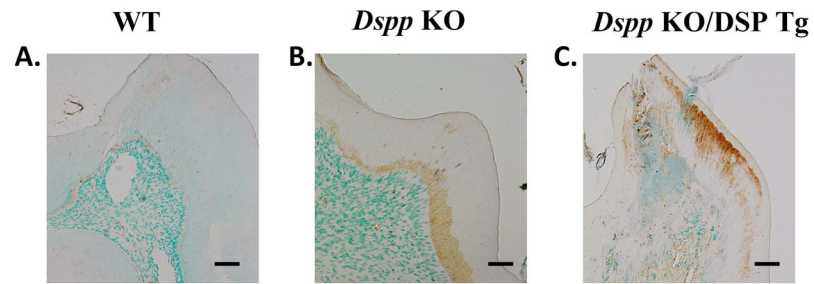
Figure 8. Biglycan immunostaining of predentin in 3-month-old mice
The WT mice (A) showed a narrow predentin zone demarcated by the brown positive biglycan staining. In the *Dspp* KO mice (B), anti-biglycan activity revealed a widened predentin layer and an irregular dentin-predentin border as compared to the WT mice (A). The results from the *Dspp* KO/DSP Tg mice (C) indicated that nearly the entire predentin-dentin complex in these mice was made of the unmineralized predentin with anti-biglycan staining extending and covering most of the structure. Bar: A-H = 100 µm.