Loss of MMP-2 disrupts skeletal and craniofacial development and results in decreased bone mineralization, joint erosion and defects in osteoblast and osteoclast growth

Rebecca A. Mosig1, Oonagh Dowling1, Analisa DiFeo1, Maria Celeste M. Ramirez1, Ian C. Parker1, Etsuko Abe2,3, Janane Diouri7, Aida Al Aqeel8, James D. Wylie9, Samantha A. Oblander9, Stephen B. Doty7, Robert J. Majeska4, Mitchell B. Schaffler4 and John A. Martignetti1,5,6,*

1Department of Genetics and Genomic Sciences, 2Mount Sinai Bone Program, 3Department of Medicine, 4Department of Orthopedics, 5Department of Oncological Sciences, 6Department of Pediatrics, Mount Sinai School of Medicine, New York, NY 10029, USA, 7Mineralized Tissue Laboratory, Hospital for Special Surgery, New York, NY 10021, USA, 8Riyadh Armed Forces Hospital, Riyadh, Kingdom of Saudi Arabia, 9Department of Biomedical Engineering and Orthopedic Research Center, Lerner Research Institute, Cleveland Clinic Foundation, Cleveland, OH 44195, USA, 10Department of Molecular, Cellular and Developmental Biology, Yale University, New Haven, CT 06520, USA and 11Department of Experimental Medicine and Pathology, La Sapienza University, Rome, Italy

Received December 27, 2006; Revised and Accepted March 13, 2007

The ‘vanishing bone’ or inherited osteolysis/arthritis syndromes represent a heterogeneous group of skeletal disorders characterized by mineralization defects of affected bones and joints. Differing in anatomical distribution, severity and associated syndromic features, gene identification in each ‘vanishing bone’ disorder should provide unique insights into genetic/molecular pathways contributing to the overall control of skeletal growth and development. We previously described and then demonstrated that the novel autosomal recessive osteolysis/arthritis syndrome, multicentric osteolysis with arthritis (MOA) (MIM #605156), was caused by inactivating mutations in the \textit{MMP2} gene [Al Aqeel, A., Al Sewairi, W., Edress, B., Gorlin, R.J., Desnick, R.J. and Martignetti, J.A. (2000) Inherited multicentric osteolysis with arthritis: A variant resembling Torg syndrome in a Saudi family. \textit{Am. J. Med. Genet.}, 93, 11–18.]. These \textit{in vivo} results were counterintuitive and unexpected since previous \textit{in vitro} studies suggested that MMP-2 overexpression and increased activity, not deficiency, would result in the bone and joint features of MOA. The apparent lack of a murine model [Itoh, T., Ikeda, T., Gomi, H., Nakao, S., Suzuki, T. and Itohara, S. (1997) Unaltered secretion of beta-amyloid precursor protein in gelatinase A (matrix metalloproteinase 2)-deficient mice. \textit{J. Biol. Chem.}, 272, 22389–22392.] has hindered studies on disease pathogenesis and, more fundamentally, in addressing the paradox of how functional loss of a single proteolytic enzyme results in an apparent increase in bone loss. Here, we report that \textit{Mmp2}–/– mice display attenuated features of human MOA including progressive loss of bone mineral density, articular cartilage destruction and abnormal long bone and craniofacial development. Moreover, these changes are associated with markedly and developmentally restricted decreases in osteoblast and osteoclast numbers \textit{in vivo}. \textit{Mmp2}–/– mice have ~50% fewer osteoblasts and osteoclasts than control littermates at 4 days of life but these differences have nearly resolved by 4 weeks of age. In addition, despite normal cell numbers \textit{in vivo} at 8 weeks of life, \textit{Mmp2}–/– bone marrow cells are unable to effectively support

*To whom correspondence should be addressed at: Mount Sinai School of Medicine, 1425 Madison Ave, PO Box 1498, New York, NY 10029, USA. Tel: +1 2126596744; Fax: +1 2128492638; Email: john.martignetti@mssm.edu

1Present address: Department of Molecular Biology and Microbiology, Case Western Reserve University, Cleveland, OH 44106, USA.
osteoblast and osteoclast growth and differentiation in culture. Targeted inhibition of MMP-2 using siRNA in human SaOS2 and murine MC3T3 osteoblast cell lines resulted in decreased cell proliferation rates. Taken together, our findings suggest that MMP-2 plays a direct role in early skeletal development and bone cell growth and proliferation. Thus, Mmp2−/− mice provide a valuable biological resource for studying the pathophysiological mechanisms underlying the human disease and defining the in vivo physiological role of MMP-2.

INTRODUCTION

The multicentric osteolyses or ‘vanishing bone’ syndromes are a group of hereditary autosomal dominant and recessive skeletal disorders characterized by marked and progressive bone loss and joint destruction resulting in skeletal deformities and significant functional impairment (1,2). The first case of ‘disappearing bone’ disease was originally described by Jackson in 1838 (3) and since then, a number of clinically distinguishable forms have been described under many different designations including idiopathic osteolysis, hereditary multicentric osteolysis and carpal and tarsal agenesis (1,2). These rare syndromes, although sharing many phenotypic characteristics, can differ from each other in mode of transmission and in clinical features, including the presence or absence of mental retardation and nephropathy and the extent and anatomic distribution of osteolysis. On the basis of clinical and radiographic features and inheritance pattern, the International Skeletal Dysplasia Registry provided a useful comparative classification scheme of the multicentric disorders into four groups (4). Most notably, each of these rare Mendelian disorders provides the unique opportunity to define the genetic/molecular pathways contributing to normal osteoblast/osteoclast development and function.

We and another group previously described the novel autosomal recessive disorder multicentric osteolysis with arthropathy (MOA) (MIM #605156) in unrelated consanguineous Saudi Arabian families (5–8). Since then, a number of non-Arabian families with the disorder have been identified confirming the disease’s pan-ethnic nature (9–10). On the basis of its clinical features, we placed MOA (5) in the group of diseases associated with carpal, tarsal and interphalangeal destruction, namely Torg (MIM #259600), Winchester (MIM #277950), Francois (MIM #221800) and Whyte-Hemingway syndromes (4). However, MOA is clinically distinguished from the other inherited osteolyses/arthritides syndromes, and in particular the other subgroup of carpal, tarsal disorders by the time-dependent severity of osteolysis and osteopenia and resultant joint contractures and ankylosis (5–10). In addition, characteristic MOA facial features include proptosis, frontal bossing, hypertelorism, brachycephaly and a bulbous nose with flattened nasal bridge. MOA affecteds usually present with symptoms in the first 2 years of life with the development and progression of painful swellings with decreased range of motion or deformity of the proximal interphalangeal joints of hands, feet and wrists (5–8). Also, although MOA affecteds may have normal birth weights, they also have decreased growth rates and are in the bottom decile of height and weight during early childhood.

Using linkage analysis and positional cloning and then molecular modeling and biochemical analysis, we recently demonstrated that MOA was caused by inactivating mutations in MMP2 (12). This finding was unexpected for two reasons. First, previous in vitro studies suggested that MMP-2 overexpression and increased activity, not deficiency, would result in osteolysis and arthritis. Indeed, MMP-2 had originally been isolated from the culture media of rheumatoid arthritis synovial tissue (11). Second, the Mmp2−/− mouse was originally characterized as overtly normal (13) (discussed subsequently).

Our discovery that MMP-2 deficiency results in a disorder characterized by apparent tissue destruction in the form of osteolysis and arthritis suggested a more complex role for this gelatinase in normal bone development and joint maintenance processes than predicted based on previous in vitro studies. The MMPs, a group of structurally related, secreted and membrane-associated proteinases, are involved in degrading the structural components of the ECM as well as other extracellular and non-matrix proteins (14–23). In recent years, the view of this family of proteases, including MMP-2, as simply ‘destructive enzymes’ has evolved to now appreciate that MMPs can alter the microenvironment, modulate the activity of bioactive molecules and regulate the activity of other proteases, thereby playing roles in cell attachment, proliferation, differentiation and apoptosis (14–25).

Prior to our original report demonstrating mutations in the human MMP2 gene, Mmp2−/− mice had been generated to examine the role of MMP-2 in amyloid precursor protein (APP) processing and secretion in vivo (13). In these mice, the wild-type promoter and first exon of the Mmp2 gene were replaced with a pgk-neo cassette by gene targeting. These mice had no differences in APP processing ability and were described as overtly normal. Curiously, Mmp2−/− mice were ~15% smaller at birth than control littermates (13). Given the genetic basis of the human disease, we sought to investigate the murine growth/skeletal phenotype in a targeted fashion by specifically focusing our studies on a possible ‘sub-clinical’ skeletal phenotype. Our findings reveal that Mmp2−/− mice partially recapitulate the human disease, as they have marked age-related bone density loss, bone abnormalities, craniofacial defects, sclerotic cranial sutures and articular cartilage destruction. Moreover, and potentially identifying the cellular basis of MMP-2-mediated skeletal defects, we show that these changes are associated with developmentally restricted changes in osteoclast and osteoclast numbers in vivo, defects in cultured Mmp2−/− bone marrow cells to support osteoblast and osteoclast differentiation in culture, and that targeted MMP-2 inhibition using siRNA in cultured cell lines suppresses osteoblast proliferation.

RESULTS

Mmp2−/− mice have early and persistent craniofacial defects

The Mmp2−/− mice used in these studies were originally generated and described by Itoh et al. (13). In accord with
those original studies, and similar to the human MOA affecteds, Mmp2\textsuperscript{2/2} homozygous mice were smaller and had markedly decreased MMP-2 expression and zymographic activity, whereas Mmp2\textsuperscript{+/+} mice had half normal levels and activity (data not shown). In addition, we noted that all Mmp2\textsuperscript{2/2} mice had a distinctive facial appearance when compared with Mmp2\textsuperscript{+/+} and Mmp2\textsuperscript{2/2} littermates. Mmp2\textsuperscript{2/2} mice had shortened, broad snouts and were hyperteloric with narrower, taller skulls (Fig. 1A). These differences were noted as early as 10 days after birth and many persisted throughout life. Caliper measurements on 4-week-old mice revealed that the snouts of Mmp2\textsuperscript{2/2} mice were \textasciitilde15\% shorter (P < 0.05), whereas intercanthal distances were increased by \textasciitilde10\%, tending towards statistical significance (P = 0.08, Fig. 1).

Direct examination of the craniofacial bones by microcomputed tomography (\(\mu\)CT) confirmed these findings and identified a number of additional features (Fig. 1B–E). First, and as shown in Figure 1C, coronal, sagittal and lambdoid sutures were prominent and sclerotic, similar to the radiological finding shared with a majority of MOA affecteds (5). Second, upper and lower jaw lengths, nose length and overall skull lengths were decreased in Mmp2\textsuperscript{2/2} mice and all were \textasciitilde10\% shorter by 12 weeks. Third, and confirming visual inspection of the skulls, we noted that the area between the right and left frontal–squamosal intersection at the temporal crest, ‘midcranial width’ in Figure 1E, was also wider in Mmp2\textsuperscript{2/2} mice and this difference increased with age. Mid-cranial width differences continued to increase from \textasciitilde10\% at 4 weeks to nearly 25\% at 24 weeks. Fourth, we also detected a significant decrease in bone volume (13.4\%) present in 24-week-old Mmp2\textsuperscript{2/2} mice. Finally, and providing the first suggestion of a transient nature to some of the skeletal features, increased intercanthal distances and skull heights of just below 10\% noted at 4 weeks of age were no longer statistically different by 12 weeks.

Mmp-2 deficiency results in erosive articular cartilage defects

In addition to craniofacial changes, MOA is also characterized by early onset arthritis and joint contractures. These are generally first noted in carpal and tarsal joints and spread proximally as the disease progresses. Most strikingly, although Mmp2\textsuperscript{2/2} mice displayed no obvious difficulties in ambulation and/or maintaining joint flexibility throughout growth and into adulthood, even to 2 years of age, direct histological examination revealed clear evidence of joint abnormalities. The knee joints of all 12-week-old Mmp2\textsuperscript{2/2} mice examined (n = 5) revealed articular cartilage destruction and erosion of the underlying bone surface (Fig. 2B and C), resulting in the loss of the smooth tibial and femoral surfaces present in all age-matched controls (n = 6; Fig. 3A). These findings suggest a similarity with the rheumatoid pannus present in rheumatoid arthritis, to which the arthritic changes in MOA have been compared (5,6). In addition, these findings highlight a likely misinterpretation in the literature that suggested that articular cartilage destruction noted in Mmp2\textsuperscript{2/2} mice was solely secondary to exogenous antibody induction and not the result of MMP-2 deficiency (26).

Bone mineral density decreases in a time-dependent manner

Given these findings and the fact that MOA affecteds also have marked osteopenia, we next investigated the quality of Mmp2\textsuperscript{2/2} mouse bones. As shown in Figure 3, X-ray analysis suggested decreased radio-opacity in Mmp2\textsuperscript{2/2} mouse skeletons. In order to quantify and better characterize the possible temporal changes in bone mineral density (BMD), we used dual energy X-ray absorptometry (DEXA) over a series

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{image.png}
\caption{Mmp2\textsuperscript{2/2} mice have abnormal craniofacial development. (A) Frontal and dorsal views of 12-week-old Mmp2\textsuperscript{2/2}, Mmp2\textsuperscript{+/+} and Mmp2\textsuperscript{2/2} mice. (B–D) Lateral, superior and inferior views, respectively, of \(\mu\)CT images of 6-month-old Mmp2\textsuperscript{2/2}, Mmp2\textsuperscript{+/+} and Mmp2\textsuperscript{2/2} mice. (E) Tabulated \(\mu\)CT measurements between paired Mmp2\textsuperscript{2/2} and Mmp2\textsuperscript{+/+} mice showing those differences reaching statistical significance (P < 0.05).}
\end{figure}
unexpectedly, given the chronic nature of the human syndrome, many of these differences were no longer apparent in bones from 4-week-old Mmp2−/− mice (Fig. 3I). This apparent resolution occurred during a developmental period in mice normally correlating with rapid growth and comparable to the age when human MOA affecteds experience rapid bone loss. Overall, the cortical, trabecular and cell density differences between Mmp2−/− (n = 7) and Mmp2+/+ (n = 7) marrow cavities at this time point had by now mostly resolved. Nonetheless, stigmata of the abnormal Mmp2−/− cortical growth remained and appearing as streaky, hypostaining infiltrating regions enhanced under polarizing light. At 12 weeks of age, these histological differences had almost completely disappeared (Fig. 3J).

Early Mmp2−/− marrow has transiently decreased osteoblast and osteoclast numbers in vivo

To investigate the cellular basis of these findings, we analyzed specific cellular components of the marrow cavity by immunohistochemistry and directly quantitated cellular amounts by Bioquant image analysis. We used procollagen I as a marker of cells actively producing ECM, which corresponded to active osteoblasts, and cathepsin K as a marker of osteoclasts. Osteoblast and osteoclast numbers were dramatically and transiently reduced in the immediate post-natal period in Mmp2−/− mice (Fig. 4). Specifically, at 4 days, the Mmp2−/− osteoblast (Fig. 4B, C and H; P < 0.005) and osteoclast (Fig. 4D, E and H; P < 0.005) numbers per trabecular bone surface area were decreased by more than half (Fig. 4H). At 4 and 12 weeks, no significant differences in numbers of either cell type were identified (data not shown). Consistent with these immunohistochemical findings, proliferating cell nuclear antigen (PCNA) staining, a general marker of cell proliferation status, revealed an ~60% decrease in the number of proliferating cells in Mmp2−/− marrow at 4 days (Fig. 4F–H; P < 0.05). No significant changes at either 4 or 12 weeks were seen (data not shown).

Mmp2−/− bone marrow stromal cells and calvaria are ineffective in supporting osteoblast and osteoclast growth ex vivo

Given these in vivo findings, we next analyzed the effect of MMP-2 loss on the formation of osteoblastic and osteoclastic cells in explanted bone marrow cell (BMC) cultures. In each of the assays used, Mmp2−/− BMC was ineffective in supporting osteoblast or osteoclast growth despite the fact that comparable numbers of cells were isolated and plated from both marrow sources. In the first set of assays, analyzing osteoblast growth from marrow of 8-week-old mice, Mmp2+/+ cells formed numerous, large, alkaline phosphatase-positive osteoblast colonies. In marked contrast, only relatively infrequent and small colonies were identified in Mmp2−/− cultures (Fig. 5A). When we modified the culturing procedure to reduce the washing stringency following plating of BMCs, to account for possible differences in adherence, Mmp2−/− alkaline phosphatase-positive colonies were now identified (Fig. 5B), but these smaller colonies still represented <20% of control (P < 0.0005, Fig. 5C).
In agreement with these findings revealing defects in Mmp2−/− osteoblastic potential from BMC, we also consistently obtained smaller cell yields and observed lower in vitro growth rates using calvarial cell populations isolated from 4-day-old Mmp2−/− mice. Notably, Mmp2+/+ osteoblasts reached confluence in 8 days, undergoing 1.5 population doublings, whereas Mmp2−/− cells plated at the same density increased their numbers by only 50% over the same time period (Fig. 5D and E).

To assess osteoclast growth potential, Mmp2+/+ and Mmp2−/− BMC or splenic cells were harvested and cultured in the presence of receptor activator of NFκB ligand (RANKL) and M-CSF. Osteoclasts, identified as multinucleated, tartrate-resistant acid phosphatase (TRAP)-positive cells, were then counted (Fig. 5F–H). The number of osteoclasts detected in cultures derived from Mmp2−/− BMC (P < 0.00005) and splenic cells (P < 0.0005) were reduced by ~60% compared with Mmp2+/+ osteoclasts, despite being similarly harvested, cultured and assayed (Fig. 5H).

**Targeted siRNA-mediated MMP2 knockdown decreases osteoblast growth in cultured cell lines**

Having identified these differences in Mmp2−/− osteoblasts both in vivo and ex vivo from mice which were globally deficient in MMP-2, we wondered whether targeted loss of this enzyme in cultured wild-type cells, devoid of supporting stromal cells and environment, would also affect their growth. Therefore, we experimentally inhibited MMP-2 expression in two well-characterized osteoblastic cell lines, human SaOS2 and mouse MC3T3, by siRNA-mediated gene silencing. Independent pools of siRNA oligos specific to human MMP2 or mouse Mmp2 (siMMP2) were used to target multiple regions of the mRNA transcript. Using transient transfection in SaOS2 cells, MMP2 mRNA levels were maximally reduced 80% at 48 h post-transfection and 65% by 72 h (Fig. 6A). MMP-2 protein activity levels in conditioned media were reduced by >70% at both these time points (Fig. 6B). The effect of targeted downregulation of
MMP-2 was an ~30% reduction of cellular proliferation at 48 h after transfection (P < 0.05) and ~20% reduction at 72 h (P < 0.05, Fig. 6C).

To examine the ability of exogenous MMP-2 to compensate for reduced endogenous expression, stable siRNA expressing SaOS2 cell lines were generated and two independent clones characterized. Stable siMMP2 clones A and B showed ~70% reduction of the MMP2 RNA transcript and protein, and cellular proliferation was reduced in each by ~35% (P < 0.05, Fig. 6G) regardless of the presence or absence of FBS, which contains high levels of MMP-2. We next analyzed the effect of exchanging the conditioned media between siMMP2 cells and control, empty vector cells on proliferation. Again, no effect was seen on proliferation rates as siMMP2 clones still proliferated at rates ~50% less than empty vector control cells (P < 0.05, Fig. 6G).

The general nature of this effect on cellular proliferation was then shown in the second cell line. In siMMP2-transfected MC3T3 cells, Mmp2 RNA levels were reduced by ~70% at 24 h and ~50% at 48 h (Fig. 6D) with a greater than 50% decrease in protein activity at both time points (Fig. 6E). Similar to the transient transfection results obtained in the SaOS2 cell line, MMP-2 inhibition in MC3T3 cells reduced proliferation rates by 25% at both 24 and 48 h time points (P < 0.05, Fig. 6F).

**DISCUSSION**

A targeted approach to the molecular basis of skeletal development can begin with the genetic analysis of a monogenic syndrome whose major phenotype derails the normal patterns of skeletal growth and homeostasis. Each monogenic trait can
activity levels are reduced by infected with siMMP-2. (C and /C)
empty-vector-containing virus (E), regardless of the addition of exogenous siMMP2 (A and B) compared with control clones infected with MMP2 in the culture media. Proliferation rates are significantly reduced in SaOS2 clones stably expressing P

Figure 6. MMP-2 inhibition by siRNA decreases the proliferation of osteoblast cell lines. (A and D) MMP-2 mRNA expression levels in (A) SaOS2 and (D) MC3T3 cells transfected with pools of siRNA targeting MMP-2 (siMMP-2) are reduced by up to 80% compared with cells transfected with an unrelated siRNA control (siNTC). (B and E) Conditioned media MMP-2 activity levels are reduced by >50% in (B) SaOS2 and (E) MC3T3 cells transfected with siMMP-2. (C and F) Proliferation rates as measured by [3H]thymidine incorporation are reduced in (C) SaOS2 and (F) MC3T3 cells transfected with siMMP-2 compared with those transfected with siNTC. *P < 0.05. (G) Proliferation rates are significantly reduced in SaOS2 clones stably expressing siMMP2 (A and B) compared with control clones infected with empty-vector-containing virus (E), regardless of the addition of exogenous MMP2 in the culture media. *P < 0.05.

First and foremost, these studies begin to unravel the paradox generated by our initial gene discovery studies in MOA, namely, ‘How does functional loss of a single proteolytic enzyme result in a ‘vanishing bone’ disorder?’ Taken together, the in vivo and ex vivo defects observed in Mmp2−/− osteoblasts and osteoclasts and in cultured osteoblast cell lines following siRNA-mediated MMP-2 inhibition support the hypothesis that MMP-2 directly affects osteoblast and osteoclast growth. Most intriguingly, this deficit is not recovered with the replacement of MMP-2 in the growth media. A question which therefore arises from these studies and is consonant with the increasing recognition of the importance of the MMP family in complex cellular activities (beyond their initially defined roles in matrix degradation) including cell attachment, proliferation, differentiation and apoptosis (30–34) is the identity of the physiologically relevant MMP-2 substrates that can induce these effects on osteoblasts and osteoclasts. Ultimately, the availability of a murine model and MMP-2 deficient cell lines will provide the framework resources for addressing these questions.

As we have shown, many of the clinical features distinctive of MOA are replicated and paralleled by Mmp2−/− mice. These include craniofacial defects, osteopenia and joint defects consistent with an underlying arthritis. Interestingly, histological evidence of arthritis in Mmp2−/− mice had been previously noted but was believed to be the result of an increased response to arthritogenic insult, and was therefore not appreciated as a direct result of enzymatic deficiency (26). In these studies, Mmp2−/− mice were injected with an arthritogenic monoclonal antibody (mAB) cocktail and then clinically and histologically graded for evidence of arthritic changes. Curiously, only mAB treated Mmp2−/− mice were examined but not untreated Mmp2−/− control mice (26). Thus, given our present findings that Mmp2−/− mice suffer from arthritic erosions, the previous results and their original interpretation must be re-examined.

Similarly, MT1-MMP deficient mice possess defects in both endochondral and intramembranous bone formation and, as we originally noted (12), share a number of features consistent with MOA (41,42). Notwithstanding that MT1-MMP deficient mice die within a few weeks of age, these mice develop craniofacial dysmorphia, osteopenia, arthritis, dwarfism and soft-tissue fibrosis. These analogous findings are particularly important since MT1-MMP is known to be a critical activator of MMP-2. Physiological activation is regulated by a cell surface interaction and achieved following the formation of a tri-molecular complex between MMP-2, MT1-MMP and tissue inhibitor of metalloproteinase (TIMP)-2 (43,44). As would be expected, cultured fibroblasts from these mice were unable to fully activate pro-MMP-2 (41,42). Since it was believed that MMP-2 knockouts lacked a phenotype, the skeletal consequences of MT1-MMP deletion have been ascribed solely to the loss of MT1-MMP activity. In light of our MMP-2 human and mouse results, this interpretation should be re-examined. Of note, TIMP-2 knockout mice, also deficient in pro-MMP-2 activation, appear phenotypically normal and develop and procreate indistinguishably from wild-type littermates (45,46). To date, no skeletal investigations have been reported.

theoretically illuminate one component in the complex, multifactorial pathway underlying normal and pathological bone biology. Using this paradigm, a number of highly relevant and completely unexpected targets have been identified from rare inherited skeletal diseases. For example, familial expansile polyostotic osteosarcoma (MIM #174810), results from mutations in RANK, a molecule essential in osteoclast formation (27). Osteoporosis—pseudoglioma syndrome (MIM #259770) and the ‘high bone mass trait’ (MIM #601884) are caused by inactivating and activating mutations, respectively, in the low-density lipoprotein receptor-related protein 5 (LRP5), a Wnt co-receptor critical to Wnt-mediated osteoblastic proliferation and differentiation (28,29). Moreover, the initial identification of many genes now known to regulate osteoblast and osteoclast differentiation, growth and function—Runx2 (CBFA, OSX2) (cleidocranial dysplasia), Msx2 (cleidocranial dysplasia, Boston-type), Sox9 (campomelic dysplasia), FGFRs 1–3 (Pfeiffer syndrome, Crouzon and achondroplasia, cranial dysplasia, Boston-type), Sox9 (campomelic dysplasia), Msx2 (cleidocranial dysplasia, Boston-type), Sox9 (campomelic dysplasia), Runx2 (CBFA, OSX2) (cleidocranial dysplasia), Msx2 (cleidocranial dysplasia, Boston-type), Sox9 (campomelic dysplasia), FGFRs 1–3 (Pfeiffer syndrome, Crouzon and achondroplasia, cranial dysplasia, Boston-type), Sox9 (campomelic dysplasia), Runx2 (CBFA, OSX2) (cleidocranial dysplasia), Msx2 (cleidocranial dysplasia, Boston-type), Sox9 (campomelic dysplasia), FGFRs 1–3 (Pfeiffer syndrome, Crouzon and achondroplasia, cranial dysplasia, Boston-type), Sox9 (campomelic dysplasia), Runx2 (CBFA, OSX2) (cleidocranial dysplasia), Msx2 (cleidocranial dysplasia, Boston-type), Sox9 (campomelic dysplasia), FGFRs 1–3 (Pfeiffer syndrome, Crouzon and achondroplasia, cranial dysplasia, Boston-type), Sox9 (campomelic dysplasia), Runx2 (CBFA, OSX2) (cleidocranial dysplasia), Msx2 (cleidocranial dysplasia, Boston-type), Sox9 (campomelic dysplasia), FGFRs 1–3 (Pfeiffer syndrome, Crouzon and achondroplasia, cranial dysplasia, Boston-type), Sox9 (campomelic dysplasia), Runx2 (CBFA, OSX2) (cleidocranial dysplasia), Msx2 (cleidocranial dysplasia, Boston-type), Sox9 (campomelic dysplasia), FGFRs 1–3 (Pfeiffer syndrome, Crouzon and achondroplasia, cranial dysplasia, Boston-type), Sox9 (campomelic dysplasia), Runx2 (CBFA, OSX2) (cleidocranial dysplasia), Msx2 (cleidocranial dysplasia, Boston-type), Sox9 (campomelic dysplasia), FGFRs 1–3 (Pfeiffer syndrome, Crous...
A particularly intriguing aspect of the Mmp2−/− mouse phenotype is the apparent time-dependent resolution of certain aspects of the skeletal changes. Indeed, a recent study by Inoue et al. (47) of aged Mmp2−/− mice failed to detect a statistically significant difference in osteoblast and osteocyte activity, and instead identified specific failure of osteocytic canalicular formation. (47). Persistent changes, such as the craniofacial and skeletal anomalies, were similar to those reported here. Although unexpected, developmentally limited tissue and cellular phenotypes are not unprecedented in studies of MMP deficiency. For example, Mmp-9, along with Mmp-2, is one of two known gelatinases in the MMP family and shares extensive structural similarities to the MMP2 gene. The only developmentally apparent abnormalities noted in Mmp9−/− mice were a 10% shorter long bone length and alteration of endochondral bone formation (48). In these mice, the growth abnormality resulted from lengthened zones of hypertrophic cartilage in chondrocytes owing to impaired vascular invasion and delayed apoptosis of terminal hypertrophic chondrocytes. In addition, Mmp-9 is required for invasion of osteoclasts and may be mediated through the ability of MMP-9 to make VEGF bioavailable from the ECM (49,50). Beyond this example, mutations in Mmp13 result in the human disease spondopetaphyseal dysplasia, Missouri type [SEMD-(MO)] (OMIM 602111), and engineered deletion of Mmp13 in mice results in similar skeletal defects (51). Interestingly, in humans, the radiographic changes seen in metaphyses, epiphyses and vertebrae during childhood resolve in adults (52) and defects in murine Mmp13−/− growth plates resolve by 12 weeks, whereas increases in trabecular bone persist for longer but are then also lost by 1 year (51). In both cases, it was postulated that upregulation of compensatory pathways allowed this resolution in the absence of critical MMPs.

The counterintuitive finding that loss of a proteolytic enzyme causes a vanishing bone and arthritic syndrome in humans (12) began our investigation into a more complex role for MMP-2 in bone biology. Knowledge of the human disease, coupled with findings in a species as evolutionally distant as zebrafish, wherein loss of MMP-2 leads to defects (53), made the apparent lack of a mouse phenotype particularly enigmatic. In this study of the Mmp2−/− mouse, we demonstrate that, despite an attenuation of the MOA phenotype, many aspects of the disease, including abnormal craniofacial development, bone and joint growth and BMD deficiency, are recapitulated and maintained in the mice. Post-natal bone growth and development is retarded, due at least in part to a proliferation defect of the marrow precursor cells, osteoblasts and osteoclasts both in vivo and in vitro. Interestingly, a previous report had also demonstrated that MMP-2 inhibition could regulate proliferation, albeit in glomerular mesangial cells (54). To our knowledge, ours is the first report demonstrating a direct effect of MMP-2 loss on osteoblasts and osteoclasts and suggest that, in turn, these findings may ultimately provide novel insight into the mechanisms coupling osteoblast bone formation and osteoclast resorption that underlies normal and pathological bone remodeling.

**MATERIALS AND METHODS**

**Animals**

Generation of the Mmp2−/− mice used in these studies was described by Itoh et al. (13) by targeted deletion of the promoter, 5′-UTR, and exon 1 of the Mmp2 gene. Genotyping was done by PCR of genomic DNA extracted from tail snips. Primers amplifying exon 1 were used to confirm the presence of the wild-type allele, whereas neo cassette primers targeted the null allele. Exon 1 forward: CAGACCTTCCCTGGTGGCT; exon 1 reverse: CACCATGCCCCATCATCAAG; neomycin forward: AAGGATCTCTGTGTCATCTACCTGCTCC; neomycin reverse: AAGAACTCAGTCACGAAGCGGATAGAA GG. The reaction conditions for exon 1 primers were: 95°C for 10 min, followed by 35 cycles of 95°C for 30 s, 55°C for 30 s, 72°C for 45 s and a final extension at 72°C. Reaction conditions for neomycin primers were identical except for an annealing temperature of 62°C.

All procedures were performed in accordance with an institutionally approved protocol for the use of animals in research.

**Micro-computed tomography**

Skulls were scanned on a General Electric Explore Locus μCT. The scans were done at 80 kV peak and 500 μA with an exposure time of 3000 ms. Two hundred views were done in a 1° increment with a frame average of 2 over 180°. Scans were reconstructed using GE reconstruction software yielding a post-reconstruction resolution of 20 μm.

**Histology and immunohistochemistry**

Bones were isolated from age-matched Mmp2−/− and Mmp2+/+ mice and fixed in 10% phosphate-buffered formalin overnight and decalcified in cold EDTA. Four-micrometer thick sagittal sections were stained with standard H&E. Sections were stained with antibodies to procollagen I, cathepsin K or PCNA (Santa Cruz Biotechnology, Inc.) and counterstained with hematoxylin. Positive cells were counted using Bioquant TCW software (Nashville, TN, USA) and normalized to the trabecular bone surface area.

**Cell culture and siRNA silencing**

**Osteoblasts**. Marrow from femurs and tibias of 8-week mice was extracted in αMEM with FBS and penicillin/streptomycin antibiotic. Cells were allowed to adhere and the non-adherent population removed. After 2 days in culture, cells were re-plated in osteoblast differentiation media contain 1 μM ascorbic acid and 5 μM dexamethasone at 30 000 cells/ml. Cell colonies formed were stained for alkaline phosphatase with 200 U/ml collagenase type I. Calvaria was isolated, rinsed with PBS and briefly decalcified in EDTA. After serial digestions with collagenase, fractions 2–5 were pooled, washed in αMEM and plated at 8 × 10^4 cells/ml in osteoblast differentiation media contain 1 μM ascorbic acid and 5 μM dexamethasone.
SiMMP2 oligo pools targeting either human MMP2 or mouse Mmp2 were obtained from Dharmacon, Inc. Transfections were done in serum-free media, using lipofectamine 2000 (Invitrogen, San Diego, CA, USA) and 100 pmol of oligo per well of a 12-well plate as previously described (55).

Forward and reverse sequences for MMP-2 shRNA construct were 5'-GATCCCGACAGTGATAGTGCCCTTCTTGAAGAGCTCCTGTCGGG-3' and 5'-AGCTTTCCAAGAGACAGTGGATGATGCCTTTTCTTGAGCTTTTCCAAAAAGACAGTGGATGATGCCTTTTCTTGAGCTTTTCCAAAAAGACAGTGGATGATGCCTTTTCT. Oligonucleotides were cloned into pSUPER.retro vector (Oligoengine, Seattle, WA, USA) according to manufacturer’s recommendations. Briefly, forward and reverse oligonucleotides were annealed and then ligated into the BglII–HindIII cleavage site within the pSUPER.retro vector pre-linearized with the same restriction enzymes. pSUPER.retro-siMMP2 vector or empty vector was transiently transfected into Phi-NX (‘Phoenix’) packaging cell line to produce ecotropic retroviral supernatants. Phoenix cells were cultured in DMEM supplemented with 10% FBS. The day before transfection, Phoenix cells were seeded in 10 cm dishes (3×10⁶ cells/dish) in order to reach 60% confluence at the time of transfection. Cells were transfected with 10 μg of viral vector DNA using Lipofectamine 2000 according to manufacturer’s protocol (Invitrogen). At 48 h after transfection, culture medium was filtered through a 0.45 μm filter and the viral supernatant was used for SaOS2 cell infection after addition of 4 μg/ml of polybrene (Sigma). After infection, SaOS2 cells were incubated at 37°C in 5% CO₂ for 5 h, followed by a second round of infection. After a second 5 h incubation, medium was changed with fresh medium and SaOS2 cells were allowed to recover for 48 h at 37°C in 5% CO₂. Infected cells were selected by adding puromycin (1 μg/ml) and clones were picked and grown under further selection. MMP2 expression in empty vector and siMMP2-infected SaOS2 cells was analyzed by real-time polymerase chain reaction (RT-PCR) and zymography as described below.

Osteoclasts. Bone marrow cells and splenic cells were harvested and plated at 6.25×10⁶ cells/cm² in the presence of 30 ng/ml M-CSF and 100 ng/ml RANKL to induce osteoclast format. After 9 days, cells were fixed in 4% paraformaldehyde and stained for TRAP activity (Sigma).

RNA isolation and quantitative RT-PCR
RNA was collected (Qiagen RNAeasy Mini Kit) and treated with DNase (Qiagen). A total of 0.5 μg of RNA was reverse-transcribed per reaction using first-strand complementary DNA synthesis with random primers (Iscript, Bio-Rad Laboratories). Quantitative RT-PCR was performed on an ABI PRISM 7900HT sequence detection system (Applied Biosystems). Cycle number values were normalized with both GAPDH and β-actin values. Values were analyzed as fold change compared with siNTC or empty vector control values. Data shown are the average of three separate experiments done in triplicate. Statistical significance was determined by comparing fold change using the unpaired, two-tailed, Student’s t-test assuming equal variances.

Gelatin zymography
Five micrograms of conditioned media was run on 10% gelatin zymograms gels (Novex), which were developed and stained according to manufacturer’s instructions as described previously (12).

Proliferation assay
About 1 μCi/ml [³H]thymidine (Amersham) was added to transiently transfected or stable cell lines with either siNTC or siMMP2 and incubated for 8 h. Cells were washed four times with ice-cold PBS and solubilized in 0.25% sodium hydroxide/0.25% SDS. After neutralization with hydrochloric acid (1 N), disintegrations per minute were estimated by liquid scintillation counting.

For conditioned media replacement assays of siMMP2 stable lines, cells were plated at equal densities. The following morning, media were removed and cells washed two times with serum-free media. Serum-free conditioned media from control empty vector clone (E) was added to siMMP2 clones (A and B), and serum-free conditioned media from siMMP2 clones added to control clones. Proliferation assay was performed over 4 h as described above after a 30 min pre-incubation in the ‘replaced’ media.

ACKNOWLEDGEMENTS
The work reported here was supported by NIH award AR47074 (to S.S.A.). Micro-CT was facilitated by the Imaging Core established through award of a Musculoskeletal Research Core Center to the Cleveland Clinic (NIH AR50953 to V. Hascall and S.S.A.). R.A.M. is supported by Pharmacological Sciences Training Grant GM-62754.

Conflict of Interest statement. The authors state no conflicts of interest.

REFERENCES


