HtrA1 Inhibits Mineral Deposition by Osteoblasts

REQUIREMENT FOR THE PROTEASE AND PDZ DOMAINS

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HtrA1 is a secreted multidomain protein with serine protease activity. In light of increasing evidence implicating this protein in the regulation of skeletal development and pathology, we investigated the role of HtrA1 in osteoblast mineralization and identified domains essential for this activity. We demonstrate increased HtrA1 expression in differentiating 2T3 osteoblasts prior to the appearance of mineralization. HtrA1 is subsequently down-regulated in fully mineralized cultures. The functional role of HtrA1 in matrix calcification was investigated using three complementary approaches. First, we transfected a full-length HtrA1 expression plasmid into 2T3 cells and showed that overexpression of HtrA1 delayed mineralization, reduced expression of Cbfal and collagen type I mRNA, and prevented BMP-2-induced mineralization. Secondly, knocking down HtrA1 expression using short interfering RNA induced mineral deposition by 2T3 cells. Third, by expressing a series of recombinant HtrA1 proteins, we demonstrated that the protease domain and the PDZ domain are essential for the inhibitory effect of HtrA1 on osteoblast mineralization. Finally, we tested whether HtrA1 cleaves specific matrix proteins that are known to regulate osteoblast differentiation, mineralization, and/or BMP-2 activity. Full-length recombinant HtrA1 cleaved recombinant decorin, fibronectin, and matrix Gla protein. Both the protease domain and the PDZ domain were necessary for the cleavage of matrix Gla protein, whereas the PDZ domain was not required for the cleavage of decorin or fibronectin. Type I collagen was not cleaved by recombinant HtrA1. These results suggest that HtrA1 may regulate matrix calcification via the inhibition of BMP-2 signaling, modulating osteoblast gene expression, and/or via the degradation of specific matrix proteins.

Mammalian HtrA1 is a member of the family of HtrA (high temperature requirement) proteins that were originally identified in bacteria (1). These proteins are characterized by the presence of a trypsin-like serine protease domain and either one or two PDZ domains. However, in contrast to bacterial HtrA, mammalian HtrA1 is secreted, and it also contains an IGFBP4/mac25-like domain and a Kazal-type inhibitor domain at the N terminus (2, 3). Evidence is now accumulating to suggest that HtrA1 is involved in the development and progression of several pathologies. HtrA1 has been found to exhibit properties of a tumor suppressor protein, as its expression is down-regulated in many cancer cell lines (2,4), low levels of HtrA1 are detected in cancerous tissue compared with normal tissues (4, 5), and overexpression of this protein inhibits tumor cell growth in vivo and in vitro (4, 5). HtrA1 expression is also markedly up-regulated in several diseases, including rheumatoid arthritis and osteoarthritis (3,6–8), Alzheimer disease (9), and Duchenne muscular dystrophy (10). In addition, a single-nucleotide polymorphism in the gene encoding HtrA1 has been shown to increase susceptibility to age-related macular degeneration (11, 12). However, the mechanisms by which HtrA1 exerts its effects are still not understood.

We demonstrated previously that HtrA1 mRNA is up-regulated as vascular pericytes undergo osteogenic differentiation and deposit a mineralized matrix in vitro (13). Consistent with these in vitro data, HtrA1 is expressed at sites of skeletal development in mouse tissues in vivo (6, 14), and its expression appears to be down-regulated in fully calcified bone compared with osteoid or newly formed bone matrices (6). However, the potential role of HtrA1 in osteogenesis has not yet been defined.

Osteogenesis is a complex process that is regulated by growth factors and extracellular matrix proteins acting in an interdependent manner (15–17). Interestingly, HtrA1 has been shown to inhibit the activity of members of the TGF-β family of proteins (14) that are known to regulate osteogenic differentiation and mineral deposition (18–20). In addition, recombinant HtrA1 lacking the IGFBP/mac25 and Kazal-type inhibitor domains has been reported to degrade several proteins that...
have also been implicated in regulating bone formation and/or mineralization either directly or indirectly (6, 7, 21).

The purpose of this study was to test the hypothesis that HtrA1 regulates mineral deposition in osteoblasts and to identify the domains essential for this activity. Several complementary approaches were taken. First, we determined the expression pattern of HtrA1 in differentiating 2T3 osteoblast cells, an in vitro model system of bone formation (22–25). Second, the effects of overexpressing and knocking down expression of HtrA1 were assessed with respect to matrix mineralization, and the response of HtrA1 overexpressing cells to BMP-2 was determined. Third, by generating recombinant full-length HtrA1 (rHtrA1) and a series of domain-specific deletion constructs, we identified which domains were essential for the inhibitory activity of HtrA1 on mineralization, and we identified potential proteolytic substrates of HtrA1. The results of these studies highlight a novel role for HtrA1 in the regulation of mineralization.

**EXPERIMENTAL PROCEDURES**

**Materials**—Unless otherwise stated, all cell culture medium and growth additives were obtained from BioWhittaker. Puromycin hydrochloride, chondroitinase ABC, β-casein, Alizarin red S, and human plasma fibronectin were obtained from Sigma. Geneticin (G418 sulfate) was purchased from Invitrogen, and 45Ca-labeled CaCl₂ was from GE Healthcare. Human recombinant BMP-2 was purchased from R&D Systems. Recombinant human decorin was kindly donated by Dr. Paul Bishop (Wellcome Trust Centre for Cell Matrix Research, University of Manchester) and Dr. Marian Young (NIDCR, National Institutes of Health, Bethesda). The plasmid construct pMGpcJ, containing a 398-bp fragment of the full-length pMGPcJ, was a kind gift from Dr. Stephen Harris (University of Texas Southwestern Medical Center, Dallas). Anti-decorin rabbit antiserum, raised against recombinant human decorin, was kindly donated by Dr. Peter Shioi (University of Manchester).

**Preparation of Conditioned Medium for Protein Expression Analysis**—2T3 cells were a kind gift from Dr. Paul Bishop (University of Texas Southwestern Medical Center, Dallas). Anti-decorin rabbit antiserum, raised against recombinant human decorin, was kindly donated by Dr. Peter Shioi (University of Manchester).

**Preparation of RNA and Real Time Quantitative PCR Analysis**—Total RNA was isolated from cultured cells using TRIzol™ reagent (Invitrogen) and incubated with turbo DNA-free DNase I (Ambion) to remove any contaminating DNA. cDNA was prepared by reverse transcription of 1 µg of DNase-treated RNA and random hexamers (Invitrogen). Reverse transcription reaction efficiency was tested by performing PCR using primers for the housekeeping gene β-actin, at 95°C for 5 min, 57°C for 30 s, 72°C for 30 s, for 30 cycles followed by 72°C for 4 min. Forward (F) and reverse (R) primers recognizing β-actin (GenBank™ accession number NM_007393, F, AGC CAT GTA CGT AGC CAT CC; R, CTC TCA GCT GTG GTG GTG AA), Cbfa-1 (GenBank™ accession number AF012834, F, GCA GTT CCC AAC AGG TTC AT; R, CAC TCT GCC TTT GGG AAG AG), and the α1 chain of collagen type I (GenBank™ accession number U08020, F, CAC CCT CAA GAG CCT GAG CT; R, GCT TCT TTT CTT TGG GGT TC) were designed using the primer-3 program. Real time PCR analysis was then performed for β-actin, Cbfa-1, and collagen type I in a 25-µl reaction using a pre-mixed SYBR Green PCR master mix (Applied Biosystems), 15 pmol of primer pairs, and 5 µl of cDNA and the ABI PRISM 7000 sequence detection system (Applied Biosystems). Cycling parameters were as before but for 40 cycles. The amplification efficiencies of the test genes and the reference gene, β-actin, were approximately equal as determined by serial dilution of cDNA prepared from wild-type day 3 cultures. Each sample was run in triplicate, and each sample set was analyzed twice. Amplification curves were checked for normal base-line and threshold levels during each run. A comparative method was used to determine RNA levels in which data for test genes (Δ) were normalized to levels of β-actin (CT) within each sample, and relative mRNA levels were determined using the equation 2((ΔCT)).
a further 24 h. The resulting conditioned medium was collected and centrifuged (5 min, 4000 × g) to remove cell debris. Supernatants were dialyzed against 50 mM ammonium bicarbonate over 72 h at 4 °C and freeze-dried. Protein samples were resuspended in 1 × loading sample buffer (62.5 mM Tris/HCl, pH 6.8, 1% SDS, 5% glycerol), and total protein concentrations were determined using a BCA protein assay (Pierce), with bovine serum albumin as the standard.

Preparation of Cell Lysates for Protein Expression Analysis— Cultures were washed with PBS and proteins extracted using lysis buffer (20 mM Tris/HCl, pH 8.0, 150 mM NaCl, 1% (v/v) Nonidet P-40, 10% glycerol, 1 mM EDTA, 5 mM sodium fluoride, and 0.1% (v/v) SDS) containing the following protease inhibitors: leupeptin (1 μg/ml), aprotinin (1 μg/ml), pepstatin A (1 μg/ml), phenylmethylsulfonyl fluoride (1 mM), and sodium orthovanadate (1 mM) for 20 min on ice. Samples were centrifuged (5 min, 10,000 × g at 4 °C) and protein concentrations determined in the supernatants as described above.

Generation of Full-length HtrA1 cDNA—Total RNA was isolated from MC3T3-E1 cells using TRIzol™ reagent (Invitrogen) according to the manufacturer’s protocol, and cDNA was synthesized using avian myeloblastosis virus reverse transcriptase and oligo(dT) primers. HtrA1 cDNA was generated by PCR using oligonucleotide primers designed to the full coding region of murine HtrA (GenBank™ accession number NM_019564). Expand High Fidelity Taq polymerase (Roche Applied Science) and 10% Me2SO were used in all reactions. The full-length product was generated from three separate DNA fragments that were generated from two overlapping 5’ and 3’ sequences. The 5’ PCR product (515 bp) was generated using the forward primer 5’-AAC GGA TCC GTC ATG CAG TCC CTG CGT A-3’ and reverse 5’-AGT TGT ACT TAT GAC GCA AAC TGT T-3’.

Western blotting of conditioned media and by Northern blotting. HtrA1 mRNA and protein in selected clones was determined by cloning or by dilution cloning after 2–3 weeks. Expression of HtrA1 mRNA and protein in selected clones was determined by Western blotting of conditioned media and by Northern blotting using 15 μg total RNA and an [α-32P]CTP-labeled HtrA1 cDNA probe. Northern blotting was performed as described (28).

Generation of HtrA1 siRNA Expression Plasmid—HtrA1 RNA interference effector molecules were delivered using the siRNA expression vector pSUPERIOR.neo+ GFP (Oligo-Engine). The HtrA1 19-nt mRNA target sequence 5’-GGU UGA GCU GAA GAA UGG A-3’ was taken from sequences provided with pre-designed siRNA 21-mer duplexes (Ambion). Preliminary experiments showed this sequence to be the most effective at knocking down the transient expression of recombinant HtrA1 in HEK293 cells. Two 60-bp oligodeoxynucleotides were designed to include the unique 19-nt sequence in both the sense and antisense direction, separated by a 9-nt spacer sequence. The sense strand of the pSuperior-HtrA1-siRNA insert is 5’-GAT CCC CGG TTG AGC TGA AGA ATG GAt tca gaT CCA TTC TTC AGC TCA ACC TTT TTA -3’. The 5’ end of the sense strand oligonucleotide corresponds to a BgIII site and the 3’ end to a thymidine termination sequence (T5) and HindIII corresponding nucleotides. The forward and reverse oligonucleotides were annealed and ligated into BgIII/ HindIII linearized pSUPERIOR.neo+ GFP vector, downstream of the H1 promoter, according to the manufacturer’s protocol. The identities of the inserted sequences were confirmed by DNA sequencing.

Transfection of 2T3 Cells with pSUPERIOR siRNA Plasmids—2T3 cells were plated 24 h prior to transfection at 4 × 105 cells/10-cm dish. Cells were transfected with 10 μg of the pSuperior-HtrA (pSuperHtrA) and pSuperior-empty vector (pSuperEV) plasmids using a calcium phosphate kit (Profection® kit, Promega). After 30 h, cells were passaged 1:2, and transfected cells were selected with 400 μg/ml G418. Neomycin-resistant cell populations were expanded, and HtrA1 expression was assessed by Western blotting of conditioned media. To confirm that knockdown of HtrA1 was specific, samples of conditioned medium and cell lysates from both cell populations were also assessed for global protein expression and for the expression of β-actin.

Cloning and Expression of HtrA1 Recombinant Proteins—Recombinant murine HtrA proteins were produced using a mammalian episomal expression vector (pCEP-His) and 293-EBNA cells (29). pCEP-His has been modified from the pCEP-pu/AC7 vector (30) to incorporate an N-terminal His, tag following a signal peptide of the BM40 protein (31). HtrA1 cDNA, without the sequence encoding for its signal peptide, was amplified from plND/mHtrA1 using the primers 5’-GCT GAC TTG CCG TCG GCC GAG GG-3’ and 5’-GTC GAC CTC CTG CCT CTT AG-3’. PCR conditions were 95 °C for 3 min followed by 30 cycles of 95 °C for 15 s, 52 °C for 30 s, and 72 °C for 2 min, with a final extension for 4 min at 72 °C. Both PCR products were ligated into pCR2.1-TOP cloning vector (Invitrogen) and sequences confirmed using Big Dye™ terminator cycle sequencing ready reaction kit and an ABI 377 automated sequencer (Applied Biosystems Inc). These cDNA constructs were digested with HindIII (creating fragments 1–3) and through a series of ligations generated the full-length product of 1460 bp in pCR2.1. The resultant full-length HtrA1 cDNA was first ligated into pND (Invitrogen) as an EcoRI-digested fragment (pND-mHtrA1) and subsequently into mammalian expression vector pcDNA3.1 as a KpnI/XbaI fragment generating the pcDNA3.1-mHtrA1 expression plasmid.

Overexpression of HtrA1 in 2T3 Osteoblasts—2T3 cells were stably transfected with pcDNA3.1-mHtrA1 expression plasmid using the FuGENE 6 reagent (Roche Applied Science). A FuGENE 6:DNA ratio of 3:1 and 1 μg of DNA/10 cm² of cell layer was used. After 16 h, medium was replaced with fresh growth medium containing 400 μg/ml G418. Drug-resistant colonies developed from single cells were selected either by ring cloning or by dilution cloning after 2–3 weeks. Expression of HtrA1 mRNA and protein in selected clones was determined by Western blotting of conditioned media and by Northern blotting using 15 μg total RNA and an [α-32P]CTP-labeled HtrA1 cDNA probe. Northern blotting was performed as described (28).
final PCR products were cloned into pCR2.1-TOPO and sequences confirmed. HtrA1 inserts were released by Sall digestion and cloned into XhoI-digested pCEP-His to produce pCEP-His/HtrA1 (H) and pCEP-His/HtrA1ΔPDZ (HΔP). Site-directed mutagenesis (QuickChange XL site-directed mutagenesis kit, Stratagene), using primers 5′-CAT TTA TGG AAA TGC CGG AGG CCC GTT AG-3′ and 5′-CTA ACG GGC CTC CGG CAT TTC CAT AAT TGA TG-3′ (3) introduced a point mutation into pCEP-His/HtrA1, which represents a mutation of the 328-serine residue to an alanine. The resulting mutated construct pCEP-His/MutHtrA1 (MH) was used as a template to amplify MutHtrA1ΔPDZ (MHΔP) using the same primer pair and PCR conditions as for HtrA1ΔPDZ. 293-EBNA cells were plated 24 h prior to transfection in 10-cm dishes at a density of 4 × 10⁴/cm² in Dulbecco’s modified Eagle’s medium supplemented with 10% (v/v) FBS, 1 mM sodium pyruvate, 50 units/ml penicillin, and 50 μg/ml streptomycin. Cells were transfected with the recombinant HtrA1 expression constructs using FuGENE 6 as described above. Cells were also transfected with pCEP-His-empty vector as a control. To select transfected cells, medium was replaced after 16 h with fresh medium containing 5 μg/ml puromycin. Cells were maintained thereafter in medium containing 0.5 μg/ml puromycin and 250 μg/ml G418.

Cloning and Expression of Recombinant Matrix Gla Protein—An expression construct encoding an N-terminally FLAG epitope-tagged mouse MGP protein (N-FLAG-MGP) was generated by PCR from the template pMGPcJ, using the forward and reverse primers 5′-GCC CCG CTA GCT GAC TAC AAG GAC GAT GAC AAAG AGT TGA CCT ACAC AAC GAA-3′ and 5′-CTC GAG ACT AGT GGA TCC AAC ACT TCA GTA A-3′. The forward primer incorporated the sequence of the FLAG epitope (DYKDDDDK). PCR conditions were 35 cycles of 92 °C for 45 s, 65 °C for 90 s, and 72 °C for 45 s. The 381-bp product was ligated as an Nhel/Xhol fragment into the episomal expression vector pCEP-Pu/AC7 (30), and the frame of the insert was confirmed by DNA sequencing. The pCEP-Pu-MGP construct was transfected into 293-HEK cells as described above for 293-EBNA cells. Transfected cells were selected and maintained in medium containing 5 and 0.5 μg/ml puromycin, respectively.

Preparation and Analysis of Recombinant Proteins—To generate recombinant HtrA proteins, transfected 293-EBNAs were maintained in α-minimal Eagle’s medium containing 10% (v/v) FBS, 2 mM L-glutamine, 50 units/ml penicillin, 50 μg/ml streptomycin, 0.5 μg/ml puromycin, and 250 μg/ml G418. Cells were expanded into 225-cm² tissue culture flasks, and at 90–100% confluence were prepared for conditioned media collection as described above. After 24 h, the conditioned media were centrifuged (4000 × g, 5 min) to remove cell debris. Supernatants, containing recombinant proteins, were concentrated ~50-fold using Amicon Ultra centrifugal filter columns with a 10-kDa molecular mass cut-off (Millipore), filtered through a 0.22-μm syringe filter, and stored at −80 °C. Medium collected from empty vector-transfected control cells was treated in the same manner. An aliquot (0.5 ml) of each concentrated conditioned medium was dialyzed against 50 mM ammonium bicarbonate, freeze-dried, resuspended in 50 μl of 1× loading sample buffer, and the total protein concentrations were determined using a BCA protein assay. To assay for serine protease activity, 20 μl of each concentrated recombinant protein was incubated with 5 μg of β-casein in 50 mM Tris/HCl, pH 8.0, at 37 °C for 2 h. Products were separated by SDS-PAGE (12% polyacrylamide) and stained with Coomassie Brilliant Blue (Sigma). The conditioned medium from 293-EBNA cells expressing FLAG-MGP was prepared as above and concentrated using Amicon Ultra centrifugal columns with a 5-kDa molecular weight cut-off.

Digestion of Matrix Proteins by Recombinant HtrA1—GAG chains were removed from human recombinant decorin (0.2 μg) by incubation with chondroitinase ABC (0.005 units) in 0.1 M Tris/HCl, pH 8.0, 0.03 mM sodium acetate at 37 °C for 16 h. Concentrated recombinant HtrA1 proteins or empty vector controls (20 μl) were added directly to the digested decorin and incubated for a further 16 h at 37 °C. Samples were subjected to SDS-PAGE (10% polyacrylamide) under reducing conditions and protein products detected by Western blotting. Human plasma-derived fibronectin (0.2 or 10 μg in PBS) was incubated with HtrA1 proteins or empty vector controls (20 μl) at 37 °C for 16 h. Products were separated by SDS-PAGE under reducing conditions on 4–12% BisTris acrylamide gels (Invitrogen) and either analyzed by Western blotting (0.2 μg) or by silver staining using SilverQuest™ silver staining kit (Invitrogen). rMGP (40 μl of concentrated conditioned medium) was incubated with HtrA1 proteins as described and products separated on 18% polyacrylamide gels and detected by Western blotting using an anti-FLAG M2 monoclonal antibody. Type I collagen was prepared from rat tail tendons and diluted to 2 mg/ml in 0.1% acetic acid. To neutralize the collagen prior to analysis, 118 μl sodium bicarbonate was added per 1 ml of collagen (volume ratio 2:17). Concentrated recombinant HtrA1 proteins (20 μl) were incubated with 10 μg of type I collagen for 16 h at 37 °C. Products were separated on 4–12% BisTris acrylamide gels and stained with Coomassie Brilliant Blue.

Immunoblotting—Protein samples were separated by SDS-PAGE under reducing conditions and transferred to nitrocellulose membranes that were subsequently blocked overnight at 4 °C with 0.1% (w/v) Tween in PBS (PBS-T) containing 5% (w/v) milk powder or 5% (w/v) bovine serum albumin (HtrA1 blots only). All primary antibodies were incubated with membranes for 1 h at room temperature in 5% (w/v) milk powder in PBS-T (anti-decorin rabbit antiserum, 1:1000; anti-FLAG M2 monoclonal, 1:1000; anti-His monoclonal, 1:3000; and anti-β-actin monoclonal (1:4000)) or in 5% (w/v) bovine serum albumin in PBS-T (anti-HtrA1 goat polyclonal, 1:50). To confirm the specificity of the HtrA1 antibody, it was preincubated, prior to blotting, with an HtrA1 blocking peptide for 1 h at room temperature using a peptide/antibody concentration of 4:1. Incubation with appropriate secondary horseradish peroxidase-conjugated antibodies was performed for 1 h in PBS-T with 5% (w/v) dry milk (swine anti-rabbit, 1:1000; rabbit anti-mouse, 1:1000) or 5% (w/v) bovine serum albumin (donkey anti-goat, 1:1000). Membranes were washed five times for 10 min with PBS-T after each incubation, and reactivity was detected using an enhanced
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**RESULTS**

**HtrA1 Is Secreted by 2T3 Osteoblasts**—Previously we reported the up-regulation of HtrA1 expression in vascular pericytes undergoing osteogenic differentiation and mineralization (13). Here we report for the first time the temporal pattern of secretion of HtrA1 protein by 2T3 osteoblasts over a 17-day culture period. Fig. 1A shows the Alizarin red staining pattern of 2T3 osteoblasts, grown to confluency (day 3, panel i) and then cultured in differentiation medium for a further 14 days (day 17). Multilayering of differentiated cells and condensed cellular areas was apparent after 10 days (Fig. 1A, panel ii). Cultures began to deposit a calcified matrix after 13 days (Fig. 1A, panel iii), which was extensively mineralized after 17 days (Fig. 1A, panel iv), as demonstrated by positive staining with Alizarin red. Serum-free conditioned media were collected from 2T3 cultures at confluence (day 3) (panel i) and after 10 (panel ii), 13 (panel iii), and 17 (panel iv) days in culture (bar = 500 μm). Calcification is demonstrated at day 13 and 17, by positive staining with Alizarin red. B, Western blot analysis of HtrA1 expression in 2T3 cells. Serum-free conditioned media (CM), containing the secreted HtrA1 protein, was collected from 2T3 cultures at confluence and on days 10, 13, and 17. Equivalent volumes of conditioned media (CM) from each time point (1.5 ml from 5 ml total) were concentrated by freeze-drying and immunblotted using an anti-HtrA1 polyclonal (upper panel). The blot was stripped and stained with India ink to show protein loading (lower panel). HtrA1 protein was detected in 2T3 conditioned media at confluence and was markedly up-regulated in post-confluent cultures (day 10), prior to the appearance of mineralized nodules. HtrA1 was then dramatically down-regulated following the onset of mineralization (day 13). Small amounts of HtrA1 were detected in heavily mineralized cultures (day 17). Preincubation of the HtrA1 antibody with an HtrA1 immunization (blocking) peptide confirmed its specificity (data not shown). The blot was stripped and stained with India ink to show protein loading (Fig. 1B, lower panel). It is important to note that the mineralization of the 2T3 wild-type cells did not occur at exactly the same time points in every experiment. However, down-regulation of HtrA1 expression was always observed with the onset of mineralization.

**HtrA1 Overexpression Inhibits Osteoblast Mineral Deposition in Vitro**—To determine the functional role of HtrA1 in osteoblast mineralization, clones of 2T3 cells stably overexpressing HtrA1 were generated and selected for further characterization based on HtrA1 mRNA and protein expression levels, relative to wild-type controls (Fig. 2, A and B). Clone 2 (C2) consistently demonstrated the highest overexpression of HtrA1 (Fig. 2, A and B). The effect of HtrA1 overexpression on the expression of early markers of the osteogenic phenotype was examined. Real time quantitative PCR analysis revealed reduced expression of Cbfa1 and collagen type I at confluence in C2 cells compared with wild-type cells (Fig. 2, C and D). There was no difference in Cbfa1 or collagen type I mRNA expression between the wild-type cells or C2 cells on day 21 (Fig. 2, C and D). The effect of HtrA1 overexpression on mineral deposition was investigated by Alizarin red staining (Fig. 2E) and quantified by measuring 45Ca incorporation into the cell layer (Fig. 2F). In these experiments, a high level of mineralization was already apparent in 2T3 wild-type cells after 10 days, which was further increased on days 13 and 17 (Fig. 2E). Markedly reduced Alizarin red staining was observed in C2 cells at time points when wild-type cells were heavily mineralized (Fig. 2E). A significant reduction in 45Ca incorporation into the matrix by C2 cells compared with wild-type cells was also observed at these time points (Fig. 2F, p < 0.001). The data presented in Fig. 2, E and F, is representative of three separate experiments. A second clone exhibited an intermediate level of expression of HtrA1 compared with C2 and wild-type cells. In these cells, some mineralization was detected at the later time points, suggesting that the effects of HtrA1 on mineralization may be dose-dependent (see supplemental material).

**HtrA1 Overexpression Prevents BMP-2-induced Mineralization**—BMP-2 accelerates the appearance of mineralized nodules in 2T3 cells (22, 23), and HtrA1 has been demonstrated to inhibit signaling of BMP family members in vitro (14). Therefore, to investigate whether the overexpression of HtrA1 could suppress the effect of BMP-2 on osteoblast mineralization, wild-type cells and C2 cells were cultured in the presence of recombinant BMP-2. Treatment of wild-type cells with BMP-2 (10 ng/ml) increased the level of mineral deposition, as shown by Alizarin red staining after 13 days (Fig. 2G, panels i and iii). In contrast, mineralization was not induced by BMP-2 in C2 cells that markedly overexpress HtrA1 (Fig. 2G, panels ii and...
Knockdown of HtrA1 Expression Using siRNA Increases Osteoblast Mineralization—The above studies show that marked overexpression of HtrA1 inhibits matrix mineralization by osteoblasts. Therefore, complementary experiments using siRNA to knock down the expression of HtrA1 in these cells were conducted, and the effects on mineralization were determined. To enable delivery of siRNA effector molecules targeted against HtrA1 over long periods, 2T3 cells were stably transfected with the pSuperior-HtrA1 siRNA expression vector. Cells transfected with the pSuperior empty vector were used as controls. Populations of the resulting antibiotic-resistant cells (pSupHtrA1 and pSupEV) were grown to confluence (day 3) and then cultured in mineralization medium for a further 7 days (until day 10). HtrA1 expression was determined on day 4 by Western blotting of equivalent volumes of conditioned medium (Fig. 3A). Knockdown of HtrA1 expression (~90%) was confirmed in the pSupHtrA1 cells compared with the control pSupEV cells (Fig. 3A). In contrast, equivalent expression of β-actin was demonstrated in pSupEV and pSupHtrA1 cell lysates at this time point (Fig. 3B). In addition, staining of the membranes with India ink confirmed that the HtrA1 siRNA was not down-regulating protein expression per se. Mineralization was markedly enhanced when HtrA1 expression was knocked down compared with control cells (Fig. 3C). The data presented are representative of two separate experiments with duplicate samples.

The Protease Domain and the PDZ Domain Are Essential for the Inhibition of Osteoblast Mineralization by HtrA1—To further investigate the effects of HtrA1 on osteoblast mineralization and to determine which of the individual domains of this protein are important for its function, a series of histidine-tagged recombinant HtrA1 proteins were expressed using 293-EBNA cells (Fig. 4A). Immunoblotting of concentrated 293-EBNA medium using a His tag reactive antibody confirmed the production of these proteins (Fig. 4B). These images are representative of four separate experiments. No mineralization was detected in the C2 cells even when BMP-2 was added at higher doses (see supplemental material).
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![Image](357x26 to 384x38)

FIGURE 3. Knockdown of HtrA1 expression increases osteoblast mineralization. 2T3 osteoblasts were stably transfected with pSupHtrA1 siRNA expression vector and pSupEV control plasmid. Cultures were treated from confluence (day 3) with mineralization media for a further 7 days (day 10), and the extent of mineralization and levels of HtrA1 expression were determined. A, after 4 days in culture, conditioned medium was collected, concentrated, and 1-ml equivalents were analyzed for HtrA1 expression by Western blotting. A reduction in HtrA1 expression is evident in pSupHtrA1 cells. India ink staining of equivalent samples shows protein loading of pSupHtrA1 and pSupEV at this time point. B, Western blotting of cell lysates (10 μg) confirmed equal expression of β-actin in pSupHtrA1 and pSupEV cells. Equal protein loading was confirmed by staining of the membrane with India ink. C, phase contrast images of Alizarin red stained pSupEV (panel i) and pSupHtrA1 (panel ii) cells after 10 days in culture (bar = 200 μm).
lacked protease activity (MH, MHΔP), almost completely degraded both intact decorin and the decorin core protein (Fig. 6B). Immunoblotting with an anti-FLAG antibody against the FLAG-MGP fusion protein showed full-length recombinant MGP migrating with an approximate molecular mass of 15 kDa (Fig. 6B, lower panel, first lane). Following incubation with conditioned medium containing HtrA1, a product of ~12 kDa was detected, indicating cleavage of MGP at the C terminus. MGP was not cleaved by conditioned medium containing either HΔP or the mutant proteins (MH, MHΔP). In contrast, none of the recombinant HtrA1-containing conditioned medium degraded acid-solubilized type I collagen (Fig. 6C).

**DISCUSSION**

This study demonstrates for the first time that HtrA1 plays a key role in the regulation of mineral deposition by osteoblasts. First, we have shown that HtrA1 is temporally expressed during osteoblast differentiation in vitro. Second, we demonstrated that mineralization is inhibited by marked overexpression of HtrA1 and is promoted by knocking down HtrA1 expression using siRNA. Furthermore, overexpression of HtrA1 down-regulated Cbfα1 and type I collagen mRNA at confluence and prevented the induction of mineralization by BMP-2. Third, by expressing a series of recombinant HtrA1 proteins, we demonstrated that the protease domain and the PDZ domain are essential for the inhibitory effect of HtrA1 on osteoblast mineralization. We also identified that HtrA1 cleaves decorin, fibronectin, and matrix Gla protein, but not type I collagen, and that the PDZ domain in addition to the protease domain is required for the cleavage of MGP.

We propose that HtrA1 may inhibit mineralization by multiple mechanisms that may not be mutually exclusive, including (i) modulating expression of specific osteoblast genes, (ii) altering BMP-2 and/or TGF-β activity, and/or (iii) cleaving specific matrix proteins that regulate differentiation or bone matrix formation either directly or indirectly.

The temporal pattern of HtrA1 expression demonstrated in this study is consistent with the in vivo expression reported by Tsuchiya et al. (6). We demonstrate increased HtrA1 expression prior to the appearance of mineralized nodules, and its subsequent down-regulation in fully mineralized cultures of 2T3 osteoblasts. In vivo, HtrA1 expression is associated with the appearance of the ossification center in mouse tissue, and HtrA1 is deposited in the matrix of embryonic and adult bone, with osteoid and newly

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formed bone matrices appearing to accumulate larger amounts of HtrA1 than fully calcified bone (6). Together, these studies demonstrate that the expression of HtrA1 in vitro and in vivo is tightly regulated both temporally and spatially.

In this study, we demonstrate that overexpression of HtrA1 in osteoblasts or the addition of recombinant full-length HtrA1 to the culture medium markedly delays matrix mineralization, whereas mineralization is increased by knocking down HtrA1 expression. Overexpression of HtrA1 also prevented BMP-2-induced mineralization. Oka et al. (14) have previously shown that HtrA1 binds to and inhibits in vitro signaling mediated by several TGF-β family proteins, including BMP-2. This group reported that neither TGF-β, BMP-2, nor BMP-4 can be degraded by HtrA1 and that signals from the constitutively active BMP-4 receptor, caBMPR-1B, are not inhibited by this protease (14). Taken together, these results suggest that HtrA1 may inhibit BMP-2-induced mineralization of 2T3 cells by inhibiting the interaction of BMP-2 with its receptor, thereby preventing downstream signaling. This suggestion is consistent with the results of a previous study in which the inhibition of BMP signaling in 2T3 osteoblasts by overexpression of Smad ubiquitin regulatory factor 1 (Smurf1) also inhibited BMP-2-induced mineralized bone nodule formation in vitro (44).

Previous studies have shown that Cbfα1 (a master regulator of osteoblast differentiation) and type I collagen play key roles in the early stages of 2T3 osteoblast differentiation (22, 44–47). The expression of these genes is down-regulated in cells induced to overexpress Smurf1 or a truncated type IB BMP
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![Images](image1.png)

**FIGURE 5. Recombinant HtrA1 decreases osteoblast mineralization, and the protease and PDZ domains are essential for this inhibition.** In each experiment, concentrated recombinant proteins were added to 2T3 cultures from confluence (day 3), in equivalent amounts, i.e., equal total protein and equal amounts of recombinant HtrA1 proteins. Conditioned media from empty vector (EV)-transfected EBNA-293 cells were used to normalize total protein levels. HtrA1 proteins were added in excess (200-fold) of the highest protein levels present in mineralizing 2T3 cultures. A, 2T3 cells were treated with empty vector (panels i and iv), MutHtrA1 (panels ii and v), and HtrA1 (panels iii and vi) conditioned media (total protein = 10 μg) for 7 days (day 10). Alizarin red staining shows decreased mineral deposition in cells treated with HtrA1 relative to cells treated with MutHtrA1 and control EV-conditioned media.

receptor, resulting in reduced osteoblast differentiation and mineral deposition (44, 45). Interestingly, we demonstrate that HtrA1 overexpression also results in the down-regulation of these genes in confluent osteoblasts, suggesting that one of the mechanisms by which HtrA1 may inhibit mineralization is by modulating osteoblast gene expression, although further studies are required to confirm this hypothesis.

Further investigation of the functional role of HtrA1 highlighted the importance of both the protease and the PDZ domains for the inhibitory effect of recombinant HtrA1 on osteoblast mineralization. Previous studies have shown that the protease domain, but not the PDZ domain, is essential for the inhibition of TGF-β signaling by HtrA1 (14). In fact, proteins lacking the PDZ domain exhibited increased inhibitory activity on TGF-β signaling. Therefore, we investigated whether specific matrix proteins that are also known to regulate bone formation, mineralization, and/or growth factor availability were cleaved by HtrA1 and whether the protease and PDZ domains were essential for this activity.

We demonstrated that conditioned medium containing full-length recombinant HtrA1 cleaves decorin, fibronectin, and matrix Gla protein (MGP) but not type I collagen. Analogous to the treatment of 2T3 osteoblasts with recombinant HtrA1, anti-fibronectin antibodies inhibited the formation of mineralized nodules in osteoblasts in vitro (47). Furthermore, the binding of the fibronectin RGD sequence to cell-surface integrin receptors has been shown to be required for osteoblast differentiation (36). Recently, fibronectin was shown to be crucial for the regulation of TGF-β and LTBP-1 (latent transforming growth factor-binding protein) incorporation into the extracellular matrix of osteoblasts (35). Therefore, it is tempting to speculate that degradation of fibronectin by HtrA1 may disrupt matrix assembly and enhance TGF-β activity, thereby leading to an inhibition of mineral deposition.

There are conflicting reports on the role of decorin in osteoblast differentiation and mineralization. The majority of evidence suggests that decorin is an inhibitor of mineralization in bone and that its removal or fragmentation is necessary for mineralization to occur (48–50). However, the opposite effect has been demonstrated in the vasculature, where decorin has been shown to promote mineral deposition (51). Furthermore, the defective bone phenotype observed in biglycan and decorin double knock-out mice (52) highlights the importance of decorin in the formation of a mineralized bone matrix. Although the decorin knock-out alone does not exhibit any striking bone phenotype, the decrease in bone mass in the double knock-out is more severe than in the biglycan single knock-out. Bi et al. (53) reported that TGF-β was not properly sequestered in the extracellular matrix of these decorin/biglycan

*Bar = 500 μm. 8. mineral deposition was quantified by 45Ca incorporation into the cell layer over 24 h. After 10 days, cultures treated with HtrA1 show a 1.8- and a 2-fold decrease in calcium deposition, relative to EV- and MutHtrA1-treated cells, respectively. Statistical analysis was performed using one-way analysis of variance and Tukey’s post hoc test. *, p < 0.001 (n = 3). C, 2T3 cells were treated with EV (panels i and iv), HtrA1ΔPDZ (panels ii and v), and HtrA1 (panels iii and vi) conditioned media (total protein = 20 μg) for 10 days (day 13). Alizarin red staining shows decreased mineral deposition in cells treated with HtrA1, compared with cells treated with HtrA1ΔPDZ- or EV-conditioned media (bar = 500 μm).*
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which it exerts these effects are still not fully understood (54–59). Several studies have shown that MGP functions as an inhibitor of mineralization in vitro and in vivo and that the Gla domains are required for this activity (42, 54, 60). However, the Gla domains of MGP also bind BMP-2 (56–58, 61–64), and it has been shown that the ability of MGP to regulate mineral deposition and osteogenic differentiation is dependent on the relative levels of MGP and BMP-2 present (57, 58). In this study we demonstrate that a small domain at the C terminus of MGP is cleaved by HtrA1, and this cleavage requires both the PDZ and protease domains of HtrA1. MGP has been shown to undergo processing at the C terminus, resulting in a 77–83-amino acid protein depending on species, although the protease(s) responsible for this cleavage have not been identified. Whether MGP is a physiological substrate for HtrA1 in vivo or whether cleavage of MGP by HtrA1 only occurs under pathological conditions is not known. As MGP binds to the matrix via amino acid residues 61–77 in its C-terminal domain (61, 65), it is tempting to speculate that HtrA1-mediated cleavage of MGP at the C terminus may enhance its ability to bind to the matrix, which, in turn, may inhibit BMP-2 signaling and mineralization, although further studies are required to test this hypothesis.

In conclusion, we demonstrate a novel function for HtrA1, as an inhibitor of matrix mineralization. HtrA1 may regulate mineralization either by modulating osteoblast gene expression, inhibiting growth factor signaling, and/or by cleaving specific matrix proteins. We suggest that the precise mechanism of action of HtrA1 may depend on the relative levels of individual growth factors and matrix proteins present and that perturbation in the levels of these molecules may result in aberrant mineral deposition. We further suggest that the de-regulation of HtrA1 expression in vivo may contribute to pathologies associated with aberrant mineral deposition.

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double knock-out mice, thereby altering the fate of bone marrow stromal cells, resulting in reduced bone formation and mineralization. It is possible that degradation of decorin by HtrA1 could release TGF-β bound within the matrix, making it available to interact with its receptor. Alternatively, HtrA1 may prevent decorin from sequestering TGF-β in the matrix and away from the cell-surface receptors. Either way this would result in decreased osteoblast mineralization.

MGP has been implicated in the regulation of both cell differentiation and mineralization, although the mechanism by...
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