Mutant Dentin Sialophosphoprotein Causes Dentinogenesis Imperfecta

Journal of Dental Research
2019, Vol. 98(8) 912–919
© International & American Associations
for Dental Research 2019
Article reuse guidelines:
sagepub.com/journals-permissions
DOI: 10.1177/0022034519854029
journals.sagepub.com/home/jdr

T. Liang¹, H. Zhang¹, Q. Xu¹, S. Wang¹, C. Qin¹, and Y. Lu¹

Abstract

Dentin sialophosphoprotein (DSPP) is an extracellular matrix protein highly expressed by odontoblasts in teeth. DSPP mutations in humans may cause dentinogenesis imperfecta (DGI), an autosomal dominant dentin disorder. We recently generated a mouse model (named “*Dspp*^{P19L/+} mice”) that expressed a mutant DSPP in which the proline residue at position 19 was replaced by a leucine residue. We found that the *Dspp*^{P19L/+} and *Dspp*^{P19L/P19L} mice at a younger age displayed a tooth phenotype resembling human DGI type III characterized by enlarged dental pulp chambers, while the teeth of older *Dspp*^{P19L/+} and *Dspp*^{P19L/P19L} mice had smaller dental pulp chambers mimicking DGI type II. The teeth of *Dspp*^{P19L/+} and *Dspp*^{P19L/P19L} mice had a narrower pulp chamber roof predentin layer, thinner pulp chamber roof dentin, and thicker pulp chamber floor dentin. In addition, these mice also had increased enamel attrition, accompanied by excessive deposition of peritubular dentin. Immunohistochemistry, in situ hybridization, and real-time polymerase chain reaction analyses showed that the odontoblasts in both *Dspp*^{P19L/+} and *Dspp*^{P19L/P19L} mice had reduced DSPP expression, compared to the wild-type mice. We also observed that the levels of DSPP expression were much higher in the roof-forming odontoblasts than in the floor-forming odontoblasts in the wild-type mice and mutant mice. Moreover, immunohistochemistry showed that while the immunostaining signals of dentin sialoprotein (N-terminal fragment of DSPP) were decreased in the dentin matrix, they were remarkably increased in the odontoblasts of the *Dspp*^{P19L/+} and *Dspp*^{P19L/P19L} mice. Consistently, our in vitro studies showed that the secretion of the mutant DSPP was impaired and accumulated within endoplasmic reticulum. These findings suggest that the dental phenotypes of the mutant mice were associated with the intracellular retention of the mutant DSPP in the odontoblasts of the DSPP-mutant mice.

Keywords: odontoblast(s), genetics, tooth development, extracellular matrix, odontogenesis, mineralized tissue/development

Introduction

Dentin sialophosphoprotein (DSPP) is a noncollagenous extracellular matrix protein highly expressed by odontoblasts. DSPP is synthesized as a large single protein, which is proteolytically cleaved into an amino-terminal fragment called dentin sialoprotein (DSP) and a carboxyl-terminal fragment known as dentin phosphoprotein (DPP) (MacDougall et al. 1997; Zhu et al. 2012). DSP is a proteoglycan containing 2 glycosaminoglycan chains (Ritchie et al. 1994; Zhu et al. 2010), whereas DPP is a highly phosphorylated and acidic protein (George et al. 1996; Ritchie and Wang 1996). While in vitro studies suggest that DSPP may be cleaved by astacin proteases, including bone morphogenetic protein 1 (BMP1), meprin α , and meprin β , transgenic animal studies demonstrate that only BMP1/Tolloid-like 1 proteinase (mTLL1) are responsible for the proteolytic cleavage of DSPP (von Marschall and Fisher 2010; Tsuchiya et al. 2011; Arnold et al. 2017; Zhang et al. 2017).

Human genetic studies have demonstrated that mutations in 1 allele of the *DSPP* gene may cause autosomal-dominant dentinogenesis imperfecta (DGI) type II (OMIM #125490), characterized by pulp chamber obliteration; DGI type III (OMIM #125500), featured by pulp chamber enlargement and thinner dentin; or dentin dysplasia (DD) type II (OMIM #125420). However, unlike human DGI/DD patients, deletion of both

Dspp alleles (*Dspp*^{-/-}) in mice is necessary to produce a tooth phenotype similar to that observed in human DGI type III patients (Sreenath et al. 2003). These human and mouse genetic studies suggest that the loss of DSPP function may not be the only mechanism that accounts for human DGI/DD cases. New animal models that express the equivalent of human mutant DSPP are required to study the pathogenic effects of mutant DSPP on dentin formation.

Human genetic studies have revealed that the same DSPP mutations can cause both DGI type II and type III. One such mutation is c.50C>T, which leads to the substitution of proline residue at position 17 (P17) with a leucine residue (p.P17L); the P17L substitution caused DGI type II in a Chinese family (Li et al. 2012) and DGI type III in a Korean family (Lee et al.

¹Department of Biomedical Sciences and Center for Craniofacial Research and Diagnosis, Texas A&M University College of Dentistry, Dallas, TX, USA

A supplemental appendix to this article is available online.

Corresponding Author:

Y. Lu, Department of Biomedical Sciences and Center for Craniofacial Research and Diagnosis, Texas A&M University College of Dentistry, 3302 Gaston Ave., Room 436, Dallas, TX 75246, USA.
Email: ylu@tamhsc.edu

2013). In human DSPP, P17 is the second residue from the N-terminus of secreted DSPP and in the “isoleucine-proline-valine (IPV)–like tripeptide motif”; it has been thought that the IPV-like motif plays an essential role in the secretion of many acidic proteins, including DSPP (von Marschall et al. 2012). Among the 43 DGI/DD-causing DSPP mutations reported, 15 directly or indirectly alter this IPV-like motif (Hart and Hart 2007; Bloch-Zupan et al. 2016; Li et al. 2017). However, it remains to be determined why the same DSPP mutations can cause both DGI types II and III in humans.

In this study, we generated a mouse model (referred to as “*Dspp*^{P19L/+}” mice) that expressed a mutant DSPP, in which the proline residue at position 19 was replaced by a leucine residue (p.P19L). The proline residue at position 19 in mouse DSPP is the second amino acid residue from the signal peptide cleavage site, and it corresponds to the proline residue P17 in human DSPP. Our findings showed that the *Dspp*^{P19L/+} and *Dspp*^{P19L/P19L} mice recapitulated the dental phenotype of human patients and displayed an age-dependent tooth phenotype—a DGI type III–like phenotype in the younger mice and a DGI type II–like defect in the older mice. Moreover, the dental phenotype of these DSPP-mutant mice was associated with the accumulation of the DSPP within the odontoblasts.

Materials and Methods

Generation of *Dspp*^{P19L/+} Mouse Model

The *Dspp*^{P19L/+} mouse model was generated on a C57BL/6 genetic background, as shown in Appendix Figure 1. The *Dspp*^{P19L/+} and *Dspp*^{P19L/P19L} mice were fertile and bred normally. Both male and female mice were used for the analyses of the tooth phenotypes, as there was no phenotypic difference between sexes. The animal protocols were approved by the Institutional Animal Care and Use Committee of Texas A&M University College of Dentistry (Dallas, TX).

Plain X-ray Radiography and Micro-Computed Tomography

The left mandibles were dissected from 3-, 8-, and 24-wk-old *Dspp*^{+/+}, *Dspp*^{P19L/+}, *Dspp*^{P19L/P19L} mice and processed for plain X-ray radiography and micro-computed tomography (μCT) analysis, as previously described (Bouxsein et al. 2010; Gibson et al. 2013; Zhang et al. 2018). Three to 5 independent mice were analyzed for each genotype. Dentin was analyzed together with cementum as they were indistinguishable by density. Detailed procedures are provided in the Appendix Methods and Materials.

Resin-Casted Backscattered and Acid-Etched Scanning Electron Microscopy

The 3- and 24-wk-old mouse left mandibles were further processed for resin-casted backscattered scanning electron microscopy (SEM) and acid-etched SEM in a JEOL JSM-6010 LA SEM (JEOL), as previously described (Gibson et al. 2013;

Zhang et al. 2018). Two independent mice were analyzed for each genotype of 3- and 24-wk-old mice.

Sample Processing and Histological Analysis

The right mandibles from 1- and 3-wk-old *Dspp*^{+/+}, *Dspp*^{P19L/+}, and *Dspp*^{P19L/P19L} mice were harvested and processed for hematoxylin and eosin (H&E) staining and other histological analyses, as previously described (Gibson et al. 2013; Meng et al. 2015; Zhang et al. 2018). Roof and floor predentin thicknesses were measured at the central region of the sagittal sections through the center of the mandibular first molars. Four independent mice were analyzed for each genotype of mice.

In situ Hybridization

In situ hybridization (ISH) was performed to detect DSPP transcript using a digoxigenin (DIG)–labeled antisense complementary RNA (cRNA) probe for mouse *Dspp*, as previously described (Gibson et al. 2013; Meng et al. 2015; Zhang et al. 2018).

Immunohistochemistry

Immunohistochemistry (IHC) was carried out to detect DSP/DSPP using a rabbit anti-DSP polyclonal antibody (recognizing both DSP and full-length DSPP), as previously described (Gibson et al. 2013; Meng et al. 2015; Zhang et al. 2018).

Quantitative Real-Time Polymerase Chain Reaction

Quantitative real-time polymerase chain reaction (qRT-PCR) was performed to analyze the DSPP messenger RNA (mRNA) levels of mouse molars as we previously described (Gibson et al. 2013). Each value was normalized using the value of glyceraldehyde 3-phosphate dehydrogenase (GAPDH) as an internal control. qRT-PCR was done in triplicate for each gene. Four independent mice were analyzed for each genotype.

DNA Constructs, DNA Transfection, Western Blotting Analysis, and Immunofluorescent Staining

A DNA construct expressing a hemagglutinin (HA)–tagged normal mouse DSPP (DSPP-HA) or expressing an HA-tagged mouse mutant P19L-DSPP (P19L-DSPP-HA) was generated (Appendix Fig. 2A). Detailed procedures for DNA construct generation, transfection, Western blotting, and immunofluorescent staining are provided in the Supplemental Appendix.

Statistical Analysis

Student's *t* test was employed to compare the difference between 2 groups. One-way analysis of variance (ANOVA) was conducted to compare the differences among 3 groups. If significant differences were found by 1-way ANOVA, the Bonferroni method was used as post hoc. The quantified results were

Figure 1. Plain X-ray radiographic and micro-computed tomography (μ CT) analyses of the mandibular molars. **(A)** The images shown are representative plain X-ray radiographs of the mandibular molars of 3-wk-old (a–c), 8-wk-old (d–f), and 24-wk-old (g–i) *Dspp*^{+/+} (a, d, and g), *Dspp*^{P19L/+} (b, e, and h), and *Dspp*^{P19L/P19L} (c, f, and i) mice. The arrows point to the proximal cusp of the mandibular first molars (g–i). Note the sharp cusps in the *Dspp*^{+/+} mice and the blunted cusps in the *Dspp*^{P19L/+} and *Dspp*^{P19L/P19L} mice (g–i). M1, first molar; M2, second molar; M3, third molar. Scale bar: 500 μ m. **(B)** Shown are the representative 3-dimensional reconstructed μ CT images (sagittal sections) of the dentin/cementum of the mandibular first molars of 3-wk-old (a'–c'), 8-wk-old (d'–f'), and 24-wk-old (g'–i') *Dspp*^{+/+} (a', d', and g'), *Dspp*^{P19L/+} (b', e', and i'), and *Dspp*^{P19L/P19L} (c', f', and i') mice. The *Dspp*^{P19L/+} and *Dspp*^{P19L/P19L} mice showed a reduced thickness in the pulp chamber roof dentin and an increased thickness in the pulp chamber floor dentin compared to the *Dspp*^{+/+} mice at 3 ages examined. The *Dspp*^{P19L/+} and *Dspp*^{P19L/P19L} mice developed a smaller pulp chamber, compared to the *Dspp*^{+/+} mice, by 24 wk. Scale bar: 200 μ m.

represented as mean $\pm$ standard deviation (SD). $P < 0.05$ was considered statistically significant.

Results

The *Dspp*^{P19L/+} and *Dspp*^{P19L/P19L} Mice Developed a Tooth Phenotype Resembling Both Human DGI Type III and Type II

We first examined the overall phenotype of the mandibular molars of the 3-, 8-, and 24-wk-old *Dspp*^{P19L/+} and *Dspp*^{P19L/P19L} mice by plain X-ray radiography and μ CT. At age 3 wk, the *Dspp*^{P19L/+} and *Dspp*^{P19L/P19L} mice displayed a tooth phenotype that mimicked human DGI type III, manifested by significantly enlarged pulp chambers, a significant decrease in the thickness of pulp chamber roof dentin, and a significant increase in the thickness of pulp chamber floor dentin, compared to the age- and sex-matched *Dspp*^{+/+} mice (Fig. 1A: a–c, Fig. 1B: a'–c', and Table). With age, the pulp chambers of all 3 groups of mice decreased in size and showed no difference at 8 wk, but the pulp chambers of the *Dspp*^{P19L/+} and *Dspp*^{P19L/P19L} mice became significantly smaller than those of the *Dspp*^{+/+} mice by 24 wk (Fig. 1A: d–f, Fig. 1B: d'–f', and Table). Nevertheless, the *Dspp*^{P19L/+} and *Dspp*^{P19L/P19L} mice consistently manifested a thinner roof dentin and a thicker floor dentin at the ages of 8 and 24 wk compared to the *Dspp*^{+/+} mice. Meanwhile, the volume and density of the dentin/cementum were significantly reduced in the 3-wk-old *Dspp*^{P19L/+} and *Dspp*^{P19L/P19L} mice, compared to the *Dspp*^{+/+} mice (Table). At the ages of 8 and 24 wk, although the dentin/cementum volume remained smaller in the *Dspp*^{P19L/+} and *Dspp*^{P19L/P19L} mice, no significant difference was observed; however, the density of the dentin/cementum was constantly decreased at all ages examined (Table). Consistently, all the mandibular molars displayed severe occlusal enamel attrition with age, as indicated by the blunt cusps observed in the *Dspp*^{P19L/+} and *Dspp*^{P19L/P19L} mice, compared to the sharp cusps in the *Dspp*^{+/+} mice (Fig. 1A and Appendix Fig. 3). It is of note that about 20% of the mandibular second molars (M2) in the *Dspp*^{P19L/+} and *Dspp*^{P19L/P19L} mice developed pulp necrosis and showed severe discoloration; the necrotized pulp had severe infection, which spread to the periodontium, leading to alveolar bone loss (Appendix Fig. 3).

Collectively, these results demonstrated that the *Dspp*^{P19L/+} and *Dspp*^{P19L/P19L} mice displayed an age-dependent tooth phenotype—a DGI type III-like phenotype in the younger mice and a DGI type II-like defect in the older mice.

Table. Micro-Computed Tomography Analysis of the Mandibular First Molars.

	3 wk	8 wk	24 wk
Roof dentin thickness (μm)			
<i>Dspp</i> ^{+/+}	143.61 $\pm$ 9.82	236.30 $\pm$ 11.72	248.57 $\pm$ 22.77
<i>Dspp</i> ^{P19L/+}	89.09 $\pm$ 12.90 ^a	138.25 $\pm$ 32.71 ^a	167.80 $\pm$ 21.63 ^a
<i>Dspp</i> ^{P19L/P19L}	83.16 $\pm$ 5.65 ^a	104.50 $\pm$ 20.39 ^a	144.56 $\pm$ 18.71 ^a
Floor dentin thickness (μm)			
<i>Dspp</i> ^{+/+}	25.77 $\pm$ 2.81	120.64 $\pm$ 18.53	142.74 $\pm$ 10.98
<i>Dspp</i> ^{P19L/+}	39.64 $\pm$ 7.58 ^a	192.26 $\pm$ 7.44 ^a	217.21 $\pm$ 14.78 ^a
<i>Dspp</i> ^{P19L/P19L}	43.81 $\pm$ 7.72 ^a	202.81 $\pm$ 8.62 ^a	217.85 $\pm$ 35.46 ^a
Pulp volume (mm^3)			
<i>Dspp</i> ^{+/+}	0.3354 $\pm$ 0.0089	0.1355 $\pm$ 0.0065	0.1310 $\pm$ 0.0062
<i>Dspp</i> ^{P19L/+}	0.3568 $\pm$ 0.0124 ^a	0.1236 $\pm$ 0.0079	0.0940 $\pm$ 0.0172 ^a
<i>Dspp</i> ^{P19L/P19L}	0.3805 $\pm$ 0.0078 ^{a,b}	0.1309 $\pm$ 0.0066	0.0751 $\pm$ 0.0147 ^a
Dentin/cementum volume (mm^3)			
<i>Dspp</i> ^{+/+}	0.3886 $\pm$ 0.0128	0.8051 $\pm$ 0.0277	0.9981 $\pm$ 0.0357
<i>Dspp</i> ^{P19L/+}	0.3490 $\pm$ 0.0251 ^a	0.7767 $\pm$ 0.0331	0.9579 $\pm$ 0.0640
<i>Dspp</i> ^{P19L/P19L}	0.3573 $\pm$ 0.0128 ^a	0.7364 $\pm$ 0.0454	0.9253 $\pm$ 0.0733
Dentin/cementum density ($\text{mg HAp}/\text{cm}^3$)			
<i>Dspp</i> ^{+/+}	989.35 $\pm$ 11.05	1,071.83 $\pm$ 10.74	1,108.78 $\pm$ 9.45
<i>Dspp</i> ^{P19L/+}	951.89 $\pm$ 19.55 ^a	1,027.24 $\pm$ 11.57 ^a	1,071.04 $\pm$ 6.88 ^a
<i>Dspp</i> ^{P19L/P19L}	953.81 $\pm$ 11.70 ^a	1,029.79 $\pm$ 17.07 ^a	1,077.33 $\pm$ 26.71

n = 3–5. Values are mean $\pm$ SD.

HAp, hydroxyapatite.

^aStatistically different from *Dspp*^{+/+} (α = 0.05).

^bStatistically different from *Dspp*^{P19L/+} (α = 0.05).

Alterations in the Dentinal Tubules and Odontoblast Processes in the *Dspp*^{P19L/+} and *Dspp*^{P19L/P19L} Mice

Next, we analyzed the changes in the dentinal tubules and odontoblast processes in the *Dspp*^{P19L/+} and *Dspp*^{P19L/P19L} mice by SEM. Backscattered SEM revealed that, at the age of 3 wk, the peritubular dentin was more readily observed in the *Dspp*^{P19L/P19L} mice compared to the *Dspp*^{+/+} and *Dspp*^{P19L/+} mice (Fig. 2A–C). By 24 wk, both *Dspp*^{P19L/+} and *Dspp*^{P19L/P19L} mice showed an excessive deposition of the peritubular dentin, so that the dentinal tubules became smaller or even diminished compared to the *Dspp*^{+/+} mice (Fig. 2D–F). Acid-etched SEM showed the comparable distribution of odontoblast processes that resided in the dentinal tubules in all 3 groups of mice at the age of 3 wk (Fig. 2G–I). By 24 wk, the odontoblast processes with terminal branches were still evident near the dentinoenamel junction (DEJ) in the *Dspp*^{+/+} mice. In contrast, the odontoblast processes were barely observed in the dentinal tubules of the *Dspp*^{P19L/+} and *Dspp*^{P19L/P19L} mice (Fig. 2J–L). Taken together, these findings suggested that with age, the odontoblast processes retracted in the *Dspp*^{P19L/+} and *Dspp*^{P19L/P19L} mice, which was accompanied by continual deposition of peritubular dentin so that the dentinal tubules became smaller or completely occluded.

Pathological Changes in the Odontoblasts in the *Dspp*^{P19L/+} and *Dspp*^{P19L/P19L} Mice

Then, we examined the histological changes in the mandibular first molars of the *Dspp*^{P19L/+} and *Dspp*^{P19L/P19L} mice by H&E

staining. At the age of 1 wk, the roof predentin thickness was similar among the 3 groups of mice (Fig. 3A–C, A'–C'; Appendix Table 2). Interestingly, by 3 wk, the thickness of the pulp chamber roof predentin was significantly reduced in the *Dspp*^{P19L/+} and *Dspp*^{P19L/P19L} mice compared to the *Dspp*^{+/+} mice, whereas the thickness of the pulp chamber floor predentin was not different among the 3 groups (Fig. 3D–F, D'–F', D''–F''; Appendix Table 2). Moreover, there was a significant difference in the roof predentin thickness between the *Dspp*^{P19L/+} and *Dspp*^{P19L/P19L} mice (Appendix Table 2). No obvious difference in odontoblast morphology was observed among the 3 groups of mice at the age of 1 wk (Fig. 3A'–C'). By 3 wk, the roof odontoblasts in the *Dspp*^{+/+} mice were columnar in shape, with the nucleus located at the basal end of each odontoblast (Fig. 3D'). In contrast, the roof odontoblasts became shorter in the *Dspp*^{P19L/+} and *Dspp*^{P19L/P19L} mice (Fig. 3E' and F'). These results suggested that the defective dentin was associated with the pathological changes in the odontoblasts of the *Dspp*^{P19L/+} and *Dspp*^{P19L/P19L} mice.

An Accumulation of DSPP Protein within the Odontoblasts Accompanied by a Decreased Level of DSPP mRNA in the *Dspp*^{P19L/+} and *Dspp*^{P19L/P19L} Mice

To explore the molecular pathogenesis underlying the dentin defects associated with mutant P19L-DSPP, we analyzed the expression of DSPP in 1- and 3-wk-old *Dspp*^{P19L/+} and *Dspp*^{P19L/P19L} mice at the mRNA and protein levels. ISH showed that DSPP

Figure 2. Scanning electron microscopy (SEM) analyses of the dentin in the mandibular first molars. Shown are the representative backscattered (A–F) and acid-etched (G–L) SEM images of the mandibular first molars of 3-wk-old (A–C and G–I) and 24-wk-old (D–F and J–L) *Dspp*^{+/+} (A, D, G, and J), *Dspp*^{P19L/+} (B, E, H, and K), and *Dspp*^{P19L/P19L} (C, F, I, and L) mice. Backscattered SEM images show the peritubular dentin (like a halo surrounding each dentinal tubule) in light gray and intertubular dentin (located between dentinal tubules) in dark gray. Inset in C shows the enlarged view of a dentinal tubule (boxed area), and the arrow points to the peritubular dentin; inset in F shows the enlarged view of the boxed area, and the arrow points to a completely occluded dentinal tubule. Acid-etched SEM revealed similar distribution of odontoblast processes in the *Dspp*^{+/+} (G), *Dspp*^{P19L/+} (H), and *Dspp*^{P19L/P19L} (I) at the age of 3 wk. By 24 wk, the odontoblast processes with terminal branches were still present near the dentinoenamel junction in the *Dspp*^{+/+} mice (J). In contrast, the odontoblast processes were barely found in the dentinal tubules of the *Dspp*^{P19L/+} (K) and *Dspp*^{P19L/P19L} (L) mice. d, dentin; DEJ, dentinoenamel junction; p, pulp. Scale bars: 10 μm.

expression was markedly reduced in the odontoblasts of 1- and 3-wk-old *Dspp*^{P19L/+} and *Dspp*^{P19L/P19L} mice compared to the *Dspp*^{+/+} mice (Fig. 4A: a–c and a'–c'; Appendix Fig. 4A–C, A'–C', and A''–C''). qRT-PCR analyses using the total RNA extracted from 3-wk-old mouse molars further confirmed that the DSPP mRNA level was reduced by 88% in the *Dspp*^{P19L/+} mice compared to the *Dspp*^{+/+} mice (Fig. 4B). Immunohistochemistry showed that DSP/DSPP immunostaining signals were detected in both odontoblasts and dentin matrixes in 1- and 3-wk-old mice (Fig. 4A: d–f and d'–f'; Appendix Fig. 4D–F, D'–F', and D''–F''). However, the signals were much stronger within the odontoblasts of the DSPP-mutant mice compared to the *Dspp*^{+/+} mice. In contrast, the intensity of DSP/DSPP signals was moderately weaker in the dentin matrixes of the *Dspp*^{P19L/+} mice and was much lower in the dentin matrixes of the *Dspp*^{P19L/P19L} mice compared to the *Dspp*^{+/+} mice. It is of note that both ISH and IHC showed that the DSPP expression was remarkably higher in the roof-forming

odontoblasts than in the floor-forming odontoblasts in 3-wk-old wild-type or DSPP-mutant mouse molars (Appendix Fig. 4). Moreover, anti-DSP Western blotting analysis of the total proteins extracted from the dental pulps and dentin matrixes of first molars revealed that the mutant P19L-DSP/DSPP proteins showed a similar migrating pattern as the normal DSP/DSPP proteins (Fig. 4C). Consistently, we found that the secretion of P19L-DSPP-HA in the transfected cells in vitro was impaired, but the secreted P19L-DSPP-HA proteins showed a migrating pattern that was similar to that of the normal DSPP-HA proteins as judged by Western blot (Appendix Fig. 2B). In addition, we found that normal DSPP-HA was mainly colocalized with GM130 protein, a Golgi complex residential protein, whereas P19L-DSPP-HA was predominantly colocalized with calreticulin, an endoplasmic reticulum (ER) residential protein, when expressed in an odontoblast-like cell line (Fig. 4D). Altogether, these results suggested that P19L-DSPP, unlike normal DSPP, was not efficiently exported from the ER to the Golgi complex, resulting in its accumulation within the ER in the odontoblasts.

Discussion

While various mutations in the human *DSPP* gene may cause DGI or DD in an autosomal dominant trait, there is no appropriate animal model to study the underlying molecular pathogenic mechanisms. In this study, we generated a novel mouse model, which expresses P19L-DSPP—a mouse equivalent of human mutant P17L-DSPP. We found that the *Dspp*^{P19L/+} and *Dspp*^{P19L/P19L} mice displayed tooth phenotypes resembling the features of both human DGI type II and type III patients.

The *Dspp*^{P19L/+} and *Dspp*^{P19L/P19L} mouse molars displayed an age-dependent abnormality in the size of the dental pulp chamber. The mandibular first molars of the *Dspp*^{P19L/+} and *Dspp*^{P19L/P19L} mice formed larger pulp chambers before postnatal 3 wk and then gradually acquired smaller pulp chambers as the mice aged compared to the *Dspp*^{+/+} mice. In addition, the *Dspp*^{P19L/+} and *Dspp*^{P19L/P19L} mouse molars had reduced thickness of pulp chamber roof dentin and increased pulp chamber floor dentin thickness compared to the *Dspp*^{+/+} mice. As the floor dentin of the mandibular first molars started to form at about postnatal 2 wk (Shimazu et al. 2009), the larger pulp chamber before postnatal 3 wk must be primarily due to the reduction of roof dentin formation, whereas the smaller pulp chamber in older mice must be attributed to the accelerated deposition of the floor dentin in the *Dspp*^{P19L/+} and *Dspp*^{P19L/P19L} mice. This change in the size of dental pulp chambers over age recapitulates that of human DGI patients bearing the corresponding mutation (i.e., p.P17L)—enlarged dental pulp chambers in children (Lee et al.

2013) and obliterated pulp chambers in adults (Li et al. 2012). Follow-up studies are necessary to determine if the children with enlarged dental pulp chambers will indeed acquire smaller/obliterated dental pulp chambers with age.

It is a very interesting finding that the effects of mutant P19L-DSPP on the odontoblasts and dentin formation of the pulp chamber roof were different from those on the pulp chamber floor. It has been known that roof dentinogenesis differs from floor dentinogenesis in molars: in roof dentin formation, the odontoblast differentiation is induced by presecretory ameloblasts of the enamel organ, whereas in the floor dentin formation, the odontoblast differentiation is induced by the epithelial cells of the epithelial diaphragm, which is similar to Hertwig's epithelial root sheath. In this study, we found that the roof-forming odontoblasts expressed a higher level of DSPP than the floor-forming odontoblasts in both wild-type and DSPP-mutant mice. Therefore, we postulate that the different effects of P19L-DSPP on the chamber roof dentin formation and floor dentin formation were due to the difference in the levels of P19L-DSPP expression between the roof-forming and floor-forming odontoblasts in the DSPP-mutant mice. However, why different levels of P19L-DSPP had different effects on dentin formation remains to be further studied.

The DSPP-mutant mice manifested accelerated enamel attrition and increased peritubular dentin deposition. The *Dspp*^{P19L/+} and *Dspp*^{P19L/P19L} mice showed significant reduction in the thickness of pulp chamber roof dentin and compromised dentin quality. Therefore, the accelerated enamel attrition may be secondary to the defective dentin, which was unable to provide sufficient support for the overlying enamel. In addition, as DSPP is transiently expressed by presecretory ameloblasts (D'Souza et al. 1997; Verdelis et al. 2016), it was very likely that the mutant P19L-DSPP may also affect enamel formation, causing intrinsic enamel defects. As a consequence of rapid enamel attrition, the underlying dentin was exposed, so that the odontoblast processes retreated and the dentinal tubules were gradually completely filled with the peritubular dentin. Future studies are necessary to examine the pathogenic effects of the P19L-DSPP on enamel development.

The tooth phenotypes of the DSPP-mutant mice were associated with intracellular accumulation of the mutant P19L-DSPP. The *Dspp*^{P19L/+} and *Dspp*^{P19L/P19L} mice showed similarly reduced levels of DSPP mRNA. Previous studies have demonstrated that the expression of DSPP was upregulated by the BMP2 signaling pathway (Chen et al. 2008). However, we did not observe an apparent difference in the levels of phosphorylated Smad1/5/8, the BMP2 signaling effectors, by immunohistochemistry in the pulp cells of all 3 groups of mice at the

Figure 3. Hematoxylin and eosin (H&E) staining of the mandibular first molars. Shown are the representative H&E staining images of the mandibular first molars of 1-wk-old (A–C) and 3-wk-old (D–F) *Dspp*^{+/+} (A and D), *Dspp*^{P19L/+} (B and E), and *Dspp*^{P19L/P19L} (C and F) mice. A'–F' are the higher magnification views of the areas marked in black boxes in A–F, respectively. D'–F' are the higher magnification views of the areas marked in white boxes in D–F, respectively. d, dentin; e, enamel; od, odontoblast; pd, predentin. Scale bars: 200 μm in A–F, 20 μm in A'–C', and 20 μm in D'–F' and D''–F''.

age of 3 wk (Appendix Fig. 5), suggesting that BMP2 signaling was not affected. While a small amount of mutant DSPP protein was secreted into the dentin matrix, there was an accumulation of DSPP (normal/mutant) protein in the odontoblasts of the DSPP-mutant mice. Consistently, our in vitro studies demonstrated that the secretion of P19L-DSPP was impaired and the mutant P19L-DSPP was accumulated within the ER. Nevertheless, the secreted P19L-DSPP proteins showed a similar migrating pattern as the normal DSPP proteins as judged by Western blot, suggesting that the secreted mutant and normal DSPP proteins underwent similar posttranslational modifications and proteolytic processing. Interestingly, the *Dspp*^{P19L/+} and *Dspp*^{P19L/P19L} mice showed remarkably increased expression of dentin matrix protein 1 (Appendix Fig. 6A–F and

Figure 4. Intracellular retention of the mouse mutant P19L-dentin sialophosphoprotein (DSPP) in the endoplasmic reticulum (ER). **(A)** In situ hybridization analyses of DSPP messenger RNA (mRNA) levels and immunohistochemical staining of dentin sialoprotein (DSP)/DSPP protein. Shown are the representative in situ hybridization of DSPP mRNA (a–c, signal in purple) and immunohistochemical staining of DSP/DSPP protein (d–f, signal in brown) of the mandibular first molars of 1-wk-old *Dspp*^{+/+} (a and d), *Dspp*^{P19L/+} (b and e), and *Dspp*^{P19L/P19L} (c and f) mice. a'–f' are the higher magnification views of the areas marked in black boxes in a–f, respectively. d, dentin; od, odontoblast. Scale bars: 200 μ m in a–f, 50 μ m in a'–c', and 20 μ m in d'–f'. **(B)** Quantitative polymerase chain reaction analysis of DSPP mRNA levels in 3-wk-old *Dspp*^{+/+} and *Dspp*^{P19L/+} mouse molars. *n* = 4. The mRNA levels of *Dspp*^{+/+} mice are set as 1; values are mean $\pm$ SD. *Statistically different from *Dspp*^{+/+} (α = 0.05). **(C)** Anti-DSPP Western blotting analyses of total proteins extracted from the dental pulps and dentin

D'–F'; Appendix Table 3) but no apparent changes in other dentin matrix proteins, including type I collagen, bone sialoprotein, osteocalcin, and osteopontin (Appendix Fig. 6G–I; Appendix Table 3). Taken together, our findings suggest that the dental defects observed in the *Dspp*^{P19L/+} and *Dspp*^{P19L/P19L} mice were most likely caused by the accumulated mutant DSPP protein within the ER but was not due to the loss of DSPP function.

DSPP is highly acidic as it contains a large number of aspartate and glutamate residues (Prasad et al. 2010). If the full-length DSPP was accumulated in the ER, the highly acidic DSPP may be very unfavorable to the ER, which may cause a high level of ER stress, leading to pathologic unfolded protein response (UPR) (Chakrabarti et al. 2011). It has been demonstrated that the UPR is involved in various inherited connective tissue diseases caused by mutations in the genes encoding extracellular matrix proteins (Boot-Handford and Briggs 2010; Brookes et al. 2014). Further studies are needed to determine if the mutant DSPP incurred ER stress in the odontoblasts in the DSPP-mutant mice.

In summary, we have successfully generated a mouse model with point mutations in the *Dspp* gene that leads to a single amino acid substitution in the DSPP protein. The new mouse model developed tooth phenotypes resembling those of human DGI patients who carried the corresponding mutation in the *DSPP* gene. Further studies are warranted to determine the molecular pathogenesis of P19L-DSPP in odontoblasts to develop potential preventative treatment modalities for DGI.

matrixes of 14-wk-old *Dspp*^{+/+} and *Dspp*^{P19L/P19L} mouse first molars. Note that the mutant P19L-DSP/DSPP proteins showed a similar migrating pattern as the normal DSP/DSPP proteins on the Western blot. **(D)** Coimmunofluorescent staining of 171A11 mouse odontoblast-like cells transfected with DSPP-hemagglutinin (HA) or P19L-DSPP-HA construct with an anti-HA monoclonal antibody (a and b, signal in red) along with an anti-GM130 (a', signal in green) or anticalreticulin (anti-CALR; b', signal in green) polyclonal antibody. GM130 marked the cis-Golgi complex and calreticulin labeled the ER, and nuclei were stained with DAPI (blue). a'' is the merge of a, a', and DAPI staining, and b'' is the merge of b, b', and DAPI staining. c is a 171A11 cell transfected with DSPP-HA construct and immunofluorescently labeled with anti-HA and anti-CALR antibodies. d is a 171A11 cell transfected with P19L-DSPP-HA and immunofluorescently labeled with anti-HA and anti-GM130 antibodies. Note that DSPP-HA was mainly colocalized with GM130 protein, whereas P19L-DSPP-HA was predominantly colocalized with calreticulin. Scale bars = 7.5 μ m in D.

Author Contributions

T. Liang, contributed to design, data acquisition, analysis, and interpretation, drafted and critically revised the manuscript; H. Zhang, contributed to data acquisition, analysis, and interpretation, drafted and critically revised the manuscript; Q. Xu, contributed to data acquisition, critically revised the manuscript; S. Wang, contributed to data acquisition, drafted the manuscript; C. Qin, contributed to conception and data interpretation, drafted and critically revised the manuscript; Y. Lu, contributed to conception, design, and data interpretation, drafted and critically revised the manuscript. All authors gave final approval and agree to be accountable for all aspects of the work.

Acknowledgments

This work was supported by National Institutes of Health (NIH)/ National Institute of Dental and Craniofacial Research (NIDCR) grants DE027345 (to Y.L. and C.Q.), DE023365 (to Y.L.), and DE022549 (to C.Q.). We thank Applied StemCell, Inc. for generating the P19L-DSPP knock-in founder mice. The authors declare no potential conflicts of interest with respect to the authorship and/ or publication of this article.

ORCID iD

Y. Lu <https://orcid.org/0000-0003-3692-1736>

References

- Arnold P, Koopmann L, Peters F, Birkenfeld F, Goff SV, Damm T, Qin C, Moali C, Lucius R, Becker-Pauly C. 2017. Deficiency of the DSPP-cleaving enzymes meprin alpha and meprin beta does not result in dentin malformation in mice. *Cell Tissue Res*. 367(2):351–358.
- Bloch-Zupan A, Huckert M, Stoetzel C, Meyer J, Geoffroy V, Razafindrakoto RW, Ralison SN, Randrianaiwa JC, Ralison G, Andriamasinoro RO, et al. 2016. Detection of a novel DSPP mutation by NGS in a population isolate in Madagascar. *Front Physiol*. 7:70. Erratum in: *Front Physiol*. 2016;7:304.
- Boot-Handford RP, Briggs MD. 2010. The unfolded protein response and its relevance to connective tissue diseases. *Cell Tissue Res*. 339(1):197–211.
- Bouxein ML, Boyd SK, Christiansen BA, Guldberg RE, Jepsen KJ, Muller R. 2010. Guidelines for assessment of bone microstructure in rodents using micro-computed tomography. *J Bone Miner Res*. 25(7):1468–1486.
- Brookes SJ, Barron MJ, Boot-Handford R, Kirkham J, Dixon MJ. 2014. Endoplasmic reticulum stress in amelogenesis imperfecta and phenotypic rescue using 4-phenylbutyrate. *Hum Mol Genet*. 23(9):2468–2480.
- Chakrabarti A, Chen AW, Varner JD. 2011. A review of the mammalian unfolded protein response. *Biotechnol Bioeng*. 108(12):2777–2793.
- Chen S, Gluhak-Heinrich J, Martinez M, Li T, Wu Y, Chuang HH, Chen L, Dong J, Gay I, MacDougall M. 2008. Bone morphogenetic protein 2 mediates dentin sialophosphoprotein expression and odontoblast differentiation via NF- κ B signaling. *J Biol Chem*. 283(28):19359–19370.
- D'Souza RN, Cavender A, Sunavala G, Alvarez J, Ohshima T, Kulkarni AB, MacDougall M. 1997. Gene expression patterns of murine dentin matrix protein 1 (Dmp1) and dentin sialophosphoprotein (DSPP) suggest distinct developmental functions in vivo. *J Bone Miner Res*. 12(12):2040–2049.
- George A, Bannon L, Sabsay B, Dillon JW, Malone J, Veis A, Jenkins NA, Gilbert DJ, Copeland NG. 1996. The carboxyl-terminal domain of phosphophoryn contains unique extended triplet amino acid repeat sequences forming ordered carboxyl-phosphate interaction ridges that may be essential in the biomineralization process. *J Biol Chem*. 271(51):32869–32873.
- Gibson MP, Zhu Q, Wang S, Liu Q, Liu Y, Wang X, Yuan B, Ruest LB, Feng JQ, D'Souza RN, et al. 2013. The rescue of dentin matrix protein 1 (Dmp1)-deficient tooth defects by the transgenic expression of dentin sialophosphoprotein (DSPP) indicates that DSPP is a downstream effector molecule of Dmp1 in dentinogenesis. *J Biol Chem*. 288(10):7204–7214.
- Hart PS, Hart TC. 2007. Disorders of human dentin. *Cells Tissues Organs*. 186(1):70–77.
- Lee SK, Lee KE, Song SJ, Hyun HK, Lee SH, Kim JW. 2013. A DSPP mutation causing dentinogenesis imperfecta and characterization of the mutational effect. *Biomed Res Int*. 2013:948181.
- Li D, Du X, Zhang R, Shen B, Huang Y, Valenzuela RK, Wang B, Zhao H, Liu Z, Li J, et al. 2012. Mutation identification of the DSPP in a Chinese family with DGI-II and an up-to-date bioinformatic analysis. *Genomics*. 99(4):220–226.
- Li F, Liu Y, Liu H, Yang J, Zhang F, Feng H. 2017. Phenotype and genotype analyses in seven families with dentinogenesis imperfecta or dentin dysplasia. *Oral Dis*. 23(3):360–366.
- MacDougall M, Simmons D, Luan X, Nydegger J, Feng J, Gu TT. 1997. Dentin phosphoprotein and dentin sialoprotein are cleavage products expressed from a single transcript coded by a gene on human chromosome 4. Dentin phosphoprotein DNA sequence determination. *J Biol Chem*. 272(2):835–842.
- Meng T, Huang Y, Wang S, Zhang H, Dechow PC, Wang X, Qin C, Shi B, D'Souza RN, Lu Y. 2015. Twist1 is essential for tooth morphogenesis and odontoblast differentiation. *J Biol Chem*. 290(49):29593–29602.
- Prasad M, Butler WT, Qin C. 2010. Dentin sialophosphoprotein in biomineralization. *Connect Tissue Res*. 51(5):404–417.
- Ritchie HH, Hou H, Veis A, Butler WT. 1994. Cloning and sequence determination of rat dentin sialoprotein, a novel dentin protein. *J Biol Chem*. 269(5):3698–3702.
- Ritchie HH, Wang LH. 1996. Sequence determination of an extremely acidic rat dentin phosphoprotein. *J Biol Chem*. 271(36):21695–21698.
- Shimazu Y, Sato K, Aoyagi K, Nango N, Aoba T. 2009. Hertwig's epithelial cells and multi-root development of molars in mice. *J Oral Biosci*. 51(4):210–217.
- Sreenath T, Thyagarajan T, Hall B, Longenecker G, D'Souza R, Hong S, Wright JT, MacDougall M, Sauk J, Kulkarni AB. 2003. Dentin sialophosphoprotein knockout mouse teeth display widened predentin zone and develop defective dentin mineralization similar to human dentinogenesis imperfecta type III. *J Biol Chem*. 278(27):24874–24880.
- Tsuchiya S, Simmer JP, Hu JC, Richardson AS, Yamakoshi F, Yamakoshi Y. 2011. Astacin proteases cleave dentin sialophosphoprotein (DSPP) to generate dentin phosphoprotein (DPP). *J Bone Miner Res*. 26(1):220–228.
- Verdelis K, Szabo-Rogers HL, Xu Y, Chong R, Kang R, Cusack BJ, Jani P, Boskey AL, Qin C, Beniash E. 2016. Accelerated enamel mineralization in DSPP mutant mice. *Matrix Biol*. 52–54:246–259.
- von Marschall Z, Fisher LW. 2010. Dentin sialophosphoprotein (DSPP) is cleaved into its two natural dentin matrix products by three isoforms of bone morphogenetic protein-1 (BMP1). *Matrix Biol*. 29(4):295–303.
- von Marschall Z, Mok S, Phillips MD, McKnight DA, Fisher LW. 2012. Rough endoplasmic reticulum trafficking errors by different classes of mutant dentin sialophosphoprotein (DSPP) cause dominant negative effects in both dentinogenesis imperfecta and dentin dysplasia by entrapping normal DSPP. *J Bone Miner Res*. 27(6):1309–1321.
- Zhang H, Jani P, Liang T, Lu Y, Qin C. 2017. Inactivation of bone morphogenetic protein 1 (BMP1) and Tolloid-like 1 (TLL1) in cells expressing type I collagen leads to dental and periodontal defects in mice. *J Mol Histol*. 48(2):83–98.
- Zhang H, Xie X, Liu P, Liang T, Lu Y, Qin C. 2018. Transgenic expression of dentin phosphoprotein (DPP) partially rescued the dentin defects of DSPP-null mice. *PLoS One*. 13(4):e0195854.
- Zhu Q, Gibson MP, Liu Q, Liu Y, Lu Y, Wang X, Feng JQ, Qin C. 2012. Proteolytic processing of dentin sialophosphoprotein (DSPP) is essential to dentinogenesis. *J Biol Chem*. 287(36):30426–30435.
- Zhu Q, Sun Y, Prasad M, Wang X, Yamaoka AK, Li Y, Feng J, Qin C. 2010. Glycosaminoglycan chain of dentin sialoprotein proteoglycan. *J Dent Res*. 89(8):808–812.