Published in final edited form as:

Matrix Biol. 2011 May ; 30(4): 281–289. doi:10.1016/j.matbio.2011.04.003.

Divergent Upstream Osteogenic Events Contribute to the Differential Modulation of MG63 Cell Osteoblast Differentiation by MMP-1 (Collagenase-1) and MMP-13 (Collagenase-3)

Takayuki Hayami, Yvonne L. Kapila, and Sunil Kapila

The University of Michigan, 1011 North University Avenue, Ann Arbor, Michigan, 48109

Takayuki Hayami: thayami@umich.edu; Yvonne L. Kapila: ykapila@umich.edu; Sunil Kapila: skapila@umich.edu

Abstract

Previously we showed that MMP-1 (collagenase-1) and MMP-13 (collagenase-3) differentially regulate the expression of osteoblastic markers in a heterogenous population of primary human periodontal ligament cells. The mechanisms for these differential responses are not known, but may result from divergence in regulation of early osteogenic transcription factors. The purpose of this study was to elucidate where in the hierarchy of osteoblast-specific transcription factors and markers the differences in MMP-1- and -13-mediated regulation of osteoblastic differentiation arise. We found that the overexpression of MMP-1 resulted in significant decreases in BMP-2, Dlx5, AP, OP and BSP and increases in TGF- β 1 and MSX2. In contrast, MMP-13 overexpression resulted in significant decreases in Runx2, OP and BSP, and increases in TGF- β 1, MSX2 and OC. The knockdown of MMP-1 caused significant increases in all osteoblastic markers. MMP-13 knockdown produced significant increases only in TGF- β 1, MSX2 and Osx, but decreases in Runx2 and OC. Suppression of both MMPs together resulted in significant increases of all osteoblastic markers except Runx2. MMP-1 had a more robust and generalized effect in regulating osteoblast transcription factors and markers than MMP-13. Finally, of the markers and transcription factors assayed, Runx2 is the most early stage transcription factor induced by suppression of MMP-1, while Osx and MSX2 are the most early stage transcription factors regulated by MMP-13. These data show that MMP-1's and -13's differential regulation of osteoblastic markers in MG63 cells likely results from their modulation of divergent signaling pathways involved in osteoblastic differentiation.

Keywords

MMP-1; MMP-13; osteoblast differentiation; transcription factors; alkaline phosphatase

© 2011 Published by Elsevier B.V. and International Society of Matrix Biology.

Address for correspondence, Sunil Kapila, PhD., 1011 North University Avenue, The University of Michigan, Ann Arbor, Michigan, 48109-1078, skapila@umich.edu, Tel: +1-734-647-8750, Fax: +1-734-763-8100.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

Conflict of interest

The authors have no conflict of interest.

1. Introduction

Matrix metalloproteinases (MMPs) are zinc-dependent endopeptidases that are capable of degrading most extracellular matrix proteins. They are classified based partly on substrate specificity and are thereby categorized as collagenases, gelatinases, stromelysins, membrane-type MMPs and others. Collagenases are key enzymes responsible for degradation of triple-helical fibril collagens, including type I collagen, which is a major organic component of bone tissue and important for differentiation of osteogenic cells into mature osteoblasts (Andrianarivo et al., 1992; Mizuno et al., 2000). There are three isoforms of collagenase, MMP-1 (collagenase-1), -8 (collagenase-2) and -13 (collagenase-3). Of these collagenases, osteoblasts are known to express both MMP-1 and -13 (Partridge et al., 1996; Varghese and Canalis, 1997).

Several studies have demonstrated that collagenases substantially and negatively regulate the expression of osteoblastic markers in osteogenic cells. Thus the incubation of MC3T3-E1 or human osteoblastic cells with IL-1 β inhibits collagen synthesis (Ikeda et al., 1988), alkaline phosphatase (AP) activity, osteocalcin (OC) expression, and mineral deposition concomitant with the induction of collagenase (Chien et al., 1999). In contrast, the dexamethasone-mediated suppression of MMP-1 expression and activity results in increased AP activity and OC mRNA levels in human periodontal ligament (PDL) cells (Hayami et al., 2007; Shiga et al., 2003). More conclusive and direct evidence for the role of collagenases in modulating osteoblastic differentiation is provided by studies in which the addition of exogenous bacterial collagenase or human MMP-1 downregulate AP activity in osteogenic cells (Franceschi et al., 1994; Hayami et al., 2008).

Further dissection of this phenomenon has shown that the two collagenases that are expressed by osteogenic cells namely MMP-1 and -13, regulate different markers of osteoblastic differentiation in human PDL cell isolates (Hayami et al., 2008) that are known to contain osteogenic cells (Huang et al., 2009; Xu et al., 2009). More specifically, on assaying a limited number of osteoblast transcription factors and markers in PDL cell cultures, it was shown that MMP-1 modulates very early to late stage osteoblastic markers, whereas MMP-13 regulates a small subset of markers of osteoblast differentiation, suggesting a differential regulation of osteoblastogenesis by these collagenases. However, there is no information currently available about where in the known signaling process of transcription factors involved in the progressive differentiation of osteoblasts that MMP-1 and -13 modulate this process. Furthermore, since cells from the PDL represent a heterogeneous population in which the proportion of osteogenic cells vary between cell isolates, the resulting variability in robustness of response between experiments makes this cell system unsuitable to perform mechanistic studies. Therefore, the current studies were performed using MG63 osteoblastic cell lines to specifically elucidate where in the hierarchy of osteoblast-specific transcription factors and markers the differences in MMP-1- and -13-mediated regulation of osteoblastic differentiation arise.

2. Results

2.1. Overexpression of MMP-1 and -13 decreases AP activity

In order to determine if MMP-1 and -13 modulate the osteoblastic phenotype in MG63 cells, we performed the initial characterization of the cell's responses to the overexpression of these MMPs by assaying for AP. Quantitative RT-PCR, Western blots and activity assays for MMP-1 and -13 on cells transiently transfected with their respective cDNAs demonstrated specific and significant increases in the expression of these collagenases (Figs. 1A to F) throughout the experimental period (data not shown). Specifically cDNA transfected cells showed a very robust 11-fold overexpression of mRNA for MMP-1 and 50-

fold overexpression for MMP-13. Additionally, MMP-1 cDNA transfected cells showed 2.0-fold higher MMP-1 activity than empty vector transfected cells (Baseline, 0.30 ng/ μ g $\pm$ 0.09) (Fig. 1E). Similarly, MMP-13 cDNA transfected cells showed 2.3-fold higher MMP-13 activity than empty vector transfected cells (Baseline, 0.05 ng/ μ g $\pm$ 0.01) (Fig. 1F). Transfection of the cells with either MMP cDNAs had no effect on cell viability (data not shown). Finally, AP activity was significantly reduced by 0.6- and 0.7-fold in cells transfected with MMP-1 or -13 cDNA, respectively, compared to empty vector transfected cells, demonstrating that these proteinases indeed modulate a key marker of osteoblastic differentiation (Fig. 1G). However, type I collagen levels in cell-matrix extract were not affected in cells overexpressing MMP-1 or MMP-13 (Fig. 1H).

2.2. Overexpression of MMP-1 and -13 differentially regulate osteoblastic marker expression

Next we examined the effects of MMP-1 and -13 overexpression on osteoblast transcription factors and markers that span a broad spectrum from very early to late stages of osteoblastic differentiation. Since preliminary time-course studies showed that transforming growth factor- β 1 (TGF- β 1), Msh homeobox 2 (MSX2), bone morphogenetic protein 2 (BMP-2) and distal-less homeobox 5 (Dlx5) were maximally modulated within a few minutes, Runx2, osterix (Osx) and AP at 36 hours, and all of the other markers at 72 hours (data not shown), these time-points were selected for subsequent assays for the respective markers.

MMP-1 overexpression resulted in a significant reduction in BMP-2 (0.8-fold), Dlx5 (0.7-fold), Osx (0.8-fold), AP (0.7-fold), osteopontin (OP; 0.4-fold), OC (0.8-fold) and bone sialoprotein (BSP; 0.5-fold), a significant induction in TGF- β 1 (1.1-fold) and MSX2 (1.5-fold), and no-change in Runx2 mRNA expression (Fig. 2A). In contrast, MMP-13 overexpression caused a significant reduction in Runx2 (0.8-fold), AP (0.7-fold), OP (0.7-fold), and BSP (0.7-fold), and a significant increase in TGF- β 1 (1.15-fold) and MSX2 (1.5-fold) mRNA expression, while the expression levels of BMP-2, Dlx5 and Osx remained largely unchanged. Unexpectedly, overexpression of MMP-13 caused a significant increase in OC gene expression (1.3-fold) (Fig. 2B). Taken together, these findings show that overexpression of MMP-1 and -13 differentially regulate osteoblast transcription factors and the downstream expression of osteoblast markers.

2.3. MMP-1 and/or MMP-13 suppression promotes an increase in AP activity

Since overexpression of collagenases, particularly MMP-1 suppressed key markers of osteoblastic differentiation, we next determined if suppression of these proteinases enhances osteoblastic differentiation, and also whether the divergent differentiation pathways observed on overexpression of MMP-1 and -13 are similarly seen when these enzymes are suppressed. Successful, robust and specific suppression of MMP-1 and / or MMP-13 transcription and translation was achieved through transient transfection of their respective siRNAs (Fig. 3A to D) over the entire duration of the experiment (data not shown). The suppression of MMP-1 or -13 also resulted in a specific decrease of about 0.4- to 0.3-fold in the respective enzymatic activities (baseline activity 0.71 ng/ μ g $\pm$ 0.08 for MMP-1 and 0.04 ng/ μ g $\pm$ 0.01 for MMP-13) (Figs. 3 E and F). Double transfection with MMP-1 and -13 siRNA resulted in similar levels of decreases in each enzymes activity as when either MMP-1 or -13 siRNA alone were transfected. The suppression of MMP-1 or -13 resulted in a 1.5- and 1.3-fold increase in AP activity, respectively, compared to controls (Fig. 3G). Cells double transfected with MMP-1 and -13 siRNA showed a 100% increase in AP activity (Fig. 3G). However, transfection of siRNAs to either or both MMPs did not modulate the levels of type I collagen in cell-matrix extracts (Fig. 3H).

2.4. Suppression of MMP-1 and -13 differentially enhance the expression of osteoblast markers

Given that knockdown of MMP-1 or -13 or both upregulated AP activity in MG63 cells we surmised that other osteoblast-related growth factors, osteoblast-specific transcription factors, and markers were also regulated by suppression of these MMPs. As with the MMP-1 and -13 overexpression studies, suppression of each MMP modulated different osteoblastic markers and produced a different magnitude of change in the expression levels of individual osteoblastic genes compared to controls. Thus suppression of MMP-1 enhanced BMP-2 (1.5-fold), Dlx5 (1.3-fold), Runx2 (1.3-fold), Osx (1.7-fold), AP (1.1-fold), OP (1.6-fold), OC (1.4-fold) and BSP (1.4-fold) expression, and produced no-change in TGF- β 1 and MSX2 gene expression (Fig. 4A). Inhibiting MMP-13 alone produced a significant increase in TGF- β 1 (1.5-fold), MSX2 (1.4-fold) and Osx (1.3-fold) expression, and produced no-change in BMP-2, Dlx5, OP, and BSP expression (Fig. 4B). Consistent with the overexpression experiment, the inhibition of this MMP caused a significant decrease in Runx2 (0.7-fold) and OC (0.7-fold). Suppression of both MMP-1 and -13 resulted in similar changes in the osteoblastic genes as that seen with MMP-1 knockdown, albeit of a higher magnitude. The exception to this similarity was that Runx2 expression, which was slightly elevated by MMP-1 suppression alone was not modulated by suppression of both MMPs. Overall, the knockdown of both MMPs caused large increases in BMP-2 (1.9-fold), Dlx5 (1.7-fold), Osx (2.4-fold), AP (1.5-fold), OP (1.6-fold), and OC (1.6-fold), a moderate increase in BSP (1.4-fold) expression, but produced no-change in TGF- β 1, MSX2 and Runx2 expression (Fig. 4C).

3. Discussion

Osteoblasts undergo a progressive differentiation from mesenchymal progenitors to preosteoblasts to immature osteoblasts to mature osteoblasts and finally to osteocytes, with each stage being characterized by different phenotypes and expression of specific osteogenic transcription factors and makers. It is currently known that osteoblast differentiation is mediated via at least two main pathways (Ryoo et al., 2006). The first of these upstream regulatory pathways involve the initial differentiation of mesenchymal progenitor cells into preosteoblasts, that sequentially require the transcription factors Dlx5 and Runx2, which in turn are induced by secreted factors such as BMP-2 (Fig. 5). At the preosteoblast stage, Dlx5 independent of or via Runx2 induces Osx. These pathways then contribute to the differentiation of the cells into immature osteoblasts marked by enhanced expression of AP and OP, and subsequently into mature osteoblasts that are characterized by increased expression of OC and BSP. Also, between the mesenchymal progenitor stage to immature osteoblast stage of differentiation MSX2, which can be induced by TGF- β 1, enhances proliferation and suppresses osteoblast differentiation. Although various secreted bioactive agents, including BMP-2 and TGF- β 1, are known to positively or negatively regulate these differentiation pathways, our findings demonstrate for the first time that the modulation of osteoblast differentiation is also mediated by MMP-1, and to a lesser extent by MMP-13, via their regulation of early osteoblast transcription factors.

Our findings show relatively robust and consistent effects of MMP-1 in the regulation of very early, mid and late stage osteoblast specific transcription factors and markers. Furthermore, overexpression or suppression of MMP-1 had consistently opposite effects on the suppression or induction, respectively, of all the differentiation transcription factors and downstream osteoblast markers with the exception of Runx2 (Figs. 5A and B). In contrast, overexpression of MMP-13 did not modulate the very early stage transcription factor Dlx5 or its effector BMP2, and slightly albeit significantly suppressed the early stage transcription factor Runx2 and the markers AP, OP and BSP. Surprisingly, the overexpression of MMP-13 increased the transcription of the OC gene, which is consistent with previous

findings on the discordant regulation of OC and AP in osteoblast precursors (Wenstrup et al., 1996). Suppression of MMP-13 enhanced the expression of *Osx*, AP and pro-proliferative factors *MSX2* and its inducer TGF- β 1. Thus, the modulation of pro-osteoblastic responses of MG63 cells to MMP-1 suppression appear to occur via *Dlx5* / *Runx2* or *Dlx5* / *Osx* or both pathways, while that mediated by MMP-13 occurs via the more downstream *Osx* pathway. The reasons for the apparent disparity in findings between the effects of MMP-1 and -13 on osteoblast differentiation are not clear, but suggest that it may involve mechanisms other than their shared common function of collagenolysis.

A classic mechanism important in facilitating osteoblastic differentiation involves accretion of collagen (Andrianarivo et al., 1992; Franceschi and Iyer, 1992; Lynch et al., 1995; Mizuno et al., 2000). Collagen may mediate its effects on osteoblast differentiation via integrin signaling (Globus et al., 1995; Jikko et al., 1999; Reyes and Garcia, 2004; Schneider et al., 2001; Xiao et al., 1998) or by appropriate presentation of stimulatory factors to the cell (Aronow et al., 1990; Beck et al., 1998; Franceschi and Iyer, 1992; Franceschi et al., 1994; Torii et al., 1994). Given this mechanism, it is plausible that increased levels of the collagenases MMP-1 and -13 diminish or delay osteoblast differentiation by disrupting the accumulation of the collagenous matrix. However, because the stimulated induction of type I collagen during osteoblast differentiation occurs after 24 hours (Franceschi and Iyer, 1992), the relatively slow accretion of collagen, as well as our observations on the changes in very early osteogenic genes despite the lack of change in type I collagen in cell-matrix extracts (Fig. 1H and Fig. 3H) by MMP-1 and -13, suggest that this mechanism is not responsible for our findings.

Instead the modulation of very early transcription factors by these MMPs combined with the consistent and opposite effects of overexpression or suppression of MMP-1 on osteoblast differentiation suggest a receptor-mediated mechanism for the actions of MMP-1 and possibly -13 on osteoblast differentiation. Indeed, cleavage of the MMP-1 receptor, protease activated receptor-1 (PAR-1) by thrombin increases proliferative responses in rat primary osteoblast cells (Abraham and MacKie, 1999); findings that are consistent with ours on the effects of MMP-1 on the proliferation transcription factor *MSX2* and its effector TGF- β 1 (Fig. 5). Furthermore, our recent studies showing that MMP-1 dose-dependently rescues the AP activity in MG63 cells in which PAR-1 expression had been suppressed provides evidence that MMP-1 interactions with PAR-1 are likely involved in regulating osteoblastic differentiation (Hayami et al., 2010).

The regulation of MMP-13 during osteoblastic differentiation has been studied extensively (D'Alonzo et al., 2002; Jimenez et al., 1999; Johansson et al., 1997; Tuckermann et al., 2000; Winchester et al., 1999). MMP-13 is an important marker for mature osteoblasts and hypertrophic chondrocytes during endochondral ossification (Johansson et al., 1997), its levels increase continuously during osteoblastic differentiation (D'Alonzo et al., 2002; Tuckermann et al., 2000; Winchester et al., 1999), its promoter contains *Runx2* binding site and it is regulated by *Runx2* (Jimenez et al., 1999), and MMP-13 knockout mice fail to undergo normal ossification (Inada et al., 2004). Because of these findings MMP-13 is considered important in bone matrix turnover during bone formation and remodeling (Jimenez et al., 1999). However, despite the intricate regulation of MMP-13 during osteoblast differentiation, its role in regulating osteoblastic differentiation or markers has not been previously elucidated. We show that MMP-13 contributes significantly to regulation of specific osteoblast markers and is involved in facilitating osteoblast differentiation. Thus, MMP-13 is not only regulated during osteoblast differentiation, but it in turn likely regulates osteoblastic genes, as shown in our studies.

The reasons for the differences in osteogenic responses of MG63 cells to MMP-1 and -13, which are largely similar to those found previously with the osteogenic PDL cells (Hayami et al., 2008), are not clear. Although these differences may be attributed to the differential degradation of the collagen matrix- accretion of which is a requisite for osteoblast differentiation- by the more potent and the higher concentrations of MMP-1 vs MMP-13 (Fujimoto and Iwata, 2001; Knauper et al., 1996), the regulation of very early osteoblastic genes and the unchanged cell-matrix collagen levels by these MMPs suggests that this mechanism may not be the reason for the observed differences. Also, the somewhat varied and relatively upstream modulation of osteoblast differentiation by MMP-1 and -13 suggest early divergence in their mechanisms of induction of osteoblast differentiation. It is plausible that alternative mechanisms such as receptor-mediated activity of MMP-1 (Hayami et al., 2010), or the intricate involvement of MMP-13 but not MMP-1 in the intrinsic physiologic functions of osteoblast differentiation (Parikka et al., 2005; Pei et al., 2006; Selvamurugan et al., 2006; Varghese et al., 2005; Zaragoza et al., 2006) as well as other unknown mechanisms may explain the differences between the observed effects of MMP-1 and -13 on early osteoblast differentiation.

MMPs play a major role in the progression of osteoarthritis and other bone destructive diseases (Martel-Pelletier et al., 2001), and chondrocytes, fibroblasts, and osteoblasts are the primary sources of enzymes responsible for this degradation (Burrage et al., 2006; Holliday et al., 2003; Sakao et al., 2009). Our findings suggest that the presence of elevated levels of MMP-1 or -13 or both contributes to the inhibition or delay in osteoblast differentiation, which in turn is likely to diminish the pool of mature osteoblasts available for repair and regeneration of bone. This mechanism of MMP-1-and -13-mediated inhibition of normal osteoblastogenesis in limiting bone repair following disease warrants validation through further *in vivo* studies. In contrast, the upregulation of osteoblastic markers following suppression of MMP-1, and to a lesser extent of MMP-13, suggest the potential role of specific MMP inhibitors in bone regeneration and bone tissue engineering. Indeed, it is plausible that the known therapeutic effects of chemically modified tetracyclines such as doxycycline and other MMP inhibitors in contributing to bone regeneration (Almazin et al., 2009; Gomes and Fernandes, 2007; Gomes et al., 2008; Sasaki et al., 1999) may result partially through this mechanism.

4. Experimental Procedures

4.1. Cell culture

Human osteoblastic-like MG63 cells were cultured in alpha-minimum essential medium (α MEM) with 10% fetal bovine serum (FBS) at 37°C in 5% CO₂ and antibiotics (1x penicillin and streptomycin) in humidified air. Cells were plated at 2.0×10^4 cells/cm² in six-well plates in α MEM with 10% FBS without antibiotics for transfection of cDNA or siRNA.

4.2. MMP overexpression with cDNA transfection

For collagenase overexpression, 2 μ g/well of MMP-1 cDNA which was inserted into pBluescript KS (gift from Dr. Goldberg of Washington University in St. Louis) (Goldberg et al., 1986) or MMP-13 cDNA which was inserted into pEE12 (gift from Dr. Lopez-Otin of Universidad de Oviedo in Oviedo, Spain) (Knauper et al., 1996) or empty plasmid (Stratagene, La Jolla, CA, USA) was transfected into cells using Fugene® HD (Roche Diagnostic, Basel, Switzerland) according to manufacturer's instructions for 24 hours. Cells were then cultured in serum free medium for five minutes, 36 hour or 72 hours, and counted and lysed for AP activity assay, or mRNA was extracted for quantitative reverse transcriptase-polymerase chain reaction (qRT-PCR) for TGF- β 1, MSX2, BMP-2, Dlx5,

Runx2, Osx, AP, OP, OC, BSP, MMP-1 and MMP-13. MMP-1 and -13 in cell-conditioned media were also assayed by Western blotting and MMP-1 and -13 specific activity assays. Collagen I levels in cell-matrix extract were also assayed by Western blots. Cell viability was determined by trypan blue staining.

4.3. Suppression of MMP-1 and -13 with siRNA transfection

To suppress collagenase, Stealth™ Select 3 RNAi Set (Invitrogen Corp., Carlsbad, CA, USA) was used to target three sites located on MMP-1 or MMP-13 mRNA as described previously (Hayami et al., 2008). A Stealth™ Negative Control (Invitrogen) siRNA was used as a negative control. Briefly, 250 nmol/well of control siRNA or MMP-1 siRNA or MMP-13 siRNA or both MMP-1 and -13 siRNAs were transfected into MG63 cells using Lipofectamine™ 2000 (Invitrogen) according to the manufacturer's instructions. Cell-conditioned media, cell lysates and total mRNA were collected and assayed as described above.

4.4. Total Protein

For standardization of subsequent assays, total protein in cell-conditioned medium was measured by a Bradford microassay (Bio-Rad, Hercules, CA, USA), and in cell lysates using the BCA protein assay (Pierce, Rockford, IL, USA) according to the manufacturers' instructions.

4.5. Western blots

Western blots were used to identify and compare the changes in MMP-1, MMP-13, and type I collagen expression in cDNA or siRNA transfected cells. Conditioned medium (for MMPs) or cell lysates (for type I collagen) standardized by total protein were electrophoretically resolved on 10% SDS-PAGE gels and transferred to PVDF membranes. Non-specific binding was blocked with 2.5% dry non-fat milk in tris-buffered saline with 0.1% Tween 20 (TBST), membranes washed and incubated either with rabbit anti-human MMP-1 (Calbiochem, San Diego, CA, USA) or MMP-13 (Abcam Inc., Cambridge, MA, USA) or rabbit anti-type I collagen (Calbiochem) antibodies. Beta-actin (primary antibody from Abcam Inc.) was used as a loading control for cell lysates. After further washes with TBST, the membranes were incubated with peroxidase-conjugated goat anti-rabbit secondary antibody (Sigma-Aldrich, St. Louis, MO, USA). The membranes were washed again and the bands visualized by enhanced chemiluminescence (SuperSignal West Pico, Pierce, Rockford, IL, USA). Images of the blots were captured by a scanner (V700 Photo Scanner, EPSON, Long Beach, CA, USA) and the bands were analyzed by video-densitometry as described previously (Shiga et al., 2003).

4.6. Alkaline phosphatase activity assay

AP activity was assayed in the cell lysates by enzymatic conversion of p-nitrophenylphosphate substrate to p-nitrophenol (Sigma-Aldrich) as described previously (Shiga et al., 2003). The amount of p-nitrophenol produced was measured spectrophotometrically at a wavelength of 410 nm using a microplate spectrofluorometer (Spectramax M2, Molecular Devices, Sunnyvale, CA, USA) against a standard curve and the results were standardized by cell number per minute.

4.7. MMP-1 and MMP-13 activity assays

The levels of active MMP-1 and -13 in the cell-conditioned medium were determined by Fluorokine® E enzyme activity assay (R&D Systems, Minneapolis, MN, USA) according to manufacturer's instructions. Briefly, 200 µl of samples or standards were incubated in 96-well plates coated with MMP-1 or -13 monoclonal antibodies. The wells were washed, and

the fluorogenic substrate linked to a quencher molecule, was added and incubated for 20 hours at 37 °C. The fluorescence resulting from the cleavage of the substrate between the fluorophore and quencher by the antibody-bound MMP was read at an excitation wavelength of 320 nm and emission wavelength of 405 nm using a microplate spectrofluorometer (Spectramax M2, Molecular Devices). The RFU for each sample was determined and converted to ng of active MMP-1 or -13 using standard curves, and the amount of MMP was standardized by total protein in the medium.

4.8. Quantitative reverse transcriptase-polymerase chain reaction

MMP-1, MMP-13, TGF- β 1, MSX2, BMP-2, Dlx5, Runx2, Osx, AP, OP, OC, BSP and GAPDH mRNA levels were assayed by qRT-PCR. Reverse transcription was performed on 50 ng total RNA in the initial mix with MuLV reverse transcriptase according to the manufacturer's instructions (High-Capacity cDNA Reverse Transcription Kits, Applied Biosystems, Foster City, CA, USA). All primers and probes (GAPDH Cat # Hs99999905-m1; MMP-1 Cat # Hs00233958-m1; MMP-13 Cat # Hs00233922-m1; TGF- β 1 Cat # Hs00171257-m1; MSX2 Cat # Hs00751239-s1; BMP-2 Cat # Hs00154192-m1; Dlx5 Cat # Hs00193291-m1; Runx2 Cat # Hs00231692-m1; Osx Cat # Hs01866874-m1; AP Cat# Hs01029144-m1; OP Cat# Hs00157093-m1; OC Cat # Hs00609452-m1; BSP Cat # Hs00173720-m1) were obtained commercially (TaqMan® Gene Expression Assay, Applied Biosystems) and amplified using a kit following the manufacturers instructions (TaqMan® Gene Expression Master Mix, Applied Biosystems). Amplification was done under the following conditions: 50°C, 2 min; 95°C, 10 min; followed by 50 cycles of 94°C, 15 s and 60°C, 1 min. Data were analyzed by $\Delta\Delta$ ct method using the ABI Prism 7500 SDS 1.2 Software (Applied Biosystems). GAPDH was used as an internal control.

4.9. Statistical analysis

All experiments were performed a minimum of three times each using three wells and all assays were performed in triplicate. All quantitative data were derived as mean fold change ($\pm$ SD) relative to baseline control levels. The effects of siRNA or cDNA transfection, on the expression of AP, MMP-1, MMP-13 and each osteoblastic marker was determined by a two-way ANOVA and the intergroup differences were analyzed by Fisher's PLSD test with the level of significance set at $P < 0.05$.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Abbreviations

AP	alkaline phosphatase
BMP-2	bone morphogenetic protein-2
BSP	bone sialoprotein
Dlx5	distal-less homeobox 5
MSX2	Msh homeobox 2
Osx	osterix
OP	osteopontin
OC	osteocalcin
Runx2	runt-related transcription factor 2

TGF- β 1 transforming growth factor- β 1
MMP matrix metalloproteinase

Acknowledgments

We wish to thank to Dr. Gregory I. Goldberg of Washington University in St. Louis for providing MMP-1 cDNA, and Dr. Carlos Lopez-Otin of Universidad de Oviedo in Oviedo, Spain for providing MMP-13 cDNA. This study was supported by NIH R01 DE16671.

References

- Abraham LA, MacKie EJ. Modulation of osteoblast-like cell behavior by activation of protease-activated receptor-1. *J Bone Miner Res.* 1999; 14:1320–1329. [PubMed: 10457264]
- Almazin SM, Dziak R, Andreana S, Ciancio SG. The effect of doxycycline hyclate, chlorhexidine gluconate, and minocycline hydrochloride on osteoblastic proliferation and differentiation in vitro. *J Periodontol.* 2009; 80:999–1005. [PubMed: 19485832]
- Andrianarivo AG, Robinson JA, Mann KG, Tracy RP. Growth on type I collagen promotes expression of the osteoblastic phenotype in human osteosarcoma MG-63 cells. *J Cell Physiol.* 1992; 153:256–265. [PubMed: 1429847]
- Aronow MA, Gerstenfeld LC, Owen TA, Tassinari MS, Stein GS, Lian JB. Factors that promote progressive development of the osteoblast phenotype in cultured fetal rat calvaria cells. *J Cell Physiol.* 1990; 143:213–221. [PubMed: 2332447]
- Beck GR Jr, Sullivan EC, Moran E, Zerler B. Relationship between alkaline phosphatase levels, osteopontin expression, and mineralization in differentiating MC3T3-E1 osteoblasts. *J Cell Biochem.* 1998; 68:269–280. [PubMed: 9443082]
- Burrage PS, Mix KS, Brinckerhoff CE. Matrix metalloproteinases: role in arthritis. *Front Biosci.* 2006; 11:529–543. [PubMed: 16146751]
- Chien HH, Lin WL, Cho MI. Interleukin-1 β -induced release of matrix proteins into culture media causes inhibition of mineralization of nodules formed by periodontal ligament cells in vitro. *Calcif Tissue Int.* 1999; 64:402–413. [PubMed: 10203417]
- D'Alonzo RC, Kowalski AJ, Denhardt DT, Nickols GA, Partridge NC. Regulation of collagenase-3 and osteocalcin gene expression by collagen and osteopontin in differentiating MC3T3-E1 cells. *J Biol Chem.* 2002; 277:24788–24798. [PubMed: 11964409]
- Franceschi RT, Iyer BS. Relationship between collagen synthesis and expression of the osteoblast phenotype in MC3T3-E1 cells. *J Bone Miner Res.* 1992; 7:235–246. [PubMed: 1373931]
- Franceschi RT, Iyer BS, Cui Y. Effects of ascorbic acid on collagen matrix formation and osteoblast differentiation in murine MC3T3-E1 cells. *J Bone Miner Res.* 1994; 9:843–854. [PubMed: 8079660]
- Fujimoto, N.; Iwata, K. Use of EIA to measure MMPs and TIMPs. In: Clark, IM., editor. *Martix Metalloproteinase Protocols*. Totowa: Humana Press Inc; 2001. p. 347–358.
- Gennari L, Bilezikian JP. Glucocorticoid-induced osteoporosis: hope on the HORIZON. *Lancet.* 2009; 373:1225–1226. [PubMed: 19362657]
- Globus RK, Moursi A, Zimmerman D, Lull J, Damsky C. Integrin-extracellular matrix interactions in connective tissue remodeling and osteoblast differentiation. *ASGSB Bull.* 1995; 8:19–28. [PubMed: 11538547]
- Goldberg GI, Wilhelm SM, Kronberger A, Bauer EA, Grant GA, Eisen AZ. Human fibroblast collagenase. Complete primary structure and homology to an oncogene transformation-induced rat protein. *J Biol Chem.* 1986; 261:6600–6605. [PubMed: 3009463]
- Gomes PS, Fernandes MH. Effect of therapeutic levels of doxycycline and minocycline in the proliferation and differentiation of human bone marrow osteoblastic cells. *Arch Oral Biol.* 2007; 52:251–259. [PubMed: 17141175]

- Gomes PS, Santos JD, Fernandes MH. Cell-induced response by tetracyclines on human bone marrow colonized hydroxyapatite and Bonelike. *Acta Biomater.* 2008; 4:630–637. [PubMed: 18291737]
- Hayami T, Kapila YL, Kapila S. MMP-1 (collagenase-1) and MMP-13 (collagenase-3) differentially regulate markers of osteoblastic differentiation in osteogenic cells. *Matrix Biol.* 2008; 27:682–692. [PubMed: 18755271]
- Hayami T, Kapila YL, Kapila S. MMP-1's regulation of osteoblastic differentiation occurs via protease-activated receptor-1. *J Dent Res.* 2010; 89:921. Spec Iss A. [PubMed: 20519491]
- Hayami T, Zhang Q, Kapila Y, Kapila S. Dexamethasone's enhancement of osteoblastic markers in human periodontal ligament cells is associated with inhibition of collagenase expression. *Bone.* 2007; 40:93–104. [PubMed: 16934542]
- Holliday LS, Vakani A, Archer L, Dolce C. Effects of matrix metalloproteinase inhibitors on bone resorption and orthodontic tooth movement. *J Dent Res.* 2003; 82:687–691. [PubMed: 12939351]
- Huang CY, Pelaez D, Dominguez-Bendala J, Garcia-Godoy F, Cheung HS. Plasticity of stem cells derived from adult periodontal ligament. *Regen Med.* 2009; 4:809–821. [PubMed: 19903001]
- Ikeda E, Kusaka M, Hakeda Y, Yokota K, Kumegawa M, Yamamoto S. Effect of interleukin 1 beta on osteoblastic clone MC3T3-E1 cells. *Calcif Tissue Int.* 1988; 43:162–166. [PubMed: 3141017]
- Inada M, Wang Y, Byrne MH, Rahman MU, Miyaura C, Lopez-Otin C, Krane SM. Critical roles for collagenase-3 (Mmp13) in development of growth plate cartilage and in endochondral ossification. *Proc Natl Acad Sci U S A.* 2004; 101:17192–17197. [PubMed: 15563592]
- Jikko A, Harris SE, Chen D, Mendrick DL, Damsky CH. Collagen integrin receptors regulate early osteoblast differentiation induced by BMP-2. *J Bone Miner Res.* 1999; 14:1075–1083. [PubMed: 10404007]
- Jimenez MJ, Balbin M, Lopez JM, Alvarez J, Komori T, Lopez-Otin C. Collagenase 3 is a target of Cbfa1, a transcription factor of the runt gene family involved in bone formation. *Mol Cell Biol.* 1999; 19:4431–4442. [PubMed: 10330183]
- Johansson N, Saarialho-Kere U, Airola K, Herva R, Nissinen L, Westermarck J, Vuorio E, Heino J, Kahari VM. Collagenase-3 (MMP-13) is expressed by hypertrophic chondrocytes, periosteal cells, and osteoblasts during human fetal bone development. *Dev Dyn.* 1997; 208:387–397. [PubMed: 9056642]
- Knauper V, Lopez-Otin C, Smith B, Knight G, Murphy G. Biochemical characterization of human collagenase-3. *J Biol Chem.* 1996; 271:1544–1550. [PubMed: 8576151]
- Komori T. Regulation of osteoblast differentiation by transcription factors. *J Cell Biochem.* 2006; 99:1233–1239. [PubMed: 16795049]
- Lynch MP, Stein JL, Stein GS, Lian JB. The influence of type I collagen on the development and maintenance of the osteoblast phenotype in primary and passaged rat calvarial osteoblasts: modification of expression of genes supporting cell growth, adhesion, and extracellular matrix mineralization. *Exp Cell Res.* 1995; 216:35–45. [PubMed: 7813631]
- Martel-Pelletier J, Welsch DJ, Pelletier JP. Metalloproteases and inhibitors in arthritic diseases. *Best Pract Res Clin Rheumatol.* 2001; 15:805–829. [PubMed: 11812023]
- Mizuno M, Fujisawa R, Kuboki Y. Type I collagen-induced osteoblastic differentiation of bone-marrow cells mediated by collagen-alpha2beta1 integrin interaction. *J Cell Physiol.* 2000; 184:207–213. [PubMed: 10867645]
- Parikka V, Vaananen A, Risteli J, Salo T, Sorsa T, Vaananen HK, Lehenkari P. Human mesenchymal stem cell derived osteoblasts degrade organic bone matrix in vitro by matrix metalloproteinases. *Matrix Biol.* 2005; 24:438–447. [PubMed: 16098718]
- Partridge NC, Walling HW, Bloch SR, Omura TH, Chan PT, Pearman AT, Chou WY. The regulation and regulatory role of collagenase in bone. *Crit Rev Eukaryot Gene Expr.* 1996; 6:15–27. [PubMed: 8882305]
- Pei Y, Harvey A, Yu XP, Chandrasekhar S, Thirunavukkarasu K. Differential regulation of cytokine-induced MMP-1 and MMP-13 expression by p38 kinase inhibitors in human chondrosarcoma cells: potential role of Runx2 in mediating p38 effects. *Osteoarthritis Cartilage.* 2006; 14:749–758. [PubMed: 16549373]
- Reyes CD, Garcia AJ. Alpha2beta1 integrin-specific collagen-mimetic surfaces supporting osteoblastic differentiation. *J Biomed Mater Res A.* 2004; 69:591–600. [PubMed: 15162400]

- Ryoo HM, Lee MH, Kim YJ. Critical molecular switches involved in BMP-2-induced osteogenic differentiation of mesenchymal cells. *Gene*. 2006; 366:51–57. [PubMed: 16314053]
- Sakao K, Takahashi KA, Arai Y, Saito M, Honjo K, Hiraoka N, Asada H, Shin-Ya M, Imanishi J, Mazda O, Kubo T. Osteoblasts derived from osteophytes produce interleukin-6, interleukin-8, and matrix metalloproteinase-13 in osteoarthritis. *J Bone Miner Metab*. 2009; 27:412–423. [PubMed: 19333684]
- Sasaki T, Ohyori N, Debari K, Ramamurthy NS, Golub LM. Effects of chemically modified tetracycline, CMT-8, on bone loss and osteoclast structure and function in osteoporotic states. *Ann N Y Acad Sci*. 1999; 878:347–360. [PubMed: 10415740]
- Schneider GB, Zaharias R, Stanford C. Osteoblast integrin adhesion and signaling regulate mineralization. *J Dent Res*. 2001; 80:1540–1544. [PubMed: 11499509]
- Selvamurugan N, Jefcoat SC, Kwok S, Kowalewski R, Tamasi JA, Partridge NC. Overexpression of Runx2 directed by the matrix metalloproteinase-13 promoter containing the AP-1 and Runx/RD/Cbfa sites alters bone remodeling in vivo. *J Cell Biochem*. 2006; 99:545–557. [PubMed: 16639721]
- Shiga M, Kapila YL, Zhang Q, Hayami T, Kapila S. Ascorbic acid induces collagenase-1 in human periodontal ligament cells but not in MC3T3-E1 osteoblast-like cells: potential association between collagenase expression and changes in alkaline phosphatase phenotype. *J Bone Miner Res*. 2003; 18:67–77. [PubMed: 12510807]
- Torii Y, Hitomi K, Tsukagoshi N. L-ascorbic acid 2-phosphate promotes osteoblastic differentiation of MC3T3-E1 mediated by accumulation of type I collagen. *J Nutr Sci Vitaminol (Tokyo)*. 1994; 40:229–238. [PubMed: 7965212]
- Tuckermann JP, Pittois K, Partridge NC, Merregaert J, Angel P. Collagenase-3 (MMP-13) and integral membrane protein 2a (Itm2a) are marker genes of chondrogenic/osteoblastic cells in bone formation: sequential temporal, and spatial expression of Itm2a, alkaline phosphatase, MMP-13, and osteocalcin in the mouse. *J Bone Miner Res*. 2000; 15:1257–1265. [PubMed: 10893674]
- Varghese S, Canalis E. Regulation of collagenase-3 by bone morphogenetic protein-2 in bone cell cultures. *Endocrinology*. 1997; 138:1035–1040. [PubMed: 9048606]
- Varghese S, Rydziel S, Canalis E. Bone morphogenetic protein-2 suppresses collagenase-3 promoter activity in osteoblasts through a runt domain factor 2 binding site. *J Cell Physiol*. 2005; 202:391–399. [PubMed: 15389594]
- Wenstrup RJ, Witte DP, Florer JB. Abnormal differentiation in MC3T3-E1 preosteoblasts expressing a dominant-negative type I collagen mutation. *Connect Tissue Res*. 1996; 35:249–257. [PubMed: 9084663]
- Winchester SK, Bloch SR, Fiocco GJ, Partridge NC. Regulation of expression of collagenase-3 in normal, differentiating rat osteoblasts. *J Cell Physiol*. 1999; 181:479–488. [PubMed: 10528234]
- Xiao G, Wang D, Benson MD, Karsenty G, Franceschi RT. Role of the alpha2-integrin in osteoblast-specific gene expression and activation of the Osf2 transcription factor. *J Biol Chem*. 1998; 273:32988–32994. [PubMed: 9830051]
- Xu J, Wang W, Kapila Y, Lotz J, Kapila S. Multiple differentiation capacity of STRO-1+/CD146+ PDL mesenchymal progenitor cells. *Stem Cells Dev*. 2009; 18:487–496. [PubMed: 18593336]
- Zaragoza C, Lopez-Rivera E, Garcia-Rama C, Saura M, Martinez-Ruiz A, Lizarbe TR, Martin-de-Lara F, Lamas S. Cbfa-1 mediates nitric oxide regulation of MMP-13 in osteoblasts. *J Cell Sci*. 2006; 119:1896–1902. [PubMed: 16636074]

Figure 1.

Overexpression of MMP-1 or MMP-13 suppresses AP activity in MG63 cells. Cells were transiently transfected with empty control vector (control), or plasmid containing MMP-1 cDNA (MMP-1) or MMP-13 cDNA (MMP-13), and then cultured for 72 hours in serum-free media following which mRNA were retrieved MMP-1 and -13 qRT-PCR (A and B, respectively). Cell conditioned medium was utilized for MMP-1 and -13 Western blots (C and D, respectively) and activity assays (E and F, respectively). Cell lysates were assayed for AP activity (G), and type I collagen protein levels by western blotting (H). Data represents mean fold change $\pm$ SD of three experiments each performed in triplicate. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

Figure 2.

MMP-1 and -13 differentially modulate pro-osteogenic and pro-proliferative transcription factors and markers in MG63 cells. Cells were transiently transfected with empty vector or plasmids containing MMP-1 cDNA or MMP-13 cDNA. The mRNA was retrieved after 0 hour, 36 hours or 72 hours of culture in serum free medium and subjected to qRT-PCR for TGF- β 1, MSX2, BMP-2, Dlx-5, Runx2, Osx, AP, OP, OC and BSP. Relative to control baseline levels (dashed line), MMP-1 overexpression produced a statistically significant decrease in mRNA levels of BMP-2, Dlx5, Osx, AP, OP, OC and BSP and an upregulation in mRNA levels of the pro-proliferative markers TGF- β 1 and MSX2 (A). Overexpression of MMP-13 resulted in a significant decrease in Runx2, AP, OP and BSP mRNA levels and an increase in TGF- β 1, MSX2 and OC levels (B). Data represent mean $\pm$ SD of three experiments each performed in triplicate. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ relative to control.

Figure 3.

Suppression of endogenous MMP-1 or MMP-13 or both MMPs upregulates AP activity in MG63 cells. Cells were transiently transfected with control siRNA, or MMP-1 siRNA and/or MMP-13 siRNA, and then cultured for 72 hours in serum-free media following which mRNA were retrieved MMP-1 and -13 qRT-PCR (A and B, respectively). Cell conditioned medium was utilized for MMP-1 and -13 Western blots (C and D, respectively) and activity assays (E and F, respectively). Cell lysates were assayed for AP activity (G) and type I collagen protein levels by western blotting (H). Data represent mean $\pm$ SD of three experiments each performed in triplicate. Statistically significant differences are represented

as follows: a = relative to control; b = relative to MMP-1 siRNA transfection; c = relative to MMP-13 siRNA transfection.

Figure 4.

Pro-osteogenic and pro-proliferative transcription factors and markers are differentially modulated by suppression of MMP-1 and/or MMP-13 in MG63 cells. Cells were transiently transfected with control siRNA, or MMP-1 siRNA and/or MMP-13 siRNA. The mRNA was retrieved after 0 hour, 36 hours or 72 hours of culture in serum free medium and subjected to qRT-PCR for TGF- β 1, MSX2, BMP-2, Dlx5, Runx2, Osx, AP, OP, OC and BSP. Relative to control baseline levels (dashed line), MMP-1 suppression produced a statistically significant increases in mRNA levels for BMP-2, Dlx5, Runx2, Osx, AP, OP, OC and BSP and no change in the levels of pro-proliferative markers TGF- β 1 and MSX2 (A). Suppression of MMP-13 resulted in significant increase in TGF- β 1, MSX2, and Osx, and a decrease in Runx2 and OC mRNA levels (B). Suppression of both MMPs resulted in significant increase in BMP2, Dlx5, Osx, AP OC and BSP (C). Data represent mean $\pm$ SD of three experiments each performed in triplicate. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ relative to control.

Figure 5.

Model for the regulation of osteoblastic differentiation by MMP-1 and -13. The differentiation transcription factors (represented in black letters) Runx2 and Osx control mesenchymal stem cell differentiation into immature osteoblasts. Dlx5 acts either upstream of Runx2 at the mesenchymal progenitor cell stage or through an independent pathway that regulates Osx at the presosteoblast/immature osteoblast stage of differentiation. In turn, the downstream markers of immature and mature osteoblasts AP, OP, OC and BSP (represented in red letters) are induced by these transcription factors. The transcription factor MSX2 enhances precursor cell proliferation thereby limiting differentiation between the mesenchymal progenitor cell and immature osteoblast stages. Secreted growth factors and other molecules such as BMP2 and TGF- β 1 (represented in purple) that are known to induce the expression of transcriptional factors involved in osteoblast differentiation (Dlx5 and Runx2) or proliferation factor (MSX2) further modulate the progression of osteoblastic differentiation. (A) Effects of MMP-1 and -13 overexpression on osteoblast differentiation. The findings of this study show that the overexpression of enzymes of the MMP family, namely MMP-1 and -13 (represented in blue and orange, respectively) also differentially modulate osteoblast differentiation by apparently downregulating the upstream differentiation transcription factors and enhancing the proliferation transcription factor MSX2 and its inducer TGF- β 1 to decrease the expression of downstream osteoblast markers. (B) Effects of MMP-1 and -13 Suppression on Osteoblast Differentiation. In contrast, the suppression of MMP-1 and, to a lesser extent the downregulation of MMP-13, enhance pathways that increase differentiation transcription factors and downstream osteoblast markers. Adapted from references (Gennari and Bilezikian, 2009; Komori, 2006; Ryoo et al., 2006).