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Multiple myeloma–derived MMP-13 mediates osteoclast fusogenesis and osteolytic disease

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Multiple myeloma (MM) cells secrete osteoclastogenic factors that promote osteolytic lesions; however, the identity of these factors is largely unknown. Here, we performed a screen of human myeloma cells to identify pro-osteoclastogenic agents that could potentially serve as therapeutic targets for ameliorating MM-associated bone disease. We found that myeloma cells express high levels of the matrix metalloproteinase MMP-13 and determined that MMP-13 directly enhances osteoclast multinucleation and bone-resorptive activity by triggering upregulation of the cell fusogen DC-STAMP. Moreover, this effect was independent of the proteolytic activity of the enzyme. Further, in mouse xenograft models, silencing MMP-13 expression in myeloma cells inhibited the development of osteolytic lesions. In patient cohorts, MMP-13 expression was localized to BM-associated myeloma cells, while elevated MMP-13 serum levels were able to correctly predict the presence of active bone disease. Together, these data demonstrate that MMP-13 is critical for the development of osteolytic lesions in MM and that targeting the MMP-13 protein—rather than its catalytic activity—constitutes a potential approach to mitigating bone disease in affected patients.

Introduction

Multiple myeloma (MM) arises as a consequence of the clonal expansion of malignant plasma cells within the BM compartment, an event associated with bone destruction and significant comorbidities including severe pain, pathologic fractures, and spinal cord compression (1). MM cells are commonly found in association with sites of active bone resorption, where they secrete a variety of pro-osteoclastogenic agents (1, 2). Current agents such as biphosphonates for the treatment of MM bone disease are aimed at spe-

Results

MMP-13 expression and regulation in MM cells. While screening human MM cell lines for their ability to express MMPs, RPMI 8266 cells were noted to secrete MMP-13 at levels that far exceeded those...
In vivo, BM stromal cells (BMSCs) are known to support MM cell growth and survival via both contact-dependent and -independent mechanisms (20, 21). Hence, we next determined whether BMSCs contribute to MMP-13 upregulation in MM cells. For this purpose, GFP+ RPMI 8266 cells were cocultured with BMSCs in direct contact or, alternatively, in Transwell dishes, thereby precluding direct cell-cell contact. Under both coculture conditions, MMP-13 was significantly upregulated relative to that detected in supernatants from MM or BMSC cultures alone (Figure 1B), demonstrating that direct cell contact is not required for MMP-13 upregulation. Further, MM cells are the major source of MMP-13 production in the coculture system, as the MMP13 mRNA of other MMPs (e.g., MMP-1, -2, -3, -7, -8, and -12). Though MMP-1, -2, -3, -7, and -8 levels all fell within detectable ranges, MMP-13 expression was approximately 300- to 400-fold higher than that of all other MMPs assessed (Figure 1A). Further, increased MMP-13 expression was not confined to RPMI 8266 cells and could be detected similarly in all tested human MM cell lines, as determined by either MMP-13 fluorometric assay (Supplemental Figure 1A; supplemental material available online with this article; doi:10.1172/JCI80276DS1) or collagen zymography (Supplemental Figure 1B). Of note, when assessed by zymography, only trace amounts of the active form, as opposed to the pro-form, of MMP-13 were detected (Supplemental Figure 1B).
level in purified GFP+ RPMI 8266 cells was more than 100-fold higher than that detected in BMSCs (Figure 1C). Similar results were obtained when any of the panel of human MM cell lines were incubated with BMSC-conditioned medium (CM). Further, the upregulation of MMP-13 could be almost completely blocked by adding a neutralizing Ab against IL-6 to the cultures (Figure 1D), suggesting that IL-6 is the key cytokine that induces MMP-13 in BMSC CM. In accordance with this finding, direct treatment of MM cells with exogenous IL-6 upregulated MMP13 transcription and protein expression (Figure 1, E and F).

Figure 2. MMP-13 mediates MM-induced OCL fusion and bone resorption. (A) Mouse nonadherent BM cells were cocultured with STGM1-EV or STGM1-MMP-13–KD #1 cells in Transwell dishes with M-CSF (10 ng/ml) and RANKL (50 ng/ml) for 4 days, with pro–MMP-13 added as indicated. OCLs were stained for TRAP and images captured as described in Methods. Scale bars: 100 μm. Data are representative of 3 independent experiments. CT, control. (B) Mouse OCL size (left panel), number of nuclei per OCL (middle panel), and number of OCLs per field (right panel) were assessed by microscopy and ImageJ software (NIH). Data represent the mean ± SEM (n = 3). *P ≤ 0.05 and **P ≤ 0.01, by ANOVA. (C) Mouse nonadherent BM cells were cultured with the indicated concentrations of pro–MMP-13 or vehicle during OCL formation in the presence of M-CSF (10 ng/ml) and RANKL (50 ng/ml). TRAP+ OCLs were scored microscopically. Scale bars: 100 μm. Data are representative of 4 independent experiments. (D) Mouse OCL size (left panel), number of nuclei per OCL (middle panel), and number of OCLs per field (right panel) were determined as above. Data represent the mean ± SEM (n = 3). P ≤ 0.0001, by linear regression (left panel) and Poisson regression (middle panel); P > 0.05 by Poisson regression (right panel). (E) Human mononuclear BM cells were cultured with the indicated concentrations of MMP-13 or vehicle during OCL formation in the presence of M-CSF (10 ng/ml) and RANKL (50 ng/ml). OCL numbers were determined by CD51/CD61 staining with 23c6 Ab and scored microscopically. Scale bars: 100 μm. Data are representative of 3 independent experiments. (F) Human OCL size, number of nuclei per OCL, and number of OCLs per well (right panel) were determined as above. Data represent the mean ± SEM (n = 3). P ≤ 0.0001, by linear regression (left panel) and Poisson regression (middle panel); P > 0.05 by Poisson regression (right panel).
MMP-13 mediates MM-induced OCL fusion and bone resorption. To assess the potential impact of MM-derived MMP-13 on OCL function, mouse BM mononuclear cells (the source of OCL progenitors) were cultured in the lower compartment of Transwell dishes with murine 5TGM1 MM cells seeded in the upper compartment in the absence or presence of IL-6. Either IL-6 or 5TGM1 cells alone stimulated OCL formation (Supplemental Figure 2, A and B). However, in the presence of both IL-6 and 5TGM1 cells, OCL formation was significantly enhanced, both in terms of increased average size and nuclei numbers (Supplemental Figure 2, A and B), indicating that IL-6-stimulated MM cellssecreted soluble factors that are capable of promoting OCL fusion and activity (2). To determine the degree to which MMP-13 participates in pro-osteoclastogenic activity, the metalloproteinase was silenced in mouse STGMI MM cells by lentiviral shRNA transduction (confirmed by quantitative reverse transcription PCR [qRT-PCR]; Supplemental Figure 3). While coculture with STGMI empty vector-transduced (EV-transduced) cells significantly increased OCL fusion relative to medium alone, 5TGM1–MMP-13–knockdown #1 (5TGM1–MMP-13–KD #1) cells largely lost the ability to induce OCL fusion, as reflected in the decreased OCL size and nuclei numbers. Underlining the specificity of the silencing strategy, OCL fusion was rescued by exogenous pro–MMP-13 (Figure 2, A and B).

A required role for MMP-13 in controlling STGMI-induced osteoclastogenesis raised the possibility that the proteinase either modulates MM function or acts as an osteoclastogenic factor. In OCL formation assays, pro–MMP-13 directly enhanced mouse (Figure 2, C and D) as well as human OCL formation in a concentration-dependent manner (Figure 2, E and F). As observed in the intact MM cell model, pro–MMP-13 increased both OCL size and nuclei numbers (Figure 2, D and F) without affecting pre-OCL proliferation (Supplemental Figure 4A). As a result of increased fusion, we observed a trend toward decreased OCL numbers (Figure 2D and Supplemental Figure 2B). Time course assays adding MMP-13 on days 1 and 2 only, on days 3 and 4 only, or on days 1–4 of OCL culture revealed that the presence of MMP-13 is critical during the entire culture period in order to induce OCL fusion (Supplemental Figure 4B), without significant effects on cell proliferation (Supplemental Figure 4C). Consistent with the increase in OCL size and nuclei numbers, the bone-resorptive activity of OCLs also increased as a function of MMP-13 concentration (Supplemental Figure 4, D and E). By contrast, supplementing cultures with either MMP-1, MMP-2, or MMP-9, three other MMPs whose expression has been linked previously to tumor-related bone metastasis (22), exerted no significant effects on OCL development (Supplemental Figure 5, A and B).

The ability of exogenous MMP-13 to support osteoclastogenesis as well as OCL activity raised the possibility that OCL progenitors may express MMP-13 as an endogenously derived pro-osteoclastogenic agent. Indeed, WT, but not Mmp13−/−, BM-derived mononuclear cells expressed Mmp13 as assessed by qRT-PCR (Supplemental Figure 6A). Furthermore, OCL formation from Mmp13−/− BM-derived mononuclear cells was significantly impaired relative to that observed in WT cells, as evidenced by significant decreases in OCL size, nuclei numbers (Supplemental Figure 6, B and C), and bone-resorptive activity (Supplemental Figure 6, D and E). As predicted, defects in Mmp13−/− OCL formation were reversed by supplementing the cultures with exogenous pro–MMP-13 (Supplemental Figure 6, B and C), without affecting pre-OCL proliferation rates (Supplemental Figure 6F). Taken together, these data indicate that MMP-13 modulates OCL fusion and bone-resorptive activity in both a cell-autonomous and a non–cell-autonomous manner.

MMP-13 induces OCL formation independently of its proteolytic activity by an ERK1/2–DC-STAMP–dependent process. MMP-induced activities are most frequently associated with the conversion of the proenzyme to a mature form that is able to hydrolyze target substrates (8, 9). Hence, as the MMP-13 zymogen enhanced OCL fusion and function in our in vitro cultures, we sought to determine (a) whether pro–MMP-13 is processed into its catalytically active form by BM-derived mononuclear cells and (b) whether proteolytic activity is required for the induction of OCL formation. Unexpectedly, the conversion of the MMP-13 zymogen to its active form was not detected in BM mononuclear cell cultures in the CM (Figure 3A, upper panel) or in association with the OCL surface (Figure 3A, lower panel), suggesting that MMP-13 enzymatic activity is not necessarily required for the stimulation of osteoclastogenic activity. Indeed, neither of the 2 specific and potent MMP-13 enzymatic inhibitors CP544439 (IC50 = 0.7 nM) (23) or CAS 544678-85-5 (IC50 = 8 nM) (24) affected MMP-13–induced OCL formation (Figure 3, B and C) at concentrations that completely blocked MMP-13 enzymatic activity (Supplemental Figure 7A).

To confirm that the proteolytic activity of MMP-13 is not required for induction of OCL fusion and activity, an enzymatically inactive mutant of MMP-13 was generated by introducing an E223A mutation within the zinc-binding motif of the proteinase (Figure 4A and ref. 25). As expected, the MMP-13 WT protein rapidly underwent autoactivation in the presence of p-aminophenylmercuric acetate (a process dependent on an intact catalytic domain), while the MMP-13 E223A mutant remained locked in its proform state, as confirmed by a fluorometric assay that showed an absence of MMP-13 enzymatic activity (Figure 4B) or by collagen zymography (Supplemental Figure 7B). Nevertheless, both MMP-13 WT and MMP-13 E223A showed similar abilities to promote OCL formation (Figure 4, C and D) and bone-resorptive activity (Figure 4, E and F), confirming that MMP-13 proteolytic activity is not required for the induction of osteoclastogenesis. Following knockin of MMP-13 WT or MMP-13 E223A into 5TGM1 MMP-13–KD cells (Supplemental Figure 8A), OCL induction was rescued in Transwell coculture assays (Supplemental Figure 8, B and C). While recent studies have suggested that proteinase-independent activities of selected MMPs may reside within the C-terminal hemopexin domain (26–30), neither the recombinant MMP-13 hemopexin nor the recombinant procatalytic domain alone (Supplemental Figure 9A) were able to reconstitute the activity of the full-length enzyme (Supplemental Figure 9, B and C).

DC-STAMP, a master fusogen essential for normal OCL function, is induced by RANKL via NFATc1 (31, 32). Consistent with the MMP-13–dependent inductive activity of OCL fusion, both the WT and E223A mutant forms of the proteinase upregulated NFATc1 and DC-STAMP expression at both the protein and mRNA levels in developing OCLs (Figure 4G and Supplemental Figure 10, A and B). Flow cytometric staining confirmed the induction of cell-surface DC-STAMP expression by MMP-13.
Supplemental Figure 10C and refs. 33, 34). Consistent with these findings, Mmp13−/− OCLs also generated lower expression levels of NFATc1 and DC-STAMP, both of which were partially rescued by exogenous MMP-13 (Supplemental Figure 11). Interestingly, MMP-13 has recently been reported to activate ERK1/2 signaling in chondrocytes (35). As ERK1/2 can serve as a potential regulator of the NFATc1/DC-STAMP axis (36), we sought to determine whether MMP-13 drives osteoclastogenesis through this signal transduction cascade. Indeed, both MMP-13 WT and the E223A mutant directly activated ERK signaling in BM-derived pre-OCLs (Supplemental Figure 12A). Further mechanistic studies indicated that both MMP-13 WT and the E223A mutant upregulated TRAF6 polyubiquitination (ref. 37 and Supplemental Figure 12B) and induced phosphorylation of the ERK upstream kinases c-Raf and MEK1/2, as well as the downstream target substrate p90RSK (refs. 38, 39, and Supplemental Figure 12C), indicating that MMP-13 upregulates ERK signaling via an enzymatic activity–independent mechanism. More important, following the inhibition of ERK signaling with U0126 (40), the ability of either MMP-13 WT or the E223A mutant to induce NFATc1 and DC-STAMP upregulation and OCL fusion was blocked (Figure 4, G and H). Taken together, these data indicate that pro–MMP-13 functions as a secreted activator of an ERK signal transduction cascade that induces both NFATc1 and DC-STAMP expression.

**MMP-13 drives MM-induced osteolysis in vivo.** To assess the role of MMP-13 in the development of MM-associated bone disease in vivo, we next used an intratibial 5TGM1 murine model that recapitulates myeloma tumor growth coupled with severe osteolysis (41). To this end, GFP-expressing 5TGM1 cells were lentivirally transduced with EV control or 2 distinct shRNA expression vectors...

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**Figure 3. MMP-13–dependent osteoclastogenesis proceeds independently of pro–MMP-13 activation.** (A) Mouse nonadherent BM cells were cultured for 4 days with M-CSF (10 ng/ml) and RANKL (50 ng/ml) to induce OCL differentiation in the absence or presence of pro–MMP-13 (200 ng/ml). Top panel: culture media were collected at the indicated times after the addition of pro–MMP-13. Processing of pro–MMP-13 to mature/active MMP-13 was assessed by Western blotting. Pro– and active recombinant MMP-13 protein standards were loaded as controls. Lower panel: OCL lysates isolated from cells incubated with or without pro–MMP-13 were analyzed by Western blotting. Data are representative of 4 independent experiments. WCL, whole-cell lysate. (B) Mouse nonadherent BM cells were cultured as described in A, in the absence or presence of pro–MMP-13 and the MMP-13 synthetic inhibitor CP544439 or CAS 544678-85-5, at the indicated concentrations. OCL formation was assessed by TRAP staining. Scale bars: 100 μm. Data are representative of 3 independent experiments. (C) OCL size, number of nuclei per OCL, and number of OCLs per field in B were determined by microscopy and ImageJ analysis. Data represent the mean ± SEM (n = 3). *P ≤ 0.05, by ANOVA.
directed against MMP-13 (MMP-13–KD #1 and MMP-13–KD #2), thereby acting to efficiently silence metalloproteinase expression (confirmed by qRT-PCR and collagen zymography; Supplemental Figure 13, A and B), without affecting 5TGM1 cell proliferation (Supplemental Figure 13C). 5TGM1-GFP-EV, 5TGM1-GFP–MMP-13–KD #1, or 5TGM1-GFP–MMP-13–KD #2 cells were injected intratibially into Rag2−/− mice (41), and after a 4-week incubation period, tibiae were harvested for histological analysis, followed by micro-quantitative CT (micro-qCT). As shown in Figure 5, intratibial injection of 5TGM1-GFP-EV cells disrupted normal
hematopoiesis in association with the development of extensive lytic lesions, resulting in the destruction of trabecular bone. MMP-13–KD #1 and –KD #2 MM cells also infiltrated the BM, but without completely replacing hematopoietic centers. Most important, in contrast to control 5TGM1-GFP-EV cells, bone structure was maintained with markedly less bone loss when recipient mice were inoculated with MMP-13–KD #1 or –KD #2 MM cells (Figure 5, top panel). As expected, while enlarged TRAP+ OCLs were observed adjacent to the 5TGM1-GFP-EV tumor, only small TRAP+ OCLs were detected in mice injected with 5TGM1-GFP–MMP-13–KD #1 or –KD #2 cells (Figure 5, bottom panel). Quantification of OCL numbers and size confirmed that MMP-13–KD decreased OCL size as determined by the OCL surface to bone surface ratio (Supplemental Figure 14).

Bone micro-qCT and morphological analysis of the tibiae and adjacent femurs demonstrated that 5TGM1-GFP-EV myeloma cells not only induced lytic bone lesions, but also resulted in trabecular bone loss (Figure 6A). By contrast, MMP-13–KD #1 or –KD #2 MM cells significantly inhibited the formation of MM-induced bone lesions (Figure 6A). These effects were further supported by our morphological analyses of trabecular bones (Figure 6B), in which MMP-13–KD #1 or –KD #2 MM cells significantly increased the bone volume fraction (bone volume/total volume [BV/TV]; EV 0.0573 vs. KD #1, 0.0723 and KD #2, 0.0987), connective density (Con.D.: EV 50.53 vs. KD #1, 83.69 and KD #2, 142.0 mm3), trabecular numbers ( Tb.N.: EV 3.081 vs. KD #1, 3.440 and KD #2, 3.368 mm3), and bone mineral density (BMD: EV 125.3 vs. KD #1, 150.9 and KD #2, 181.9 mg/cm3), while decreasing the bone surface to bone volume ratio (BS/TV: EV 74.44 vs. KD #1, 71.53 and KD #2, 64.01 mm3) and the structure model index (SMI: EV 3.257 vs. KD #1, 3.023 and KD #2, 2.610). Hence, silencing of MM-derived MMP-13 inhibits osteolysis in vivo, further confirming its critical role in MM bone disease.

In a second set of animal experiments, we focused on the effects of MMP-13 silencing on MM tumor progression. Four weeks after intratibial injection of 5TGM1-GFP-EV or 5TGM1-GFP MMP-13–KD #1 cells, serum levels of mouse IgG2b were measured to assess tumor burden, while tibiae were harvested for histological analysis as well as GFP imaging, followed by micro-qCT. Similarly, the BM infiltration of MMP-13–KD #1 MM cells caused markedly less bone loss compared with that seen with STGMI-GFP-EV cells (Supplemental Figure 15A, top panel) and the formation of smaller OCLs in juxtaposition to MM cells (Supplemental Figure 15A, bottom panel). Despite the fact that MMP-13 had no direct effect on MM cell growth, as demonstrated by in vitro 3H-thymidine incorporation assay (Supplemental Figure 15B), the decreased OCL activity and subsequent bone disease led to a slightly lower MM tumor burden in vivo, as reflected by quantitative fluorescence imaging (Supplemental Figure 15, C and D) and IgG2b secretion (Supplemental Figure 15E). Consistent with the previous results, micro-qCT analysis again showed that MMP-13 silencing blocked the formation of MM-induced bone lesions in both the tibiae and adjacent femurs (Supplemental Figure 16).

MMP-13 expression in patients’ plasma cells and serum. Given the key role played by MMP-13 in regulating MM-induced osteoclastogenesis in our model systems, we next sought to determine whether MMP-13 expression levels might serve as an index of bone disease in patients. MMP-13 expression was first monitored in marrow biopsies of a cohort of patients with MM and controls. In tissue arrays of 11 normal biopsy samples and 10 MM samples, MMP-13 expression was specifically and highly expressed in primary MM BM samples (Figure 7A, bottom panel; 9 of 10 samples), but not in any of the normal control samples (Figure 7A, top panel; 0 of 11 samples). Given that MMP-13 is a secreted protein, we considered the possibility that serum levels of the metalloproteinase could reflect the presence of active bone disease. As such, we determined MMP-13 serum levels in 32 patients with MM and in 6 healthy donors. As shown in Figure 7B, 10 of 18 patients with MM-associated bone disease showed positive serum expression levels of MMP-13, while none of the 14 MM patients without bone disease had detectable levels of MMP-13. Further, MMP-13 was not detected in any of the 6 healthy donors. Thus, consistent
with a pivotal role for MMP-13 in MM-associated bone disease, serum MMP-13 levels were specific for bone disease status in a significant subset of affected patients.

Discussion
Following their homing to the BM compartment, MM cells secrete OCL activation factors that contribute to the formation of bony lesions in affected patients (1–6, 42). Here, we demonstrate that IL-6–mediated MM-BMSC interactions induce MMP-13 expression that, in turn, serves as a potent osteoclastogenic factor capable of promoting the generation of bone-resorptive, multinucleated OCLs. Unexpectedly, in contrast to previous studies that have sought to identify potential roles for MMPs in bone-resorptive disease states, MMP-13 exerts its pro-osteoclastogenic effects independently of a requirement for proteolytic activity by acting as a DC-STAMP–inducing secretagogue. Complementing these in vitro studies, the pathophysiological relevance of our findings are highlighted by the demonstration that silencing MMP-13 expression in MM cells exerts bone-sparing effects in a mouse MM disease model. In extending these findings to human populations, MMP-13 was readily detected at both the MM-BM interface and in the serum of a subset of patients with active bone disease.
MM cells have long been known to express multiple MMPs that extend beyond MMP-13, including MMP-1, MMP-2, MMP-7, MMP-8, MMP-9, and MT1-MMP (43–46). The biologic rationale for the expression of multiple MMPs by MM cells remains unclear, but studies performed to date have most frequently stressed their role in supporting the tissue-trafficking activity of the neoplastic clones as they encounter basement membrane and interstitial matrix barriers in vivo (44, 45). While no specific functions have been assigned previously to MM-derived MMP-13, recent studies have proposed roles for the metalloproteinase in promoting tumor-associated osteolytic lesions in breast cancer as well as giant-cell tumors (15, 47–50). In each of these cases, however, the preferred mode of MMP-13–dependent activity is the proteolysis of downstream targets thought to play direct or indirect roles in bone resorption, including type I collagen, RANKL, osteoprotegerin, galectin 3, and TGF-β (15, 47–51). By contrast, all of the pro-osteoclastogenic effects associated with the WT proteinase in our studies could be recapitulated by a catalytically inactive E223A MMP-13 mutant. At first glance, the proposition that a catalytically inactive MMP mediates unique biological activities seems counterintuitive. More recently, however, a small subset of MMP family members, including catalytically inactive forms of MMP-3, MMP-9, and MT1-MMP, have been shown to affect a range of cell responses, ranging from cell motility to energy metabolism and cytokine expression (26, 52–55). Interestingly, MT1-MMP has been recently reported to support normal OCL function via a Rac1-dependent process that proceeds independently of the proteolytic activity of the enzyme (55). These latter observations, taken together with those presented here for MMP-13, suggest that OCL function may be modulated by a subset of catalytically inactive MMPs expressed in both a cell-autonomous and non–cell-autonomous fashion. Indeed, consistent with the pro-osteoclastogenic effects of exogenous MMP-13 found in vitro, Mmp13–/– OCLs also showed defects in form and function in vitro. Underlining these effects, Mmp13–/– mice have previously been reported to show an osteopetrotic phenotype with increased trabecular bone volume and bone mineral density (16, 56, 57), along with increased callus bone volume and bone mineral density during active bone remodeling following fracture (10). Given these findings, we considered the possibility that MMP-13 might also affect osteoblast (OB) formation or MM cell behavior itself, as both cell types express the metalloproteinase (Supplemental Figure 17A). However, MMP-13 supplementation did not affect OB differentiation, MM proliferation, or MM responses to the antineoplastics bortezomib or pomalidomide (Supplemental Figure 17B and Supplemental Figure 18, respectively). Similarly, MMP-13 silencing in MM cells showed no inhibitory effects on in vitro MM growth or MM-induced OB inhibition in vivo (Supplemental Figure 13C and Supplemental Figure 17C, respectively).
In light of the finding that MMP-13 induces OCL activation independently of its enzymatic activity, we assessed the effects of MMP-13 inhibition on MM bone lytic lesion and tumor progression by silencing MMP-13 expression, as opposed to using inhibitors directed against its enzymatic activity. Confirming our in vitro studies, MMP-13–KD significantly inhibited the development of bone lytic lesions in vivo. Although MMP-13–KD did not affect MM cell growth during in vitro culture, we observed a modest inhibition of MM tumor growth in the in vivo setting. This is in accordance with data showing that activated OCLs contribute to adjacent MM growth (20). As such, the inhibition of MM growth in vivo most likely occurs as a consequence of effects secondary to decreased OCL activation within the bone microenvironment following MMP-13 silencing.

In considering the potential mechanisms by which MMP-13 regulates osteoclastogenesis in normal as well as neoplastic states, our attention centered on DC-STAMP, a critical fusogenic protein (35). Efforts are currently under way to identify the MMP-13 receptor(s) that are operative in OCL progenitors, with preliminary cross-linking and mass spectroscopic studies having identified a number of potential cell-surface ligands whose expression will require silencing on a one-by-one basis (our unpublished observation). These studies will, by necessity, need to proceed cautiously, as several MMPs including MMP-13 can regulate diverse cell functions by trafficking to the nuclear compartment to exert transcriptional effects (61, 62).

To date, all efforts to link MMPs to cancer-induced osteolytic states including MM have emphasized the importance of proteolytic activity and the identification of targeted substrates. Not surprisingly, these studies have also accelerated efforts to identify new therapeutics that can specifically inhibit a single, selected MMP target (63). Our findings, however, support an alternate, and previously unsuspected, role for the MMP-13 zymogen as a MM-derived pro-osseoclastogenic secretagogue. Consonant with our in vitro and mouse model studies, MMP-13 could be detected in the BM compartment of almost all patients with MM in the small cohort of 10 patients studied. While serum MMP-13 levels would not necessarily be predicted to reflect events operative at the OCL-bone interface, we were surprised to find that nearly 60% of the 18 patients with MM identified with active bone disease who were monitored in our study registered detectable blood levels of MMP-13. At this juncture, we cannot rule out the possibility that the cohort of patients with bony lesions and undetectable serum MMP-13 levels are no longer actively remodeling bone. As such, further studies are underway to monitor serum MMP-13 levels in tandem with markers of active bone turnover. In any case, our findings suggest that, in contrast to interventions designed to target pro–MMP-13–OCL interactions, MMP-13 catalytic activity inhibitors would be predicted to exert little, if any, effect on MM-associated bone disease.

**Methods**

*Animals.* C57BL/6 WT mice were purchased from The Jackson Laboratory. C57BL/6 Mmp13–/– mice were maintained as previously described (57). For the 5TGM1 intratibial MM bone disease model, 8- to 10-week-old C57BL/6 Rag2–/– female mice were purchased from Taconic.

*Patients’ samples and analysis.* BM aspirates were obtained from patients with MM, while serum samples were obtained from either healthy controls or MM patients with or without clinically confirmed (by x-ray or CT) bone disease. MMP-13 levels were determined using a human MMP-13 ELISA kit (EMD Millipore).

For BM and tissue samples, tissue microarray slides (US Biomax) were prepared from formalin-fixed, paraffin-embedded normal and MM tissues. One slide included 21 well-characterized, randomly selected samples from 10 MM patients and 11 normal costal tissues. Five-micrometer sections of 1.5-mm-diameter cores were stained using a human MMP-13 (hMMP-13) Ab (clone VIIIa2; Calbiochem), combined with Dako EnVision Systems HRP (DAB).

*Cells.* The human MM cell lines RPMI-8226, H929, and U266 were purchased from the ATCC. MM.1R and OPM2 were provided by Klaus Podar (Dana-Farber Cancer Institute, Boston, Massachusetts, USA). All cell lines were cultured as previously described (64, 65). The murine MM cell line 5TGM1-GFP was cultured in IMDM medium with L-glutamine, penicillin-streptomycin, and 10% FBS. The mouse monocyte/macrophage cell line RAW264.7 and the pre-OB cell line MC3T3-E1 subclone 4 were purchased from the ATCC and cultured in α-MEM without ascorbic acid (Life Technologies) supplemented with L-glutamine, penicillin-streptomycin, and 10% FBS, respectively. Mouse primary BMSCs were collected by culturing adherent mouse BM cells in Iscove’s modified Dulbecco’s medium (IMDM) medium with L-glutamine, penicillin-streptomycin, and 10% FBS until confluence was reached. Human peripheral blood mononuclear cells (PBMCs) were isolated by ficoll gradient separation, then enriched by Monocyte Enrichment Cocktail (STEMCELL Technologies) according to the manufacturer’s instructions (34).

*Luminex screening assay.* Cell culture supernatants were screened for secreted MMPs (MMP-1,-2,-3,-7,-8,-12, and -13) or cytokines using Luminex assays (Luminex Core Facility at the University of Pittsburgh). The bead set conjugated with a capture Ab specific to a unique target protein was mixed with a 50-μl cell culture supernatant sample.
After washing, secondary biotin-conjugated Ab was added to form a capture sandwich immunoassay. Streptavidin-R-phycocerythrin solution was added and incubated for 30 minutes. A Lumienx array reader was used to determine MMP and cytokine concentrations on the basis of 7-point standard curves.

**Coculture experiments.** Primary BMSCs from patients with MM were seeded in 96-well plates at 4 × 10⁴ cells per well in RPMI 1640 supplemented with 10% FCS. After 24 hours, human MM cells (4 × 10⁴ cells/well) were seeded on the BMSC layer and cocultured for an additional 48 hours. MMP-13 concentrations in recovered supernatants were determined using a human MMP-13 Fluorokine E Kit (R&D Systems). To confirm the cell source of MMP-13 in cocultures, human BMSCs were precultured at a concentration of 3 × 10⁴ cells per well in 24-well plates for 24 hours. GFP⁺ RPMI 8226 cells (1 × 10⁵ cells/well) were then seeded onto the stromal cell monolayer for 48 hours. MMP-13 expression in RPMI 8226 cells and BMSCs was measured by qRT-PCR following the separation of GFP⁺ BMSCs from GFP⁺ RPMI 8226 cells by flow cytomtery. β-actin was used as a loading control.

**Transwell assays.** BMSCs (3 × 10⁴ cells/well) were cultured for 24 hours in the lower chambers of 24-well Transwell plates. MM cells (1 × 10⁴ cells/well) were seeded in the upper chamber and cocultured for an additional 48 hours. MMP13 mRNA expression in MM cells was measured by qRT-PCR. For MM-OCL Transwell coculture assays, mouse nonadherent BM cells (1.5 × 10⁶ cells/well) were cultured in the lower chambers of 24-well Transwell plates with murine macrophage-CSF (mM-CSF) (10 ng/ml) and mRANKL (50 ng/ml) (both from R&D Systems). 5TGM1 EV or 5TGM1-MMP-13-KD cells (3 × 10⁵ cells/well) were seeded in the upper chamber and cocultured for 4 days.

**BMSC CM.** BMSCs (5 × 10⁵) isolated from patients with MM were cultured in 100-mm dishes for 24 hours in 10% FCS-DMEM media. The cells were then washed with PBS and cultured with fresh 5% FCS-DMEM media for a 3-day incubation period. The CM was collected by centrifugation (500 g for 15 minutes) and cell-free aliquots stored at −80°C until use.

**OCL formation and bone resorption assays.** For mouse cell studies, 1.5 × 10⁵ nonadherent BM mononuclear cells from C57BL/6 mice (4–6 weeks of age) were cultured in 96-well plates in α-MEM supplemented with 10% FBS, 10 ng/ml mM-CSF, and 50 ng/ml mRANKL (R&D Systems) in the presence or absence of recombinant human (rh) pro-MMP-1,-2,-9 or -13 (R&D Systems). To investigate MMP-13–induced ERK1/2 signaling in pre-OCLs, CD11b+ cells were purified by CD11b microbeads (Miltenyi Biotec) and cultured for 3 days. The cells were then fixed in formalin and stained for TRAP using a TRAP staining kit (Sigma-Aldrich). TRAP⁺ cells containing 3 or more nuclei were counted as OCLs. For bone resorption pit assays, 2 × 10⁵ BM mononuclear cells were seeded on dentin slices in 96-well plates and treated as described above for 21 days. The number of OCLs per well, the number of nuclei per OCL, and OCL size were quantified using 40X-2000X Digital Microscope with Scopimage 9.0 software (Bioimager) or Magnafire 4.1 software (Optronics).

**OB differentiation.** MC3T3 E1 cells (1 × 10⁵) were seeded in 24-well culture dishes in OB differentiation media (α-MEM without ascorbic acid, supplemented with 10% FBS, 1% penicillin-streptomycin, 50 μg/ml L-ascorbic acid, and 2 mM β-glycerophosphate), with or without MMP-13 (200 ng/ml), for up to 14 days. Media was changed every 3 days, and, at the times indicated in Supplemental Figure 17B, cultures were fixed in 4% paraformaldehyde for 10 minutes and stained for alkaline phosphatase or alizarin red.

**Immunoblot analysis.** Immunoblotting was performed as described previously (64, 67) using the following Abs: anti–MMP-13 (ab39012; Abcam); anti–DC-STAMP (clone 1A2; EMD Millipore); anti-NFATc1 (7A6) and anti-TRAF6 (H274) (both from Santa Cruz Biotechnology Inc.); anti-ubiquination (Ub-i; Thermo Fisher Scientific); anti-phosphorylated-c-Raf (anti-p-c-Raf) (Ser338) (56A6), anti-p-MEK1/2 (Ser217/221), anti-p-ERK1/2 (D13.14.4E), anti-p-p90RSK (Ser380) (D3H11), and anti-Erk2 (9108) (all from Cell Signaling Technology); and β-actin (Sigma-Aldrich).

**qRT-PCR analysis.** Total RNA was isolated with TRizol reagent (Thermo Fisher Scientific) and cDNA generated with SuperScript III reverse transcriptase (Invitrogen), followed by SYBR Green–based (Invitrogen) qRT-PCR assays performed as previously described (64). Primer pairs were as follows: human MMP13: forward, 5′-GATGGGCCCTCTGGGCTGCT; reverse, 5′-TGGCCGAATCTCATGCGGAC; human β-actin: forward, 5′-CGAGCACAGAGCTCTGCTTGTG; reverse, 5′-CGACGAGGGCCGGGATATCAT; mouse Mmp13: forward, 5′-GGTTCCAGGCGATGAAAGCCTCC; reverse, 5′-GGTGTCGAGGCGGCGAAGA; and mouse β-actin: forward, 5′-AACCTAAGGCCAACCCTGCTA; reverse, 5′-CGACGAGGCTACAGGAGCAC. Mouse Nfatc1 and Destm mRNA were analyzed by TaqMan qRT-PCR (Life Technologies).

**Flow cytometric assay.** For flow cytometric analysis, human PBMCs or mouse OCLs were harvested, washed once with FACS buffer (HBSS buffer supplemented with 0.5% BSA and 0.1% Na3), and incubated with primary Ab mouse anti–DC-STAMP clone 1A2 (EMD Millipore) and Fc receptor blocker anti-CD16/CD32 (BD Pharmingen) at room temperature for 20 minutes. Cells were washed with FACS buffer and finally incubated with FITC-conjugated goat anti-mouse secondary Ab (Invitrogen) at room temperature for 20 minutes. FACS data were acquired using a BD FACSCanto System and analyzed by FlowJo software (54, 67).
Expression constructs and site-directed mutagenesis. WT human MMP13 cDNA was generated by PCR using cDNA prepared from RPMI 8266 cells as a template with the following primers: forward, 5′-GACTGGAATTCCACTGACTCCAGGGGTCCTGGCCT-3′; reverse, 5′-GACTTCTGAGGCAACACCAAAATGGAATTTGC-3′. PCR products were then cloned into pcDNA6/myc-His B (Invitrogen) after EcoRI-XhoI digestion. GFP MMP-13–KD cells were transduced with an EV lentiviral control or an MMP-13–targeting shRNA following primers: forward, 5′-GAGGTCTTCCTCAGA-3′; reverse, 5′-TAAAGGAGTTCGGCAATGCTGGCAGCAACAGG-3′. Similarly, MMP-13 procatelolic domain (aa 1-267) constructs were generated by PCR using pcDNA6/myc-His B-MMP-13 WT as a template with the following primers: forward, 5′-GACTGGAATTCCACTGACTCCAGGGGTCCTGGCCT-3′; reverse, 5′-GACTTCTGAGGCAACACCAAAATGGAATTTGC-3′. MMP-13 procatalytic domain (aa 1-267) construct was generated by mutagenesis PCR with a pcDNA6/myc-His A–MMP-13 WT construct as a template and the following primers: forward, 5′-TCTGGAAGAGACCCTGGGAGCATGAAGACCCCAAC-3′; reverse, 5′-GACTGGAATTCCACTGACTCCAGGGGTCCTGGCCT-3′; followed by cloning into pcDNA6/myc-His A after EcoRI-XhoI double digestion. An MMP-13 hemopexin domain (deletion 37-267) construct was generated by mutagenesis PCR with a pcDNA6/myc-His B-MMP-13 WT construct as a template and the following primers: forward, 5′-TCTGGAAGAGACCCTGGGAGCATGAAGACCCCAAC-3′; reverse, 5′-GACTGGAATTCCACTGACTCCAGGGGTCCTGGCCT-3′. WT construct as a template using the following primers: forward, 5′-GACTTCTGAGGCAACACCAAAATGGAATTTGC-3′; reverse, 5′-GACTGGAATTCCACTGACTCCAGGGGTCCTGGCCT-3′. MMP-13 is 60 kD, while that of activated MMP-13 is 48 kD (69).

Lentivirus infection and MMP-13 knockdown and knockin. To silence MMP-13 expression in mouse MM cells, STGMI-GFP cells were transduced with an EV lentiviral control or an MMP-13-targeting shRNA lentiviral construct 1 (5′-CCGGGTCATCCATTTTCCTCCAGAAATTAGCGAATGAGCTTCTTCTG-3′) or an MMP-13-targeting shRNA lentiviral construct 2 (5′-CCGGGACTCTGAAATCCATACACTCAGGAATGGTATGATTTCTTACATTTTGTG-3′) (provided by Robert W. Sobol, Hillman Cancer Center, Pittsburgh, Pennsylvania, USA). STGMI-GFP cells were incubated with lentiviral particles and polybrene (8 μg/ml for 16 hours) and then washed. A second infection was repeated 24 hours later. Cells were selected by culturing for 14 days in puromycin (8 μg/ml). MMP-13 protein and mRNA levels were determined in control and silenced cells by Western blotting and qRT-PCR, respectively. To knock down MMP-13 WT or the E223A mutant into STGMI-GFP MMP-13–KD cells, MMP-13 WT or the E223A mutant was inserted into a pLHCX retroviral vector (Clontech). Retroviruses were packed by Phoenix-AMPHO cells (ATCC), and STGMI-GFP MMP-13–KD cells were transduced as described above. Cells were selected by culturing for 14 days in hygromycin B (100 μg/ml). MMP-13 protein was determined by Western blot analysis.

Protein purification. pcDNA6/myc-His B-MMP-13 WT, MMP-13 E223A, as well as MMP-13 procatelolic domain (aa 1-267) and hemopexin domain (deletion aa 37-267) constructs were transfected into HEK-293 cells using Lipofectamine 2000 (Invitrogen) and cultured in Opti-MEM serum-free medium (Thermo Fisher Scientific). Cell culture media were collected 48 hours after transfection, concentrated by ultrafiltration, and resuspended in PBS. Protein purity and concentration were determined by Coomassie Brilliant Blue R-250 staining following SDS-PAGE and a Bradford protein assay, respectively. The proteolytic activity of WT and E223A mutant MMP-13 was determined with an MMP-13 Fluorokine E Kit (R&D Systems) after pretreatment with 1 mM 4-aminophenylmercuric acetate (Sigma-Aldrich) at 37°C for 1 hour, according to the manufacturer’s instructions, and its activity compared with commercially obtained MMP-13 protein (R&D Systems) as an internal standard. In all cell cultures, MMP-13 was used at a concentration of 200 ng/ml unless stated otherwise.

H-thymidine incorporation assay. STGMI-1-GFP EV or MMP-13-KD cells (5 × 10^4 cells) were incubated in 96-well plates in 200 μl IMDM containing 10% FBS at 37°C and 5% CO₂ for 2 days. DNA synthesis was measured by 3H-thymidine incorporation (NEP Products, PerkinElmer Life and Analytical Sciences). Cells were pulsed with 3H-thymidine (1 μCi/well or 0.037 MBq) for the last 8 hours of culture, harvested onto glass fiber filter mats (Wallac) using an automatic cell harvester (Harvester 96, Mach III; Tomtec), and counted using a Wallac TriLux Beta Plate Scintillation Counter (PerkinElmer Life and Analytical Sciences) (64).

Cell proliferation assay. MM cells (1 × 10^5) or CD11b⁺ mouse BM mononuclear cells (1 × 10^5) were incubated in 96-well plates in 100 μl culture medium with bortezomib (100 nM), pomalidomide (2 μM), or vehicle, with or without MMP-13 (200 ng/ml), at 37°C and 5% CO₂ for up to 3 days. DMSO was added in the control group. Ten microliters of Premixed WST-1 Cell Proliferation Reagent (Clontech, Takara Bio) was added to each well for 4 hours and absorption determined in an ELISA reader (BioTek Instruments).

Collagen zymography. MM cells, RAW264.7 cells, MC3T3-E1 clone 4, or mouse primary BMSCs were cultured in standard medium until confluent and then switched to Opti-MEM I serum-free medium (Thermo Fisher Scientific) for 3 days. Medium was collected and concentrated by Amicon ultracentrifugal filtration (EMD Millipore). The concentrated supernatants were analyzed by 10% SDS-PAGE gel containing 0.5% collagen type I as previously described, and gels were stained with Coomassie Brilliant Blue R250 (Thermo Fisher Scientific) and then de-stained (68). In selected experiments, 0.4 μg purified MMP-13 WT or E223A mutant protein was incubated in MMP-13 reaction buffer, with or without 1 mM APMA, for 20 minutes at 37°C and analyzed by collagen zymography. The molecular weight of pro-MMP-13 is 60 kD, while that of activated MMP-13 is 48 kD (69).

Mouse MM model. STGMI-GFP EV or MMP-13-KD cells (1 × 10^5 cells) were bilaterally injected into both tibiae of C57BL/6 Rag2⁻/⁻ female mice as described previously (41, 70). Mice were sacrificed 4 weeks later, and tibiae were collected for GFP imaging and then fixed in 4% neutral-buffered formalin for micro-qCT analysis. Following micro-qCT scanning, the same tibiae were decalcified with 10% EDTA and subjected to histologic analysis. Tumor burden was assessed by serum analysis of the myeloma-specific IgG2b.

Micro-qCT analysis. Tissue samples were scanned using a micro-qCT system (VivaCT 40; Scanco Medical). For trabecular microstructure, 100 slices, corresponding to a 1.05-mm region underneath the growth plate, were obtained with 10.5 μm spatial resolution. For the cortical bone, 50 slices, corresponding to a 1.05-mm region starting in a region that was 56% of the tibia length from its proximal end, were obtained with 21 μm spatial resolution. A global threshold technique was applied to seg-
ment the grayscale image into binarized images. Both the trabecular and cortical compartments were analyzed with a semiautomatic contouring technique to assess microstructural parameters (71).

Bone histology and immunohistochemical staining. Following micro-qCT, the sameibia were decalcified in 10% EDTA (pH 7.4) buffer for 2 weeks at 4˚C and embedded in paraffin. Bone sections (5 μm thickness) were stained with H&E and for tartrate-resistant acid phosphatase (TRAP) using a Leukocyte Acid Phosphatase Kit (Sigma-Aldrich). For OB staining, paraffin sections underwent 10 mM sodium citrate antigen retrieval, followed by staining with anti-osteocalcin Ab (ab93876; Abcam) and EnVision® Systems HRP (DAB) (Dako) according to the manufacturer’s instructions.

Statistics. Quantitative data are presented as the mean ± SD or SEM, as indicated in the figure legends. Statistical significance was assessed by a 2-tailed Student’s t test or a Wilcoxon rank-sum test, as appropriate, for comparisons between 2 groups. In experiments with more than 2 experimental groups, Westfall’s adjustment for multiple comparisons (72) was applied to pairwise comparisons following ANOVA. Count data were analyzed by Poisson ANOVA (73), with a similar adjustment for multiple comparisons. The relationship between quantitative variables was characterized by linear or Poisson regression, depending on the nature of the response variable. A P value of 0.05 or less was considered significant, and a P of 0.01 or less was considered highly significant.

Study approval. All animal procedures were reviewed and approved by the IACUC of Columbia University (protocols AC-AAAE9803 and AC-AAAN5201). BM aspirates were obtained with informed consent from healthy controls and MM patients under an IRB-approved tissue repository protocol at the University of Pittsburgh Cancer Institute (UPCI 86-22) and Columbia University Medical Center (AAAL5045).

Author contributions

26. Correia AL, Mori H, Chen EI, Schmitt FC, Bissell MJ. The hemopexin domain of MMP13 is responsible for mammary epithelial invasion and morphogenesis through extracellular interaction with...