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Transgenic Expression of *Dspp* Partially Rescued the Long Bone Defects of *Dmp1*-null Mice

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Abstract

Dentin matrix protein 1 (DMP1) and dentin sialophosphoprotein (DSPP) belong to the Small Integrin-Binding Ligand *N*-linked Glycoprotein (SIBLING) family. In addition to the features common to all SIBLING members, DMP1 and DSPP share several unique similarities in chemical structure, proteolytic activation and tissue localization. Mutations in, or deletion of *DMP1*, cause autosomal recessive hypophosphatemic rickets along with dental defects; *DSPP* mutations or its ablation are associated with dentinogenesis imperfecta. While the roles and functional mechanisms of DMP1 in osteogenesis have been extensively studied, those of DSPP in long bones have been studied only to a limited extent. Previous studies by our group revealed that transgenic expression of *Dspp* completely rescued the dentin defects of *Dmp1*-null (*Dmp1*^{-/-}) mice. In this investigation, we assessed the effects of transgenic *Dspp* on osteogenesis by analyzing the formation and mineralization of the long bones in *Dmp1*^{-/-} mice that expresses a transgene encoding full-length DSPP driven by a 3.6-kb rat *Col1a1* promoter (referred as “*Dmp1*^{-/-};*Dspp*-Tg mice”). We characterized the long bones of the *Dmp1*^{-/-};*Dspp*-Tg mice at different ages and compared them with those from *Dmp1*^{-/-} and *Dmp1*^{+/+} (normal control) mice. Our analyses showed that the long bones of *Dmp1*^{-/-};*Dspp*-Tg mice had a significant increase in cortical bone thickness, bone volume and mineral density along with a remarkable restoration of trabecular thickness compared to those of the *Dmp1*^{-/-} mice. The long bones of *Dmp1*^{-/-};*Dspp*-Tg mice underwent a dramatic reduction in the amount of osteoid, significant improvement of the collagen fibrillar network, and better organization of the lacunocanalicular system, compared to the *Dmp1*^{-/-} mice. The elevated levels of biglycan, bone sialoprotein and osteopontin in *Dmp1*^{-/-} mice were also noticeably corrected by the transgenic expression of *Dspp*. These findings suggest that DSPP and DMP1 may function synergistically within the complex milieu of bone matrices.

Keywords

Bone; Dentin Matrix Protein 1; Dentin Sialophosphoprotein; Mineralization; Transgenic Mice

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INTRODUCTION

The organic components in the extracellular matrix (ECM) of bone are composed of collagen type I and a number of non-collagenous proteins (NCPs). One family of NCPs is the Small Integrin-Binding Ligand N-linked Glycoprotein (SIBLING) family, which consists of osteopontin (OPN), bone sialoprotein (BSP), dentin matrix protein 1 (DMP1), dentin sialophosphoprotein (DSPP) and matrix extracellular phosphoglycoprotein (MEPE) [1]. These SIBLING family members play important biological roles in the formation and mineralization of bone and dentin [1-4], as evidenced by the observations that mutations in or ablation of their genes are associated with developmental abnormalities in the two tissues [5-7].

DMP1, first cloned from a rat odontoblast cDNA library, has been identified in dentin, bone and cementum as well as in some non-mineralized tissues [8-10]. In the appendicular skeleton, DMP1 is expressed by osteocytes, osteoblasts and hypertrophic chondrocytes [11-13]. *Dmp1*-deficient mice displayed severe defects in the cartilage and bone, which resembled the manifestations of autosomal recessive hypophosphatemic rickets (ARHR), a human hereditary disease caused by mutations in the *DMP1* gene. This condition was characterized by the elevation of serum fibroblast growth factor 23 (FGF23) and a reduction of serum phosphorus, along with malformed and hypomineralized bone and dentin [14, 15]. Although DSPP was originally thought to be exclusively expressed by odontoblasts, the dentin-forming cells, later on its expression was detected in bone, cementum and certain non-mineralizing tissues including the salivary glands and kidneys [16-19]. Our previous studies showed that the expression level of DSPP in the rat long bone is approximately 1/400th of that in the rat dentin [19]. Mouse and human genetic studies have associated *DSPP* mutations or its ablation with dentinogenesis imperfecta, characterized by thinner dentin, enlarged pulp chamber and widened predentin [5, 6, 20, 21]. While *Dspp* knockout mice have severe defects in the formation and mineralization of dentin, the changes in the long bones of *Dspp*-deficient mice are mild [22].

Certain pieces of evidence suggest a possible functional relationship between DMP1 and DSPP [9, 16, 23]. The dentin defects in *Dmp1*^{-/-} mice are similar to those in *Dspp*^{-/-} mice [20, 24] and the dentin of the *Dmp1*^{-/-} mice has a reduced level of DSPP expression [24]. *In vitro* studies revealed that DMP1 localizes in the nucleus during the differentiation of odontoblasts and is bound to and activates the *Dspp* promoter in odontoblast cell lines [25]. While DMP1 has been shown to play a crucial role in osteogenesis [14, 26, 27] and chondrogenesis [7], *Dspp* ablation in mice or its mutations in human dentinogenesis imperfecta subjects do not cause severe defects in the long bone, although *Dspp*-knockout mice have obvious alveolar bone abnormalities [22, 28]. Previously, we showed that the transgenic expression of *Dspp* rescued the dentin and alveolar bone defects of *Dmp1*^{-/-} mice [29]. To further elucidate the molecular mechanisms by which DMP1 interacts with DSPP in osteogenesis, we systematically characterized the long bones of *Dmp1*^{-/-} mice that express a transgene encoding full-length DSPP driven by a type I collagen promoter to determine whether or how much the transgenic expression of *Dspp* would rescue the long bone defects of *Dmp1*-deficient mice.

Results

In this study, the mice heterozygous for *Dmp1* (*Dmp1^{+/-}*) were used as normal controls for comparison, as these mice do not manifest any developmental abnormalities compared to wild type mice [7, 10, 14].

Expression of *Dspp* in the long bones of *Dmp1^{+/-}*, *Dmp1^{-/-}* and *Dmp1^{-/-};Dspp-Tg* mice

In order to compare the DSPP expression in the long bones of normal control (*Dmp1^{+/-}*), *Dmp1^{-/-}* and *Dmp1^{-/-};Dspp-Tg* mice, real-time quantitative polymerase chain reaction (RT-qPCR), immunohistochemical (IHC) staining and *in situ* hybridization (ISH) were performed. RT-qPCR analyses (Fig. 1a) showed that the DSPP mRNA level in the long bone of *Dmp1^{-/-}* mice was reduced by approximately one-third compared to the *Dmp1^{+/-}* mice, while the DSPP mRNA level in the long bone of the *Dmp1^{-/-};Dspp-Tg* mice was approximately 20-fold greater than that of the *Dmp1^{+/-}* mice. IHC revealed that, compared to the *Dmp1^{+/-}* mice (Fig. 1b), the long bone of the *Dmp1^{-/-}* mice (Fig. 1c) had weaker anti-DSP signals, while the long bone of the *Dmp1^{-/-};Dspp-Tg* mice (Fig. 1d) demonstrated a much stronger anti-DSP immunoreactivity than either the *Dmp1^{+/-}* mice or the *Dmp1^{-/-}* mice, consistent with the observations of the RT-qPCR analyses. *In situ* hybridization analyses showed that osteoblasts in the trabeculae immediately adjacent to the growth plate area had stronger signals for DSPP mRNA than the cortical bone in diaphysis regions. Compared to the *Dmp1^{+/-}* mice (Fig. 1e), DSPP mRNA was downregulated in the *Dmp1^{-/-}* mice (Fig. 1f). The *Dmp1^{-/-};Dspp-Tg* mice (Fig. 1g) had much elevated level of DSPP mRNA in the same region. These data confirmed the high expression level of the transgenic *Dspp* in the long bone of *Dmp1^{-/-};Dspp-Tg* mice.

Transgenic expression of *Dspp* lengthened the long bones of *Dmp1^{-/-}* mice

Plain X-ray radiography showed that compared to the femurs of 3-month-old *Dmp1^{+/-}* control mice (Fig. 2a), the femurs of the *Dmp1^{-/-}* mice (Fig. 2b) were shorter and had wider metaphyses. The femurs of *Dmp1^{-/-};Dspp-Tg* mice (Fig. 2c) were longer than those of the *Dmp1^{-/-}* mice, with the metaphyses of the former slightly narrower than the latter. The density of the cortical bone in *Dmp1^{-/-};Dspp-Tg* mice also appeared higher than the *Dmp1^{-/-}* mice, indicating a higher level of mineralization in the former than in the latter, which was further confirmed by micro-computed tomographic (μ -CT) analyses (see below). However, the femurs of the *Dmp1^{-/-} Dspp-Tg* mice were notably shorter than those of the *Dmp1^{+/-}* mice. At 6 months of age, the femurs of the *Dmp1^{+/-}* mice (Fig. 2d) were long and thin with parallel cortical plates; the degree of difference in the femur length between the *Dmp1^{-/-}* mice (Fig. 1e) and *Dmp1^{-/-};Dspp-Tg* mice (Fig. 2f) was similar to the 3-month-old mice, with the proximal and distal metaphyses becoming much narrower in the latter than in the former mice. The density of the femurs in the 6-month-old *Dmp1^{-/-};Dspp-Tg* mice, as reflected by radiopacity, was much higher than that of the *Dmp1^{-/-}* mice at the same age, indicating that as the animal aged, the improvement of mineralization by the transgenic *Dspp* further advanced. The quantitative analyses of the changes in femur length (Fig. 2g) showed that at 3 months of age, the average femur lengths of the *Dmp1^{+/-}*, *Dmp1^{-/-}* and *Dmp1^{-/-};Dspp-Tg* mice were 15.6 mm, 11 mm and 12.9 mm, respectively. At 6 months of age, the average femur lengths of the *Dmp1^{+/-}*, *Dmp1^{-/-}* and *Dmp1^{-/-};Dspp-Tg*

mice were 17.6 mm, 13.5 mm and 15 mm, respectively. The femur lengths were reduced to 70% of normal in the *Dmp1^{-/-}* mice, which increased to 82% of the normal in *Dmp1^{-/-};Dspp*-Tg mice at 3 months, an 11% increase in length. At 6 months of age, the femur length increased by 8% in *Dmp1^{-/-};Dspp*-Tg mice compared to the *Dmp1^{-/-}* mice. At either age point, the femur length differences between *Dmp1^{-/-};Dspp*-Tg and *Dmp1^{-/-}* mice were statistically significant ($P < 0.05$, $n = 5$). The tails of the *Dmp1^{-/-};Dspp*-Tg mice were also longer than the those of *Dmp1^{-/-}* mice but were shorter than the *Dmp1^{+/-}* mice (data not shown).

Transgenic expression of *Dspp* partially rescued the cortical bone defects in *Dmp1^{-/-}* mice

The three-dimensional imaging evaluation from the low resolution micro-computed tomographic (μ -CT) scans of whole femurs showed that the femurs of the *Dmp1^{+/-}* mice had no apparent surface porosities (Fig. 3a); the femurs of the *Dmp1^{-/-}* (Fig. 3b) and *Dmp1^{-/-};Dspp*-Tg mice (Fig. 3c) had reduced length and demonstrated a lower mineral content as reflected by the surface porosities at 3 months of age. Although noticeably more porous than the *Dmp1^{+/-}* mice (Fig. 3d), the femurs of *Dmp1^{-/-};Dspp*-Tg mice (Fig. 3f) had an obvious decrease in porosity at 6 months compared to the *Dmp1^{-/-}* (Fig. 3e) mice. In contrast to the smooth and well-organized femurs of the *Dmp1^{+/-}* mice (Fig. 3g and j), the longitudinal view of the femurs revealed that at both 3 and 6 months of age, those of the *Dmp1^{-/-}* (Fig. 3h and k) and *Dmp1^{-/-};Dspp*-Tg mice (Fig. 3i and l) had inner surface porosities and structural disorganization of the cortical bone.

High-resolution μ -CT scans of the cortical bone in the midshaft region of the femoral diaphysis were done for detailed imaging and quantitative analyses. At either 3 or 6 months of age, the cortical layer of the *Dmp1^{-/-}* mouse femurs (Fig. 4b and h) was dramatically thinner than the *Dmp1^{+/-}* mice (Fig. 4a and g). At 6 months, the cortical thickness of *Dmp1^{-/-};Dspp*-Tg mouse femurs (Fig. 4c and i) was similar to the *Dmp1^{+/-}* mice. The quantification of the cortical thickness for 3-month-old (Fig. 4d) and 6-month-old (Fig. 4j) mice showed that the cortical thickness in the *Dmp1^{-/-};Dspp*-Tg mice was restored to nearly the normal level at both ages. The bone volume fraction, expressed as the ratio of bone volume to the total volume (BV/TV), had significantly improved in the femurs of the *Dmp1^{-/-};Dspp*-Tg mice at 3 and 6 months of age compared to the *Dmp1^{-/-}* mouse femurs, although the BV/TV ratios in both types of mice were lower than in the *Dmp1^{+/-}* mice (Fig. 4e and k). When the BV/TV of the *Dmp1^{+/-}* mice was taken as 1, the BV/TV ratio of *Dmp1^{-/-}* mice was lower than of the *Dmp1^{+/-}* mice by 23% at 3 months, while that of the *Dmp1^{-/-};Dspp*-Tg mice was lower than *Dmp1^{+/-}* mice by 13%. At 6 months, the BV/TV ratio of *Dmp1^{-/-}* mice was lower than for the *Dmp1^{+/-}* mice by 23%, while that of the *Dmp1^{-/-};Dspp*-Tg mice was lower by 8%. Based on these data, we calculated that the transgenic expression of *Dspp* rescued the BV/TV reduction of *Dmp1^{-/-}* mice by 42% at 3 months and 63% at 6 months. There was a significant increase in the apparent density and material density of the cortical bone in the *Dmp1^{-/-};Dspp*-Tg mouse femurs compared to those of the *Dmp1^{-/-}* mice at 3 months of age (Fig. 4f and l). Our calculations revealed that the transgenic expression of *Dspp* rescued the apparent density reduction in the femurs of *Dmp1^{-/-}* mice by 30% at 3 months and 45% at 6 months; it corrected the material density reduction of *Dmp1^{-/-}* mice by 34% at 3 months and 32% at 6 months. Nevertheless, both

the apparent density and material density of the *Dmp1^{-/-};Dspp*-Tg mice were still significantly lower than the *Dmp1^{+/-}* mice at either time point. These findings indicated that the transgenic expression of *Dspp* significantly increased the bone volume and mineral density of cortical bone of *Dmp1^{-/-}* mice.

The trabecular thickness but not the trabecular number was significantly rescued by the transgenic expression of *Dspp*

To analyze the trabeculae in these mice, we performed high-resolution μ -CT scans of the femoral distal metaphysis regions. At 3 and 6 months, the trabecular images showed a drastic reduction in trabecular bone in the *Dmp1^{-/-}* mice (Fig. 5b and h) compared to the control mice (Fig. 5a and g), which was marginally improved in the *Dmp1^{-/-};Dspp*-Tg mice (Fig. 5c and i). The quantitative analyses revealed that the number of trabeculae in the *Dmp1^{-/-}* mice was lower than the *Dmp1^{+/-}* mice by 58% at 3 months and 53% at 6 months, while that of the *Dmp1^{-/-};Dspp*-Tg mice was lower by 53% at 3 months and 42% at 6 months (Fig. 5d and j). We calculated that the transgenic expression of *Dspp* rescued the trabecular number reduction of *Dmp1^{-/-}* mice by 8% at 3 months and 21% at 6 months. The trabecular bone thickness in the *Dmp1^{-/-}* mice was greater than for the *Dmp1^{+/-}* mice by 40% at 3 months and 24% at 6 months, while that in the *Dmp1^{-/-};Dspp*-Tg mice increased by 7% at 3 months and 2% at 6 months (Fig. 5e and k) compared to the *Dmp1^{+/-}* mice. At 6 months, the transgenic expression of *Dspp* near completely rescued the trabecular thickening defects of the *Dmp1^{-/-}* mice. The trabecular separation (spacing) in the *Dmp1^{-/-}* mice was greater than in the *Dmp1^{+/-}* mice by 149% at 3 months and 87% at 6 months, while that in the *Dmp1^{-/-};Dspp*-Tg mice had increased by 106% at 3 months and 57% at 6 months (Fig. 5f and l). The relatively lower level of rescue for trabecular spacing might be attributed to the limited correction of the trabecular bone numbers as described above (Fig. 5d and j).

Transgenic expression of *Dspp* significantly improved the morphology and ultrastructure of the long bone in *Dmp1^{-/-}* mice

Hematoxylin and eosin (H&E) staining of femurs from 3-month-old mice showed that in comparison with the *Dmp1^{+/-}* mice (Fig. 6a), the femurs of the *Dmp1^{-/-}* mice (Fig. 6b) were shorter and wider, along with presence of irregular trabeculae. There was a remarkably improved development of the trabecular bone network in the *Dmp1^{-/-};Dspp*-Tg mice (Fig. 6c) compared to the *Dmp1^{-/-}* mice. The overly-widened growth plate (red arrows) in the *Dmp1^{-/-}* mice was reversed to a nearly normal thickness in the *Dmp1^{-/-};Dspp*-Tg mice. The reduced thickness of the cortical bone in the *Dmp1^{-/-}* mice (Fig. 6e) was also reversed in the *Dmp1^{-/-};Dspp*-Tg mice (Fig. 6f) to a level similar to that of the *Dmp1^{+/-}* mice (Fig. 6d). Also, the cortical bone of *Dmp1^{-/-}* mice (Fig. 6e) had numerous areas that were hypomineralized or osteoid-like (arrow heads). To assess the collagen structure of the cortical bone, we performed Picrosirius red staining and visualized the collagen fibers under polarized light. When examined under polarized light, the larger collagen fibers were bright yellow or orange, and the thinner ones, including reticular fibers, were green. Compared to the *Dmp1^{+/-}* mice (Fig. 6g), the collagen fiber network in the cortical bone of the *Dmp1^{-/-}* mice (Fig. 6h) appeared disorganized and sparse. The collagen fiber organization in the femoral cortical bone of the *Dmp1^{-/-};Dspp*-Tg mice (Fig. 6i) had remarkably improved, although it was not as well oriented and dense as in the *Dmp1^{+/-}* mice. The results of

histological evaluation provided further support to the conclusion drawn from the μ -CT data regarding the restoration of cortical bone thickness and structural organization.

To further confirm the partial rescue of the *Dmp1*-deficient defects by the *Dspp* transgene, we analyzed the osteocyte lacunocanalicular system of the femurs using the resin-casted scanning electron microscopy (SEM) approach. The osteocyte lacunae (red arrows) in the femurs of the *Dmp1*^{+/-} mice (Fig. 7a) were spindle-shaped, while the osteocyte lacunae in the *Dmp1*^{-/-} mice (Fig. 7b) appeared enlarged, which was probably due to an accumulation of osteoid in the bone of these mice. The lacunae in the *Dmp1*^{-/-};*Dspp*-Tg mice (Fig. 7c) were smaller than those in the *Dmp1*^{-/-} mice but larger than in the *Dmp1*^{+/-} control mice. Acid-etched SEM imaging showed that the osteocytes in the *Dmp1*^{-/-} mice (Fig. 7e) exhibited a marked decrease in the number of canaliculi (red asterisks). The canaliculi in the long bones of the *Dmp1*^{-/-};*Dspp*-Tg mice (Fig. 7f) were more numerous than in the *Dmp1*^{-/-} mice, but were not as well oriented as in the control (Fig. 7d). Fluorescein isothiocyanate (FITC) is a fluorescent stain that binds to the osteocytes and canaliculi but not to the mineralized matrix. Thus, it helps to visualize the unaltered cellular structure of osteocytes. The FITC staining showed well-organized and evenly spaced osteocytes with well oriented canaliculi in the *Dmp1*^{+/-} mice (Fig. 7g). The osteocytes in the *Dmp1*^{-/-} mice (Fig. 7h) were round to ovoid and had a marked reduction in the number of canaliculi. The *Dmp1*^{-/-};*Dspp*-Tg mice (Fig. 7i) exhibited smaller osteocytes than the *Dmp1*^{-/-} mice with a significant rise in the number of canaliculi although they were not as well oriented as in the *Dmp1*^{+/-} controls. Thus, the *Dspp* transgene partially corrected the lacunocanalicular defects of the *Dmp1*-deficient mice.

The transgenic expression of *Dspp* partially corrected the altered levels of certain ECM molecules

Immunohistochemistry (IHC) results confirmed the presence of DMP1 signals around the osteocyte lacunae and in the bone ECM of the *Dmp1*^{+/-} mice (Fig. 8a) and its loss in *Dmp1*^{-/-} mice (Fig. 8b) and *Dmp1*^{-/-};*Dspp*-Tg mice (Fig. 8c). Biglycan is present in the unmineralized matrix (osteoid), and anti-biglycan IHC is often used to reveal osteoid [30]. The anti-biglycan IHC analyses showed remarkably increased biglycan in the matrix of the cortical bone of *Dmp1*^{-/-} mice (Fig. 8e). The matrix of the *Dmp1*^{-/-};*Dspp*-Tg mice (Fig. 8f) had much less biglycan (osteoid) than the *Dmp1*^{-/-} mice, but more than the *Dmp1*^{+/-} mice (Fig. 8d). These findings substantiated the improved mineralization of the *Dmp1*^{-/-};*Dspp*-Tg mouse long bones. The levels of BSP, OPN and MEPE were higher in the long bones of the *Dmp1*^{-/-} mice (Fig. 8h,k and n) while the levels of these proteins in the long bones of the *Dmp1*^{-/-};*Dspp*-Tg mice (Fig. 8i, l and o) were only slightly more elevated than those of the *Dmp1*^{+/-} mice (Fig. 8g, j and m). Compared to the *Dmp1*^{+/-} mice (Fig. 8p), the FGF23 level was elevated in the *Dmp1*^{-/-} mice (Fig. 8q), and the FGF23 level in the *Dmp1*^{-/-};*Dspp*-Tg mice (Fig. 8r) was slightly lower than in the *Dmp1*^{-/-} mice, and higher than in the *Dmp1*^{+/-} controls.

The RT-qPCR analyses showed that the BSP mRNA level (Fig. 9a) was elevated by 9 folds while that of the OPN (Fig. 9b) increased to 11 folds in *Dmp1*^{-/-} mice compared to that of the *Dmp1*^{+/-} mice. The mRNA levels of both BSP and OPN in the *Dmp1*^{-/-};*Dspp*-Tg mice

were reduced to close to normal. The mRNA level of MEPE (Fig. 9c), which increased to about 5 folds in *Dmp1*^{-/-} mice, was restored to normal in the *Dmp1*^{-/-};*Dspp*-Tg mice. Similarly, the collagen Type 1 alpha 1 (Col1a1) expression (Fig. 9d) was reduced almost to the normal level in *Dmp1*^{-/-};*Dspp*-Tg mice.

The *in situ* hybridization analyses demonstrated relatively strong signals for DMP1, BSP, OPN and Col1a1 mRNAs in osteoblasts (arrows in Fig. 10) of the newly formed bone immediately subjacent to the epiphyseal growth plates in the *Dmp1*^{+/-} mice. There was a complete lack of DMP1 mRNA in the *Dmp1*^{-/-} (Fig. 10b) and *Dmp1*^{-/-};*Dspp*-Tg mice (Fig. 10c), consistent with the immunohistochemistry results. In agreement with the immunohistochemistry and RT-qPCR data, the *in situ* hybridization analyses showed that compared to the *Dmp1*^{+/-} controls (Fig. 10d and g), the BSP and OPN mRNA levels were higher in the *Dmp1*^{-/-} mice (Fig. 10e and h), whereas the levels of these molecules in the *Dmp1*^{-/-};*Dspp*-Tg mice (Fig. 10f and i) were similar to those of the *Dmp1*^{+/-} mice. The Col1a1 mRNA level was also higher in the *Dmp1*^{-/-} mice (Fig. 10k) than in the *Dmp1*^{+/-} controls (Fig. 10j), while the Col1a1 level in *Dmp1*^{-/-};*Dspp*-Tg mice (Fig. 10l) was similar to the *Dmp1*^{+/-} mice.

Discussion

DMP1 and DSPP share several unique similarities [4, 19, 31]. Both are cleaved by bone morphogenetic protein 1/tolloid-like metalloproteinases into their NH₂-terminal and COOH-terminal fragments [10, 32-35]. Their NH₂-terminal fragments mainly exist as proteoglycans [36-38], whereas their COOH-terminal fragments are highly phosphorylated and promote mineralization [39, 40]. They show similar localization patterns in bone and tooth [41, 42]. Additionally, *Dmp1*-null mice and *Dspp*-null mice manifest similar dentin defects [20, 24]. These striking similarities between DMP1 and DSPP indicate that these two molecules may have synergistic or redundant roles in the formation and mineralization of hard tissues.

While the role of DMP1 in osteogenesis has been extensively studied, the function of DSPP has been studied only to a limited extent. The loss of *Dspp* in mice leads to an age-related mineralization defect; however, the abnormalities in the long bone of the *Dspp*-deficient mice are mild [22]. Recently, we showed that the transgenic expression of DSPP completely rescued the dentin and alveolar bone defects of *Dmp1*^{-/-} mice but failed to correct the elevated level of serum FGF23 and the reduction of serum phosphorus level in the *Dmp1*-deficient animals [29]. The *in vitro* luciferase assay studies by our group and others revealed that DMP1 enhanced the promoter activity of the *Dspp* gene in odontoblast-like cells or C3H10T1/2 mesenchymal cells transfected with DMP1-expressing constructs [25, 29]. These findings indicate that DSPP may be a downstream target of DMP1 during the formation of dentin and alveolar bone.

In this study, we observed that the transgenic expression of *Dspp* led to the significant rescue of the long bone defects in *Dmp1*-null mice. While the introduction of transgenic *Dspp* into the *Dmp1*-deficient background led to a remarkable correction of the long bone defects caused by *Dmp1* ablation, the impact of transgenic *Dspp* on different bone parameters reflecting various aspects of bone quality were divergent. The transgenic expression of

DSPP rescued the bone volume reduction and hypomineralization defects in the cortical bone to a much greater extent than the correction of the trabecular number loss in the metaphysis region of *Dmp1*-deficient femurs. DMP1 plays broader roles than DSPP; the former is involved in gene regulation, FGF23 dynamics and phosphorus metabolism, in addition to mineralization-promoting effect, while the latter primarily participates in the matrix mineralization aspect of dentinogenesis and osteogenesis. The trabecular bone number is the most important indicator regarding the healthy status of trabeculae. The trabecular bones in the metaphysis region undergo faster remodeling and have a greater turnover rate than the cortical bone. Thus the formation of trabecular bones in this region may be more vulnerable to alterations of factors such as a reduction of the serum phosphorus level than is the cortical bone, which may be partially responsible for the observation that the rescue of trabecular number loss was not as significant as that of the cortical bones in the *Dmp1*^{-/-}; *Dspp*-Tg mice.

In addition to the cortical and trabecular bone changes, the partial restoration of the osteocyte defects by the *Dspp* transgenic expression was also significant. The number and orientation of canaliculi and the osteocyte morphology in the *Dmp1*^{-/-}; *Dspp*-Tg mice showed a remarkable improvement over the *Dmp1*^{-/-} mice. Our immunohistochemistry, RT-qPCR and *in situ* hybridization analyses of BSP, OPN and MEPE showed that all these molecular markers were elevated in the *Dmp1*^{-/-} mice, but returned to levels close to normal in *Dmp1*^{-/-}; *Dspp*-Tg mice. The correction of altered levels of these ECM molecules by the transgenic expression of *Dspp* may facilitate the osteocyte maturation process and improve the collagen fiber organization, which may subsequently lead to the improvement in mineralization and matrix morphology in the long bones of *Dmp1*^{-/-}; *Dspp*-Tg mice. These findings suggest that DSPP may play an important role for maintaining the morphology and function of osteocytes via its relationship to certain ECM proteins in bone.

Both DMP1 and DSPP are acidic proteins that can attract Ca²⁺ and promote the formation and growth of hydroxyapatite (HA) crystals on the collagen matrix, facilitating the conversion of osteoid to bone and predentin to dentin [4, 8, 16, 40]. We speculate that DSPP's role in promoting the deposition of HA crystals as a redundant partner of DMP1 is the principal factor contributing to the improved quality of the long bone in *Dmp1*^{-/-}; *Dspp*-Tg mice. Previous studies have shown that adding the COOH-terminal fragment of DSPP into MC3T3-E1 osteoblastic cells up-regulated the transcription of the osteoblast markers, *Runx2* and osterix [43], both of which are known to be essential transcription factors for bone formation. It was postulated that the COOH-terminal fragment of DSPP may promote the differentiation of osteogenic cells via the activation of the MAPK and Smad pathways as well as the up-regulation of bone morphogenetic proteins [43, 44]. Thus, in addition to its role of directly promoting the formation and growth of HA crystals, DSPP or its fragments may regulate certain pathways in osteogenesis, which may be another factor contributing to the improvement of bone quality in *Dmp1*-null mice. DSPP's role in regulating certain osteogenic pathways may also be responsible for the increase of long bone length in *Dmp1*^{-/-}; *Dspp*-Tg mice, which was likely due to enhanced bone formation.

Previously, we showed that the transgenic expression of DSPP completely rescued the alveolar bone defects in *Dmp1*-null mice [29], while in this study, the same transgene

partially rescued the long bone defects of the *Dmp1*^{-/-} mice. The differences in the extent of the improvement between the alveolar bone and the long bone could be attributed to the following aspects. 1) The forming alveolar bone cells originate from the neural crest, which is from the ectoderm, while the osteoblasts of the long bone are from the mesoderm. 2) The DSPP expression level in alveolar bone is much higher than in the long bone [19, 45]. 3) The alveolar bone defects in *Dspp*-null mice [28] are much more severe than the long bone defects [22], suggesting that DSPP's role in alveolar bone formation is more important than in the long bone. Clearly, future studies are warranted to elucidate the mechanisms by which the DMP1 and DSPP function differently in the formation of alveolar bone and long bone.

DSPP is proteolytically processed into NH₂-terminal and COOH-terminal fragments. *In vitro* studies have revealed that the COOH-terminal fragment of DSPP promotes the formation and growth of HA crystals [39, 46-48]. *In vivo* studies by our group showed that transgenic expression of *Dspp* NH₂-terminal fragment failed to rescue the dentin and alveolar bone defects in *Dspp*-null mice [49]. We postulate that the COOH-terminal fragment of DSPP may be the molecular variant of DSPP contributing to the significant improvement of bone quality in *Dmp1*-null mice. Future studies are warranted to further define which of the individual fragments derived from DSPP is responsible for correcting the long bone defects of *Dmp1*-null mice.

In summary, the transgenic expression of DSPP partially, but not completely, rescued the long bone defects of *Dmp1*-null mice, without a correction of serum FGF23 elevation and serum phosphorus reduction. DMP1 and DSPP are likely to have redundant roles in promoting the deposition of HA crystals during osteogenesis, which may be responsible for the significant improvement of long bone quality in *Dmp1*^{-/-};*Dspp*-Tg mice.

Materials and methods

Generation of *Dmp1*^{-/-};*Dspp*-Tg mice

The generation of mice expressing a transgene encoding the full-length form of DSPP has been described in our previous reports [35, 50]. In these transgenic mice, the DSPP transgene is downstream to the 3.6-kb rat *Col 1a1* promoter that drives the expression of the transgene in type I collagen-expressing tissues, including bone. The transgenic mouse line showing the highest level of DSPP mRNA [35] in the long bone in the wild type background was crossbred with the *Dmp1*^{-/-} mice [7, 24] to create mice that express the transgenic *Dspp* but lack *Dmp1*; these mice were referred to as “*Dmp1*^{-/-};*Dspp*-Tg mice”. The genotyping approach and primers for identifying the *Dmp1*-null alleles and *Dspp* transgene were described previously [29]. The animal protocols related to this study were approved by the Animal Welfare Committee of Texas A&M University Baylor College of Dentistry (Dallas, TX, USA). The mice heterozygous for *Dmp1* (*Dmp1*^{+/-}) of corresponding ages were used as normal controls for all experiments as these mice do not manifest any developmental abnormalities compared to the wild type mice [7, 10, 14].

Real Time Quantitative Polymerase Chain reaction (RT-qPCR)

The RT-qPCR analyses were performed to measure the mRNA levels of DSPP, BSP, OPN, MEPE and COL1a1 in the mouse long bone. Total RNA was extracted from the femurs of the 12-week-old mice of each group, treated with DNase I (Promega, Madison, WI), and purified with the RNeasy Mini Kit (Qiagen, Inc., Valencia, CA). The RNA (1 µg/ml per sample) was transcribed into cDNA by SuperScript III reverse transcriptase (Invitrogen, San Diego, CA). Specific primers and conditions used for the RT-qPCR analyses are listed in Table 1. The housekeeping gene, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), was used as the internal control. The RT-qPCR reactions were performed using the Brilliant SYBR Green QPCR Master Mix (Applied Biosystems; Foster City, CA) and the CFX-96 Real-Time PCR Detection System (Bio-Rad; Hercules, CA). The mean values from triplicate analyses were then calculated and compared.

Plain X-ray Radiography and Micro-computed Tomography (µ-CT)

The femurs from the hind limbs of 3- and 6-month-old male mice were dissected and analyzed with a Faxitron MX-20 specimen radiography system (Faxitron X-ray Corp., Buffalo Grove, IL). The measurement tool of the Faxitron software was used to measure the femur lengths using five femurs from five mice in each group (n = 5). The mean and standard deviation values from five measurements were used for quantitative comparison.

For the µ-CT analyses, the femurs were scanned using a µ-CT 35 imaging system (Scanco Medical, Basserdorf, Switzerland). The µ-CT analyses included: 1) a low-resolution scan (7.2 µm slice increment) of the whole femur from the 3-month-old and 6-month-old male mice for an overall assessment of the shape and structure; 2) a high-resolution scan (3.4 µm slice increment) of the femoral midshaft region (midway between the two epiphyses along the cranio-caudal axis, 200 slices) for analysis of the cortical bone in the 3-month-old and 6-month-old male mice; 3) a high-resolution scan (3.4 µm slice increment) of the femoral metaphysis region proximal to the distal growth plate for evaluation of the trabecular bones in the 3- and 6-month-old male mice. For trabecular bone analyses, we selected a cylinder area in the center of the metaphysis region with a radius of 100 µm and a length of 1400 µm (400 slices); the cortical shell was excluded in these trabecular bone analyses. The cortical thickness was measured using the Scanco software, and the averages were obtained from five femurs of five mice in each group (n = 5). Every 20th slice from the high-resolution scans of the femoral midshaft region of each sample were analyzed for cortical thickness. The data acquired from the high-resolution scans were used for quantitative analyses. The quantitative µ-CT parameters obtained and analyzed using the Scanco software included: ratio of bone volume to total volume (BV/TV), apparent density, material density, trabecular number (Tb.N), trabecular thickness (Tb.Th), and trabecular separation (Tb.Sp). Six femurs from six mice in each group were used for quantitative analyses of BV/TV, apparent density, material density, Tb.N, Tb.Th, and Tb.Sp.

Tissue Preparation and Histology Evaluation

Under anesthesia, the *Dmp1*^{+/-}, *Dmp1*^{-/-} and *Dmp1*^{-/-};*Dspp*-Tg mice at postnatal 6 weeks and 3 months were perfused from the ascending aorta with 4% paraformaldehyde in 0.1 M phosphate-buffered saline. The femurs were dissected and further soaked in the same

fixative for 48 h, followed by demineralization in 14% EDTA (pH 7.4) at 4°C for 2 weeks. The tissues were processed for paraffin embedding, and serial 5-µm sections were prepared. The sections were stained with hematoxylin and eosin (H&E) for routine histology analyses; these paraffin sections were also used for immunohistochemistry and *In situ* hybridization analyses (see later). Picrosirius Red staining [51] was performed to assess the morphology and organization of the collagen fibrils.

Scanning Electron Microscopy (SEM)

The tibias of 3-month-old mice from each group were dissected and fixed in 2% paraformaldehyde and 2.5% glutaraldehyde in 0.1M cacodylate buffer solution (pH 7.4) at room temperature. Four hours later, the samples were immersed in 0.1M cacodylate solution. The samples were then dehydrated in ascending concentrations of ethanol and embedded in methyl-methacrylate (MMA) resin. Microcloths with Metadi Supreme polycrystalline diamond suspensions of decreasing sizes (Buehler, Lake Bluff, IL) were used to polish the sample surfaces. The samples were coated with carbon for backscattered SEM analyses. For the resin-casted SEM, the samples were acid-etched with 37% phosphoric acid for 2–10 seconds and washed with 5.25% sodium hypochlorite for 5 minutes. The samples were then coated with gold and palladium for secondary electron image analyses. A JEOL JSM-6300 scanning electron microscope (JEOL Limited, Tokyo, Japan) was used to perform the analyses as reported previously [14].

Fluorescein Isothiocyanate (FITC) Staining

FITC, a small molecular dye, fills in the osteocyte lacunae and canaliculi, but does not stain the mineralized matrix. Thus, the dye provides a visual representation of the organization of the osteocytes and their lacunocanalicular system under a confocal microscope [52]. The tibias from each group of 3-month-old mice were dissected and dehydrated through a series of ethanol solutions from 70–100% and acetone solution, followed by 1% FITC stain (Sigma-Aldrich, St. Louis, MO) overnight, with additional dehydration and MMA embedding as described above. A cross section (500 µm thick) from the tibial midshaft region was cut with a diamond-bladed saw (Buehler), and the plastic sections were then sanded and ground to a final thickness of 30–50 µm for confocal imaging using the Leica SP2 confocal microscope (Leica TCS, Germany).

Immunohistochemistry (IHC) and Generation of Monoclonal Anti-FGF23 Antibodies

For the immunohistochemistry analyses, anti-DSP-2C12.3 monoclonal antibody [53] was used at a concentration of 2.05 µg/ml. Anti-DMP1 monoclonal antibody that recognizes the C-terminal region of DMP1 [16] was used at a concentration of 4.7 µg/ml. Anti-biglycan polyclonal antibody LF-159 [54] was used at a dilution of 1:1000. Anti-BSP monoclonal antibody 10D9.2 [55] was used at a concentration of 4.5 µg/ml. Polyclonal anti-OPN antibody [56] was used at a dilution of 1:400. Anti-MEPE polyclonal antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) was used at a concentration of 2.1 µg/ml.

Recently, we generated anti-FGF23 monoclonal antibodies which recognize both human and mouse FGF23. Briefly, five BALB/c mice were immunized with the full-length human recombinant FGF23 using a Bac-to-Bac baculovirus expression system (Invitrogen) as we

previously reported [57], and the immunization boosting was given every 3 weeks for a total of four times (Creative Biolabs, Shirley, NY). The titers of serum anti-FGF23 antibodies were determined with an enzyme-linked immunosorbent assay. One of the five mice that showed the highest titer for the anti-FGF23 activity was given a final boosting and sacrificed for cell fusion; the splenocytes from this mouse were fused with FO murine myeloma cells. The fusion cells showing positive reactions to FGF23 were screened with the Omni Hybridoma Platform (Creative Biolabs) and cloned. After testing 207 single clones, we obtained 15 positive clones. Western immunoblotting analyses in our laboratory revealed that the cultural media (supernatants) from three positive clones (hybridoma cell lines) including Clone 79 showed strong reactions to both human and mouse FGF23. Clone 79 with the IgG isotype was expanded in syngeneic mice; the ascites were collected from these mice and the “anti-FGF23-79 antibody” was isolated from the ascites by HiTrap rProtein A FF (Creative Biolabs). We further confirmed the immunoreactivity of purified anti-FGF23-79 antibody to both human and mouse recombinant FGF23 by Western immunoblotting analyses. Our IHC analyses with long bone samples from *Dmp1*^{+/-} and *Dmp1*^{-/-} mice revealed that anti-FGF23-79 antibody gave rise to highly specific signals in the osteocyte lacunae and the matrices around osteocytes, and the anti-FGF23 immunoreactivity was remarkably stronger in the *Dmp1*^{-/-} mouse bone than in the *Dmp1*^{+/-} mouse bone. The anti-FGF23-79 antibody at 2.75 µg/ml concentration was used for IHC analyses in the current study.

All the IHC experiments were carried out using the mouse on mouse kit for monoclonal antibodies and the ABC kit for polyclonal antibodies (Vector Laboratories, Burlingame, CA). The 3, 3'-diaminobenzidine (DAB) kit (Vector Laboratories) was used for color development according to the manufacturer's instructions.

***In Situ* Hybridization**

In situ hybridization was performed to assess the mRNA levels of DSPP, DMP1, BSP, OPN and COL1a1 in the femurs of 6-week-old mice for each group. Detailed information regarding the RNA probes for DSPP [16], DMP1 [16], BSP [58], OPN [58] and COL1a1 [14] has been described previously. The RNA probes were labeled with digoxigenin using a RNA labeling kit (Roche, Indianapolis, IN) and were detected by an enzyme-linked immunoassay with a specific anti-digoxigenin-alkaline phosphatase antibody conjugate and alkaline phosphatase substrate (Roche), following the manufacturer's instructions. Nuclear fast red was used for counterstaining.

Statistical Analysis

The data analyses were performed with a one-way analysis of variance for multiple group comparisons. If significant differences were found with the one-way analysis of variance, the Bonferroni method was used to determine which groups were significantly different from others. The quantified results were expressed as the mean ± S.D. P < 0.05 was considered to be statistically significant.

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HIGHLIGHTS

- Transgenic expression of *Dspp* lengthened the long bone of *Dmp1*-null mice.
- *Dspp* transgenic expression improved the cortical bone of *Dmp1*-null mice.
- *Dspp* overexpression improved the collagen network of *Dmp1*-deficient bone.
- Overexpressing *Dspp* improved the lacunocanalicular system of *Dmp1*-deficient bone.
- Transgenic *Dspp* corrected the altered levels of ECM molecules in *Dmp1*-deficient bone.

Fig. 1. Expression of *Dspp* transgene

a, RT-qPCR analyses; b-d, immunohistochemistry staining using the anti-DSP antibody; e-g, *in situ* hybridization analyses for DSPP mRNA. In the RT-qPCR analyses (a), we measured the mRNA levels of DSPP in the femurs of 3-month-old mice from each group. The DSPP mRNA level in the normal control (*Dmp1*^{+/-}) mice (green bar) was taken as 1. DSPP mRNA level in the *Dmp1*^{-/-} mice (red bar) was 30% of the *Dmp1*^{+/-} mice, while its level in the *Dmp1*^{-/-}; *Dspp*-Tg mice (blue bar) was 20 fold of the normal. The anti-DSP immunostaining exhibited signals for this protein around the osteocyte lacunae in the cortical bone in the mid-shaft region of the femurs of control mice (b). The anti-DSP signals were slightly

weaker in the cortical bone of *Dmp1^{-/-}* mice (c) than in the *Dmp1^{+/-}* controls. The cortical bone of *Dmp1^{-/-};Dspp-Tg* mice (d) demonstrated a higher level of anti-DSP immunoreactivity compared to the other two groups of mice. *In situ* hybridization analyses show the presence of DSPP mRNA (arrows) in the newly formed bone proximal to the epiphyseal growth plate of *Dmp1^{+/-}* mice (e); DSPP mRNA level was lower in *Dmp1^{-/-}* mice (f) than in the *Dmp1^{+/-}* mice. In the same region, *Dmp1^{-/-};Dspp-Tg* mice (g) showed elevated level of DSPP mRNA confirming the higher expression levels of *Dspp* transgene in these mice. Scale bar: 50 μ m in b-g.

Fig. 2. Plain X-ray radiography of femurs from the three groups of mice at the ages of 3 and 6 months

At 3 months, the femurs of the control (*Dmp1*^{+/-}) mice (a) had parallel and uniform cortical layers throughout the diaphysis of the bone and the metaphysis regions were slightly wider than the shaft. The femurs of the 3-month-old *Dmp1*^{-/-} mice (b) and *Dmp1*^{-/-}; *Dspp*-Tg mice (c) were shorter and wider than those of the *Dmp1*^{+/-} mice, and showed significant outward protrusion (irregular enlargement) in the metaphysis regions, particularly in the distal metaphysis areas. At 6 months, the length difference between the *Dmp1*^{+/-} mice (d) and *Dmp1*^{-/-} mice (e) became greater. Both the distal and proximal metaphysis regions of 6-month-old *Dmp1*^{-/-} mice (e) and *Dmp1*^{-/-}; *Dspp*-Tg mice (f) showed apparent enlargement. At either 3 or 6 months, the femurs of *Dmp1*^{-/-}; *Dspp*-Tg mice (c, f) were longer than those of the *Dmp1*^{-/-} mice (b, e). Quantitative analyses (g) showed the differences in average femur length between the *Dmp1*^{-/-}; *Dspp*-Tg and *Dmp1*^{-/-} mice at 3 months (12.94 mm vs. 11.08 mm) and 6 months (15.1 mm vs. 13.5 mm) were statistically significant. $P < 0.05$ (Student's *t*-test) was considered significant. Data represent mean $\pm$ SD ($n = 5$). When the letters above two bars are different, the difference between the two groups was statistically significant ($P < 0.05$).

Fig. 3. Micro-computed tomography (μ -CT) analyses of femurs from 3- and 6-month-old mice
a-f: full views of the femurs at lower resolution; g-l: longitudinal-section views of the femurs at lower resolution. Full views of the whole femurs revealed that at both ages, the femurs of the *Dmp1*^{+/-} mice (a, d) had a smooth surface while those of the *Dmp1*^{-/-} (b, e) and *Dmp1*^{-/-};*Dspp*-Tg (c, f) mice showed a porous appearance. Longitudinal-section views demonstrated that compared to the *Dmp1*^{+/-} controls (g, j), the inner surfaces of the femur shells of the *Dmp1*^{-/-} (h, k) and *Dmp1*^{-/-};*Dspp*-Tg (i, l) mice were rougher. At either 3 or 6 months, the bone in the *Dmp1*^{-/-};*Dspp*-Tg mice appeared to have fewer surface porosities than in the *Dmp1*^{-/-} mice in both the full and longitudinal-section views. Scale bar: a to l = 500 μ m

Fig. 4. High resolution imaging and quantitative μ -CT analyses of cortical bone

a-c: high resolution views of the cortical mid-shaft regions of femurs at 3 months; g-i: high resolution views of the cortical mid-shaft regions of femurs at 6 months. At both ages, the cortical bone in the mid-shaft region was thinner in the *Dmp1^{-/-}* mice (b, h) than in the *Dmp1^{+/-}* mice (a, g), while the femur thickness of *Dmp1^{-/-};Dspp-Tg* mice (c, i) was similar to that of *Dmp1^{+/-}* mice. Scale bars: 100 μ m in all images.

The quantitative analyses of the cortical thickness validated the imaging result at 3 months (d) and 6 months (j) of ages; the cortical thickness in *Dmp1^{-/-};Dspp-Tg* mice was restored to a level similar to that of the *Dmp1^{+/-}* mice. The bone volume fraction (BV/TV) in the diaphyseal mid-shaft region in the femurs of *Dmp1^{-/-};Dspp-Tg* mice was significantly greater than in the *Dmp1^{-/-}* mice; at 6 months, the improvement of BV/TV in the *Dmp1^{-/-};Dspp-Tg* mice was more remarkable (e, k). The bone density represented by the density of total volume (Apparent Density) and density of bone volume (Material Density) was significantly reduced in *Dmp1^{-/-}* mice compared to the *Dmp1^{+/-}* mice at 3 months (f) and 6 months (l). The transgenic expression of *Dspp* significantly improved the apparent and material densities at 3 months. At 6 months, the apparent density of the *Dmp1^{-/-};Dspp-Tg* mice was significantly higher than in *Dmp1^{-/-}* mice, but the change in the material density was not statistically significant. $P < 0.05$ (Student's *t*-test) was considered significant. Data represent mean $\pm$ SD ($n = 5$ for d and j; $n = 6$ for e, f, k and l). Different letters above two bars indicate a significant difference ($P < 0.05$) between the two groups.

Fig. 5. High resolution imaging and quantitative μ -CT analyses of trabecular bone

a-c: high resolution views of the distal metaphysis regions at 3 months; g-i: high resolution views of the distal metaphysis regions at 6 months. High resolution images of the distal metaphysis regions showed that in comparison with the *Dmp1^{+/-}* mice (a, g), *Dmp1^{-/-}* mice (b, h) and *Dmp1^{-/-};Dspp-Tg* mice (c, i) had a remarkable reduction in trabecular bone number. Scale bars: 100 μ m in all images. Colored scale bars indicate the trabecular thickness ranging from 0.00 mm (blue) to 0.07 mm (red). Quantitative analyses revealed that at 6 months (j), but not at 3 months (d), the trabecular number was significantly higher in the *Dmp1^{-/-};Dspp-Tg* mice than in *Dmp1^{-/-}* mice. The most remarkable change of trabeculae in *Dmp1^{-/-};Dspp-Tg* mice was the restoration of trabecular thickness to nearly normal level at both ages (e, k). The spacing between the trabeculae in *Dmp1^{-/-};Dspp-Tg* mice, at both 3 months (f) and 6 months (l), although not completely reduced to the normal level of *Dmp1^{+/-}* control mice, was significantly lower than that of *Dmp1^{-/-}* mice. $P < 0.05$ (Student's *t*-test) was considered significant. Data represent mean $\pm$ SD ($n = 6$ for d-l). Different letters above two bars indicate a significant difference ($P < 0.05$) between the two groups.

Fig. 6. Histological analyses of the femurs from 3-month-old mice

a-f: H&E staining; the images in the middle row are higher magnification views of boxed areas in the upper row; g-i: Picrosirius red staining of the cortical bone in the mid-shaft region of the femurs. The growth plate (red arrows) was wider and cortical bone was thinner in the *Dmp1*^{-/-} mice (b) than in the *Dmp1*^{+/-} mice (a) or *Dmp1*^{-/-}; *Dspp*-Tg mice (c). The cortical bone in the *Dmp1*^{-/-} mice (e) appeared to have more hypomineralized areas or osteoid areas (stained grey, indicated by arrowheads) than in the *Dmp1*^{+/-} mice (d) or the *Dmp1*^{-/-}; *Dspp*-Tg mice (f). Picrosirius red staining, which specifically stains collagen,

showed that while the cortical bone of *Dmp1^{+/-}* mice (g) had well-organized fibrillary network with fewer large (yellow/orange) fibers and more reticular (green) fibers. The collagen network was highly disorganized in *Dmp1^{-/-}* mice (h) with obvious reduction in the reticular fibers. *Dmp1^{-/-};Dspp-Tg* mice (i) showed a notable improvement in collagen fibril organization. Scale bars: 500 μ m in a-c; 100 μ m in d-f; 50 μ m in g-i

Fig. 7. Scanning electron microscopy (SEM) analyses of the femurs from 3-month-old mice
a-c: Backscattered SEM imaging of cortical bone from midshaft region of tibia; d-f: acid-etched SEM imaging of cortical bone from midshaft region of tibia; g-i: FITC staining of cortical bone from the same region. The backscattered SEM imaging showed spindle-shaped osteocyte lacunae that were well organized and well oriented in the bone of *Dmp1*^{+/-} mice (a). The cortical bone of *Dmp1*^{-/-} mice (b) and *Dmp1*^{-/-};*Dspp*-Tg mice (c) showed fewer and disoriented osteocyte lacunae. The acid etched SEM analyses revealed that the osteocytes in the *Dmp1*^{+/-} mice (d) were narrow with many osteocyte processes in the canaliculi that were running perpendicular to the cell bodies. The osteocyte lacunae in *Dmp1*^{-/-};*Dspp*-Tg mice (f) were slightly more enlarged and ovoid with fewer canaliculi than the in *Dmp1*^{+/-} mice, but were smaller than the rounded lacunae in *Dmp1*^{-/-} mice (e) that had lost nearly all of the canaliculi. FITC staining showed the lacunocanicular system was well organized with numerous canaliculi in the *Dmp1*^{+/-} mice (g). *Dmp1*^{-/-} mice (h) had a remarkable reduction in the number of canaliculi while the *Dmp1*^{-/-};*Dspp*-Tg mice (i)

showed a partial restoration in the number of canaliculi. Scale bars in a-c: 20 μm ; in d-f: 5 μm ; in g-i: 10 μm .

Fig. 8. Immunohistochemical analyses of the femurs from 3-month-old mice

All of the images were from the mid-shaft region of the femurs. Immunohistochemistry against DMP1 (a-c) showed that the *Dmp1*^{+/-} mice (a) demonstrated positive anti-DMP1 signals in the cortical bone, which were relatively stronger around the osteocyte lacunae, whereas there was a complete loss of signals for this molecule in *Dmp1*^{-/-} mice (b) and *Dmp1*^{-/-}; *Dspp*-Tg mice (c). Immunohistochemistry against biglycan (d-f), which often serves to reflect the amount of osteoid in the bone matrix, showed that compared to the *Dmp1*^{+/-} mice (d), the femurs of *Dmp1*^{-/-} mice (e) had much more biglycan. The level of biglycan in *Dmp1*^{-/-}; *Dspp*-Tg mice (f) was remarkably lower than in the *Dmp1*^{-/-} mice but slightly higher than in the *Dmp1*^{+/-} mice. The signals for BSP, OPN and MEPE were increased in *Dmp1*^{-/-} mice (h, k, n), compared to the *Dmp1*^{+/-} controls (g, j, m), while the levels of these proteins in *Dmp1*^{-/-}; *Dspp*-Tg mice (i, l, o) were comparable to the *Dmp1*^{+/-} mice. FGF23 level was increased in *Dmp1*^{-/-} mice (q), compared to the *Dmp1*^{+/-} mice (p). FGF23 level was slightly reduced in *Dmp1*^{-/-}; *Dspp*-Tg mice (r) compared to the *Dmp1*^{-/-} mice. Scale bars: 50 μ m in all images.

Fig. 9. Real Time Quantitative PCR (RT-qPCR) analyses

RT-qPCR analyses of RNA samples from the femurs of 3-month-old mice showed that compared to the *Dmp1*^{+/-} control, *Dmp1*^{-/-} mice had an approximately 9 fold increase of BSP mRNA level (a); in *Dmp1*^{-/-}; *Dspp*-Tg mice, BSP mRNA level was about 2 fold of the normal. The changes of OPN mRNA levels (b) showed a similar pattern. The mRNA level of MEPE (c) in *Dmp1*^{-/-} mice was 5 fold over normal, while it was restored completely back to normal in *Dmp1*^{-/-}; *Dspp*-Tg mice. Col1a1 mRNA levels (d) were restored close to normal in *Dmp1*^{-/-}; *Dspp*-Tg mice.

Fig. 10. *In Situ* hybridization analyses

In situ hybridization showed the presence of DMP1 mRNA in the osteoblasts (arrows) of newly formed trabecular bone immediately subjacent to the growth plate in the *Dmp1*^{+/-} mice (a). The *Dmp1*^{-/-} mice (b) and *Dmp1*^{-/-}; *Dspp*-Tg mice (c) had a complete lack of signals for DMP1. The same region of *Dmp1*^{-/-} mouse femurs (e, h) had elevated levels of BSP and OPN mRNA, compared to the *Dmp1*^{+/-} mice (d, g). The BSP and OPN mRNA levels in *Dmp1*^{-/-}; *Dspp*-Tg mice (f, i) were similar to the *Dmp1*^{+/-} mice. Col1a1 mRNA

level was higher in *Dmp1^{-/-}* mice (k), which was also reduced in *Dmp1^{-/-};Dspp-Tg* mice (l), to a level similar to the *Dmp1^{+/-}* mice (j). Scale bar: 50 μ m in all images.

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Table 1

Primer sequences used for Real Time quantitative PCR

Gene	Primer sequences	Product size	T _m (°C)
<i>Dspp</i>	Forward: 5'-AACTCTGTGGCTGTGCCTCT-3' Reverse: 5'-TATTGACTCGGAGCCATTCC-3'	171	59
<i>Bsp</i>	Forward: 5'-AAAGTGAAGGAAAGCGACGA-3' Reverse: 5'-GTCCTTCTGCACCTGCTTC-3'	215	52
<i>Opn</i>	Forward: 5'-TCTGATGAGACCGTCACTGC-3' Reverse: 5'-AGGTCCTCATCTGTGGCATC-3'	170	53
<i>Mepe</i>	Forward: 5'-CTGTGGATCCTTGTGAGAAT-3' Reverse: 5'-TAGAGGATTTTGGCTTTCTG-3'	199	55
<i>Collal</i>	Forward: 5'-CCTGACGCATGGCCAAGAAGA-3' Reverse: 5'-GCATTGCACGTCATCGCACA-3'	145	60
<i>Gapdh</i>	Forward: 5'-CAAAGTTGTCATGGATGACC-3' Reverse: 5'-CCATGGAGAAGGCTGGGG-3'	195	56

(Note: T_m Melting Temperature)