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HtrA1 is upregulated during RANKL-induced osteoclastogenesis, and negatively regulates osteoblast differentiation and BMP2-induced Smad1/5/8, ERK and p38 phosphorylation

Xinghuo Wu [a,b,1], Shek Man Chim [b,1], Vincent Kuek [b], Bay Sie Lim [b], Siu To Chow [b], Jinmin Zhao [c], Shuhua Yang [a], Vicki Rosen [d], Jennifer Tickner [b], Jiake Xu [b,c,*]

[a] Department of Orthopedics, Union Hospital, Tongji Medical College, Huazhong University of Science and Technology, Wuhan 430022, China
[b] School of Pathology and Laboratory Medicine, University of Western Australia, Perth, WA 6009, Australia
[c] Research Centre for Regenerative Medicine, Department of Orthopaedic Surgery, The First Affiliated Hospital of Guangxi Medical University, Guangxi 530021, China
[d] Developmental Biology, Harvard School of Dental Medicine, Boston, MA 02115, USA

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ABSTRACT

Bone remodeling is regulated by secreted factors in the bone microenvironment. However, data regarding osteoclast-derived factors that influence osteoblast differentiation are lacking. Here, we show that HtrA1 is produced as a secreted protein during osteoclastogenesis, and negatively regulates osteoblast differentiation. Exogenous addition of recombinant HtrA1 attenuates osteoblast differentiation and BMP2-induced Smad1/5/8, ERK1/2 and p38 phosphorylation in pre-osteoblasts. Our studies imply a unique mode of crosstalk in which HtrA1 is produced by both osteoclasts and osteoblasts and negatively regulates osteoblast differentiation, suggesting that HtrA1 may mediate the fine tuning of paracrine and autocrine regulations during bone remodeling processes.

1. Introduction

Bone, a specialized connective tissue, is coordinately regulated by two different types of cells, namely bone-forming osteoblasts and bone-resorbing osteoclasts [1]. Osteoblasts are differentiated from mesenchymal stem cells and are responsible for the synthesis and mineralization of bone. Osteoclasts are derived from haematopoietic stem cells, and are the principal cells for bone resorption. Through the coupling of bone resorption and formation by osteoclasts and osteoblasts, bone tissue undergoes constant remodeling [1]. Deciphering the molecular mechanisms regulating the coordinated action of osteoclasts and osteoblasts is important for our understanding of bone remodeling [1]. It is of particular interest to identify osteoclast-derived factors that regulate osteoblasts by paracrine mechanism in the bone microenvironment.

Secreted factors or ligand and receptor signaling networks are believed to play a critical role in the communication between osteoclasts and osteoblasts [1]. For example, osteoblasts control the formation and activity of osteoclasts via the receptor activator of NF-κB ligand (RANKL) system, a membrane-bound protein that is expressed by osteoblasts and binds to its receptor RANK on osteoclast precursors [2]. Semaphorin 4D is expressed by osteoclasts and inhibits bone formation by binding to its receptor Plexin-B1 on osteoblasts and regulating RhoA signaling [3]. These data point to a complex paracrine and autocrine mechanisms of intercellular communications in the bone microenvironment. In order to fully understand the crosstalk between osteoclasts and osteoblasts, novel secreted or membrane bound molecules produced by osteoclasts that is capable of influencing osteoblast differentiation remain to be vigorously uncovered.

HtrA (high temperature requirement protease A) proteins belong to a family of serine proteases, and four types of HtrA have been identified, HtrA1, HtrA2, HtrA3, and HtrA4, which share a common trimeric pyramidal architecture where each monomer comprises a serine protease domain and one or two PDZ domains [4]. The HtrA family is involved in a variety of biological functions such as mitochondrial homeostasis, and apoptosis and in the pathogenesis of several diseases including, Alzheimer's disease, age-related macular degeneration, osteoarthritis, neurodegenerative

* Corresponding author at: School of Pathology and Laboratory Medicine, The University of Western Australia M508, 35 Stirling Hwy, Crawley, WA 6009, Australia. Fax: +61 8 9346 3210.
E-mail address: jiake.xu@uwa.edu.au (J. Xu).
[1] Authors make equal contribution to this work.

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disorders, and cancer [5,6]. HtrA1 expression has been found to be temporally increased in differentiating 2T3 osteoblasts prior to the appearance of mineralization, and then down regulated in fully mineralized culture [7]. When overexpressed endogenously, HtrA1 negatively regulated osteoblast mineralization [7]. However, it is hitherto unknown in regards to the expression of HtrA1 during RANKL-induced osteoclast formation and its role as an exogenous factor in osteoclast and osteoblast differentiation. Moreover, the effect of HtrA1 on BMP signaling of small mother against decapentaplegic (Smad) and mitogen-activated protein kinases (MAPK) such as ERK1/2 and p38 remains to be elucidated.

In this study, in an attempt to hunting for osteoclast-derived factors that mediate osteoblast differentiation, we have strategically screened for novel molecules that are differentially regulated during osteoclast differentiation using microarray analysis. We discovered that HtrA1 gene expression is highly induced during RANKL-induced osteoclastogenesis, and the most prominent RANKL-responsive isoform within the HtrA family members. We observed that HtrA1 is secreted by osteoclasts and as an exogenous factor negatively regulates osteoblastic differentiation but does not affect osteoclast differentiation. Furthermore, we showed that HtrA1 inhibits BMP2-induced Smad1/5/8, ERK1/2 and p38 phosphorylation and osteoblast marker gene expression. Taken together, this study and other work show that HtrA1 is produced by both osteoclasts and osteoblasts and negatively regulates osteoblast differentiation and BMP-induced Smad and MAPK signaling.

2. Materials and methods

2.1. Media and reagents

Alpha Modified Eagles Medium (α-MEM) and fetal bovine serum (FBS) was purchased from Life Technologies (Life Technologies, Australia). Recombinant Human HtrA1/PRSS11 and bone morphogenetic protein 2 (BMP2) were purchased from R&D Systems (R&D, USA). *p*-Nitrophenyl phosphate (pNPP) and 1,25-dihydroxyvitamin D3 (Vit D3) was purchased from Sigma (Sigma, Australia). Recombinant RANKL protein was expressed and purified as previously described [8].

2.2. Cells and cell culture

KusaO cell is a mouse bone marrow stromal cell line established for studying osteoblast differentiation [9]. KusaO cells were maintained in α-MEM as previously described [10]. Primary mouse bone marrow macrophages (BMM) were isolated from C57BL/6J mice. Bone marrow was collected from femora and tibia of 8- to 12-week-old mice by flushing the bone with complete α-MEM followed by filtration through a 100 μm mesh. BMM were cultured in complete α-MEM. Primary calvarial osteoblasts were isolated from mice according to our previously described protocol [10].

2.3. In vitro osteoclastogenesis assay

BMM were seeded onto a 96-well plate (6×10^3 cells/well) with complete α-MEM medium containing M-CSF (25 ng/ml) and in the presence or absence of RANKL (50 ng/ml) for 5 days. Cells were then fixed and stained using a Tartrate-resistant acid phosphate (TRACP) kit (Sigma, Australia). TRACP positive cells with more than three nuclei were counted as osteoclasts.

2.4. RNA isolation, cDNA array, and semiquantitative reverse transcription (RT)-PCR

Total RNA was isolated from the cultures using RNA extraction kit (Qiagen, Victoria, Australia). The RNA concentration was determined by measuring the absorbance at 260 nm with a Nano-drop 2000 (Thermo Scientific), and quality assessed using Agilent Bioanalyzer. Gene expression of HtrA family members during in vitro osteoclast differentiation was detected by cDNA microarray. Microarray protocols were performed with two independent sets of paired samples of BMM and osteoclasts by mouse expression WG6 beadchip according to the manufacturer's instructions (Illumina Inc.). For RT-PCR, single stranded cDNA was prepared from 2 μg of total RNA using reverse transcriptase with oligo-dT primer. All PCR was performed using cycling parameters 94 °C, 40 s; 55 °C, 40 s; 72 °C, 40 s for 30 cycles with primers listed in Supplementary Table 1.

2.5. Real-time PCR (qPCR)

qPCR was used to define gene expression of HtrA1. qPCR was performed using SYBR green (Qiagen, Australia) and iCycler (BioRad) according to manufacturer's instructions. PCR amplification was performed using a profile with 94 °C for 1 min (denaturation), 60 °C for 30 s (annealing), 72 °C for 45 s (elongation) for a total of 38 cycles, followed at the end by 72 °C for 5 min (extension). Negative controls without RT were carried out in parallel for every PCR reaction to exclude amplification of contaminating DNA. 18S ribosomal RNA (18S) was chosen as an internal control to standardize the variability. After amplification, a dissociation analysis was performed to evaluate the presence or absence of non-specific amplifications in each PCR reaction.

2.6. In vitro osteoblastogenesis assay

KusaO cells were cultured in osteogenic differentiation medium as previously described [10]. Cells were then fixed and stained using the leukocyte alkaline phosphatase kit (Sigma, Australia) according to the manufacturer's instructions. To quantify alkaline phosphatase (ALP) activity, in cells unfixed monolayers were washed with PBS, and lysed with 1% Triton-X 100 in milliQ water. ALP activity was assessed using pNPP (70 mM) as substrate in AMP reagent buffer (420 mM 2-amino-2-methyl-1-propanol, 1 mM magnesium chloride, 1 mM zinc chloride pH 10.3), and measuring the change in absorbance at 405 nm. ALP levels were normalised to total protein concentrations as measured by Bradford assay.

2.7. Cell proliferation assay

Cell proliferation was examined using the CellTiter 96® Aqueous Non-Radioactive Cell Proliferation Assay (Sigma, Australia) according to the manufacturer's instructions.

2.8. Western blot analysis

Western blot was performed according to our previously described protocol [10]. The primary antibodies used in the experiment included anti-HtrA1 (1:250, Santa Cruz Biotechnology), anti-phospho-Smad1/5/8 (1:1000, Cell Signaling), Smad1 (1:1000, Cell Signaling), anti-phospho-ERK1/2 (1:1000, Santa Cruz Biotechnology), anti ERK1/2 (1:5000, Promega), anti-p38 MAPK (1:1000 dilution, Cell Signaling), anti-phospho-p38 MRPK (1:1000 dilution, Cell Signaling), anti-β-actin (JLA20) (DSHB University of Iowa). An anti-V-ATPase d2 antibody was generated by the Polyclonal Antibody Production Facility in the School of Molecular and Biomedical Sciences, University of Adelaide [11]. Horseradish peroxidase (HRP)-conjugated anti-mouse and anti-rabbit antibodies (1:5000 dilution, Santa Cruz Biotechnology) were used as secondary antibodies. The membranes were then visualized using the ECL system (Amersham, Australia). β-Actin was used to confirm equal protein loading.

2.9. Statistics

All measurements were collected in triplicate. Unless otherwise stated, all data are presented as the Mean ± S.D. One-way ANOVA or Student's *t*-test was used to detect differences between the various treatment groups. *P* values of less than 0.05 were considered to be statistically significant.

3. Results

3.1. HtrA family genes are differentially expressed during RANKL-induced osteoclastogenesis

To identify osteoclast-derived factors that are involved in the regulation of osteoblast differentiation, microarray analysis was performed on cDNA isolated from osteoclasts and BMM. HtrA family genes are found to be differentially expressed during RANKL-induced osteoclast differentiation, as shown by the heatmap analysis (Fig. 1A). Among all four members of the HtrA family, HtrA1 was most highly up-regulated based on microarray analysis (Fig. 1B). Multiple sequence alignment and guide tree analysis of the amino acid sequences of mouse HtrA family proteins shows that HtrA1 is most closely related to HtrA4, followed by HtrA3 and HtrA2 (Fig. 1C and D). Sequence analysis further indicates that HtrA1, HtrA3, and HtrA4 exhibit characteristics of a secreted protein; including a signal peptide sequence at the N-terminus and lack a transmembrane domain. Given that microarray and bioinformatics analyses hint that HtrA1 is most highly induced by RANKL and bears characteristics of a secreted protein, it prompted us to investigate the role of HtrA1 in osteoblast and osteoclast differentiation as an exogenous factor.

3.2. HtrA1 is highly induced by RANKL during osteoclast differentiation

The induction of HtrA1 gene by RANKL was further confirmed by qPCR analysis. The results showed a 14.68-fold increase in HtrA1 mRNA levels in osteoclasts at day 5 after RANKL treatment, as compared to day 0 (Fig. 2A). The upregulation of HtrA1 is in line with other established osteoclast marker genes, including TRACP and dendritic cell-specific transmembrane protein (DC-Stamp) (Fig. 2B and C). By comparison, we also examined the gene expression of HtrA1 during calvarial osteoblast differentiation. qPCR analysis demonstrated that HtrA1 was up-regulated during osteoblast differentiation in line with other established osteoblast markers

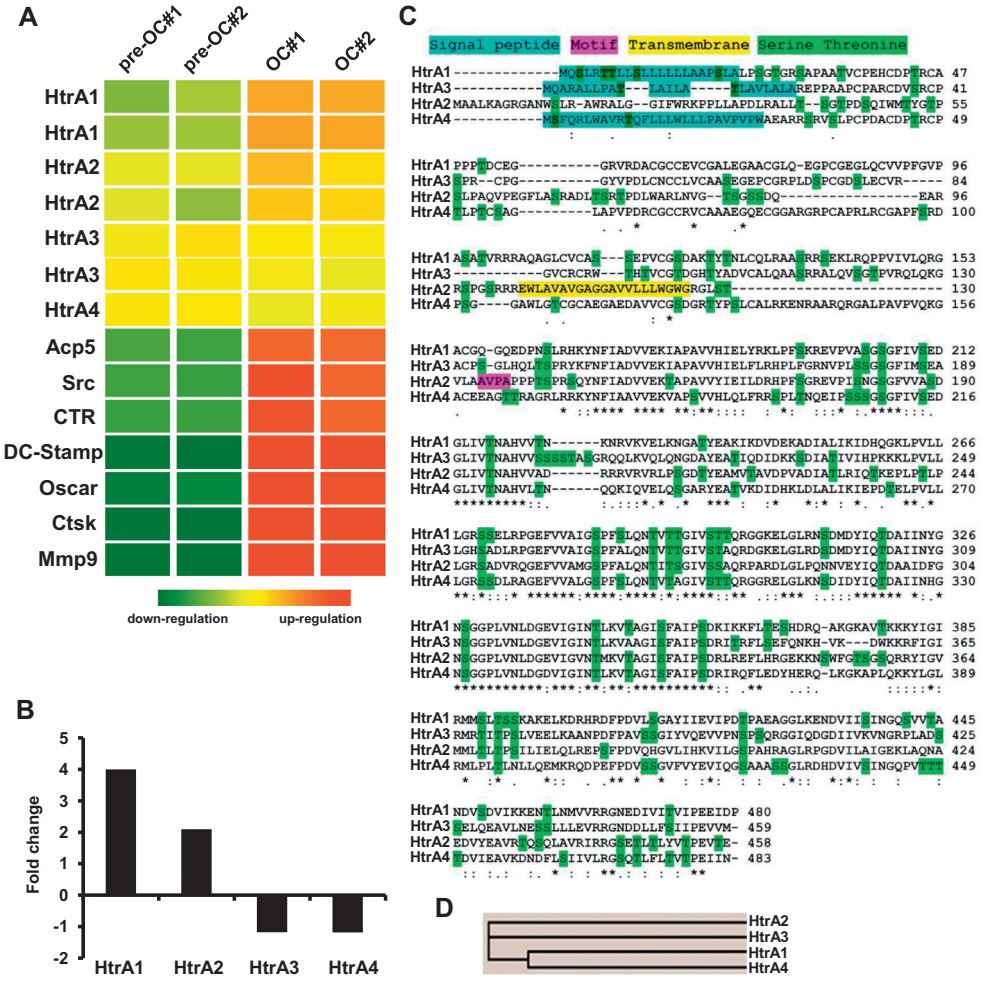

Fig. 1. Microarray analysis of HtrA family gene expression during osteoclast differentiation and multiple alignment of HtrA family amino acid sequences. (A) Total RNA from BMM cells (pre-OC) and mature osteoclasts (OC) were harvested for microarray analysis. Heatmap demonstrating the upregulation of HtrA1 and HtrA2 during osteoclast differentiation, with osteoclast specific genes. Up-regulation and down-regulation are shown in red and green respectively. (B) The average fold change (OC/pre-OC) of HtrA family members in microarray analysis. (C) Multiple alignment of full length protein sequences of HtrA family members. (D) Dendrograms of HtrA family members generated using Uniprot program.

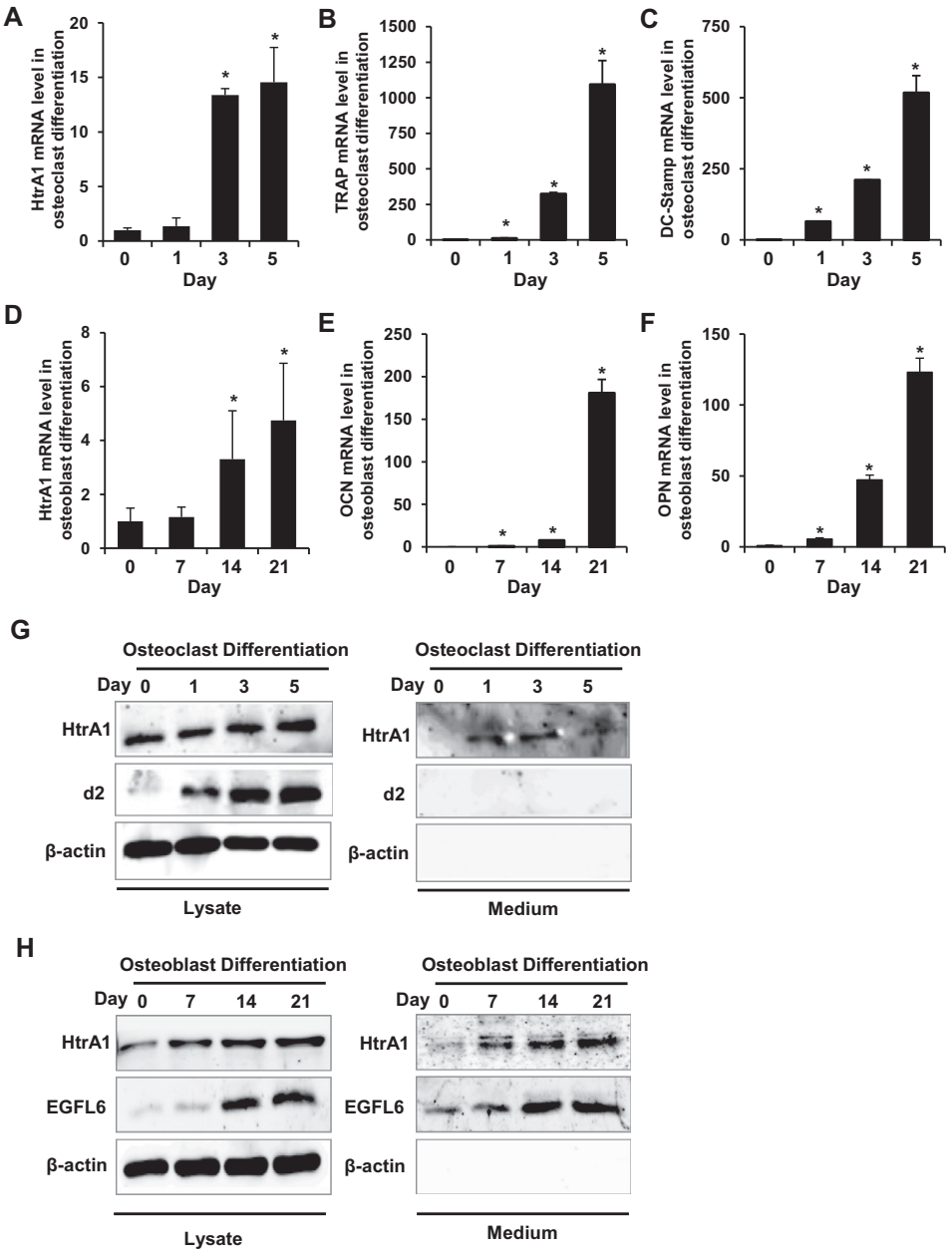

Fig. 2. HtrA1 is up-regulated during osteoclast and osteoblast differentiation. (A–C) HtrA1 is significantly up-regulated during osteoclast differentiation. Gene expression was examined by qPCR analysis using primers specific for (A) HtrA1 and osteoclast specific genes (B) TRACP and (C) DC-Stamp. (D–F) HtrA1 is significantly up-regulated during primary calvarial osteoblast differentiation. Gene expression was examined by qPCR analysis using primers specific for (D) HtrA1, and osteoblast specific genes (E) OCN and (F) OPN. The gene expressions are normalized to 18S and compared to the expression at day 0. (G and H) The protein levels of HtrA1 were up-regulated during (G) osteoclast differentiation and (H) osteoblast differentiation. $^{*}P < 0.05$.

including osteocalcin (OCN) and osteopontin (OPN). There was a 4.52-fold increase in HtrA1 mRNA levels in osteoblasts at day 21 as compared to day 0 (Fig. 2D–F).

In addition, we examined the protein expression of HtrA1 in osteoclasts. Both supernatant and cell lysates were collected and were subjected to Western blot analysis. As shown in Fig. 2G, HtrA1 was expressed in and secreted by osteoclasts. Osteoclast marker, V-ATPase d2 was used as a control [11]. Our results conclude that HtrA1 is a secreted protein induced by RANKL during osteoclastogenesis. Thus, these data document the first evidence of HtrA1 expression by osteoclasts. HtrA1 protein was also upregulated throughout osteoblast differentiation (Fig. 2H). EGFL6, a secreted factor that is known to be upregulated during osteoblast differentiation [10] was used as a control. In addition, analysis of tissue distribution revealed that the level of HtrA1 mRNA expression was highly expressed in the brain, heart and ovary (Supplementary Fig. S1).

3.3. HtrA1 inhibits osteoblast differentiation but does not affect cell proliferation

Next, we examined effect of HtrA1 as an exogenous factor on osteoblast differentiation and proliferation using KusaO cells. KusaO cell, a bone marrow stromal cell line is a subclone of Kusa cells which have been well established and widely used to study osteoblast differentiation and physiology [9,12]. We found that HtrA1 significantly inhibited BMP2-induced ALP activity by ALP staining and activity assay (Fig. 3A and B). In addition, we found

that HtrA1 also inhibited the mineralization of KusaO cells in a dose dependent manner (data not shown), which is in agreement with previous observations that forced expression of endogenous HtrA1 inhibits osteoblast mineralization [7]. Next, we sought to investigate the effects of HtrA1 on osteoblast marker gene expression. KusaO cells were cultured in osteogenic differentiation medium with BMP2, in the presence or absence of HtrA1 for 7 days. RNA was then harvested, followed by qPCR analysis. HtrA1 significantly inhibits BMP2-induced osteoblast marker gene expression including ALP, Runx2, OCN and OPN (Fig. 3C–F). Moreover, HtrA1 has no significant inductive or inhibitory effect on the proliferation of KusaO cells (data not shown).

3.4. HtrA1 inhibits BMP2-induced phosphorylation of Smad1/5/8, ERK and p38 in osteoblastic like cells

It has been demonstrated that HtrA1 acts as an antagonist to transforming growth factor-β (TGF-β) family members including BMP2 [6,13]. Interestingly, it was shown that HtrA1 inhibits BMP2-induced signaling in C2C12 myoblast cells [13]. To investigate the potential mechanism by which HtrA1 inhibits BMP2-induced osteoblast differentiation, we examined the effect of HtrA1 on BMP2 signaling pathways, Smad and MAPK in KusaO. As shown in Fig. 4A, HtrA1 significantly inhibited BMP2-induced Smad1/5/8 phosphorylation. Next, we examined the effect of HtrA1 on MAPK signaling. Previous study suggested that BMP2 induced ERK1/2 and p38 phosphorylation peaking at 2–5 h [14]. Here, we showed that both BMP2-induced ERK1/2 and p38 phosphorylation

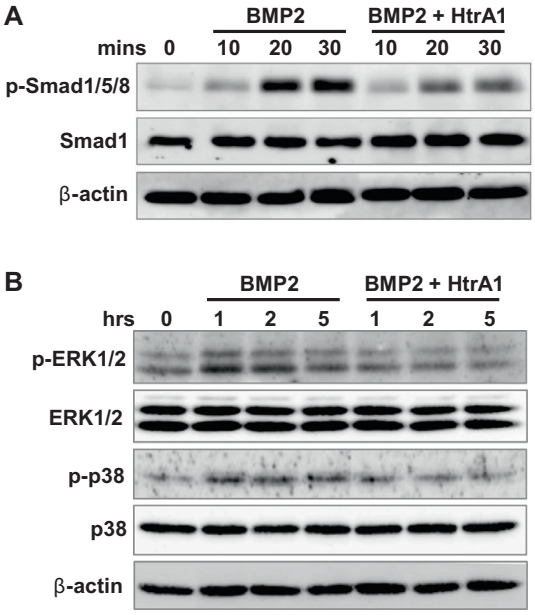

Fig. 4. HtrA1 inhibits BMP2-induced Smad1/5/8, ERK1/2 and p38 phosphorylation. The cellular phosphorylation levels of Smad1/5/8, ERK1/2 and p38 were determined by Western blot analysis after KusaO cells were stimulated with BMP2 (50 ng/ml) in the presence or absence of HtrA1 (100 ng/ml) for the indicated time points. β-Actin was used as a loading control.

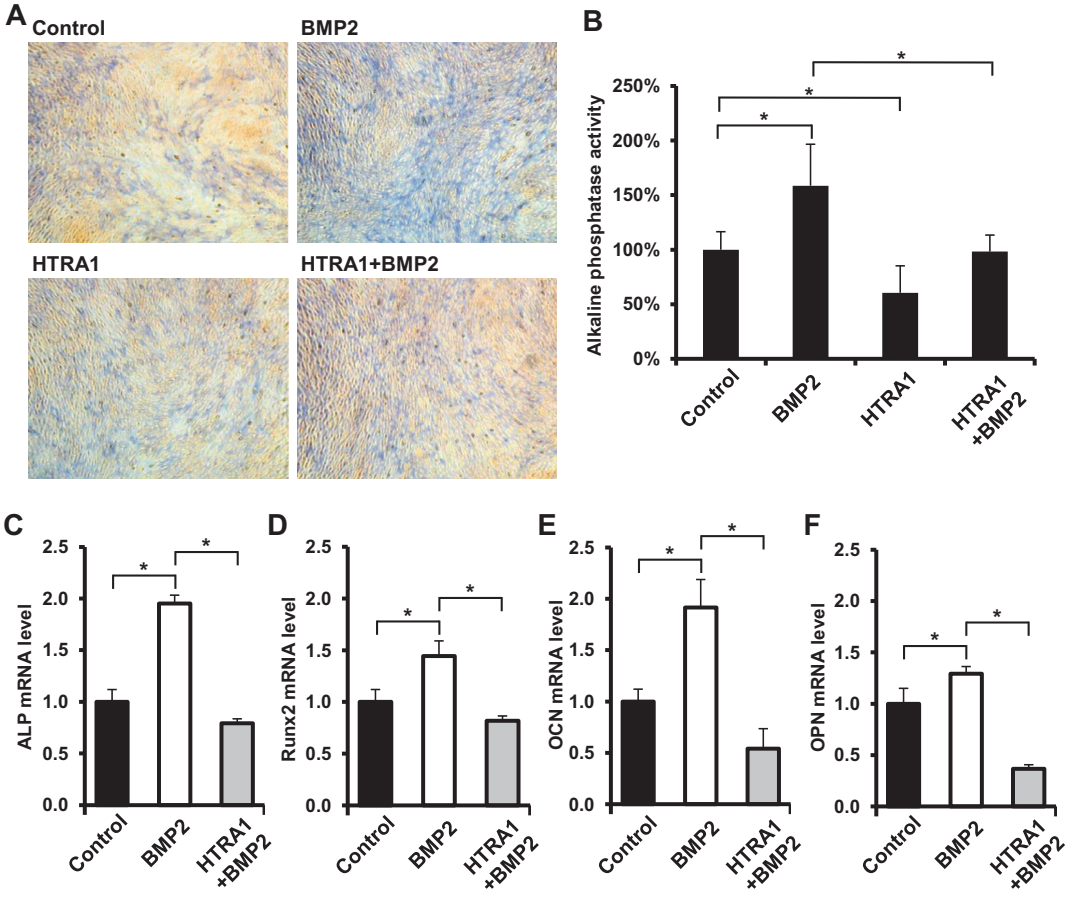

Fig. 3. HtrA1 inhibits osteoblastic-like cell differentiation and osteoblast marker gene expressions. (A) KusaO cells were cultured in osteogenic medium with HtrA1 (100 ng/ml) in the presence or absence of BMP2 (50 ng/ml) for 4 days, followed by ALP staining. (B) Quantification of ALP activity shown as a percentage relative to untreated cell lysates. (C–F) KusaO cells were cultured in osteogenic differentiation medium with BMP2 (50 ng/ml) in the presence or absence of HtrA1 (100 ng/ml) for 7 days. RNA were harvested, followed by qPCR analysis. HtrA1 significantly inhibit BMP2-induced osteoblast marker gene expression including (C) ALP, (D) Runx2, (E) OCN and (F) OPN.

was inhibited by HtrA1 (Fig. 4B). β-Actin was used as a loading control.

3.5. HtrA1 does not affect RANKL-induced osteoclastogenesis

Next, we examined the effect of HtrA1 on RANKL-induced osteoclastogenesis. BMM were incubated with different concentrations of HtrA1 in the presence or absence of RANKL, and the number of osteoclast counted. BMM cultured in the presence of M-CSF and RANKL formed typical TRACP-positive osteoclasts. Addition of HtrA1 protein into BMM cultures showed no significant effect on the formation of osteoclast as measured by the TRACP positive multinucleated cells (Fig. 5A and B). These results suggest that HtrA1 did not impact osteoclastogenesis in BMM cultures.

3.6. HtrA1 has little effects on the ratio of RANKL/OPG gene expression in osteoblast lineage

To investigate whether HtrA1 has an indirect effect on osteoclastogenesis, we examined the regulation of RANKL/OPG gene expression in osteoblasts. KusaO cells were treated with 100 ng/ml HtrA1 for 24 h. 1,25-dihydroxyvitamin D3 (Vit D3) at the concentration of 10 nM was used a as a positive control for the regulation of RANKL/OPG gene expression. Semiquantitative RT-PCR and qPCR results show that HtrA1 does not significantly alter the gene expression of RANK/OPG. As a positive control, Vit D3 induced both RANKL and OPG gene expression in KusaO cells (Fig. 5C–E).

4. Discussion

The crosstalk between osteoblasts and osteoclasts is an essential requirement for the balancing act of bone remodeling, and the disruption of coordinated activities between osteoclasts and osteoblasts contributes to bone pathogenesis and diseases [1]. Discovering novel osteoclast-derived factors that mediate osteoblasts has become increasingly important to understand the molecular mechanism of the crosstalk between osteoclasts and osteoblasts. In this study, we showed that HtrA1 is highly up-regulated in RANKL-induced osteoclastogenesis and, as a secreted factor, has a negative regulation on osteoblast differentiation, but has little impact on osteoclast differentiation.

A previous study has shown that HtrA1 is temporally up-regulated during osteoblast mineralization but inhibits mineralization by an autocrine fashion, with its expression peaking at day 17, and gradually subdued in 2T3 cell culture [7]. By comparison, using primary mouse osteoblast culture, we have found that HtrA1 is constantly unregulated during osteoblast differentiation from day 0 to 21. This discrepancy might be due to two different cell types being used. Consistent to our observations, a more recent study has also shown that HtrA1 is up-regulated by BMP2 and BMP7 during osteoblastic differentiation of C2C12 cells [15]. Nevertheless, our studies extended previous findings [7] to show that osteoclast-derived HtrA1 has an inhibitory effect on osteoblast differentiation and gene expression, in addition to its effect on osteoblast mineralization process. In addition, since HtrA1 is

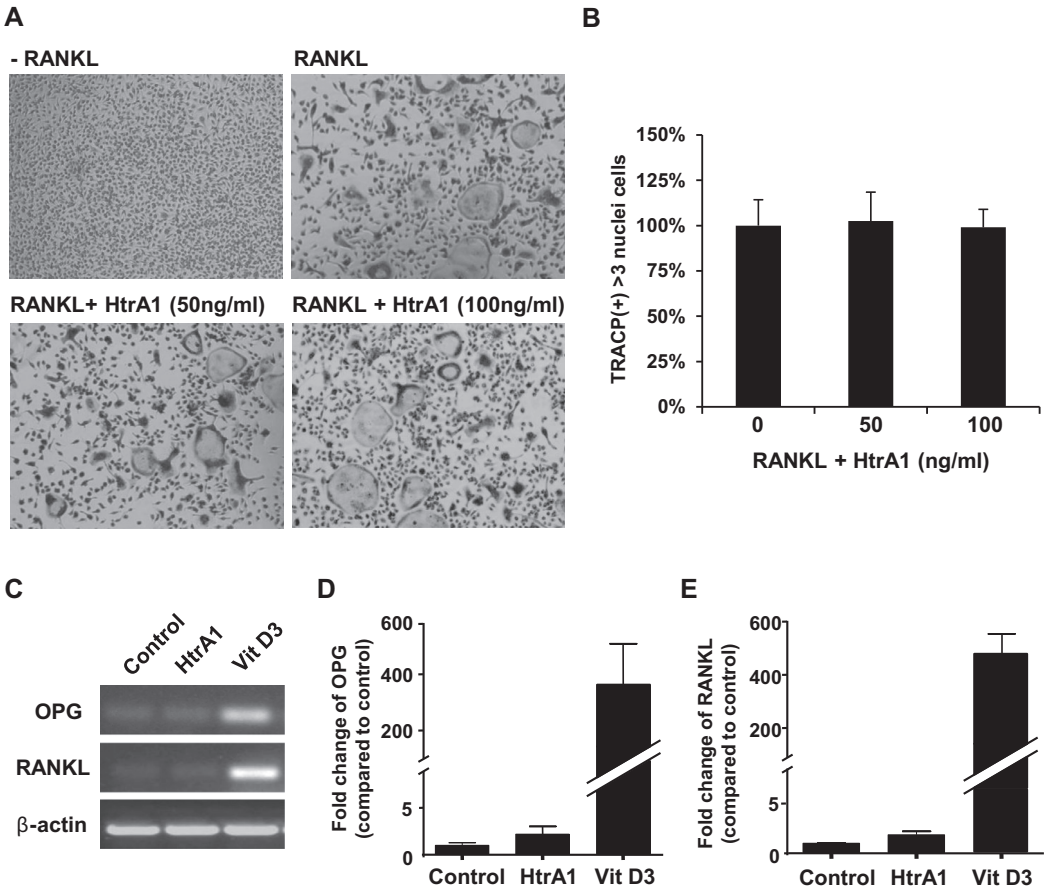

Fig. 5. HtrA1 does not have direct or indirect effects on osteoclast formation. (A) BMM cells were stimulated with RANKL to differentiate into osteoclasts in the presence or absence of HtrA1. Cells were then fixed and stained for TRACP activity. (B) Quantitation of TRACP positive multinuclear cells (>3 nuclei). (C) KusaO cells were treated with HtrA1 (100 ng/ml) for 48 h. Vit D3 (10nM) was used as a positive control. Semiquantitative RT-PCR and qPCR analysis was performed to examine the gene expression of OPG (D) and RANKL (E). Gene expression were normalized to 18S expression and presented in fold change compared to control. $^{*}P < 0.05$.

produced by osteoclasts and osteoblasts, and negatively regulates osteoblast differentiation, it implies for a paracrine mode of action in addition to its autocrine effect as previous reported [7]. Based on these new findings, a working model of HtrA1 is proposed (Fig. 6A), which highlights a unique mode of HtrA1 action that is different from other molecules that are recently implicated in the crosstalk of osteoclasts and osteoblasts.

Surprisingly, in a disagreement with our findings, a recent study has proposed that HtrA1 could positively regulate osteoblast differentiation of human mesenchymal stem cells [16]. One of the reasons to these different findings could be due to the various doses and source of HtrA1 being used. It is important to note that a very high dose of HtrA1 (5 μg/ml) was employed in Tiaden's study [16], which are 25–50 times more than what was used in this study (100–200 ng/ml). However, it is interesting to point out that TGF-β family members such as TGF-β1 and BMP4 have bidirectional dose-dependent effects on cellular activities [17–19]. Thus, HtrA1, in a dose-dependent manner might result in a biphasic effect on osteoblast differentiation. If this is the case for a biphasic effect, then it should raise an intriguing possibility that when HtrA1 is induced far in excess in bone microenvironment during RANKL-mediated osteoclastogenesis, it might confer to a positive effect on osteoblast differentiation, thus providing a fine tuning of the balancing act by osteoclast and osteoblast-mediated bone remodeling processes.

Moreover, we have found that HtrA1 can temper the BMP2-induced activation of Smad1/5/8, ERK1/2 and p38 in osteoblastic like cells and the gene expression of ALP, Runx2, OCN and OPN. Given that HtrA1 is able to bind to TGF beta family proteins to prevent receptor activation, and inhibit signaling by BMP2, BMP4, and TGF-beta1 in C2C12 cells [13], it is possible that HtrA1 inhibits the BMP2-mediated phosphorylation of Smad1/5/8, ERK1/2 and p38 and osteoblast differentiation via its direct binding and inactivation of BMP2 (Fig. 6B).

Recently, the expression of HtrA1 was also found to be increased in the articular cartilage degeneration, such as type IX collagen-deficient and type XI collagen-haploinsufficient [20,21]. Interestingly, a genetic variation at the HtrA1 gene promoter locus has been identified to be associated with spinal disc degeneration [22]. Moreover, it was reported that HtrA1 regulated angiogenesis through TGF-β family member [23]. Vascular supply is important in bone homeostasis, including subchondral region [24]. Thus it is also possible that the locally produced HtrA1 by osteoclasts and osteoblasts may contribute to the homeostasis of cartilage-subchondral bone interface. In addition, given that HtrA1 is widely expressed in many tissues; including brain, heart and ovary, it remains to be determined whether HtrA1, as a secreted protein, when is produced in excess by other cell types in disease conditions might contribute to the bone and joint pathogenesis.

The role of other HtrA family proteins HtrA2, HtrA3 and HtrA4 in bone cells is hitherto unknown. Based on sequence analyses, HtrA2 appears to be a mitochondrial like protein. HtrA3 and HtrA4 are not significantly regulated during osteoclast and osteoblast differentiation when compared to HtrA1. It was suggested that HtrA3 has overlapping biological activities to HtrA1, but can function in a complementary fashion in certain types of tissues [25]. To date, the involvement of HtrA3 in the bone physiology and disease remains to be elucidated.

In summary, our studies and other work suggest a unique mode of action of HtrA1, in which HtrA1 is produced by both osteoclasts and osteoblasts and as an exogenous factor negatively regulates osteoblast differentiation, indicative of both autocrine and paracrine mechanisms. HtrA1 does so, at least in part by suppressing BMP2-induced activation of Smad1/5/8, ERK1/2 and p38 and the gene expression of ALP, Runx2, OCN and OPN. Overall, HtrA1 may involve in tilting the balancing act during osteoclast and osteoblast-mediated bone remodeling, and thus may serve as a potential therapeutic target for the treatment of bone disorders.

Conflict of interest disclosures

All authors state that they have no conflicts of interest.

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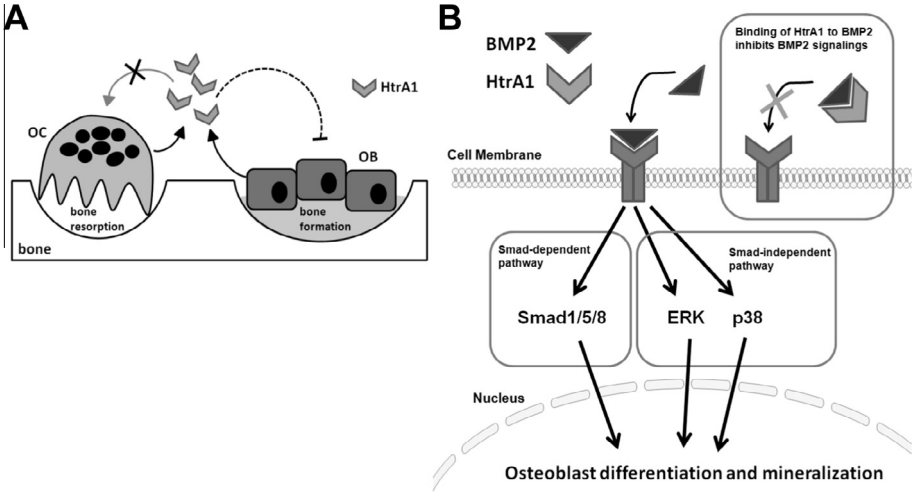

Fig. 6. Proposed model of HtrA1 function in the communication between osteoclasts and osteoblasts. (A) A unique mode of action by which HtrA1 is produced by both osteoclasts and osteoblasts and negatively regulates osteoblast differentiation. Conversely, HtrA1 has no impact on osteoclasts. (B) HtrA1 acts as an antagonist to BMP2, and inhibited BMP2-induced Smad and MAPK signaling pathways in osteoblast.

University of Western Australia between 2011 and 2012. Prof. Jiake Xu is a visiting Professor to the Research Centre for Regenerative Medicine, Guangxi Medical University in 2013.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.febslet.2013.11.022.

References

[1] Kular, J., Tickner, J., Chim, S.M. and Xu, J. (2012) An overview of the regulation of bone remodelling at the cellular level. Clin. Biochem. 45, 863–873.
[2] Boyle, W.J., Simonet, W.S. and Lacey, D.L. (2003) Osteoclast differentiation and activation. Nature 423, 337–342.
[3] Negishi-Koga, T., Shinohara, M., Komatsu, N., Bito, H., Kodama, T., Friedel, R.H. and Takayanagi, H. (2011) Suppression of bone formation by osteoclastic expression of semaphorin 4D. Nat. Med. 17, 1473–1480.
[4] Singh, N., Kuppili, R.R. and Bose, K. (2011) The structural basis of mode of activation and functional diversity: a case study with HtrA family of serine proteases. Arch. Biochem. Biophys. 516, 85–96.
[5] Zurawa-Janicka, D., Skorko-Glonek, J. and Lipinska, B. (2010) HtrA proteins as targets in therapy of cancer and other diseases. Expert Opin. Ther. Targets 14, 665–679.
[6] Canfield, A.E., Hadfield, K.D., Rock, C.F., Wylie, E.C. and Wilkinson, F.L. (2007) HtrA1: a novel regulator of physiological and pathological matrix mineralization? Biochem. Soc. Trans. 35, 669–671.
[7] Hadfield, K.D., Rock, C.F., Inkson, C.A., Dallas, S.L., Sudre, L., Wallis, G.A., Boot-Handford, R.P. and Canfield, A.E. (2008) HtrA1 inhibits mineral deposition by osteoblasts: requirement for the protease and PDZ domains. J. Biol. Chem. 283, 5928–5938.
[8] Xu, J., Tan, J.W., Huang, L., Gao, X.H., Laird, R., Liu, D., Wysocki, S. and Zheng, M.H. (2000) Cloning, sequencing, and functional characterization of the rat homologue of receptor activator of NF-kappaB ligand. J. Bone Miner. Res. 15, 2178–2186.
[9] Allan, E.H., Ho, P.W., Umezawa, A., Hata, J., Makishima, F., Gillespie, M.T. and Martin, T.J. (2003) Differentiation potential of a mouse bone marrow stromal cell line. J. Cell. Biochem. 90, 158–169.
[10] Chim, S.M. et al. (2011) EGFL6 promotes endothelial cell migration and angiogenesis through the activation of extracellular signal-regulated kinase. J. Biol. Chem. 286, 22035–22046.
[11] Feng, H. et al. (2009) Myocyte enhancer factor 2 and microphthalmia-associated transcription factor cooperate with NFATc1 to transactivate the V-ATPase d2 promoter during RANKL-induced osteoclastogenesis. J. Biol. Chem. 284, 14667–14676.
[12] Kartsogiannis, V. et al. (2008) Osteoclast inhibitory lectin, an immune cell product that is required for normal bone physiology in vivo. J. Biol. Chem. 283, 30850–30860.
[13] Oka, C. et al. (2004) HtrA1 serine protease inhibits signaling mediated by Tgfbeta family proteins. Development 131, 1041–1053.
[14] Gallea, S. et al. (2001) Activation of mitogen-activated protein kinase cascades is involved in regulation of bone morphogenetic protein-2-induced osteoblast differentiation in pluripotent C2C12 cells. Bone 28, 491–498.
[15] Bustos-Valenzuela, J.C., Fujita, A., Halcsik, E., Granjeiro, J.M. and Sogayar, M.C. (2011) Unveiling novel genes upregulated by both rhBMP2 and rhBMP7 during early osteoblastic transdifferentiation of C2C12 cells. BMC Res. Notes 4, 370.
[16] Tiaden, A.N. et al. (2012) Human serine protease HTRA1 positively regulates osteogenesis of human bone marrow-derived mesenchymal stem cells and mineralization of differentiating bone-forming cells through the modulation of extracellular matrix protein. Stem Cells 30, 2271–2282.
[17] Jacobsen, S.E., Keller, J.R., Ruscetti, F.W., Kondaiah, P., Roberts, A.B. and Falk, L.A. (1991) Bidirectional effects of transforming growth factor beta (TGF-beta) on colony-stimulating factor-induced human myelopoiesis in vitro: differential effects of distinct TGF-beta isoforms. Blood 78, 2239–2247.
[18] Kale, V.P. (2004) Differential activation of MAPK signaling pathways by TGF-beta1 forms the molecular mechanism behind its dose-dependent bidirectional effects on hematopoiesis. Stem Cells Dev. 13, 27–38.
[19] Shou, J., Murray, R.C., Rim, P.C. and Calof, A.L. (2000) Opposing effects of bone morphogenetic proteins on neuron production and survival in the olfactory receptor neuron lineage. Development 127, 5403–5413.
[20] Polur, I., Lee, P.L., Servais, J.M., Xu, L. and Li, Y. (2010) Role of HTRA1, a serine protease, in the progression of articular cartilage degeneration. Histol. Histopathol. 25, 599–608.
[21] Rosenthal, A.K., Gohr, C.M., Ninomiya, J. and Wakim, B.T. (2011) Proteomic analysis of articular cartilage vesicles from normal and osteoarthritic cartilage. Arthritis Rheum. 63, 401–411.
[22] Urano, T. et al. (2010) Association of HTRA1 promoter polymorphism with spinal disc degeneration in Japanese women. J. Bone Miner. Metab. 28, 220–226.
[23] Zhang, L. et al. (2012) High temperature requirement factor A1 (HTRA1) gene regulates angiogenesis through transforming growth factor-beta family member growth differentiation factor 6. J. Biol. Chem. 287, 1520–1526.
[24] Chim, S.M. et al. (2013) Angiogenic factors in bone local environment. Cytokine Growth Factor Rev. 24, 297–310.
[25] Tocharus, J., Tsuchiya, A., Kajikawa, M., Ueta, Y., Oka, C. and Kawaichi, M. (2004) Developmentally regulated expression of mouse HtrA3 and its role as an inhibitor of TGF-beta signaling. Dev. Growth Differ. 46, 257–274.