A CRUCIAL ROLE FOR MMP-2 IN OSTEOCYTIC CANALICULAR FORMATION AND BONE METABOLISM

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Extracellular matrix production and degradation by bone cells are critical steps in bone metabolism. Mutations of the gene encoding matrix metalloproteinase (MMP)-2, an extracellular matrix-degrading enzyme, are associated with a human genetic disorder characterized by subcutaneous nodules, arthropathy, and focal osteolysis. It is not known how the loss of MMP-2 function results in the pathology. Here, we show that Mmp2−/− mice exhibited opposing bone phenotypes due to an impaired osteocytic canalicular network. Mmp2−/− mice showed decreased bone mineral density in the limb and trunk bones, but increased bone volume in the calvariae. In the long bones, there was moderate disruption of the osteocytic networks and reduced bone density throughout life, while osteoblast and osteoclast function was normal. In contrast, aged but not young Mmp2−/− mice had calvarial sclerosis with osteocyte death. Severe disruption of the osteocytic networks preceded osteocyte loss in Mmp2−/− calvariae. Successful transplantation of wild-type periosteum restored the osteocytic canalicular networks in the Mmp2−/− calvariae, suggesting local roles of MMP-2 in determining bone phenotypes. Our results indicate that MMP-2 plays a crucial role in forming and maintaining the osteocytic canalicular network, and we propose that osteocytic network formation is a determinant of bone remodeling and mineralization.

Bone is continuously remodeled to adopt a volume appropriate for the local environment; the amount of bone deposited depends on the balance between bone formation and resorption by bone cells, osteoblasts, osteoclasts, and osteocytes (1). Osteoblasts are bone-forming cells that differentiate from mesenchymal stem cells and secrete extracellular matrix (ECM) proteins, which are subsequently mineralized. Osteoclasts are bone-resorbing cells that differentiate from hematopoietic stem cells, and degrade bone ECM proteins after demineralization in the extracellular space (Howship’s lacunae) adjacent to the ruffled borders. In contrast to osteoblasts and osteoclasts, which act at bone surfaces, osteocytes, cells of osteoblastic lineage, are embedded in bone and are terminally differentiated. Osteocytes extend their dendritic processes into the bone matrix to constitute a well-developed canalicular network with other cells. Although osteocytes are the most abundant cell type in bone tissue, their role in...
bone metabolism is not firmly established.

ECM production and degradation by bone cells are critical steps in bone metabolism (1), and disturbed ECM turnover leads to bone disease. Type I collagen is a major ECM component. Secreted type I collagen molecules are processed by propeptidases and cross-linked by lysyl oxidases into mature collagen. Mutations of genes encoding type I collagen cause the bone disease osteogenesis imperfecta (2). Type I collagens are mainly degraded by matrix metalloproteinases (MMPs), which exert their enzymatic activity at a neutral pH in a zinc-ion-dependent manner (3,4). Several MMPs are expressed in bone tissue (5-9). MMPs may play a role in osteoclastic bone resorption (4,5). Osteoblasts and osteocytes also produce MMPs such as MMP-2 and MMP-13 (7-9). Recent linkage analysis suggests that a loss of function mutation of MMP2 causes a human autosomal recessive disorder with multicentric nodulosis, arthropathy with joint erosion and osteolysis, termed NAO syndrome (10,11). This syndrome also includes facial abnormalities and generalized osteoporosis (12-14). The finding that NAO syndrome is caused by a loss of MMP-2 activity raises questions about how an ECM-degrading enzyme affects bone volume (10,11).

We previously generated mice devoid of MMP-2 by gene targeting, and observed a subtle retardation in their growth rate (15). In the present study, we found marked skeletal abnormalities in the Mmp2-/- mice with decreased mineralization of long bones but increased bone deposition with osteocytic death in the calvariae. The data suggest that mild impairment in canalicular formation affects bone mineralization, but complete disruption of the osteocytic networks enhances osteoblastic activity and increases bone formation. We propose that osteocytes, through their canalicular networks, control osteoblast function and possibly modulate secondary mineralization.

**Experimental Procedures**

**Animals** - The MMP-2-deficient mice (15) used in this study were generated previously and backcrossed 9 to 11 times to C57BL/6J mice. Homozygous mutants were obtained by crossing heterozygotes. The Mmp2-/- mice were maintained in the animal facility of the RIKEN Brain Science Institute, with dry food pellets and water available ad libitum. The Colla1<sup>OE</sup> mice (16,17) were maintained in the animal facility at the Massachusetts General Hospital. All experimental protocols were approved by the Institutional Animal Care and Use Committees.

**Radiographic measurement** - For soft X-ray radiograms, mice were subjected to X-irradiation at 25 mA for 2 s. For bone mineral density (BMD) measurement, whole bodies and isolated bones were scanned with a Lunar PIXImus2 densitometer (GE Yokogawa Medical Systems, Tokyo, Japan).

**Peripheral quantitative computed tomography (pQCT) analysis** - For pQCT analyses, isolated bones were subjected to XCT Research SA+ (Stratec Medizintechnik GmbH, Pforzheim, Germany). Femoral bones were scanned in two 0.46-mm thick slices with a 0.08-mm voxel size. The slice 1.4 mm from the distal growth plate was used for cancellous BMD, and the slice 5.5 mm from the distal growth plate was used for cortical BMD. Calvarial bones were scanned at a 0.46-mm slice thickness and 0.05-mm voxel size. The coronal slice at the middle of parietal bones was used for calvarial BMD.

**Bone histomorphometry** - Tibial and femoral bones were fixed in 70% ethanol, and the non-decalcified bones were embedded in methylmethacrylate. For cancellous bone, longitudinal sections of tibial bone (3 μm thick) were stained with toluidine blue. Measurements were made at 400x magnification on a minimum of 20 optical fields between 450 and 1650 μm from the epiphyseal growth plate, and 150 μm from the lateral cortices of the secondary spongiosa. For cortical bone, cross sections (25 μm thick) of the femoral mid-shafts were stained with toluidine blue. Measurements were made at 400x magnification on a minimum of 12 optical fields. Calvarial bones were fixed in 70% ethanol, and the non-decalcified bones were embedded in glycomethacrylate. Coronal sections (3 μm thick) were cut and stained with toluidine blue. Measurements were made at 400x magnification on a minimum of 12 optical fields at 0.3 mm from the calvarial midline to both lateral sides. The mineral apposition rate was calculated by
measuring the interval between two calcein-labeled lines following two intraperitoneal injections of calcein (4.0 mg/kg each). Nomenclature, symbols, and units used are those recommended by the Nomenclature Committee of the American Society of Bone and Mineral Research (18).

**Histology** - Bones were isolated and fixed in 4% paraformaldehyde, decalcified in 25% formic acid for 4 to 14 d at room temperature, and embedded in paraffin. For MMP-2 staining, anti-human MMP-2 monoclonal antibody 42-5D11 (Daiichi, Takaoka, Japan) was used at 10 μg/ml. For dentin matrix protein 1 (DMP1) and sclerostin (the SOST gene product) staining, anti-rat DMP1 polyclonal antibody M176 (Takara Bio, Ohtsu, Japan) and anti-mouse SOST polyclonal antibody MAB1589 (R&D Systems, Minneapolis, MN) were used at 2 μg/ml and 50 μg/ml, respectively, after 0.25% trypsin/EDTA treatment at 37°C for 15 min.

**Staining of bone canaliculi** - Bone canaliculi were stained using the previously described Bodian method with minor modifications (19,20). De-paraffinized sections were stained with protein-silver solution containing copper balls at 37°C for 24 h and reduced in 1% hydroquinone and 5% formalin for 30 min. The sections were then treated with 0.5% gold chloride solution for 50 min and 2% oxalic acid solution for 60 min. Finally, they were dehydrated and embedded for microscopic observation.

**Cell culture** - Bone marrow was flushed from the femora, and the cells were plated at 1 x 10^6 cells/well. For mineralized nodule formation, cells were cultured for 25 d in Dulbecco’s modified Eagle’s medium (D-MEM) containing 50 μg/ml ascorbic acid and 10 mM beta-glycerophosphate. The mineralized nodules were stained with alizarin red. For osteoclast formation, cells were cultured in D-MEM containing 10 nM 1, 25(OH)2 vitamin D3 and 100 nM dexamethasone for 10 d, and visualized by tartrate resistant acid phosphatase staining. Calvarial primary cells were obtained from the outgrowth cultures of bone fragments of calvariae taken from 55-wk-old Mmp2+/- and Mmp2-/- mice. The cells were reseeded at 3 x 10^4 cells/well into 96-well plates and cultured in D-MEM containing 0.5% or 5% fetal bovine serum. Three days after plating, the cells were subjected to 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay and alkaline phosphatase assay, for measuring proliferation and osteoblastic differentiation, respectively.

**Transplantation** - Calvarial periosteal tissues were stripped from 6-wk-old Mmp2+/- and Mmp2-/- mice and washed in phosphate buffered saline. The tissues were placed onto the incised heads of 10-d-old Mmp2-/- mice and the skins were stitched. Four Mmp2+/- and three Mmp2-/- donor mice, and seven Mmp2+/- recipient mice were used. Two Mmp2+/- mice, which were littermates of Mmp2-/- recipient mice, were used as positive controls. The calvarial tissues of the recipient mice were harvested 2 weeks after transplantation and subjected to fixation, decalcification, and paraffin-embedding. De-paraffinized sections were subjected to MMP-2 staining and subsequently to the Bodian method.

**Statistical analysis** - All comparisons were made with the Mann-Whitney U-test and values are expressed as means ± SEM. A p value of less than 0.05 was considered significant. n.s., not significant; *, p < 0.05; **, p < 0.01; ***, p < 0.001.

**RESULTS**

**Mmp2-/- mice have facial abnormalities and other bone alterations**

Mmp2-/- mice had facial abnormalities such as short snouts, hypertelorism, and dome-shaped heads (Fig. 1A). The skulls of 45-wk-old Mmp2+/- mice also differed from those of Mmp2+/- (wild-type) mice, with deformed parietal bones and sutures (Fig. 1B). The jaws of Mmp2-/- mice were ~ 20% smaller than those of Mmp2+/- mice (data not shown), and Mmp2-/- mice had a smaller body size (Fig. 1C) (15). Radiographic analyses also revealed osteopenia in Mmp2-/- mice (Fig. 1C). There was medullary widening with cortical thinning of the femoral bones. Spontaneous fractures often occurred in the tibiae in mice 3 mo of age and older.
Dual-energy-X-ray absorptiometry (DEXA) revealed reduced BMD per unit area in several bones of both 7- and 35-wk-old Mmp2−/− mice [Fig. 1D; 7 wk, 55.1 ± 0.6 (+/+) vs 52.3 ± 0.7 (−/−) mg/cm², p < 0.01; 35 wk, 67.4 ± 0.7 (+/+) vs 62.0 ± 0.6 (−/−) mg/cm², p < 0.01]. Osteopenia and facial abnormalities are features of NAO syndrome (12-14). Although the skulls of 45-wk-old and 60-wk-old Mmp2−/− mice had higher BMD [Fig. 1E; 85.3 ± 1.4 (+/+) vs 92.1 ± 1.8 (−/−) mg/cm², p < 0.01], the skulls of 7-wk-old Mmp2−/− mice were thicker than those of Mmp2+/+ mice (Fig. 5A and B). Sclerotic changes occurred only in the calvarial bones. Thus, we observed a novel result of the Mmp2-null mutation, increased calvarial BMD. Some patients with NAO do have sclerotic sutures (13). Heterozygous (Mmp2+/−) mice do not have skeletal abnormalities (not shown), consistent with the inheritance pattern in human NAO syndrome. Gender effects were not observed in the knockout mice, nor are they reported in NAO patients. Arthropathy, characteristic of human NAO syndrome (12-14), was not observed in Mmp2+/− mice at any age (data not shown). The Mmp2−/− mice, however, are more susceptible to antibody-induced arthritis (21), suggesting that some additional factors, environmental or genetic, could be involved in the pathogenesis of arthritis in human NAO syndrome. In addition, the focal osteolysis of NAO syndrome was not observed in the Mmp2+/− mice.

**Decreased matrix mineralization in the long bones of Mmp2−/− mice with no evidence of osteoblastic and osteoclastic dysfunction**

To analyze the bone alterations in Mmp2−/− mice in detail, we performed pQCT analyses and histomorphometric analyses on long bones in 7-wk-old Mmp2−/− mice. Long bone is composed of two structure types, cancellous and cortical bone (Fig. 2A) (1). There was no significant difference in cancellous bone matrix volume between genotypes (Fig. 2F). The pQCT analyses revealed that BMD per unit volume was significantly reduced in both cancellous and cortical bone [Fig. 2B-E; cancellous bone (D), 235.5 ± 3.8 mg/cm³ (+/+) vs 197.3 ± 3.8 mg/cm³ (−/−), p < 0.001; cortical bone (E), 928.0 ± 6.6 mg/cm³ (+/+) vs 822.2 ± 9.5 mg/cm³ (−/−), p < 0.001]. In cortical bone, there were more insufficiently mineralized regions (<690 mg/cm³; Fig. 2B and C).

Histomorphometric analyses revealed that the mineral apposition rate (MAR) was unchanged in Mmp2−/− mice [Fig. 3A, B and D]. These morphometric data suggested that the timing and rate of matrix mineralization were normal in Mmp2−/− mice. None of the parameters reflecting the in vivo properties of osteoblasts and osteoclasts was significantly different (Fig. 3A, B, and D). Furthermore, cultures of bone marrow cells obtained from Mmp2−/− mice developed normally mineralized nodules and tartrate-resistant acid phosphatase-positive osteoclasts in vitro (Fig. 4A). These in vivo and in vitro observations suggest that osteoblast and osteoclast functions are not appreciably altered in Mmp2−/− mice. Thus, the results suggest that “secondary” mineralization (22,23) is decreased in the limb bones of Mmp2−/− mice. Secondary mineralization, a slow and gradual maturation of the bone mineral component, is measured by quantitative microradiography and was not performed here. Decreases in secondary mineralization are usually due to high rates of bone turnover and a shortening of the life span of basic structural units; high rates of bone turnover, however, were not found in Mmp2−/− mice.

**Bone formation is enhanced in the calvariae of Mmp2−/− mice**

Calvarial thickness and BMD were normal at 7 wk of age [Fig. 5A and B]. Histomorphometric analyses of the calvariae demonstrated normal bone formation in Mmp2−/− calvariae. The matrix was fully mineralized in Mmp2−/− calvariae at this age. The thickness and BMD of Mmp2−/− calvaria, however, were significantly increased by 55 wk of age [Fig. 5A and B; thickness, 254 ± 7 μm (+/+) vs 376 ± 12 μm (−/−); p < 0.001, BMD, 851.7 ± 4.3 mg/cm³ (+/+) vs 1051.5 ± 3.0 mg/cm³ (−/−), p < 0.0001]. Consistent with these findings, osteoblastic activities, represented by MAR and the ratio of bone formation rate to bone surface (BFR/BS), were significantly increased at 55 wk of age (Fig. 5D), suggesting that the augmented bone
formation resulted in the sclerosis of aged Mmp2+/− calvariae. Osteoblastic cells derived from Mmp2+/− calvariae, however, had proliferation and differentiation properties identical to those of cells derived from Mmp2+/+ calvariae in vitro (Fig. 4B). Thus, in vivo-specific mechanisms likely induce calvarial osteoblast hyperactivity in aged Mmp2+/− mice.

**Osteocytic cell death is increased in the calvariae of Mmp2+/− mice, not in the long bones**

Further exploration of bone sections revealed that the ratio of empty lacunae increased with age only in Mmp2+/− calvariae [Fig. 6A and B; 3 wk, 2.8 ± 0.3 % (+/+ vs 2.3 ± 1.0 % (-/-), p > 0.05; 11 wk, 17.1 ± 1.9 % (+/+ vs 30.0 ± 3.2 % (-/-), p < 0.001; 55 wk, 20.0 ± 1.8 % (+/+ vs 52.9 ± 1.6 % (-/-), p < 0.001]. A large number of osteocytes in Mmp2+/− calvariae were positive for TdT-mediated dUTP-biotin nick end labeling at 9 wk of age, suggesting that the empty lacunae resulted from apoptotic cell death (data not shown). In contrast, the ratios of empty lacunae in Mmp2+/+ femora were comparable to those in Mmp2+/− femora at any age examined (Fig. 6A and B). There were no differences between genotypes in other bones (Fig. 6C). Thus, the calvaria-specific bone phenotype is closely associated with osteocytic cell death and is caused by the loss of the osteocytic canalicular network.

**MMP-2-deficiency affects development of the canalicular network in both long bones and calvariae**

The above data demonstrated alterations in structure and deposition of bone in long bones and calvariae in Mmp2+/− mice, in spite of the normal intrinsic properties of the osteoblasts and osteoclasts. To assess the MMP-2 sites of action in bone, immunohistochemistry was performed in sections of femora and calvariae. MMP-2 was detected in the vicinity of osteocytes in both bone types (Fig. 7A and C). One characteristic feature of bone matrix is a well-developed cellular network (1) comprised of osteocytes, which have their cell bodies in the lacunae and extensive dendritic processes in the canalicular channels throughout bone. At a higher magnification, MMP-2 was observed in and around the osteocytic lacunae as well as along the canicular channels in femora and calvaria (Fig. 7A and C). There were strong MMP-2 signals close to the bone surface, which represented newly-formed osteocytic canaliculi (Fig. 7A insert). The bone-surface mature osteoblasts extended their processes after being embedded in the bone matrix and differentiating into mature osteocytes. These findings suggest that MMP-2 contributes to form and/or maintain the osteocytic network. To examine the role of MMP-2 in the osteocytic network, we stained bone sections using the Bodian method to visualize osteocytic canaliculi (19,20). Bodian staining revealed a significant impairment of the fine, slender structures of the osteoblastic/osteocytic network in Mmp2+/− femora [Fig. 7E and G; number of connections between pairs of adjacent lacunae per section, 10.9 ± 0.6 (+/+ vs 3.7 ± 0.3 (-/-), p < 0.001; number of processes protruding from a lacuna per section, 25.9 ± 1.0 (+/+ vs 17.1 ± 0.7 (-/-), p < 0.001]. This network was disrupted in Mmp2+/− calvariae [Fig. 7F and G; number of connections, 6.4 ± 0.3 (+/+ vs 0.3 ± 0.2 (-/-), p < 0.001; number of processes, 13.0 ± 0.5 (+/+ vs 8.3 ± 0.6 (-/-), p < 0.001]. Thus, Mmp2+/− mice had a moderately disrupted osteocytic canalicual network in the femora and a severely disrupted osteocytic canalicarial network in the calvariae.

**Restored canalicular formation by wild-type periosteal transplantation**

To determine whether MMP-2 activity is required locally for osteocytic canalicular formation, we transplanted the periosteal tissues of Mmp2+/− or Mmp2+/+ mice onto the calvariae of 10-d-old Mmp2+/− mice. Two weeks after the transplantation, we examined osteocytic canalicular networks in the recipient mice. Osteocytic canalicular networks were successfully formed in one of four Mmp2+/− mice transplanted with Mmp2+/− periosteum (Fig. 8A). To avoid nonspecific osteocyte damage in the recipients, we did not irradiate the recipients before transplantation. Thus, the low rate of canalicular formation may be explained in part by immunological rejection of some of the periosteal transplants. The newly formed osteocytic canaliculi were located in areas adjacent to the transplanted periosteum; MMP-2 was also
identified in these areas by immunohistochemistry. We did not observe any formation of osteocytic canaliculi in the sections from the recipients transplanted with Mmp2\(^{-/-}\) periosteum. These results suggest that MMP-2 acts locally to form osteocytic canaliculi.

**Differential localizations of DMP-1 and sclerostin in Mmp2\(^{-/-}\) long bones and calvarial bones**

The results just described indicated differences in osteocyte canalicular networks in the long bones and calvariae of Mmp2\(^{-/-}\) mice. To study the properties of osteocytes in the two types of bone, we analyzed the expression of two representative molecules associated with osteocytes: DMP-1 and sclerostin. DMP-1 is a non-collagenous, acidic glycoprotein that presumably has a role in matrix mineralization of bones and teeth. In bone, DMP-1 is localized adjacent to osteocyte cell bodies and processes (24,25). Recent studies demonstrate that matrix mineralization is severely impaired in the long bones of Dmp1\(^{-/-}\) mice (26,27). Interestingly, we found using immunohistochemistry decreased expression of DMP-1 in long bones of young Mmp2\(^{-/-}\) mice, compared with expression in Mmp2\(^{+/+}\) mice. The reduction was particularly evident in the osteocytic processes, rather than the cell bodies, reflecting the reduced canalicular number in Mmp2\(^{-/-}\) long bones (Fig. 8B). Thus, a loss of canalicular localization of DMP-1 may underlie deficits in matrix mineralization in long bones from Mmp2\(^{-/-}\) mice. In Mmp2\(^{-/-}\) calvariae, however, the expression of DMP-1 was also reduced (Fig. 8B).

Sclerostin decreases bone formation by inhibiting osteoblast proliferation and differentiation and promoting osteoblast apoptosis (28-30). Sclerostin localized mainly on the osteocytic cell bodies in Mmp2\(^{+/+}\) calvariae. Although sclerostin immunoreactivity was not appreciably altered in long bones from Mmp2\(^{-/-}\) mice, sclerostin immunoreactivity in Mmp2\(^{-/-}\) calvariae increased on the walls of filled and empty lacunae, as well as in the osteocytic cell bodies (Fig. 8C). These changes were observed in both young and aged Mmp2\(^{-/-}\) calvariae. Sclerostin is thought to be transported from osteocytes to osteoblasts via the canalicular networks (28,29).

Sclerostin must accumulate in the osteocytic lacunae in Mmp2\(^{-/-}\) mouse calvariae since the canalicular networks are ablated or not formed. These results suggest that interrupted transport of sclerostin from osteocytes to osteoblasts removes the inhibition of osteoblastic bone formation and, in addition, augments apoptosis in Mmp2\(^{-/-}\) calvariae.

**Analysis of osteocytic canalicular networks in collagenase-resistant Coll1a1\(^{-/-}\) mice**

We demonstrated that Mmp2\(^{-/-}\) mice have decreased matrix mineralization with moderately disrupted osteocytic canalicular networks in long bones as well as increased bone formation with severely disrupted osteocytic canalicular network in calvariae. The contrasting bone phenotypes could be accounted for by differences in the magnitude of disruption of the osteocytic networks. We therefore analyzed the osteocytic canaliculi of the Coll1a1\(^{-/-}\) mice that deposit a collagenase-resistant type I collagen. Type I collagen is a major constituent of bone ECM and type I collagen is a substrate for MMP-2 (31). As Coll1a1\(^{-/-}\) mice age, the bone mass in long bones and calvariae increases, which is associated with significant osteocytic cell death and osteoblastic hyperactivity (16,17). We compared Bodian-stained sections of wild-type and Coll1a1\(^{-/-}\) mice at 3 wk of age, an early stage in which there is little sclerotic change (17). The calvarial sections of Coll1a1\(^{-/-}\) mice had severe canalicular impairment similar to that in Mmp2\(^{-/-}\) mice [Fig. 9A and B; number of connections, 8.6 ± 0.5 (+/+) vs 0.0 ± 0.0 (r/r), p < 0.001; number of processes, 18.2 ± 0.8 (+/+) vs 2.1 ± 0.3 (r/r), p < 0.001]. Similar changes were observed in the femora of Coll1a1\(^{-/-}\) mice [Fig. 9A and B; number of connections, 14.1 ± 0.6 (+/+) vs 1.5 ± 0.5 (r/r), p < 0.001; number of processes, 29.8 ± 1.6 (+/+) vs 4.0 ± 1.0 (r/r), p < 0.001]. Thus, consistent with the findings from Mmp2\(^{-/-}\) calvariae, severe disruption of osteocytic canaliculi was associated with sclerotic changes in long bones and calvariae in Coll1a1\(^{-/-}\) mice. In contrast to the Mmp2\(^{-/-}\) long bones, however, osteocyte apoptosis was prominent in the long bones in Coll1a1\(^{-/-}\) mice (17).

**DISCUSSION**
In the present study, we found alterations in bone formation, mineralization, and structure due to impaired formation of the osteocytic canalicular network in Mmp2\(^{-/-}\) mice. It is possible that MMP-2 activity results in the formation of spaces in the ECM around the canaliculi before mineralization is completed, and that osteocytes control bone mass by suppressing osteoblast activity to a steady state level through the canalicular network. Seemingly opposite changes, bone reduction in long bones and bone increase in calvariae, can be explained by the extent of impairment in each bone type; reduced canaliculi in long bones and loss of canaliculi in calvariae.

Mmp2\(^{-/-}\) mice had calvarial sclerosis that increased as the mice aged. Although we observed no differences in osteoblastic and osteoclastic activities in cells prepared from Mmp2\(^{-/-}\) mice ex vivo, osteoblastic activity was increased in Mmp2\(^{-/-}\) calvaria in vivo, consistent with findings in Coll1\(^{+/+}\) mice (16,17). Coll1\(^{+/+}\) mice have bone sclerosis with enhanced osteoblastic activity in vivo. These results strongly suggest that in vivo-specific mechanisms, presumably functioning through the osteocyte-osteoblast network, suppress osteoblastic activity (28,33) as was postulated for Coll1\(^{+/+}\) mice (17). The possibility of negative regulation of osteoblastic activity by osteocytes has recently been emphasized. Sclerostin, secreted by osteocytes, inhibits osteoblast proliferation and differentiation and promotes osteoblast apoptosis (28-30). Loss of function mutations of \(SOST\) cause sclerosteosis and Van Buchem disease (34,35). In this regard, in Mmp2\(^{+/+}\) calvariae, augmented expression of sclerostin was evident around the osteocytic lacunae, consistent with the idea that loss of the canalicular network disturbs the transport from osteocytes to osteoblasts. As a result, osteocytes exposed to excess sclerostin undergo apoptotic cell death, and osteoblasts in the periosteum in the absence of sclerostin are released from inhibition and make more bone. Furthermore, osteocytes and osteoblasts are electrically synchronized through gap junctions (1,36-38). A severely impaired canalicular network could additionally interrupt electrical signals between osteocytes and osteoblasts.

We propose, based on our results as described, that in contrast to calvariae, a moderately impaired canalicular network accounts for the reduced BMD in long bones of Mmp2\(^{-/-}\) mice. There was no osteocyte apoptosis or alteration of osteoblast activities in long bones of Mmp2\(^{-/-}\) mice suggesting that osteocyte-osteoblast communication was maintained. A moderate decrease in canalicular number might not interfere with sclerostin function or gap-junctional electrical signals through the canalicular network. What, then, are the cellular and molecular mechanisms underlying BMD reduction in the long bones of Mmp2\(^{-/-}\) mice? We propose that the osteocytic canalculi provide the primary locus for mineral exchange. Osteocytic processes increase the physical surface area of bone. A reduced number of canaliculi then leads to a reduced surface area. Mmp2\(^{-/-}\) mice had normal MAR in long bones, indicating that the rate of mineral apposition, by itself, is unchanged. Long bones of Mmp2\(^{-/-}\) mice, however, were not fully mineralized (pQCT analysis). The canalicular space is a site where mineral ions are exchanged between bone and extracellular fluid in the processes of resorption and mineralization of the organic matrix (39). The canalicular space is also a site for extracellular phosphorylation of matrix proteins which bind mineral ions (40,41). Reduced canalicular number/density in the long bones of Mmp2\(^{-/-}\) mice could result in decreased surface area and ultimately, decreased secondary mineralization (22,23). DMP-1 is one of the acidic non-collagenous phosphoproteins that binds calcium ions and interacts with type I collagen and can function as an inorganic mineral phase nucleator (24,25). Mineral/matrix ratios are significantly reduced in the long bones of Dmp1\(^{-/-}\) mice at the age of 4 and 16 wk (26,27). In the long bones of Mmp2\(^{-/-}\) mice, the decreased canalicular number may lead to a reduction in the spaces in which DMP-1 binds to type I collagen for proper matrix mineralization.

We suggest that degradation of the ECM by MMP-2 and other MMPs is involved in the process of canalicular development. Thus, in Coll1\(^{+/+}\) mice, where type I collagen cannot be cleaved by any MMP collagenase, the formation of canaliculi is impaired in calvariae as well as in long bones. The formation of canaliculi is also impaired in MT1-MMP-deficient mice (42). MT1-MMP is also a collagenase. The significance
of the observations in the MT1-MMP-deficient mice remains to be established in view of the complex phenotype of the MT1-MMP-null mice with unexplained early postnatal death, usually within weeks of birth (43, 44). It is pertinent, however, that in vitro studies indicate that cell-surface MT1-MMP converts pro-MMP-2 into its active form (3, 45); decreased activation of pro MMP-2 is reported in MT1-MMP-deficient mice (44). It is also pertinent that mechanical loading regulates the expression of MMP-2 (46, 47). Thus, it is likely that MT1-MMP and MMP-2 are both involved in the formation of the bone canalicular network.

We suggest that the differential canalicular impairment among bone types is affected by the magnitude of the redundancy of, and compensation by, another MMP such as MMP-13, which is highly expressed by osteocytes in long bones. MMP-13 can also be activated by MT1-MMP (48) and is localized along bone canalliculi (42), but we have not yet examined the canalicular network in bones of MMP-13-deficient mice (49, 50). It is not known whether humans with NAO syndrome and loss of function of MMP-2 have canalicular impairment. We also have no pertinent information to explain why the characteristic nodulosis, arthropathy, and focal osteolysis of the human disease are not observed in the Mmp2−/− mice.

In conclusion, this study established a causal relationship between the loss of MMP-2 and some components of the bone phenotype of human NAO syndrome. Osteocytes are the most abundant of the bone cell types, and form characteristic canalicular networks in the bones. Their function and significance, however, are not fully characterized. We propose possible mechanisms by which the osteocyte cellular networks contribute to bone metabolism.
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41. Suzuki, Y., Yamaguchi, A., Ikeda, T., Kawase, T., Saito, S., and Mikuni-Takagaki, Y.


**FOOTNOTES**

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The abbreviations used are: DMP-1, dentin matrix protein 1; D-MEM, Dulbecco’s modified Eagle’s medium; ECM, extracellular matrix; MMP, matrix metalloproteinase; NAO, nodulosis, arthropathy and osteolysis; DEXA, dual-energy-X-ray absorptiometry; BMD, bone mineral density; pQCT, peripheral quantitative computed tomography; MAR, mineral apposition rate; BFR/BS, ratio of bone formation rate to bone surface; ES/BS, ratio of eroded surface to bone surface.

FIGURE LEGENDS

Fig. 1. Altered skeletons in Mmp2−/− mice. A, Facial appearance of 35-wk-old Mmp2+/+ and Mmp2−/− mice. The Mmp2−/− mice have marked brachycephaly. B, Skull appearance of 45-wk-old mice. The mutant skull is distorted and oval. C, Roentgenographs of 25-wk-old Mmp2+/+ and Mmp2−/− mice. Bottom panels, femora. Shortened and osteopenic femora are observed in Mmp2−/− mice. D, Whole body bone mineral content determined by DEXA. n’s = 5 (7 wk, +/+), 5 (7 wk, −/−), 13 (35 wk, +/+), and 12 (35 wk, −/−). E, Bone mineral content of skull determined by DEXA. n’s = 10 (7 wk, +/+), 9 (7 wk, −/−), and 7 (45 wk and 60 wk for aged, +/+ and −/−). **, p < 0.01.

Fig. 2. Decreased mineralization of long bones in Mmp2−/− mice. A, Diagram of long bone. B, Representatives of cortical BMD by pQCT. C, The diagram indicates representative data showing mineral densities across the broadest diameter of the femora from Mmp2+/+ (black line) and Mmp2−/− (red line) mice. D, E, Quantification of BMD by pQCT (+/+, n = 6; −/−, n = 6). (D): cancellous bone, (E): cortical bone. F, Representative micrographs of tibia longitudinal sections at 7 wk of age. There was no significant difference in cancellous bone matrix volume between the two genotypes.

Fig. 3. Unaltered osteoblastic and osteoclastic properties in long bones of Mmp2−/− mice. A, Cancellous bone histomorphometry of 7-wk-old mice. None of the parameters reflecting osteoblastic and osteoclastic activities was altered in Mmp2−/− mice (n = 6 for each genotype). Left, MAR, representing the rate of osteoblast mineralization. Middle, BFR/BS, representing formed bone mass per year. Right, ES/BS, representing the ratio of osteoclast-eroded area per bone surface. B-D, Cortical bone histomorphometry of 7-wk-old mice. None of the parameters was changed at the endosteum or periosteum (n = 6 for each genotype). C, diagram of cortical bone. B, MAR and BFR/BS of the cortical periosteum. D, MAR, BFR/BS, and ES/BS of the cortical endosteum. Bone is resorbed only at the endosteum.

Fig. 4. Unaltered properties of osteoblastic and osteoclastic cells from Mmp2−/− mice in vitro. A, in vitro bone marrow cell cultures. Osteoblastic nodule formation and osteoclast formation rates were not different between genotypes. (n = 6 for each genotype and each assay). B, in vitro calvarial cell culture. Proliferation and osteoblastic differentiation were measured by 3-(4,5-dimethylthioazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay and alkaline phosphatase (ALP) assay, respectively. Proliferation and osteoblastic differentiation of cells from Mmp2−/− mice remained unchanged (n = 6 for each genotype).

Fig. 5. Sclerotic changes of calvarial bones of Mmp2−/− mice. A, Representative calvarial sections indicating the increased thickness of Mmp2−/− calvariae at 55 wk-old. Left end: midline suture. B, Quantification of the thickness (+/+, n = 6; −/−, n = 6; at each age) and BMD by pQCT (n = 5 per each genotype at 7 wk of age, n = 3 per each genotype at 55 wk of age). C, diagram of calvarial bone. D, upper row, MAR and BFR/BS of calvarial outer membrane. lower row, MAR and BFR/BS of calvarial inner membrane. These parameters were
significantly higher in Mmp2−/− calvaria at 55 wk of age. *, p < 0.05; **, p < 0.01; ***, p < 0.001.

Fig. 6. Osteocytic death in calvarial bones of Mmp2−/− mice. A, Hematoxylin and eosin-stained sections of 55-wk-old Mmp2+/+ and Mmp2−/− mice. Little osteocytic death was detected in Mmp2+/+ femora (upper columns); a large number of empty lacunae were detected in Mmp2−/− calvaria (lower columns). Bars, 50 μm. B, Ratio of femoral and calvarial empty lacunae at age 3 wk, 11 wk, and 55 wk of age. n = 4 ~ 10. C. The ratio of empty lacunae in various bone types at 70 wk of age. There were no significant changes in the lower jaw, clavicle, spine, and tibia (n = 3 for each genotype). ***, p < 0.001.

Fig. 7. MMP-2-deficiency affects the osteocytic network in both femoral and calvarial bones. A, Immunohistochemical staining of MMP-2 in 5-wk-old wild-type femur. Bars, 20 μm. MMP-2 signals (brown) are present in the dendritic processes. Asterisk, osteocytic lacunae. Right side of panels represents the endosteal side. B, No positive signals were detected in the Mmp2−/− femur. C, Immunohistochemical staining of MMP-2 in 5-wk-old wild-type calvaria. MMP-2 localized along the osteocytic canaliculus. D, No positive signals were detected in the Mmp2−/− calvaria. E, Bodian-stained femoral sections of Mmp2+/+ and Mmp2−/− mice, showing the disorganized osteocytic network in the mutant sections. Bars, 20 μm. F, Bodian-stained calvarial sections of Mmp2+/+ and Mmp2−/− mice, showing the complete lack of a canalicular network in the mutant sections. Bar, 20 μm. G, Quantification of osteocytic disorganization. Processes, number of processes extending from each osteocyte per section ([Femur, +/+; n = 20; −/−, n = 22]; [Calvaria, +/+; n = 24; −/− n = 16]); Connection, number of processes connecting two osteocytes per section ([Femur, +/+; n = 19; −/−, n = 18]; [Calvaria, +/+; n = 24; −/− n = 16]).

Fig. 8. MMP-2-dependent-osteocytic canalicul ar formation. A, Restored canaliculi in the calvarial bone of Mmp2−/− mouse. Calvarial bones of Mmp2−/− mice were transplanted with Mmp2+/− periosteal tissues. Immunohistochemistry for MMP-2 and Bodian-staining revealed successful reconstruction of osteocytic canalicul ar networks in the mutant calvaria. B, Immunohistochemical staining of DMP-1 in 4-wk-old Mmp2+/+ and Mmp2−/− mice. DMP-1 localization was seen in the osteocytic lacunae and canaliculi of Mmp2+/+ mice. Canalicul ar localization of DMP-1 was reduced in the bones of Mmp2−/− mice. Bar, 20 μm. C, Immunohistochemical staining of sclerostin in 8-mo-old Mmp2+/+ and Mmp2−/− mice. Localization of sclerostin was sifted from canalicul ar to lacunae and its density was increased in the calvariae of Mmp2−/− mice. Bar, 20 μm.

Fig. 9. Severe canalicul ar disruption is a common phenomenon observed in all bone types of Col1a1r/r mice. A, Bodian-stained femoral and calvarial sections of 3-wk-old wild-type (+/+) and Col1a1r/r (r/r) mice, showing complete disruption of the canalicul ar network in the Col1a1r/r sections. Bar, 20 μm. B, Quanti fi cation of the canalicul ar disorganization of Col1a1r/r mice. Processes, (Femur, +/+; n = 16; r/r, n = 10); (Calvaria, +/+; n = 10; r/r n = 12); Connection (Femur, +/+; n = 16; r/r, n = 10); (Calvaria, +/+; n = 10; r/r n = 12). ***, p < 0.001.

Fig. 10. Roles of osteocytic canalicul ar networks in bone metabolism. A, Summary of observations from Mmp2−/− and Col1a1r/r mice. B, Osteocytes extend their processes to other osteoblasts/osteocytes. Yellow circle, DMP-1. Red circle, Sclerostin. C, The osteocytic dendrites send signals to suppress osteoblastic cell activity (red arrow direction) and the canalicul ar space (lightly colored) around them is the site for matrix mineralization (yellow...
arrow direction). D, In long bones of Mmp2<sup>−/−</sup> mice, the less developed canalicular network reduces total mineralization space, leading to decreased bone matrix mineralization, while the network is sufficiently functional to regulate osteoblastic activities. Osteoblastic activity is fully suppressed by residual sclerostin. E, In calvariae of Mmp2<sup>−/−</sup> mice and bones of ColIa1<sup>−/−</sup> mice, a completely disrupted canalicular network removes the control of osteoblasts and their hyperactivity increases bone mass (darkly colored).
Figure 1

(A) +/+ -/-

(B) +/+ -/-

(C) +/+ -/-

(D) Whole body

(E) Calvaria
Figure 2

A

B

C

D

E

F

Cortical bone

Cancellous bone

Bone marrow

BMD (mg/cm³)

+(/+ -/-

1 8 15 22 29 36 43 50 57 64 71 78 85 92 99 106

1200 1000 800 600 400 200 0

BMD (mg/cm³)

Cortical bone

Bone marrow

Bone marrow

+(/+ -/-

1 8 15 22 29 36 43 50 57 64 71 78 85 92 99 106

1200 1000 800 600 400 200 0

BMD (mg/cm³)

Figure 3
Figure 4

A
Osteoblastic
Nodule Formation

Osteoclast Formation

B
MTT assay
ALP assay

0.5% FBS 5% FBS
0.5% FBS 5% FBS

n.s. n.s.
Figure 5

A

B

C

D

+/-

-/-

Thickness

7-wk

55-wk

7-wk 55-wk

***

n.s.

BMD

Suture

Calvarial bone

Brain

Skin

Inner Membrane

Outer Membrane

3m/

3m/

mm/cm/year

3m/

3m/

mm/cm/year

3m/

3m/

mm/cm/year

3m/

3m/

mm/cm/year
Figure 6

A

B

C
Figure 7

A

B

C

D

E

F

G

Connections (number)

Processes (number)

Femur Calvaria

Femur Calvaria
**Figure 8**

**A**

<table>
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<th>Donor periosteum</th>
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<th>Bodian stain</th>
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**B**

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**C**

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</table>
Figure 9

A

B

Connections

Processes

(number)

(number)

Femur  Calvaria

Femur  Calvaria

+/-r/r  r/r

+/-r/r  r/r

* * * * *
Figure 10

A

Osteocytic network

- Extremely developed
- Moderately developed
- Impaired
- Severely impaired

Calvariae

Limb & trunk bones

Mmp2

Col1

Mineralization

Osteocytic death

Osteoblastic hyperproliferation

Bone Decrease

Bone Increase

B

osteoblast

C

osteocyte

D

E
A crucial role for MMP-2 in osteocytic canalicular formation and bone metabolism

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