A Crucial Role for Matrix Metalloproteinase 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 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 caused by 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, whereas 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

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The abbreviations used are: ECM, extracellular matrix; DMP-1, dentin matrix protein 1; DMEM, Dulbecco's modified Eagle's medium; MMP, matrix metalloproteinase; NAO, nodulosis, arthropathy, and osteolysis; BMD, bone mineral density; pQCT, peripheral quantitative computed tomography; MAR, mineral apposition rate; BFR/BS, ratio of bone formation rate to bone surface.
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 9–11 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 Col1ai1−/− mice (16, 17) were maintained in the animal facility at Massachusetts General Hospital. All of the experimental protocols were approved by the Institutional Animal Care and Use Committees.

Radiographic Measurement—For soft x-ray radiograms, the 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 nondecalcified 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 400× magnification on a minimum of 20 optical fields with 450 and 1650 μm from the epiphysial growth plate and 150 μm from the lateral cortices of the secondary spongiosa. For cortical bone, cross-sections (25 μm thick) of the femoral midshafts were stained with toluidine blue. The measurements were made at 400× magnification on a minimum of 30 optical fields. Calvarial bones were fixed in 70% ethanol, and the nondecalcified bones were embedded in glycomethacrylate. Coronal sections (3 μm thick) were cut and stained with toluidine blue. The measurements were made at 400× 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). The nomenclature, symbols, and units used are those recommended by the Nomenclature Committee of the American Society of Bone and Mineral Research (18).

Histology—The bones were isolated and fixed in 4% paraformaldehyde, decalcified in 25% formic acid for 4–14 days 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 (DMP-1) and sclerostin (the SOST gene product) staining, anti-rat DMP-1 polyclonal antibody M176

![Figure 1. Altered skeletons in Mmp2−/− mice.](image-url)
Staining of Bone Canaliculi—Bone canaliculi were stained using the previously described Bodian method with minor modifications (19, 20). Deparaffinized 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 × 10^6 cells/well. For mineralized nodule formation, the cells were cultured for 25 days in Dulbecco’s modified Eagle’s medium (DMEM) containing 50 μg/ml ascorbic acid and 10 mM β-glycerophosphate. The mineralized nodules were stained with alizarin red. For osteoclast formation, cells were cultured in DMEM containing 10 nM 1, 25(OH)2 vitamin D3, and 100 nM dexamethasone for 10 days 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-week-old Mmp2+/+ and Mmp2−/− mice. The cells were cultured in DMEM. After reaching confluence, the cells were reseeded at 3 × 10^4 cells/well into 96-well plates and cultured in DMEM 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-week-old Mmp2+/+ and Mmp2−/− mice and washed in phosphate-buffered saline. The tissues were placed onto the incised heads of 10-day-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. Deparaffinized sections were subjected to MMP-2 staining and subsequently to the Bodian method.

**Statistical Analysis**—All of the comparisons were made with the Mann-Whitney U test, and the values are expressed as the means ± S.E. 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-week-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 months of age and older. Dual energy x-ray absorptiometry revealed reduced the BMD/unit area in several bones of both 7- and 35-week-old **Mmp2**/*−/−* mice (Fig. 1D; 7 weeks, 55.1 ± 0.6 (+/+) versus 52.3 ± 0.7 (−/−) mg/cm², p < 0.01; 35 weeks, 67.4 ± 0.7 (+/+) versus 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- and 60-week-old **Mmp2**/*−/−* mice had higher BMD (Fig. 1E; 85.3 ± 1.4 (+/+) versus 92.1 ± 1.8 (−/−) mg/cm², p < 0.01), the skulls of 7-week-old **Mmp2**/*−/−* mice had reduced BMD (Fig. 1E; 62.1 ± 0.5 (+/+) versus 55.8 ± 1.6 (−/−) mg/cm², p < 0.01). The calvarial bones of 55-week-old **Mmp2**/*−/−* mice were thicker than those of **Mmp2**/*+/+* mice (see Fig. 5, A 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 knock-out 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-week-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 the BMD/unit of volume was significantly reduced in both cancellous and cortical bone (Fig. 2, B–E; cancellous bone (D), 235.5 ± 3.8 mg/cm³ (+/+) versus 197.3 ± 3.8 mg/cm³ (−/−), p < 0.001; cortical bone (E), 928.0 ± 6.6 mg/cm³ (+/+) versus 822.2 ± 9.5 mg/cm³ (−/−), p < 0.001). In cortical bone, there were more insufficiently mineralized regions (<690 mg/cm³; Fig. 2, B and C).

Histomorphometric analyses revealed that the mineral apposition rate (MAR) was unchanged in **Mmp2**/*−/−* mice (Fig. 3, A, 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 osteoblastic and osteoclastic activities was altered in **Mmp2**/*−/−* mice (n = 6 for each genotype). Left panel, MAR, representing the rate of osteoblast mineralization. Middle panel, BFR/BS, representing formed bone mass/year. Right panel, ES/BS, representing the ratio of osteoclast-eroded area per bone surface. B–D, cortical bone histomorphometry of 7-week-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 the ratio of eroded surface to bone surface of the cortical endosteum (ES/BS). Bone is resorbed only at the endosteum.
MMP-2 in Bone Homeostasis

A

Osteoblastic Nodule Formation

Osteoclast Formation

B

MTT assay

ALP assay

FIGURE 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-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium 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).

ual 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 weeks of age (Fig. 5, A 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 weeks of age (Fig. 5, A and B; thickness, 254 ± 7 μm (+/+) versus 376 ± 12 μm (−/−); p < 0.001, BMD, 851.7 ± 4.3 mg/cm³ (+/+) versus 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 weeks of age (Fig. 5D), suggesting that the augmented bone formation resulted in the sclerosis of aged Mmp2−/− calvariae. Osteoblasts 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. 6, A and B; 3 weeks, 2.8 ± 0.3% (+/+) versus 2.3 ± 1.0% (−/−), p > 0.05; 11 weeks, 17.1 ± 1.9% (+/+) versus 30.0 ± 3.2% (−/−), p < 0.001; 55 weeks, 20.0 ± 1.8% (+/+) versus 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 weeks 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. 6, A 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, despite 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. 7, A 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 canalicular channels in femora and calvaria (Fig. 7, A and C). There were strong MMP-2 signals close to the bone surface, which represented newly formed osteocytic canaliculi (Fig. 7, inset). 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. 7, E and G; number of connections per section, 10.9 ± 0.6 (+/+) versus 3.7 ± 0.3 (−/−), p < 0.001; number of processes protruding from a lacuna per section, 25.9 ± 1.0 (+/+) versus 17.1 ± 0.7 (−/−), p < 0.001). This network was disrupted in Mmp2−/− calvariae (Fig. 7, F and G; number of connections, 6.4 ± 0.3 (+/+) versus 0.3 ± 0.2 (−/−), p < 0.001; number of processes, 13.0 ± 0.5 (+/+) versus 8.3 ±
0.6 (−/−), p < 0.001. Thus, Mmp2−/− mice had a moderately disrupted osteocytic canalicular network in the femora and a severely disrupted osteocytic canalicular 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-day-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 canaluli 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 canaluli.

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 noncollagenous, acidic glycoprotein that presumably has a role in matrix mineralization of bones and teeth. In bone, DMP-1 is localized adjacent to osteocyte cell

**FIGURE 5.** Sclerotic changes of calvarial bones of Mmp2−/− mice. A, representative calvarial sections indicating the increased thickness of Mmp2−/− calvariae at 55 weeks old. Left end, midline suture. B, quantification of the thickness (+/+ n = 6; −/− n = 6; at each age) and BMD by pQCT (n = 5 for each genotype at 7 weeks of age, n = 3 for each genotype at 55 weeks 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−/− calvariae at 55 weeks of age. *, p < 0.05; **, p < 0.01; ***, p < 0.001.
cell bodies in Mmp2<sup>−/−</sup> calvariae. Although sclerostin immunoreactivity was not appreciably altered in long bones from Mmp2<sup>−/−</sup> mice, sclerostin immunoreactivity in Mmp2<sup>−/−</sup> 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<sup>−/−</sup> 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<sup>−/−</sup> mouse calvariae because 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<sup>−/−</sup> calvariae.

Analysis of Osteocytic Canalicular Networks in Collagenase-resistant Col1a1<sup>r/r</sup> Mice—We demonstrated that Mmp2<sup>−/−</sup> 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 Col1a1<sup>r/r</sup> 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 Col1a1<sup>r/r</sup> 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 Col1a1<sup>r/r</sup> mice at 3 weeks of age, an early stage in which there is little sclerotic change (17). The calvarial sections of Col1a1<sup>r/r</sup> mice had severe canalicular impairment similar to that in Mmp2<sup>−/−</sup> mice (Fig. 9, A and B; number of connections, 8.6 ± 0.5 (+/+) versus 0.0 ± 0.0 (r/r), p < 0.001; number of processes, 18.2 ± 0.8 (+/+) versus 2.1 ± 0.3 (r/r), p < 0.001). Similar changes were observed in the femora of Col1a1<sup>r/r</sup> mice (Fig. 9, A and B; number of connections, 14.1 ± 0.6 (+/+) versus 1.5 ± 0.5 (r/r), p < 0.001; number of processes, 29.8 ± 1.6 (+/+) versus 4.0 ± 1.0 (r/r), p < 0.001). Thus, consistent with the findings from Mmp2<sup>−/−</sup> calvariae, severe disruption of osteocytic canaliculi was associated with sclerotic changes in long bones and calvariae in Col1a1<sup>r/r</sup> mice. In contrast to the Mmp2<sup>−/−</sup> long bones, however, osteocyte apoptosis was prominent in the long bones in Col1a1<sup>r/r</sup> mice (17).

**DISCUSSION**

In the present study, we found alterations in bone formation, mineralization, and structure caused by impaired formation of the osteocytic canalicular network in Mmp2<sup>−/−</sup> mice (Fig. 10). 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.
MMP-2 deficiency affects the osteocytic network in both femoral and calvarial bones. 5-week-old wild-type femur.

**A**, immunohistochemical staining of MMP-2 in 5-week-old wild-type femur. Bars, 20 μm. MMP-2 signals (brown) are present in the dendritic processes. Asterisk, osteocytic lacunae. The right sides of the panels represent the endosteal side. **B**, no positive signals were detected in the Mmp2<sup>+/−</sup> femur. **C**, immunohistochemical staining of MMP-2 in 5-week-old wild-type calvaria. MMP-2 localized along the osteocytic canaliculus in the mutant sections. Bars, 20 μm. **D**, no positive signals were detected in the Mmp2<sup>+/−</sup> calvaria. **E**, Bodian-stained femoral sections of Mmp2<sup>+/−</sup> and Mmp2<sup>+/−</sup> mice, showing the disorganized osteocytic network in the mutant sections. Bars, 20 μm. **F**, Bodian-stained calvarial sections of Mmp2<sup>+/−</sup> and Mmp2<sup>+/−</sup> 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/section (Femur, +/+, n = 20; −/−, n = 22; Calvaria, +/+, n = 24; −/−, n = 16). Connections, number of processes connecting two osteocytes per section (Femur, +/+, n = 24; −/−, n = 18; Calvaria, +/+, n = 24; −/−, n = 16).

**MMP-2 in Bone Homeostasis**

Mmp2<sup>−/−</sup> 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<sup>−/−</sup> mice ex vivo, osteoblastic activity was increased in Mmp2<sup>−/−</sup>-calvaria in vivo, consistent with findings in Col1a1<sup>−/−</sup> mice (16, 17). Col1a1<sup>−/−</sup> 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 (32, 33) as was postulated for Col1a1<sup>−/−</sup> 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<sup>−/−</sup> 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<sup>−/−</sup> mice. There was no osteocyte apoptosis or alteration of osteoblast activities in long bones of Mmp2<sup>−/−</sup> 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<sup>−/−</sup> mice? We propose that the osteocytic canaliculi 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<sup>−/−</sup> mice had normal MAR in long bones, indicating that the rate of mineral apposition, by itself, is unchanged. The long bones of Mmp2<sup>−/−</sup> 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 that 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 noncollagenous 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 Dmp-1−/− mice at the age of 4 and 16 weeks (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 Col1a1r/r 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-null 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 canaliculi (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<sup>−/−</sup> 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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