Roles of DMP1 Processing in Osteogenesis, Dentinogenesis and Chondrogenesis

Yao Sun^{a, b} Li Chen^c Su Ma^d Jin Zhou^e Hua Zhang^a Jian Q. Feng^a
Chunlin Qin^a

^aDepartment of Biomedical Sciences, Baylor College of Dentistry, Texas A&M Health Science Center, Dallas, Tex., USA; Departments of ^bDental Implants, ^cEndodontics, and ^dPeriodontics, School of Stomatology, Harbin Medical University, and ^eDepartment of Hematology, The First Affiliated Hospital of Harbin Medical University, Harbin, China

Key Words

Dentin matrix protein 1 • Proteolytic processing • Osteogenesis • Dentinogenesis • Chondrogenesis

Abstract

Dentin matrix protein 1 (DMP1) is an acidic protein that plays critical roles in osteogenesis and dentinogenesis. Protein chemistry studies have demonstrated that DMP1 primarily exists as processed NH₂- and COOH-terminal fragments in the extracellular matrix of bone and dentin. Our earlier work showed that the substitution of Asp²¹³ (a residue at a cleavage site) by Ala²¹³ blocks the processing of mouse DMP1 in vitro. Recently, we generated transgenic mice expressing this mutant DMP1 (designated 'D213A-DMP1'). By crossbreeding these transgenic mice with *Dmp1*-knockout (*Dmp1*-KO) mice, we obtained mice expressing the D213A-DMP1 transgene in the *Dmp1*-null background (named '*Dmp1*-KO/D213A-Tg' mice). In this study, we analyzed the long bone, mandible, dentin, and cartilage of *Dmp1*-KO/D213A-Tg mice in comparison with wild-type, *Dmp1*-KO, and *Dmp1*-KO mice expressing the normal DMP1 transgene (*Dmp1*-KO/normal-Tg). Our results showed that D213A-DMP1 was barely cleaved in the dentin matrix of *Dmp1*-KO/D213A-

Tg mice and the expression of D213A-DMP1 failed to rescue the developmental defects in *Dmp1*-null mice. Interestingly, enlarged growth plates and condylar cartilages were observed in *Dmp1*-KO/D213A-Tg mice, indicating a potential role of the full-length form of DMP1 in chondrogenesis.

Copyright © 2011 S. Karger AG, Basel

Introduction

Dentin matrix protein 1 (DMP1) was first identified using a rat odontoblast mRNA library [George et al., 1993] which was later shown to be also expressed in bone and other tissues [D'Souza et al., 1997; Hirst et al., 1997; MacDougall et al., 1998; Qin et al., 2007]. Loss of DMP1

Abbreviations used in this paper

BMP-1	bone morphogenetic protein-1
DMP1	dentin matrix protein 1
HE	hematoxylin and eosin
IHC	immunohistochemistry
ISH	in situ hybridization
KO	knockout
NCPs	noncollagenous protein
WT	wild type

Yao Sun and Li Chen contributed equally to this work.

leads to osteomalacia/rickets and dentin hypomineralization, indicating that DMP1 plays important roles in osteogenesis and dentinogenesis [Ye et al., 2004, 2005; Feng et al., 2006].

In the extracellular matrix of bone and dentin, DMP1 mainly occurs as proteolytically processed fragments originating from the NH₂-terminal and COOH-terminal regions of the DMP1 amino acid sequence, respectively. The NH₂-terminal fragment of DMP1 (designated as 'DMP1-N') exists in 2 forms, i.e. the 37-kDa core protein form and the proteoglycan form (designated as DMP1-PG), while the COOH-terminal fragment (designated as 'DMP1-C') is present as the 57-kDa fragment [Qin et al., 2003, 2006]. The full-length form of DMP1 has been detected at a lower level in the extracellular matrix of bone and dentin [Huang et al., 2008] and at a relatively high level in articular cartilage [Sun et al., 2010a, b].

In vitro mineralization studies have shown that DMP1-C and DMP1-N fragments promote the nucleation of hydroxyapatite crystals while full-length DMP1 and DMP1-PG inhibit their growth [Tartaix et al., 2004; Gericke et al., 2010]. Studies have shown that the localization of DMP1-N is different from that of DMP1-C in tooth and long bone [Maciejewska et al., 2009]. Our previous work showed that rat DMP1 is proteolytically processed into DMP1-N and DMP1-C at 4 peptide bonds: Phe¹⁸⁹-Asp¹⁹⁰, Ser¹⁹⁶-Asp¹⁹⁷, Ser²³³-Asp²³⁴, and Gln²³⁷-Asp²³⁸ [Qin et al., 2003]. An in vitro study demonstrated that bone morphogenetic protein-1 (BMP-1)/tolloid-like proteinases cleave the full-length form of rat DMP1 at peptide bond Ser¹⁹⁶-Asp¹⁹⁷ [Tartaix et al., 2004]. Moreover, the substitution of Asp²¹³ in mouse DMP1 (corresponding to Asp¹⁹⁷ in rat DMP1) effectively blocked the proteolytic processing of mouse DMP1 in eukaryotic cells [Peng et al., 2009]. Recently, we generated transgenic mice expressing the D213A-DMP1 transgene in a *Dmp1*-null background (named '*Dmp1*-KO/D213A-Tg' mice), and D213A-DMP1 was barely cleaved in bone [Sun et al., 2010c]. In this report, we analyze the phenotypic changes in the bone, dentin and cartilage expressing this mutant D213A-DMP1 in the *Dmp1*-null background.

Materials and Methods

Generation of Transgenic Mice

The details of the generation of *Dmp1*-knockout (*Dmp1*-KO) mice, *Dmp1*-KO/D213A-Tg mice, and *Dmp1*-KO mice expressing the normal DMP1 transgene (*Dmp1*-KO/*normal*-Tg) have been described in previous publications [Feng et al., 2003; Lu et al., 2007, 2010; Sun et al., 2010c].

Detection of DMP-Related Products in Dentin Matrix and Digestion of DMP1 by BMP-1

Noncollagenous proteins (NCPs) were extracted from the dentin matrix of 6-week-old wild-type (WT), *Dmp1*-KO, *Dmp1*-KO/D213A-Tg, and *Dmp1*-KO/*normal*-Tg mice. The NCPs, including DMP1, were analyzed by Stains-All staining and Western immunoblotting [Sun et al., 2010c]. Stains-All staining was employed to examine full-length DMP1 and/or its processed fragments. Western immunoblotting was carried out to confirm the presence/absence of DMP1. To test whether BMP-1 cleaves DMP1 isolated from dentin, normal DMP1 (from *Dmp1*-KO/*normal*-Tg mice) and D213A-DMP1 (from *Dmp1*-KO/D213A-Tg mice) were incubated with recombinant human BMP-1 (R&D Systems, Minneapolis, Minn., USA) as previously described [Steiglitz et al., 2004]. For Western immunoblotting, the polyclonal anti-DMP1-C-857 antibody [Maciejewska et al., 2009] was used.

X-Ray Analyses

Femurs were dissected from 1-year-old WT, *Dmp1*-KO, *Dmp1*-KO/D213A-Tg, and *Dmp1*-KO/*normal*-Tg mice. These specimens were analyzed by high-resolution X-ray radiography (piXarray 100; Micro Photonics) at 5 s/30 kV.

Histology, Immunohistochemistry, and in situ Hybridization Analyses

Hematoxylin and eosin (HE) staining was used to evaluate the phenotypic changes in the long bone, incisors, and cartilage of those mice. Immunohistochemistry (IHC) and in situ hybridization (ISH) were used to evaluate the expression of normal DMP1/D213A-DMP1 in the long bone, incisors, and cartilage of those mice. For IHC, the monoclonal anti-DMP1-C-8G10.3 antibody was used [Baba et al., 2004]. ISH was performed as previously described [Lu et al., 2007].

Results

Identification of DMP1 in Dentin Extracts and Its Digestion by BMP-1

Stains-All staining showed that the dentin of *Dmp1*-KO/D213A-Tg mice had significant amounts of full-length DMP1 and a negligible quantity of DMP1 fragments (fig. 1a). The enzyme digestion experiments showed that the normal full-length DMP1 isolated from *Dmp1*-KO/*normal*-Tg mice was cleaved by BMP-1, while the mutant D213A-DMP1 isolated from the dentin of *Dmp1*-KO/D213A-Tg mice was hard to cleave (fig. 1b).

X-Ray Analyses

The femurs of *Dmp1*-KO/D213A-Tg mice were shorter and had a lower density than those of WT or *Dmp1*-KO/*normal*-Tg mice. Thus, the bone development defects in *Dmp1*-KO/D213A-Tg mice remained similar to those in *Dmp1*-KO mice (fig. 1c).

Fig. 1. Detection of DMP1 in dentin extracts, digestion of DMP1 by BMP-1, and X-ray images of femurs. **a** NCPs were extracted from the dentin of 6-week-old WT (lane 1), *Dmp1*-KO (lane 2), *Dmp1*-KO/*D213A*-Tg (lane 3), and *Dmp1*-KO/*normal*-Tg (lane 4) mice. Stains-All staining was performed to detect DMP1-related components. Full-length DMP1 (black arrow) was clearly observed in the dentin extracts from *Dmp1*-KO/*D213A*-Tg mice (lane 3), while DMP1-N and DMP1-C fragments (black arrowheads) were seen in the dentin extracts from WT and *Dmp1*-KO/*normal*-Tg mice (lanes 1 and 4). DPP (strong blue bands signaled by a red arrowhead). **b** Digestion of DMP1 by BMP-1. The digestion products were analyzed by Western immunoblotting using

the anti-DMP1-C-857 antibody. Lane 1 was 0.2 μ g purified DMP1 extracted from rat dentin (positive control). The normal DMP1 (lane 3, from the dentin of *Dmp1*-KO/*normal*-Tg) and *D213A*-DMP1 (lane 5, from *Dmp1*-KO/*D213A*-Tg) were incubated with BMP-1. Note that most of the full-length form of normal DMP1 was processed into fragments (lane 3) by BMP-1 while *D213A*-DMP1 was not (lane 5). Also note that the full-length form of *D213A*-DMP1 appeared as doublets; at this point, we are unsure as to why it occurred as 2 bands. **c** X-ray images of the femurs of 1-year-old WT, *Dmp1*-KO, *Dmp1*-KO/*D213A*-Tg, and *Dmp1*-KO/*normal*-Tg mice. Note that the femurs of *Dmp1*-KO or *Dmp1*-KO/*D213A*-Tg mice were shorter.

Histological Analyses of Bone and Dentin

HE staining demonstrated that the bone and dentin phenotypes of *Dmp1*-KO/*D213A*-Tg mice were different from those of WT mice (fig. 2). Compared to those of WT mice of the same age, the femurs of 1-year-old *Dmp1*-KO/*D213A*-Tg mice were shorter; the metaphysis region was disorganized, and more osteoid was observed in the cortical bone area. In the incisors, the predentin in *Dmp1*-KO/*D213A*-Tg mice was wider than that in WT mice (note the distance between the 2 black arrows in fig. 2g and h).

Phenotypic Changes in the Cartilage of *Dmp1*-KO/*D213A*-Tg Mice and Expression of DMP1 in the Growth Plate of Tibia

The growth plate of *Dmp1*-KO/*D213A*-Tg mice was remarkably thicker than that of WT mice. More layers of chondrocytes were observed in the hypertrophic layer and proliferative layer in the enlarged growth plate of *Dmp1*-KO/*D213A*-Tg mice. The organization of the growth plate in *Dmp1*-KO/*D213A*-Tg mice was better than in *Dmp1*-KO mice. The condylar cartilage of the mandibles of *Dmp1*-KO/*D213A*-Tg mice was also enlarged (fig. 3). In the growth plate, the distribution of DMP1 in *Dmp1*-KO/*D213A*-Tg mice was different from that in WT

Fig. 2. Histological analyses. **a, b** HE staining of the femurs of 1-year-old WT and *Dmp1*-KO/*D213A*-Tg mice. **c, d** Enlarged views of the cortical bone (insets in **a, b**) from the diaphysis region. **e, f** The cross sections of the incisors of 1-year-old WT and *Dmp1*-KO/*D213A*-Tg mice were analyzed. **g, h** Enlarged views of the predentin/dentin region (insets in **e, f**). Compared to WT

mice, the femurs of 1-year-old *Dmp1*-KO/*D213A*-Tg mice were shorter and the metaphysis region was disorganized, with more osteoid in the cortical bone area. In the incisors, the predentin of *Dmp1*-KO/*D213A*-Tg mice was wider than that of WT mice. DE = Dentin; OD = odontoblast; PD = predentin; EN = enamel.

mice. In WT mice, signals of normal DMP1 mRNA and protein were mainly observed in cells of the hypertrophic layer, while in *Dmp1*-KO/*D213A*-Tg mice signals of DMP1 were primarily found in cells of the proliferative layer (fig. 3).

Discussion

Previously we hypothesized that proteolytic processing of DMP1 may be essential for the biological functions of this protein in biomineralization [Qin et al., 2007]. The results of this study show that the bone and dentin phenotypes of 1-year-old *Dmp1*-KO/*D213A*-Tg mice were similar to those of *Dmp1*-KO mice. In addition, the expression of this *D213A*-DMP1 failed to rescue the phenotypic changes in the long bone of 6-week-old *Dmp1*-KO mice [Sun et al., 2010c], but the normal DMP1 could [Lu et al., 2010; Sun et al., 2010c]. All of these results provide strong support to our belief that DMP1 processing is essential to the formation of bone and dentin. The present study also shows that BMP-1 is the proteinase responsible for the proteolytic processing of DMP1 at the NH₂-terminus of Asp²¹³ in mouse DMP1 [Steiglitz et al., 2004].

The growth plate and condylar cartilage in *Dmp1*-KO/*D213A*-Tg mice were remarkably thicker than those in WT and *Dmp1*-KO mice. Previous studies reported an enlargement of the growth plate cartilage in *Dmp1*-KO mice, which was believed to be a result of the decrease in serum phosphate level [Feng et al., 2006]. It has been shown that the serum phosphate level in *Dmp1*-KO/*D213A*-Tg mice is higher than in *Dmp1*-KO mice [Sun et al., 2010c], whereas the growth plate and condylar cartilage in *Dmp1*-KO/*D213A*-Tg mice is even thicker than in *Dmp1*-KO mice. Thus, a lower level of phosphate could not well explain the extraordinary thickening of the cartilage in *Dmp1*-KO/*D213A*-Tg mice at the age of 6 weeks. It has also recently become known that, compared with WT mice, the major phenotypic change in the growth plate of 6-week-old or older *Dmp1*-KO mice is disorganization but not enlargement [Lu et al., 2010; Sun et al., 2010c]. Combined with the finding that a large amount of full-length DMP1 exists in cartilage matrix [Sun et al., 2010a, b], we speculate that the full-length form of DMP1 may play a certain role in chondrogenesis. The observation that the cellular organization of cartilage in *Dmp1*-KO/*D213A*-Tg mice was much better than in *Dmp1*-KO mice also supports this speculation. Clearly, further investigations are warranted to evaluate

Fig. 3. Phenotypic changes in the cartilage of *Dmp1*-KO/*D213A*-Tg mice and expression of DMP1 in the growth plate of the tibia. All samples were from 6-week-old mice. **a–c** The growth plate of *Dmp1*-KO mice was disorganized, and the cells in the *Dmp1*-KO growth plate lost their columnar arrangement. The growth plate of *Dmp1*-KO/*D213A*-Tg mice was thicker than that of either WT or *Dmp1*-KO mice, and the organization of the growth plate in *Dmp1*-KO/*D213A*-Tg mice was better than that in *Dmp1*-KO mice. **d–f** HE staining for the mandibular condylar cartilage from WT, *Dmp1*-KO, and *Dmp1*-KO/*D213A*-Tg mice. Similar to the findings

in the growth plate, the condylar cartilage of *Dmp1*-KO/*D213A*-Tg mice was thicker than that of WT or *Dmp1*-KO mice. **g–i** ISH demonstrated positive signals of DMP1 in the chondrocytes of both WT and *Dmp1*-KO/*D213A*-Tg mice. In WT mice, the positive signals in the growth plate were mainly detected in the hypertrophic layers, while in *Dmp1*-KO/*D213A*-Tg mice the signals were mainly present in the proliferative layer. **j–l** IHC staining of growth plates revealed a distribution pattern of DMP1 protein similar to the results obtained by ISH.

the role of full-length DMP1 in the development of cartilage. As articular cartilage, mandibular condylar cartilage expresses both type I and type II collagen. Thus, it is understandable that the D213A-DMP1 transgene (driven by a type I collagen promoter) was expressed in condylar cartilage and might contribute to the formation of articular cartilage. However, we do not know why the D213A-DMP1 transgene was also expressed in the growth plate, which normally does not express type I collagen.

In summary, we strongly believe that the proteolytic processing of DMP1 is essential to its functions in mineralized and cartilaginous tissues. We further envision

that DMP1 fragments are functional forms in the mineralization of bone and dentin [Gericke et al., 2010], while the uncleaved full-length DMP1 may play a role in the development of cartilage.

Acknowledgements

This work was supported by NIH Grant DE 005092 (to C.Q.), Department of Science and Technology of Heilongjiang Province of China – Gongguan Project Grant GC09C412-1, and Department of Education of Heilongjiang Province of China – Overseas Scholar Grant 1155h013.

References

- Baba, O., C. Qin, J.C. Brunn, J.N. Wygant, B.W. McIntyre, W.T. Butler (2004) Colocalization of dentin matrix protein 1 and dentin sialoprotein at late stages of rat molar development. *Matrix Biol* 23: 371–379.
- D'Souza, R.N., A. Cavender, G. Sunavala, J. Alvarez, T. Ohshima, A.B. Kulkarni, M. MacDougall (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: 2040–2049.
- Feng, J.Q., H. Huang, Y. Lu, L. Ye, Y. Xie, T.W. Tsutsui, T. Kunieda, T. Castranio, G. Scott, L.B. Bonewald, Y. Mishina (2003) The Dentin matrix protein 1 (Dmp1) is specifically expressed in mineralized, but not soft, tissues during development. *J Dent Res* 82: 776–780.
- Feng, J.Q., L.M. Ward, S. Liu, Y. Lu, Y. Xie, B. Yuan, X. Yu, F. Rauch, S.I. Davis, S. Zhang, H. Rios, M.K. Drezner, L.D. Quarles, L.F. Bonewald, K.E. White (2006) Loss of DMP1 causes rickets and osteomalacia and identifies a role for osteocytes in mineral metabolism. *Nat Genet* 38: 1310–1315.
- George, A., B. Sabsay, P.A. Simonian, A. Veis (1993) Characterization of a novel dentin matrix acidic phosphoprotein: implications for induction of biomineralization. *J Biol Chem* 268: 12624–12630.
- Gericke, A., C. Qin, Y. Sun, R. Redfern, D. Redfern, Y. Fujimoto, H. Taleb, W.T. Butler, A.L. Boskey (2010) *J Dent Res* 89: 355–359.
- Hirst, K.L., D. Simmons, J. Feng, H. Aplin, M.J. Dixon, M. MacDougall (1997) Elucidation of the sequence and the genomic organization of the human dentin matrix acidic phosphoprotein 1 (DMP1) gene: exclusion of the locus from a causative role in the pathogenesis of dentinogenesis imperfecta type II. *Genomics* 42: 38–45.
- Huang B., I. Maciejewska, Y. Sun, T. Peng, D. Qin, Y. Lu, L. Bonewald, W.T. Butler, J. Feng, C. Qin (2008) Identification of full-length dentin matrix protein 1 in dentin and bone. *Calcif Tissue Int* 82: 401–410.
- Lu, Y., L. Ye, S. Yu, S. Zhang, Y. Xie, M.D. McKee, Y.C. Li, J. Kong, J.D. Eick, S.L. Dallas, J.Q. Feng (2007) Rescue of odontogenesis in Dmp1-deficient mice by targeted re-expression of DMP1 reveals roles for DMP1 in early odontogenesis and dentin apposition in vivo. *Dev Biol* 303: 191–201.
- Lu, Y., B. Yuan, C. Qin, Z. Cao, Y. Xie, S. Dallas, M.D. McKee, M.K. Drezner, L.F. Bonewald, Jian Q. Feng (2010) The biological function of DMP1 in osteocyte maturation is mediated by its 57-kDa C-terminal fragment. *J Bone Miner Res*, in press.
- MacDougall, M., T.T. Gu, X. Luan, D. Simmons, J. Chen (1998) Identification of a novel isoform of mouse dentin matrix protein 1: spatial expression in mineralized tissues. *J Bone Miner Res* 13: 422–431.
- Maciejewska, I., C. Cowan, K. Svoboda, W.T. Butler, R. D'Souza, C. Qin (2009) The NH₂-terminal and COOH-terminal fragments of dentin matrix protein 1 (DMP1) localize differently in the compartments of dentin and growth plate of bone. *J Histochem Cytochem* 57: 155–166.
- Peng, T., B. Huang, Y. Sun, Y. Lu, L. Bonewald, S. Chen, W.T. Butler, J.Q. Feng, R.N. D'Souza, C. Qin (2009) Blocking of proteolytic processing and deletion of glycosaminoglycan side chain of mouse DMP1 by substituting critical amino acid residues. *Cells Tissues Organs* 189: 192–197.
- Qin, C., J.C. Brunn, R.G. Cook, R.S. Orkiszewski, J.P. Malone, A. Veis, W.T. Butler (2003) Evidence for the proteolytic processing of dentin matrix protein 1: identification and characterization of processed fragments and cleavage sites. *J Biol Chem* 278: 34700–34708.
- Qin, C., R. D'Souza, J.Q. Feng (2007) Dentin matrix protein 1 (DMP1): new and important roles for biomineralization and phosphate homeostasis. *J Dent Res* 86: 1134–1141.
- Qin, C., B. Huang, J.N. Wygant, B.W. McIntyre, C.H. McDonald, R.G. Cook, W.T. Butler (2006) A chondroitin sulfate chain attached to the bone dentin matrix protein 1 NH₂-terminal fragment. *J Biol Chem* 281: 8034–8040.
- Steiglitz BM, M. Ayala, K. Narayanan, A. George, D.S. Greenspan DS (2004) Bone morphogenetic protein-1/Tolloid-like proteinases process dentin matrix protein-1. *J Biol Chem* 279: 980–986.
- Sun, Y., V. Gandhi, M. Prasad, W. Yu, X. Wang, Q. Zhu, J.Q. Feng, R.J. Hinton, C. Qin (2010a) Distribution of small integrin-binding ligand, N-linked glycoproteins (SIBLING) in the condylar cartilage of rat mandible. *Int J Oral Maxillofac Surg* 39: 272–281.
- Sun, Y., S. Ma, J. Zhou, A. Yamoah, J.Q. Feng, R.J. Hinton, C. Qin (2010b) Distribution of small integrin-binding ligand, N-linked glycoproteins (SIBLING) in the articular cartilage of the rat femoral head. *J Histochem Cytochem* 58: 1033–1043.
- Sun, Y., M. Prasad, T. Gao, X. Wang, Q. Zhu, R. D'Souza, J.Q. Feng, C. Qin (2010c) Failure to process dentin matrix protein 1 (DMP1) into fragments leads to its loss of function in osteogenesis. *J Biol Chem* 285: 31713–31722.
- Tartaix, P.H., M. Doulaverakis, A. George, L.W. Fisher, W.T. Butler, C. Qin, E. Salih, M. Tan, Y. Fujimoto, L. Spevak, A.L. Boskey (2004) In vitro effects of dentin matrix protein-1 on hydroxyapatite formation provide insights into in vivo functions. *J Biol Chem* 279: 18115–18120.
- Ye, L., M. MacDougall, S. Zhang, Y. Xie, J. Zhang, Z. Li, Y. Lu, Y. Mishina, J.Q. Feng (2004) Deletion of dentin matrix protein-1 leads to a partial failure of maturation of predentin into dentin, hypomineralization, and expanded cavities of pulp and root canal during postnatal tooth development. *J Biol Chem* 279: 19141–19148.
- Ye, L., Y. Mishina, D. Chen, H. Huang, S.L. Dallas, M.R. Dallas, P. Sivakumar, T. Kunieda, T.W. Tsutsui, A. Boskey, L.F. Bonewald, J.Q. Feng (2005) Dmp1-deficient mice display severe defects in cartilage formation responsible for a chondrodysplasia-like phenotype. *J Biol Chem* 280: 6197–6203.