Bioactive Materials 38 (2024) 399–410

Contents lists available at ScienceDirect

Bioactive Materials

journal homepage: www.keaipublishing.com/en/journals/bioactive-materials

HtrA3 paves the way for MSC migration and promotes osteogenesis

Yaru Guo [a,1], Siqin Ma [b,1], Dandan Wang [c,1], Feng Mei [d,e,1], Yusi Guo [a,1], Boon Chin Heng [f], Shihan Zhang [a], Ying Huang [a], Yan Wei [a], Ying He [a], Wenwen Liu [a], Mingming Xu [a], Xuehui Zhang [g,h,**], Lili Chen [d,e,***], Xuliang Deng [a,g,h,*]

[a] Department of Geriatric Dentistry, Peking University School and Hospital of Stomatology, Beijing, 100081, China
[b] Department of Stomatology, PLA General Hospital, First Affiliated Hospital (304 Hospital), Beijing, 100081, China
[c] Department of Pediatric Dentistry, Peking University School and Hospital of Stomatology, Beijing, 100081, China
[d] Department of Stomatology, Union Hospital, Tongji Medical College, Huazhong University of Science and Technology, Wuhan, 430022, China
[e] School of Stomatology, Tongji Medical College, Huazhong University of Science and Technology, Wuhan, 430022, China
[f] Central Laboratory, Peking University School and Hospital of Stomatology, Beijing, 100081, China
[g] NMPA Key Laboratory for Dental Materials, Department of Dental Materials & Dental Medical Devices Testing Center, Peking University School and Hospital of Stomatology, Beijing, 100081, China
[h] National Engineering Research Center of Oral Biomaterials and Digital Medical Devices, Beijing Laboratory of Biomedical Materials, Peking University School and Hospital of Stomatology, Beijing, 100081, China

ARTICLE INFO

Keywords:
Mesenchymal stem cell
Migration
High-temperature requirement A3
Osteogenesis
Mechanotransduction

ABSTRACT

Mesenchymal stem cell (MSC) migration determines the healing capacity of bone and is crucial in promoting bone regeneration. Migration of MSCs is highly dependent on degradation of extracellular matrix by proteolytic enzymes. However, the underlying mechanisms of how enzymolysis paves the way for MSCs to migrate from their niche to the defect area is still not fully understood. Here, this study shows that high-temperature requirement A3 (HtrA3) overcomes the physical barrier and provides anchor points through collagen IV degradation, paving the way for MSC migration. HtrA3 is upregulated in MSCs at the leading edge of bone defect during the early stage of healing. HtrA3 degrades the surrounding collagen IV, which increases the collagen network porosity and increases integrin β1 expression. Subsequently, integrin β1 enhances the mechanotransduction of MSCs, thus remodeling the cytoskeleton, increasing cellular stiffness and nuclear translocation of YAP, eventually promoting the migration and subsequent osteogenic differentiation of MSCs. Local administration of recombinant HtrA3 in rat cranial bone defects significantly increases new bone formation and further validates the enhancement of MSC migration. This study helps to reveal the novel roles of HtrA3, explore potential targets for regenerative medicine, and offer new insights for the development of bioactive materials.

1. Introduction

Migration of mesenchymal stem cells (MSCs) is a key process in tissue regeneration, and determines the outcome of bone defect healing [1,2]. Large bone defects are often unable to heal spontaneously via endogeneous repair mechanisms due to limited migration of MSCs to the defect area [3]. Therefore, enhancing MSC migration might be a promising strategy to promote spontaneous regeneration of larger than critical-sized bone defects. MSC migration is a spatio-temporally coordinated multi-step process that is precisely-orchestrated. Initially, quiescent MSCs reside surrounding sinusoids and blood vessels throughout the bone marrow [4]. Upon stimulation by chemokines and cytokines, MSCs can secrete proteases to break through and remodel extracellular matrix (ECM) barriers to escape from the stem cell niche

Peer review under responsibility of KeAi Communications Co., Ltd.
* Corresponding author. Department of Geriatric Dentistry, Peking University School and Hospital of Stomatology, Beijing, 100081, China.
** Corresponding author. National Engineering Research Center of Oral Biomaterials and Digital Medical Devices, Beijing Laboratory of Biomedical Materials, Peking University School and Hospital of Stomatology, Beijing, 100081, China.
*** Corresponding author. Department of Stomatology, Union Hospital, Tongji Medical College, Huazhong University of Science and Technology, Wuhan, 430022, China.
E-mail addresses: zhangxuehui@bjmu.edu.cn (X. Zhang), chenlili1030@hust.edu.cn (L. Chen), kqdengxuliang@bjmu.edu.cn (X. Deng).
[1] These authors contributed equally to this work.

https://doi.org/10.1016/j.bioactmat.2024.05.016
Received 24 January 2024; Received in revised form 6 May 2024; Accepted 7 May 2024

and potentially enter blood vessels or directly migrate to the bone forming area [5,6]. The most widely studied proteolytic enzymes are the matrix metalloproteinases (MMPs) family, which mainly display type I collagenolytic activities [7]. However, ECM is a complex network composed of approximately 300 proteins, the most represented and studied of those are collagens [8]. Only degradation of collagen I could lead to the collapse of this collagen-based microstructure and loss of enzymolysis-formed interconnected scaffold pores, through which MSCs migrate to the defect area [9]. Therefore, it is imperative to identify other proteinases that are involved in paving the way for migrating MSCs, which might provide new insights into the treatment of larger than critical-sized bone defects.

The high temperature requirement A (HtrA) family, including HtrA1-4, is stress-related serine proteases and plays an important role in cell motility, death and signal transduction, embryo implantation and maintenance of mitochondrial homeostasis via regulation of intra- and extracellular protein metabolism [10–13]. Therefore, the dysfunction of HtrA family may lead to various pathologies, including cancer, pathological mineralization, neurodegenerative diseases, and arthritic disorders [14,15]. As a member of the HtrA family, HtrA3 has the overlapping biological activities with HtrA1, which was newly identified as a positive regulator of osteogenesis and mineralization [16,17]. Studies have shown that HtrA3 is largely localized in the adult bone matrix and increases during bone injury [11]. Moreover, HtrA3 has been found to promote human trophoblast invasion during embryo implantation [18], and is able to degrade ECM proteoglycans, such as decorin and biglycan [11,19]. Therefore, we postulate that HtrA3 might be also involved in MSC migration and bone regeneration through ECM degradation and remodeling.

Here, we found up-regulation of HtrA3 in MSCs by inflammation microenvironment at the leading edge of bone repair tissues during the early healing stage. It enhanced cellular cortical protrusions, migration, and osteogenic differentiation of MSCs. In-depth research showed the underlying mechanisms: HtrA3 selectively degraded collagen IV to overcome the physical barrier and increase the expression of integrin β1 in MSCs, which improved the traction forces for MSCs to move forward. Moreover, integrin β1 enhanced MSC-ECM interaction to reorganize and stiffen cytoskeleton, thus promoting the cytoplasm-to-nuclear transport of YAP to improve the expression of osteogenesis-related proteins. Consequently, the migration and osteogenic differentiation of MSCs were enhanced and rapid osteogenesis *in vivo* was achieved upon HtrA3 administration. Given the scarcity of knowledge on HtrA3 and the critical role of MSCs in tissue regeneration, coupled with the ongoing development of collagen IV-containing biomaterials, our study helps to reveal the novel roles of HtrA3, explore potential targets for regenerative medicine, and offer new insights for the development of bioactive materials.

2. Materials and methods

2.1. Cell culture

The human primary cell lines, hBMSCs were purchased from ScienCell Research Laboratories and cultured in mesenchymal stem cell medium (MSCM; 7501; ScienCell Research Laboratories; Carlsbad, CA, USA), within a humidified chamber at 37 °C with 5 % CO_2.

Before cell culture for alkaline phosphatase (ALP) staining and Alizarin red S staining, the culture plates were coated with diluted Matrigel (1 mg ml^{-1}) for 12 h.

2.2. Lentivirus production and transfection

All lentiviral vectors encoding green fluorescent protein (GFP) for knockdown of HtrA3 were purchased from GeneChem Co., Ltd. (Shanghai, China).

Sequence of shRNA:

5′-CCGGCATCAAGATCCATCCCAA-GAACTCGAGTTCTTGGGATGGATCTTGATGTTTTT-3′

Cells transfected with scramble were treated as controls.

The lentivirus vector fused with the GFP-labeled HtrA3 gene to upregulate HtrA3 was also purchased from GeneChem Co., Ltd. (Shanghai, China). The lentiviral vector with HtrA3 fusion protein (fused to mCherry) was constructed.

One day before lentiviral transfection, hBMSCs were seeded in six-well plates at a density of 1.2×10^5 cells per well. Next, lentiviral vectors overexpressing HtrA3 were added together with 5 mg mL^{-1} polybrene (Gene Pharma) to the cell culture for 12 h. Then, the transfected cells were selected using puromycin (P8833; Sigma-Aldrich) treatment for 3 days.

2.3. RT-qPCR analysis

The total cellular RNA from hBMSCs was extracted using Trizol reagent (Invitrogen), and reverse transcription was achieved using a PCR thermal cycler (Takara). Optical 96-well reaction plates (Thermo Fisher Scientific) and optical adhesive films (Thermo Fisher Scientific) were used for PCR.

Then, the data were analyzed using QuantStudio Design & Analysis Desktop Software (Thermo Fisher Scientific). Differences in gene expression levels among the different groups were statistically analyzed. The primer sequences are listed in Table S1 (online). GAPDH served as the endogenous housekeeping control.

2.4. Wound healing assay (including matrigel)

hBMSCs were seeded in six-well plates. Scratches were made using a 1 mL pipette tip when the cells formed a confluent monolayer. Then, the scratched monolayers were washed with phosphate buffer solution (PBS) in order to remove floating cells and debris. 1 ml of Matrigel (6 mg ml^{-1}) mixed with MSCM were added to the cell surface and were allowed to gellify for 0.5 h at 37 °C. Cell migration was assessed using a digital inverted microscope after 24 h, with both the initial wound area and area covered by migrating cells being analyzed with the Image Pro Plus software.

2.5. Transwell cell invasion assay (including matrigel)

hBMSCs cell invasion assay was conducted using transwell cell culture inserts (Transwell Assay System; Corning, 3422) for 24-well plates. The upper polycarbonate membrane was coated with Matrigel (250μg ml^{-1}). Subsequently, 1×10^4 hBMSCs cells suspended in serum-free medium were added to the upper polycarbonate membrane chambers (pore size, 8 μm). The medium with 10 % (v v^{-1}) fetal bovine serum was added to the lower chambers and incubated at 37 °C. The upper polycarbonate membrane chambers were fixed with 4 % (w v^{-1}) paraformaldehyde for 10 min and then stained with 0.5 % (w v^{-1}) crystal violet for 10 min at room temperature after 24 h. Then, the cells that had not crossed the membrane were removed with a wet cotton. The cells which had migrated to the underside of the inserts were captured and counted. All experiments were performed in triplicates.

2.6. Alkaline phosphatase (ALP) staining and quantification

Cells cultured for 14 days were assessed for ALP activity. ALP staining was performed using the nitroblue tetrazolium (NBT) and 5-bromo-4chloro-3-indolyl phosphate (BCIP) (NBT/BCIP) staining kit (C3206; Beyotime) according to the manufacturer's instructions. And ALP quantification was performed using the ALP ELISA kit (A059-2; Nanjing Jiancheng Bioengineering Institute).

2.7. Alizarin red S staining and quantification

Cells cultured for 21 days were subjected to alizarin red S staining and quantification. For staining, cells were fixed in 4 % (w v^{-1}) paraformaldehyde for 10 min and then stained with 1 % (w v^{-1}) alizarin red S (A5533; Sigma Aldrich) for 0.5 h at room temperature. As for quantification, the stain was extracted with cetylpyridinium chloride (52350; Sigma-Aldrich) for 1 h, followed by quantification through spectrophotometric absorbance readings at a wave length of 570 nm.

2.8. Proliferation assay

The proliferation of hBMSCs was assessed by EdU (ethynyl-deoxyuridine) cell proliferation assay and real-time observations. The culture medium containing EdU was added 2 h before fixing cells.

2.9. Immunocytochemical staining

The samples were rinsed with PBS and fixed for 10 min in 4 % (w v^{-1}) paraformaldehyde, permeabilized for 10 min in 0.2 % (v v^{-1}) Triton X-100, and blocked for 1 h in 5 % (w v^{-1}) BSA at room temperature. Then, the samples were incubated with the following primary antibodies overnight at 4 °C: anti-HtrA3 (A14649; ABclonal), anti-YAP1 (14074S; Cell Signaling Technology), anti-RUNX2 (12556; Cell Signaling Technology), anti-BMP2 (ab284387; Abcam), and anti-integrin β1 (ab183666; Abcam) antibodies. After washing three times in PBS, the samples were probed with the secondary antibodies, Alexa Fluor 594 (ab150080; Abcam), for 1 h in the dark. The cell nuclei were stained with DAPI. Images were acquired using a confocal microscope.

2.10. Proteolysis assay of collagen IV

250 ng recombinant Collagen IV (ab7536; Abcam) was mixed with 0, 250, 400, 800 ng of rhHtrA3 solution (Abnova, Taiwan, China). The mixture was incubated at 37 °C. After 12 h, the mixture was collected for Western blot analysis.

2.11. Live cell proteolysis assay

hBMSCs spheroids were coated with DQ™ mixed gels containing 25 μg mL^{-1} DQ-collagen IV. Then, the spheroids were allowed to sprout for 24 h. Proteolysis of DQ-collagen IV was observed in live cells (green fluorescence) under confocal microscopy.

2.12. Ethics statement

All procedures were performed according to established ethical guidelines and approved by the Institutional Animal Care and Use Committee of Peking University Health Science Center (No. LA2018245) and the Experimental Animal Research Ethics Committee of Peking University Department of Medicine (Approval number: DLASBD0185). The mice were purchased from Beijing HFK Bioscience Co. Ltd. All efforts were made to minimize the suffering of animals.

2.13. Animals and surgical procedures

For the mandible defect model, healthy 5-week-old SD rats were anesthetized with 1 % (w v^{-1}) sodium pentobarbital solution. Trephine (MR.229.205.040, Meissen, Germany) was used to remove a 3 mm-diameter bone core at the center of mandibular angle. The muscle and skin incisions were then closed with suture. Penicillin G was administered to prevent infection. The mandibula were harvested either immediately (day 0) or on the 3rd and the 7th day after surgery for histological analyses.

For the calvarial defect model, a trephine drill was utilized to make two critical-sized full thickness bone defects (5 mm) in SD rats at the center of each parietal bone. The freshly-formed rat cranial defects were covered by osmotic pumps (Yuyan scientific instrument Co. Ltd, Shanghai, China), which are small enough so that they can be placed subcutaneously. 72 μL rHtrA3 solution was injected into the osmotic pump, then the flow moderator is pressed into capsules. Subsequently, 4 μg rHtrA3 was released at a speed of 1 μL h^{-1} for 3 days into the defects due to the osmotic pressure. Solvents provided by the manufacturer were used as a control. After 1, 4, and 8 weeks, the whole calvarias were harvested and analyzed using microcomputed tomography (μCT) and histology.

2.14. Morphological observations of three dimensional cultured cells

Vehicle and HtrA3 over-expressing cells were mixed with matrigel and collagen I, followed by incubation at 37 °C for 30 min to allow the mixture to gellify. The cells were captured by laser confocal microscopy after culture for 24 h and 72 h, respectively. The surface area, volume and sphericity of the cells were calculated.

2.15. Traction force microscopy

Traction force microscopy was performed on the uniform gels. The uniform gels were prepared with 0.2 μm diameter fluorescent beads and pre-polymer solutions containing 10 % acrylamide and 0.13 % bis-acrylamide crosslinker. After hBMSCs attached, the fluorescent bead images at 2, 4, 6, 8, 10, 12, 14 and 24 h were taken. Then the cells were dissolved with 0.5 % SDS. Then, bead displacement data at different time points were calculated from the analysis of the fluorescent bead images. Displacement fields were determined using iterative particle image velocimetry plugin in Fiji. The traction stresses were then calculated using the Fourier-transform traction cytometry (FTTC) method.

2.16. Statistical analyses

All results were presented as the mean ± standard error of the mean (SEM). For analysis between two groups, two-tailed unpaired Student's *t*-test was used. For multiple comparisons, Analysis of variance (ANOVA) with Fisher's least significant difference test was used. A p value < 0.05 was considered significant.

3. Results and discussion

3.1. HtrA3 is upregulated during early healing when MSCs are undergoing migration and differentiation

We first investigated the expression levels of HtrA3 within the bone defect healing area. Mandibular defects of 3 mm-diameter were fabricated within the mandibular angle region of rats. Immunohistochemical staining was used to observe the expression and distribution pattern of HtrA3 at the 0, 3rd and 7th day after surgery (Fig. 1A). The expression of HtrA3 in strol1^{+} BMSCs gradually increased after surgery around the 3rd day, but decreased at the 7th day (Fig. 1B and C). Additionally, to explore the expression patterns of HtrA3 during MSC migration *in vitro*, a wound was simulated with a BMSC monolayer, and then a thin matrigel layer was used to cover the monolayer cells to mimic the extracellular microenvironment (Fig. S1A). After 12 h, the expression of HtrA3 in cells located within the frontier area of the wound was significantly higher than that in the rear area, with the level of HtrA3 expression increasing progressively from the back to the front (Fig. S1B). When osteogenic differentiation of cultured BMSCs was initiated with osteogenic induction medium, we found that HtrA3 expression significantly increased on the 3rd day, but was then downregulated on the 7th day and 14th day, at both the mRNA and protein levels (Figs. S1C and D). This process was accompanied by upregulation of osteogenic related genes and their corresponding protein secretion, including runt-related transcription factor 2 (Runx2) and bone morphogenetic protein 2 (BMP2). These

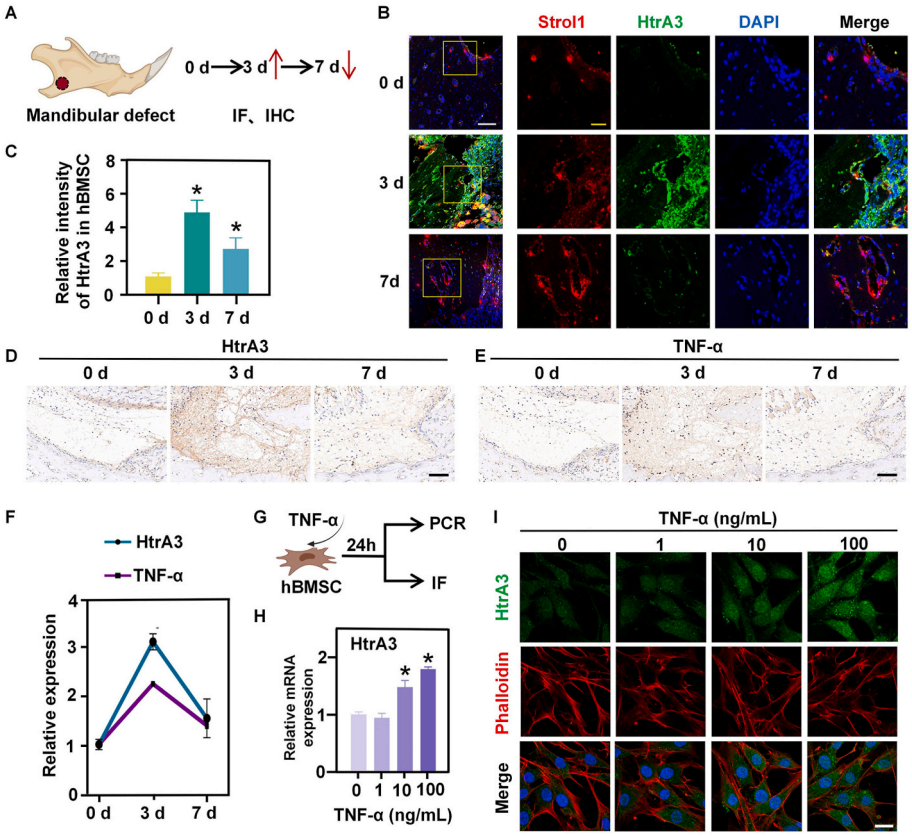

Fig. 1. HtrA3 is upregulated in the leading edge of bone defect repair during the early stage of bone healing in response to the inflammatory environment. (A) Illustration of the rat mandible bone defect model and the various timepoints for harvesting speciments post-surgery. (B) Immunohistofluorescence staining results showed that HtrA3 expression in strol1^{+} BMSCs increased after surgery around the 3rd day and decreased by the 7th day (Scale bar: left 100 μm, right 25 μm). (C) Quantification analysis of relative fluorescence intensity of HtrA3 staining in (B). Immunohistochemistry staining showed HtrA3 (D) and TNF-α (E). (Scale bar: 100 μm) (F) Quantification analysis of HtrA3 and TNF-α, indicating a similar expression pattern in the defect area. (G) Schematic of TNF-α treatment and related experiments. (H–I) qRT-PCR (H) and immunocytochemical staining (I) showed HtrA3 were significantly increased by TNF-α treatment in a concentration dependent manner (Scale bar: 25 μm). *P < 0.05.

results thus suggest that HtrA3 might play a key role in MSCs recruitment and differentiation within the bone defect area.

During the early stage of bone healing, a large number of inflammatory factors and chemokines are released by immune cells in and around the defect area, which serve to recruit various cell types to participate in bone healing [20]. TNF-α is a principal member of this repertoire of inflammatory factors and has been widely used to stimulate pro-inflammatory conditions. We found that TNF-α expression displayed a similar trend as HtrA3 expression in the defect area, with an increase on the 3rd day and a subsequent decline on the 7th day (Fig. 1D–F). We thus postulated that HtrA3 expression might be induced by TNF-α to promote MSC mobility. HtrA3 expression of BMSCs stimulated by TNF-α was thus analyzed (Fig. 1G). Utilizing qPCR and immunofluorescence, we found that HtrA3 expression at both the protein and mRNA levels were significantly upregulated by TNF-α treatment in a concentration-dependent manner (Fig. 1H and I). Moreover, we have also explored the effect of interleukin-1 (IL-1) and IL-6 on HtrA3 secretion, both of which are secreted inflammatory mediators during early stage of bone defect repair [21]. Similarly, qPCR, western bolt and immunofluorescence staining all showed that HtrA3 was also upregulated by IL-1 and IL-6 (Figs. S2A–C). Taken together, these results thus suggested that pro-inflammatory stimuli after surgery contributed to the upregulation of HtrA3, which might enhance the subsequent migration and differentiation of MSCs.

3.2. *HtrA3 enhances cellular cortical protrusions, migration, and osteogenic differentiation of MSCs in vitro*

Having established the upregulation of HtrA3 during the early stage of osteogenesis, the next step would be to investigate the effects of HtrA3 on MSC properties, including cellular shape, mobility, proliferation, and differentiation. For this purpose, a lentivirus system was established to either overexpress or knock down HtrA3 expression in hBMSCs (Figs. S3A–D). MSCs morphology modulated by HtrA3 was investigated by examination of cellular cortical protrusions in a 3D environment. After implantation in a complete 3D matrix, hBMSCs in the overexpression group exhibited more cellular cortical protrusions into the 3D microenvironment over time (Fig. 2A). The cellular surface areas, volume and sphericity were calculated with iMaris software to quantify the cellular shape variation. We found that HtrA3 overexpression in hBMSCs significantly increased the cellular surface areas and volume while decreasing their sphericity (Fig. 2B). By contrast, cells in the control group retained their spherical shape and showed smaller surface areas and volumes. The results demonstrated that HtrA3 affected cell sphericity, spreading area and cell volume by modulating the ECM. However, *in vitro* without ECM, HtrA3 was shown to have no effect on cytoskeletal functions, even after binding with TCP1α (a type of cytoskeletal molecular chaperone) [22]. We found that HtrA3 exerted negligible effects on the proliferation of hBMSCs through real-time observations (Fig. S4A) and EdU assays (Figs. S4B and C).

Then, the motility of BMSCs was assessed by a modified scratch test and transwell test (without and with matrigel). When with matrigel, in

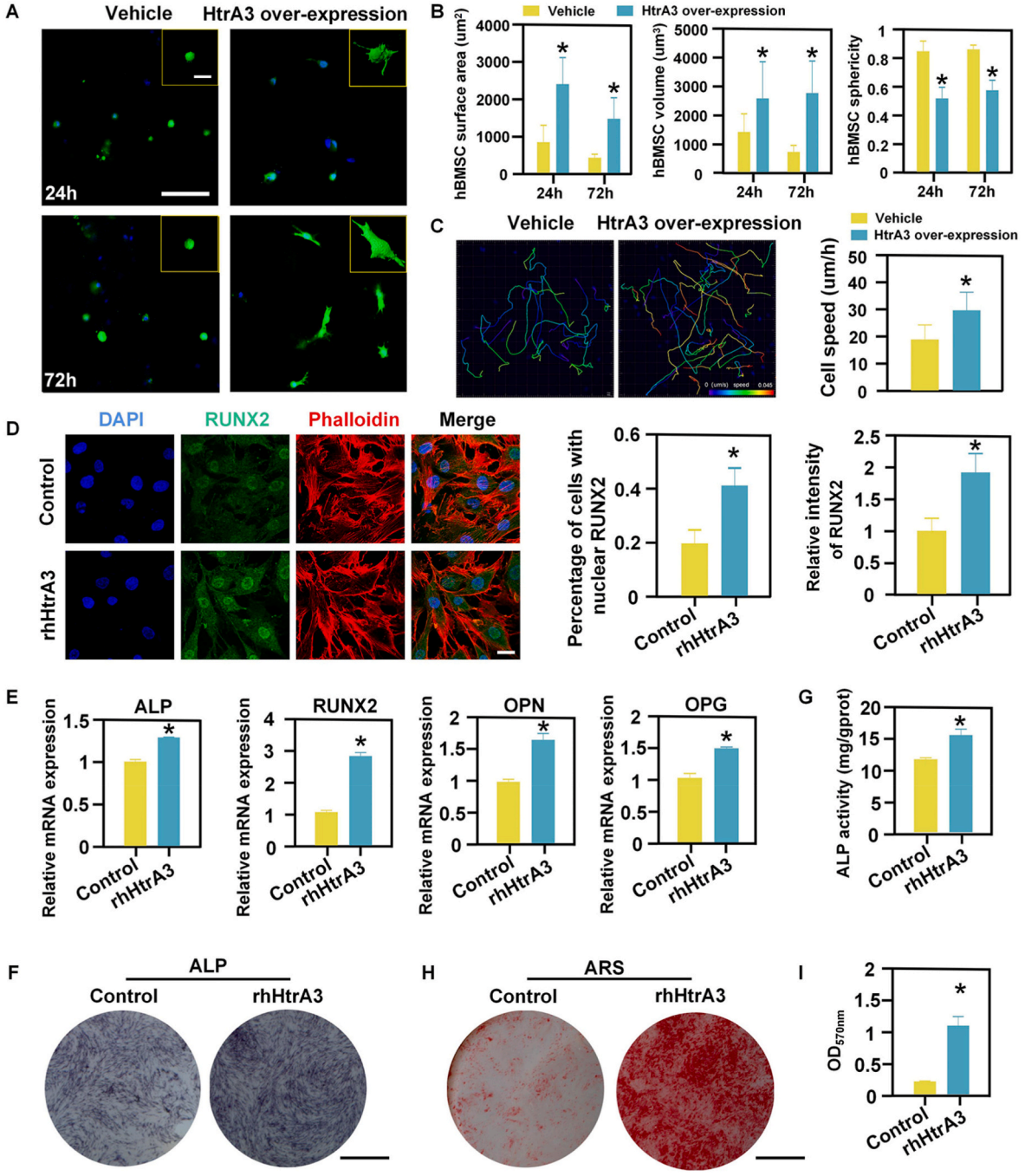

Fig. 2. HtrA3 enhances formation of cellular cortical protrusions, MSCs migration and osteogenic differentiation *in vitro*. (A) Representative microscopy images of control and HtrA3 over-expressing hBMSCs cultured in gel for 1 and 3 days. Images show cell nuclei (blue) and GFP expression in cytosol (green). Insets demonstrated 3D-reconstructed micrographs of individual cells (Scale bar: 20 μm). (B) Quantitative analysis of cell surface area, volume and sphericity were performed using Imaris software. (C) Single cell tracking showed that HtrA3 promoted the cell migration speed of hBMSCs for 24 h. (D) Immunocytochemical staining showed that RUNX2 and its nuclear translocation were significantly increased after rhHtrA3 treatment (Scale bar: 25 μm). (E) qPCR showed that treatment of hBMSCs with rhHtrA3 for 3 days promoted expression of osteogenic related markers. (F–I) ALP (F and G) and ARS (H and I) staining also showed enhanced osteogenic differentiation of MSCs upon treatment with rhHtrA3 for 3 days (Scale bar: 0.5 mm). *P < 0.05.

the wound healing assay, we observed that HtrA3-overexpressing hBMSCs migrated 1.5 times closer towards the middle of the wound (Figs. S5A and B). The HtrA3-knockdown group resulted in less reduction in the wound area (Figs. S5C and D). In the transwell assay, we observed that the number of hBMSCs from the upper chamber across the membrane in the HtrA3-overexpression group was about 4 times higher than the control group (Figs. S5E and F), while less cells across the membrane were observed in the HtrA3-knockdown group (Figs. S5G and H). However, when the scratch test and transwell assay were performed without matrigel barrier, no significant differences were found between the HtrA3-overexpressing versus the knockdown or control groups (Figs. S6A–H). Taken together, these results indicated that HtrA3 promoted the migration capacity of hBMSCs and that the interaction between cells and extracellular matrix mediated by HtrA3 played a key role in this phenomenon.

We subsequently examined the effects of HtrA3 on the osteogenic differentiation of MSCs. To follow the natural trend of HtrA3 expression, hBMSCs were treated with human recombinant HtrA3 (rHtrA3) for 3

days and the cell phenotype was analyzed by immunocytochemistry on the 7th day. We found that expression of BMP2, RUNX2 and its nuclear translocation, were significantly increased in cells cultured with rHtrA3 (Fig. 2D and S7). Similarly, the mRNA levels of osteogenic related genes, including alkaline phosphatase (*ALP*), *RUNX2*, *OPN* and *OPG*, significantly increased after rHtrA3 treatment (Fig. 2E). The diversity of MSCs lineage specificity was corroborated by upregulation in ALP production (Fig. 2F and G) and alizarin red S staining (Fig. 2H and I). These results were consistent with previous studies that indicated the critical role of cytoskeletal organization in stem cell lineage specification and osteogenic enhancement by the highly branched cellular morphology of BMSCs. However, we found that continuous HtrA3 overexpression impeded BMSC osteogenic differentiation (Fig. 3), whereas HtrA3 knockdown enhanced the osteogenic phenotype (Fig. S8). Taken together, these results indicated that HtrA3 expression followed the natural trend enhanced osteogenic differentiation of MSCs.

3.3. HtrA3 promotes MSC osteogenesis and migration in vivo

After determining that HtrA3 can affect MSC lineage commitment specificity *in vitro*, we next investigated whether HtrA3 could enhance tissue regeneration *in vivo*. In order to follow the sequential regulation of HtrA3, osmotic pumps were used to slowly release rhHtrA3 into freshly-made rat cranial defects for 3 days. At 4 and 8 weeks post-surgery, microcomputed tomography (μCT) revealed that there was more new bone formation in the defect area treated with rHtrA3 (Fig. 4A). Quantitative analysis showed higher bone volume and bone mineral density (BMD) values in the rHtrA3 group (Fig. 4B and C). The promotion of bone regeneration was further verified by HE staining and Masson's Trichrome staining. As shown in Fig. 4D, there was abundant tissue formation and massive bone regeneration in the defect area treated by rHtrA3, while thin fibroid tissue and new bone formation were observed on the control side. At 8 weeks post-surgery, there was still a large bone defect area in the control group, whereas a mature bone laminate embedded within the bone lacuna was observed in the rHtrA3 group (Fig. 4E). Moreover, immunohistochemical staining was performed at one week post-transplantation. As shown in Fig. 4F, three times more BMSCs were observed in the defect area of the rhHtrA3 versus control groups. This phenomenon thus demonstrated that rHtrA3 promoted MSCs migration during the early stages of bone defect repair. Taken together, these data thus indicated that HtrA3 was beneficial to the aggregation of MSCs during the early stages of bone defect repair and promoted subsequent bone regeneration.

3.4. HtrA3 selectively degrades ECM and increased expression of integrin β1 in hBMSCs

Having established that HtrA3 enhanced BMSC migration and osteogenesis, and the crucial role of enzymolysis in these processes, we next aimed to unravel the underlying mechanisms by identifying the substrates of HtrA3. Under quiescent condition, MSCs with osteogenic differentiation potential reside in a complex 3D niche microenvironment, composed of type I collagen-rich connective tissue as structural framework and other matrices as filler. Therefore, a mixture of collagen I and matrigel, which served as scaffold and filling matrix respectively, was utilized to mimic the niche microenvironment of MSCs. Then the artificial ECM was treated with rHtrA3 protease or control solvent. As shown in Fig. 5A, the collagen I fibers formed a three-dimensional reticular spatial structure with many pores while the matrigel exhibited a characteristic sandlike appearance. Interestingly, the cloud-like matrigel was obviously degraded by HtrA3, while the structural integrity of type I collagen fibers was maintained (Fig. 5A). By contrast, there was little variation after the control solvent treatment, suggesting that HtrA3 can degrade the ECM filler and maintain integrity of the structural framework.

The content of Matrigel is complex and are constituted of three major components including collagen IV [23]. Hence, we wonder if collagen IV serves as the specific substrate of HtrA3. To this end, we transfected BMSCs with lentivirus encoding HtrA3 recombinant fusion protein and performed a spheroid sprouting assay within DQ-collagen IV. DQ-collagen IV is normally non-fluorescent, while it would emit green fluorescence when degraded. After 24 h of culture, the red fluorescent HtrA3 fusion protein was observed to co-localize with green fluorescent collagen IV within the BMSC spheroid (white triangle arrow), thus indicating that HtrA3 directly degraded collagen IV (Fig. 5B). Additionally, protease hydrolysis assay was performed. We found that there was a decrease of collagen IV after co-incubation with HtrA3 (Fig. 5C and D). These data thus demonstrated that type IV collagen might the specific substrates of HtrA3 in matrigel.

Although ECM breakdown reduces the barrier impeding MSC

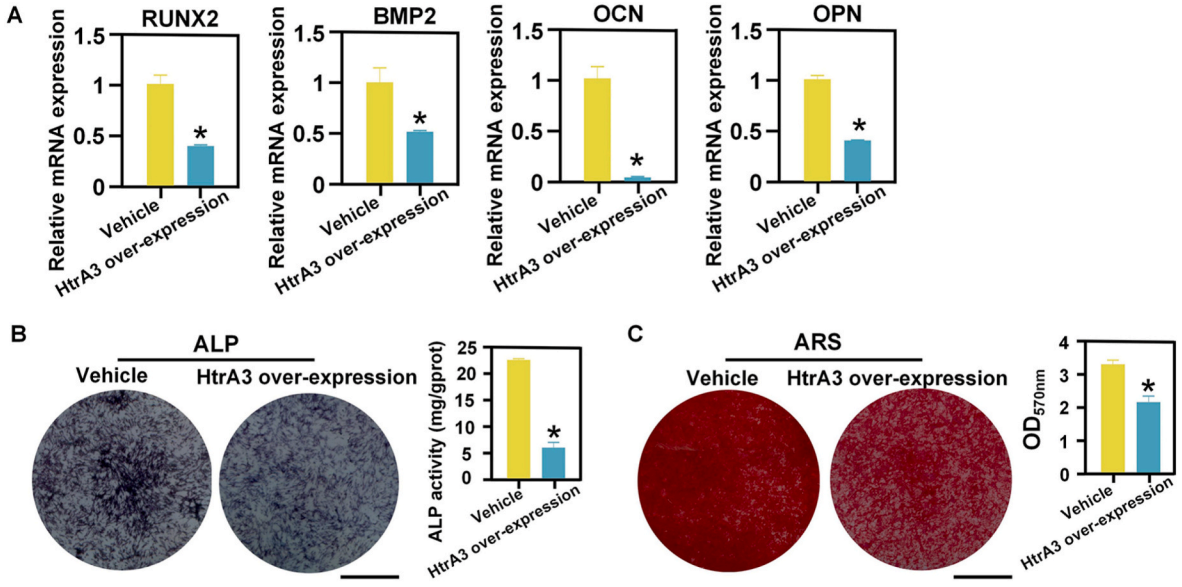

Fig. 3. HtrA3 continuous overexpression inhibited BMSC osteogenic differentiation. (A) qPCR showed that HtrA3 overexpression decreased expression of osteogenic related markers. ALP (B) and ARS (C) staining showed inhibitory effects on osteogenic differentiation in the HtrA3 overexpression group (Scale bar: 0.5 mm). *P < 0.05.

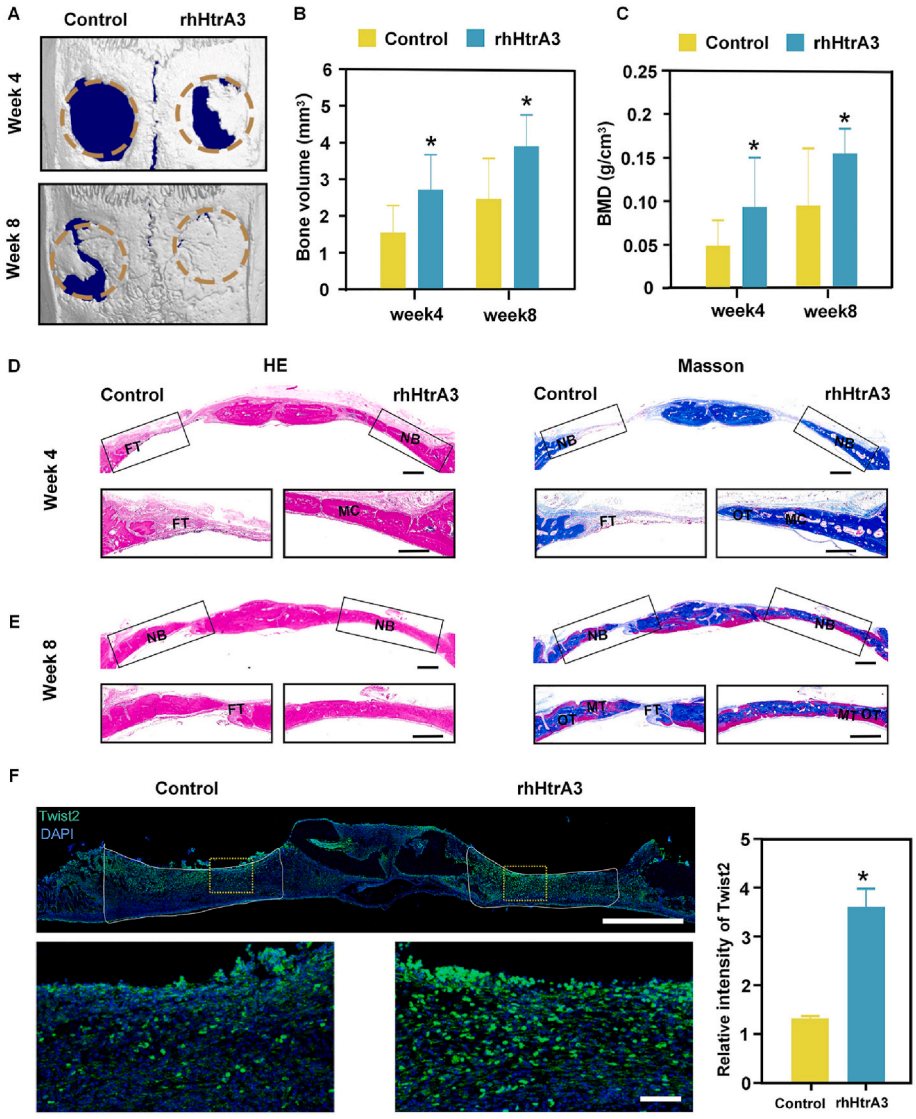

Fig. 4. HtrA3 promoted MSC migration and osteogenesis *in vivo*. (A) Representative micro-CT images of critical-sized rat calvarial full-thickness defects at week 4 and week 8 after treatment with rhHtrA3 for 3 days, indicating more new bone formation in defect area treated with rHtrA3. (B–C) Quantitative analysis of bone volume (B) and bone mineral density (C). (E-F) HE and Masson's trichrome staining of histological sections. (NB, nascent bone; FT, fibrous tissue; MC, medullary cavity; OT, osteoid tissue; MT, mineralized tissue). (F) Immunohistofluorescence staining showed that rHtrA3 promoted MSCs migration during the early stage of bone defect repair (Scale bar: up 3 mm, down 50 μm). *P < 0.05.

migration, it alone is not enough to explain MSC functional variations induced by HtrA3 overexpression, including extensive cellular cortical protrusions, improved mobility and osteogenic differentiation. Therefore, it can be speculated that ECM degradation could induce outside-in signal transduction. Integrin is one of the most important members of the membrane receptor family, which mediates transmembrane signal transduction. Among these, integrin β1 (Itgβ1) is able to combine the various isoforms of α subunits and is thus the most essential cluster of membrane receptors. To investigate whether Itgβ1 signaling was enhanced by HtrA3, immunocytochemistry showed that HtrA3 overexpression promoted the expression of Itgβ1 (Fig. 5E), while the expression of Itgβ1 in HtrA3 knockdown-MSCs decreased significantly (Fig. S9). These findings thus suggest that HtrA3 can not only degrade ECM directly, but also remodel ECM to increase expression of Itgβ1.

3.5. *HtrA3 promotes migration and osteogenic differentiation of MSCs through mechanotransduction*

We next investigated how increased expression of Itgβ1 eventually led to changes in cell behavior and lineage commitment. Through the extracellular and intracellular regions, integrin connects ligands in the ECM to the cellular actin cytoskeleton, functioning as principal sites of mechanotransduction [24,25]. As shown in Fig. 2A and B, cellular cortical protrusions induced by HtrA3 is an indication of actin cytoskeleton remodeling. This would therefore suggest that mechanotransduction might have a role in modulating the biological effects of HtrA3. To this end, we first analyzed the forces they exert on their surrounding microenvironment during their adhesion and the mechanical properties of cells after they spreading completely. Traction force microscopy (TFM) was used to acquire a cell traction.

stress map consisting of different time points from the adhesion (Fig. 6A). The total traction force at each individual time point was calculated (Fig. 6B). The results showed that HtrA3 significantly increased the total traction forces at all time points. The maximum traction force exerted by MSCs overexpressing HtrA3 was over 1.5 times of that in the control group (Fig. 6C). Moreover, the slope of total traction force-time curves at the first 2 h was calculate (l_{2h}). We found that l_{2h} was higher in HtrA3 overexpressing BMSCs as compared to the

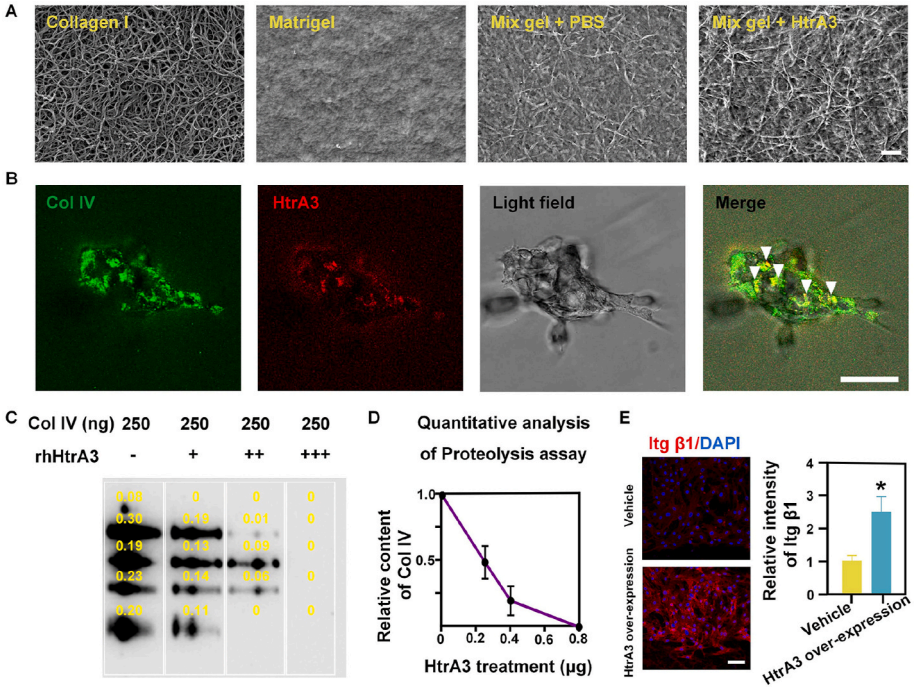

Fig. 5. HtrA3 selectively degrades ECM and increased expression of Itgβ1 in hBMSCs. (A) SEM images showed the fibrillar structure of collagen I and the cloud-like structure of Matrigel. The far right SEM images demonstrated that the fibrillar structure of the collagen-Matrigel mixture gel was exposed after HtrA3 recombinant protein treatment (Scale bar: 1 μm). (B) Co-localization of HtrA3 fusion protein of MSCs and degraded green fluorescent collagen IV (Scale bar: 50 μm). (C) Proteolysis assay results of collagen IV - HtrA3 co-culture showed the degradation of collagen IV by rhHtrA3. (D) Quantitative analysis of proteolysis assay in (C). (D) Immunocytochemical staining results of Matrigel-cultured hBMSCs showed that HtrA3 over-expression promoted the expression of Itgβ1 (Scale bar: 100 μm). *P < 0.05.

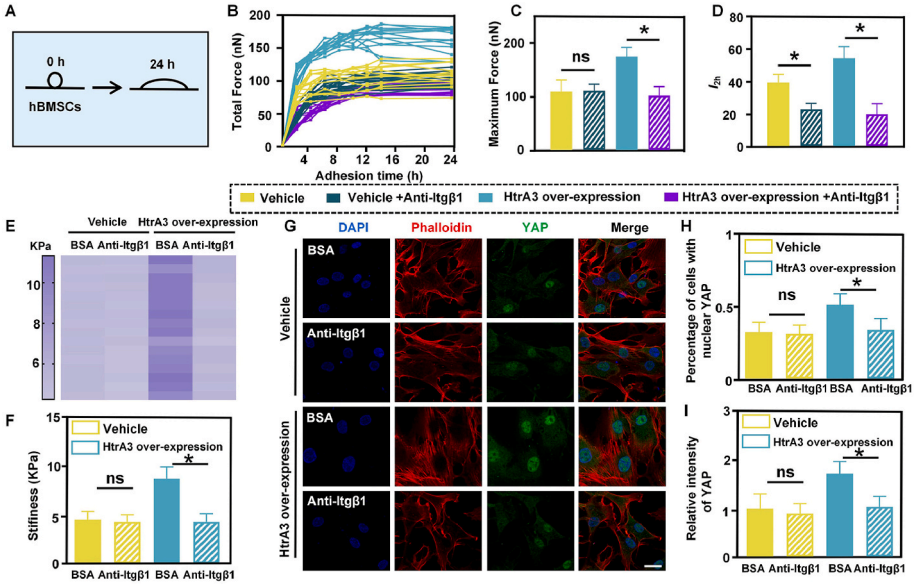

Fig. 6. HtrA3 promotes the migration and osteogenic differentiation of MSCs through mechanotransduction. (A) The diagram of detecting traction forces with TFM. (B) The curves of total traction force exerted by hBMSCs in different groups at different time durations from adhesion. The maximum of total traction force and the slope of total traction force-time curves at the first 2 h (l_{2h}) were shown in (C) and (D), respectively. (E) The typical mechanics heatmaps of celluar stiffness detected through AFM. (F) Quantitative analysis showed that celluar stiffness was increased by HtrA3 overexpression, which was abrogated by the Itg β1 antibody. (G-I) Immunocytochemical staining of YAP showed HtrA3 overexpression significantly increased YAP expression and its nuclear translocation, which were abrogated by the neutralizing Itg β1 antibody (Scale bar: 25 μm). *P < 0.05.

control group (Fig. 6D), indicating that HtrA3 significantly increased both the interaction force and interaction intensity between MSCs and the substrate. Utilizing an atomic force microscope (AFM), we detected 16 positions of each cell and constructed typical mechanics heatmaps. As shown in Fig. 6E and F, a twofold increase in cellular stiffness was found in the HtrA3 overexpression group, as compared with the control group. Blocking Itgβ1 with its neutralizing antibody absolutely eliminated the mechanical differences between the HtrA3 overexpression

group and the vehicle group, including traction forces, l_{2h} and cellular stiffness (Fig. 6A–F). Taken together, these data suggested that HtrA3 enhanced the mechanical cues from the microenvironment to BMSCs via Itgβ1.

Since the actin cytoskeleton is the primary macromolecular system within cells responsible for regulating cellular stiffness [9], we therefore examined variations in the actin cytoskeleton and its mechanoeffector, YAP [26], which nuclear transduction promotes osteogenic differentiation of BMSCs [27]. As shown in Fig. 6G–I, HtrA3 overexpression enhanced stress fiber formation, as well as the expression and cytoplasm-to-nuclear transport of YAP. Mechanotransduction blockade by neutralizing Itgβ1 antibody treatment reduced the formation of stress fibers and the YAP cyto/nuclear ratio to levels similar to the control group. Consistently, the mechanotransduction block also decreased the formation of cellular cortical protrusions, leading to a decrease in the cellular surface area and volume, as well as an increase in sphericity (Fig. 7A) and the migration of MSCs (Fig. 7B–C and Fig. S10). Consistently, the expression of BMP2, RUNX2 and its nuclear translocation in

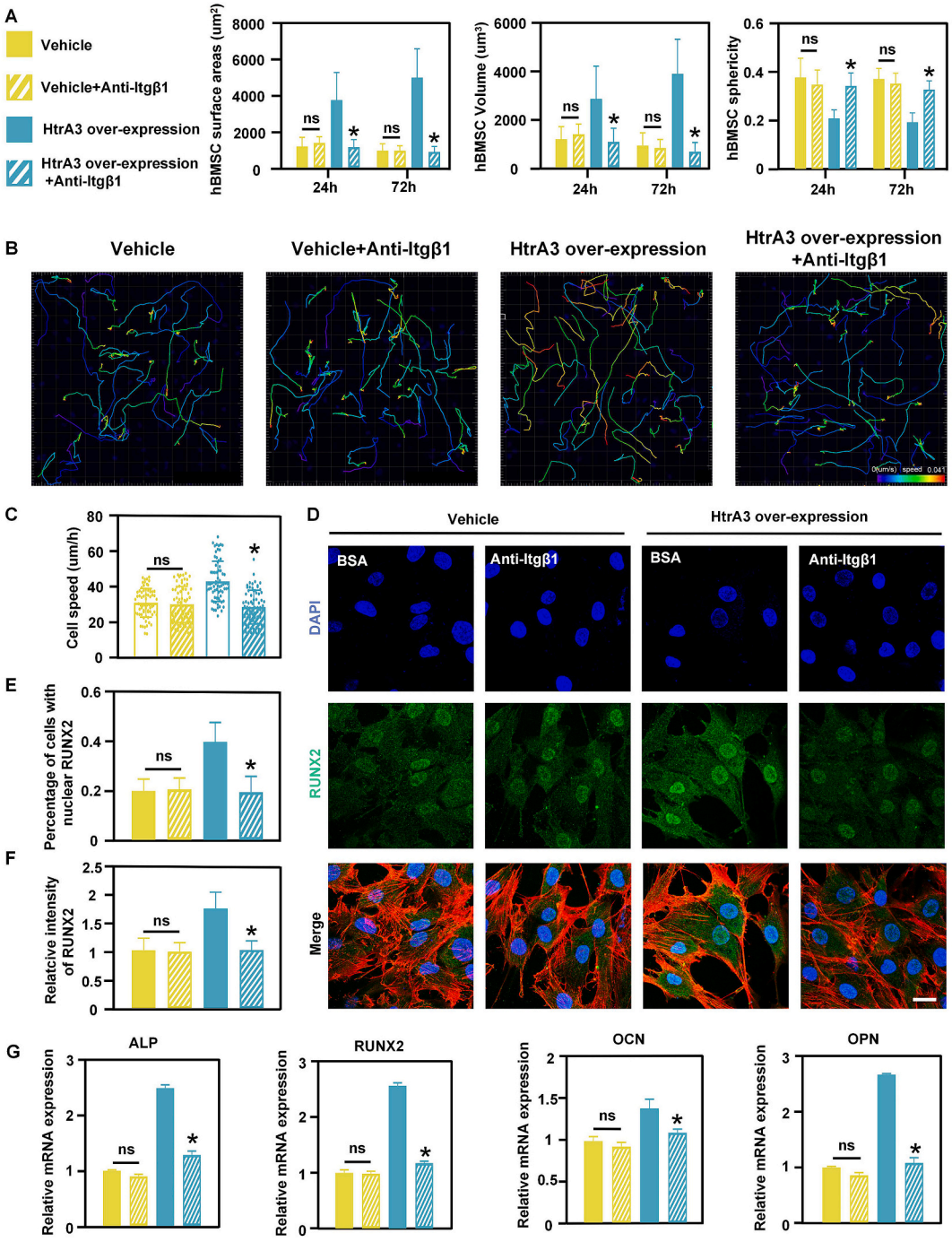

Fig. 7. Itgβ1 blocking diminishes the cellular cortical protrusions, the increased mobility and osteogenic differentiation of BMSCs induced by HtrA3 over-expression. (A) Itg β1 antibody treatment decreased the cell surface area and volume, while increasing sphericity in the HtrA3 overexpression group. (B–C) Single cell tracking showed that neutralizing Itgβ1 antibody decreased cell migration speed of HtrA3 overexpression hBMSCs. (D) The representative RUNX2 immunofluorescence staining of hBMSCs in different groups (Scale bar: 25 μm). (E-F) Quantitative analysis of RUNX2 nuclear transloaction and its fluorescence intensity. (G) qPCR analysis showed that expression of osteogenic-related genes in the rhHtrA3 group were diminished after Itg β1 antibody treatment. *P < 0.05.

the rHtrA3 group were significantly downregulated by treatment with the neutralizing Itgβ1 antibody (Fig. 7D–F and Fig. S11), indicating that osteogenic differentiation of BMSCs was impeded. Eventually, we found that the expression levels of osteogenic related genes, including *ALP*, *RUNX2*, *OCN* and *OPN*, were diminished by Itgβ1 blocking (Fig. 7G). Accordingly, these results indicated that HtrA3 enhanced the BMSC-ECM crosstalk and exerted its effect through mechanotransduction mediated by the Itgβ1-YAP signaling axis.

Taken together, the exact sequence of events and molecular mechanisms after bone defect were revealed in this study. Inflammatory micrenvironment is created immediately after tissue injury and inflammatory mediators, such as TNF-α, IL-1 and IL-6, were secreted [20,21,28,29]. Stimulated by inflammatory factors, MSCs upregulate expression of HtrA3. HtrA3 selectively degrades collagen IV to overcome the physical barrier. Moreover, HtrA3 could also increase the expression of Itgβ1 in MSCs, which on one hand improves the traction forces for MSCs to move forward, on the other remodels cytoskeleton to promote osteogenic differentiation of MSCs through mechanotransduction. Specifically, increased Itgβ1 enhances MSC-ECM interaction through its transmembrane structure, which is confirmed by the traction forces of HtrA3-overexpression MSCs towards their surrounding environment (Fig. 6A–D). Since Itgβ1 mediates physical link between the extracellular substrate and the actin cytoskeleton [30], increased MSC-ECM interaction remodels actin cytoskeleton, leading to more cellular cortical protrusions (Fig. 2A and B) and improved cellular stiffness (Fig. 6E and F). Finally, the mechanoeffector of actin cytoskeleton, YAP, transports from cytoplasm to nuclear, where it promotes the transcription of osteogenesis-related genes (Fig. 8) [26,27]. In this way, HtrA3 paves the way for MSC migrations and promote their osteogenic differentiation. Compared with other members of HtrA family, HtrA3 is still less studied at present, and most studies on it are focused on the tumor field. our study can not only reveal the novel roles of HtrA3 but also deepen our understanding of the underlying mechanisms of bone repair.

MSCs are promising candidates for adult cell therapies in regenerative medicine. To fully exert their potential, the homing capability of MSCs, allowing the cells to migrate to sites of injury and inflammation from the stem cell niche, play an important role. The wall of the bone marrow niche is composed of a luminal layer of endothelial cells and an incomplete outer lining of adventitial reticular cells [4]. The sinusoidal endothelial cells express type IV collagen and laminin and various adhesion molecules such as integrins and selectins which are involved in cell migration [31]. Hence, mobilized MSCs need to break through ECM, including collagen IV, to migrate out of their residing niche. Subsequently, MSCs flow in the circulatory system, polarize and scan the endothelium for exit cues. At the right spot, MSCs overcome the endothelial barrier, the endothelial basement membrane, and the pericyte sheath and then continue chemokine guided interstitial migration [32]. Type IV collagen, as one of the most abundant components, is a network-forming molecule and major component of the basement membrane [33,34]. Therefore, degradation of IV collagen by HtrA3 contributes to MSC migration and the *trans*-endothelial migration during their systemic homing. At present, clinical applications of MSCs have met the biggest challenges due to their inefficient homing, with only a small percentage of cells reaching the target tissue following systemic administration [32,35]. HtrA3 might be a new target for the efficiency improvement of MSC homing. Moreover, various collagen IV-containing hydrogels, such as ECM-derived hydrogel and Collagen IV-impregnated hydrogels, have been prepared to promote tissue repair [36,37]. Considering collagen IV degradation ability of HtrA3, it might be included in the design of bone repair materials for regenerative medicine and our study may offer new insights for the development of bioactive materials.

Cells are able to sense signals from the surrounding environment at any time to regulate the direction of their migration [4,20]. After injury, the concentration gradient of soluble cytokines, such as TNF-α, IL-1 and IL-6, is created, which decreased gradually with increased distance from the damaged area [21,22]. Thus, MSCs closer to the injury site would sense higher concentrations of cytokines and subsequently upregulate the expression of HtrA3. Through braking down of collagen IV and exerting traction forces towards ECM, HtrA3 transforms the injury signals to direction information for MSCs. In this way, ECM degradation ability of HtrA3 also helps MSCs to control the direction during the process of migration and cross endothelial barrier at the required site during their systemic homing.

The osteogenic differentiation of MSCs is a precisely controlled spatio-temporal process [38,39]. The results of this study stressed the importance of time-dependent HtrA3 secretion on MSC lineage fate commitment. HtrA3 treatment for only 3 days induced MSC osteogenic commitment while continuous HtrA3 overexpression hindered such differentiation. The ECM disintegration rather than remodeling might be responsible for the observed decrease in osteogenic differentiation by continuous overexpression of HtrA3. Therefore, the natural expression trends of chemokines, cytokines and proteases *in vivo* can provide us some cues for biochemical agent administration and biomaterial design to achieve better therapeutic effects.

Previous studies have shown that mice lacking TNF-α have severely impaired fracture healing, with bone formation being delayed by several weeks [40,41]. While inflammation is an essential mechanism to initiate bone healing, an efficient and timely resolution of inflammation is necessary for transition to a pro-regenerative environment [42,43]. Dysregulated inflammation, including either decreased or elevated levels of inflammatory cytokines, negatively impact fracture healing and

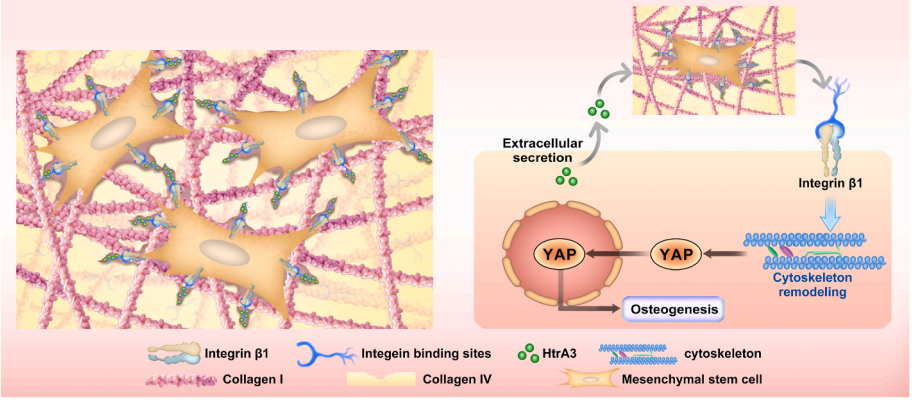

Fig. 8. Schematic diagram illustrating the underlying mechanisms by which HtrA3 promotes MSCs migration and osteogenesis. During the early stage of bone healing, HtrA3 is upregulated by inflammatory factors in MSCs and then secreted to ECM. It breaks down ECM to make room for cell movement and increases expression of integrin β1, thus enhancing BMSC-ECM crosstalk and osteogenic differentiation of MSCs.

results in increased rates of delayed bone healing and nonunion [20]. However, our understanding of how elevated or protracted inflammatory response impairs bone healing is still limited. Our present work sheds some light on this issue. HtrA3 was induced by inflammatory cytokines in a concentration-dependent manner (Fig. 1G–I). Thus, elevated acute or protracted inflammation results in continuous HtrA3 production, which is proven in our study to hinder bone regeneration. Moreover, HtrA3 sustained knockdown favored the tosteogenic commitment of MSCs (Fig. S8), indicating that HtrA3 can be a new target for the treatment of bone defect repair under some pathological conditions, including osteoarthritis, type 1 diabetes, obesity, and rheumatoid arthritis. To this end, more investigations are needed.

4. Conclusion

In summary, our work thus provides a deep insight into how HtrA3, a novel serine protease, can stimulate a pro-regenerative environment by mediating crosstalk between MSCs and ECM to promote the migration and osteogenic differentiation of MSCs. In addition, local administration of exogeneous recombinant HtrA3 in rat cranial bone defects significantly increases new bone formation. HtrA3 can potentially be a therapeutic molecule for enhancing osteogenesis in tissue repair and tissue engineering.

Ethics approval and consent to participate

All procedures were performed according to established ethical guidelines and approved by the Institutional Animal Care and Use Committee of Peking University Health Science Center (No. LA2018245) and the Experimental Animal Research Ethics Committee of Peking University Department of Medicine (Approval number: DLASBD0185). The mice were purchased from Beijing HFK Bioscience Co. Ltd. All efforts were made to minimize the suffering of animals.

CRediT authorship contribution statement

Yaru Guo: Writing – original draft, Methodology, Investigation, Funding acquisition. **Siqin Ma:** Writing – original draft, Methodology, Investigation, Formal analysis. **Dandan Wang:** Writing – original draft, Methodology, Investigation, Formal analysis. **Feng Mei:** Writing – original draft, Methodology, Investigation, Formal analysis. **Yusi Guo:** Writing – original draft, Methodology, Investigation, Formal analysis. **Boon Chin Heng:** Writing – review & editing. **Shihan Zhang:** Methodology, Data curation. **Ying Huang:** Writing – original draft, Methodology. **Yan Wei:** Writing – original draft, Methodology. **Ying He:** Methodology, Formal analysis. **Wenwen Liu:** Methodology, Funding acquisition. **Mingming Xu:** Data curation. **Xuehui Zhang:** Writing – review & editing, Supervision, Project administration, Funding acquisition, Conceptualization. **Lili Chen:** Writing – review & editing, Supervision, Project administration, Conceptualization. **Xuliang Deng:** Writing – review & editing, Supervision, Project administration, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

Yaru Guo, Siqin Ma, Dandan Wang, Feng Mei and Yusi Guo contributed equally to this work. This work was supported by the National Natural Science Foundation of China 81991505, U22A20160, 82221003, 82201123, 82022016, and 52273258; the National Key Research and Development Program of China 2021YFB3800800, the Beijing Municipal Natural Science Foundation 7222226 and China Postdoctoral Science Foundation 2021M700279 and 2023T160029. We thank Dr. Siying Qin at the National Center for Protein Sciences of Peking University for technical help with AFM.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bioactmat.2024.05.016.

References

[1] F. Nitzsche, C. Müller, B. Lukomska, J. Jolkkonen, A. Deten, J. Boltze, Concise review: MSC adhesion cascade-insights into homing and transendothelial migration, Stem Cells (Dayton) 35 (6) (2017) 1446–1460.
[2] S. He, T. Hou, J. Zhou, Q. Ai, C. Dou, F. Luo, J. Xu, J. Xing, Endothelial cells promote migration of mesenchymal stem cells via PDGF-BB/PDGFRβ-Src-Akt in the context of inflammatory microenvironment upon bone defect, Stem Cell. Int. 2022 (2022) 2401693.
[3] Q. Zhang, H. Dong, Y. Li, Y. Zhu, L. Zeng, H. Gao, B. Yuan, X. Chen, C. Mao, Microgrooved polymer substrates promote collective cell migration to accelerate fracture healing in an in vitro model, ACS Appl. Mater. Interfaces 7 (41) (2015) 23336–23345.
[4] J.L. Liesveld, N. Sharma, O.S. Aljitawi, Stem cell homing: from physiology to therapeutics, Stem Cells (Dayton) 38 (10) (2020) 1241–1253.
[5] X. Fu, G. Liu, A. Halim, Y. Ju, Q. Luo, G. Song, Mesenchymal stem cell migration and tissue repair, Cells 8 (8) (2019).
[6] Q. Yu, L. Chen, Y. You, C. Zou, Y. Zhang, Q. Liu, F. Cheng, Erythropoietin combined with granulocyte colony-stimulating factor enhances MMP-2 expression in mesenchymal stem cells and promotes cell migration, Mol. Med. Rep. 4 (1) (2011) 31–36.
[7] S.J. Atkinson, M.L. Patterson, M.J. Butler, G. Murphy, Membrane type 1 matrix metalloproteinase and gelatinase A synergistically degrade type 1 collagen in a cell model, FEBS Lett. 491 (3) (2001) 222–226.
[8] M. Paolillo, S. Schinelli, Extracellular matrix alterations in metastatic processes, Int. J. Mol. Sci. 20 (19) (2019).
[9] I. Jalilian, C. Heu, H. Cheng, H. Freittag, M. Desouza, J.R. Stehn, N.S. Bryce, R. M. Whan, E.C. Hardeman, T. Fath, G. Schevzov, P.W. Gunning, Cell elasticity is regulated by the tropomyosin isoform composition of the actin cytoskeleton, PLoS One 10 (5) (2015) e0126214.
[10] D. Beleford, R. Rattan, J. Chien, V. Shridhar, High temperature requirement A3 (HtrA3) promotes etoposide- and cisplatin-induced cytotoxicity in lung cancer cell lines, J. Biol. Chem. 285 (16) (2010) 12011–12027.
[11] J. Tocharus, A. Tsuchiya, M. Kajikawa, Y. Ueta, C. Oka, M. Kawaichi, Developmentally regulated expression of mouse HtrA3 and its role as an inhibitor of TGF-beta signaling, Dev. Growth Differ. 46 (3) (2004) 257–274.
[12] M. Ye, K. Sharma, M. Thakur, A.A. Smith, O. Buyuktanir, X. Xiang, X. Yang, K. Promnares, Y. Lou, X.F. Yang, U. Pal, HtrA, a temperature- and stationary phase-activated protease involved in maturation of a key microbial virulence determinant, facilitates Borrelia burgdorferi infection in mammalian hosts, Infect. Immun. 84 (8) (2016) 2372–2381.
[13] T. Clausen, C. Southan, M. Ehrmann, The HtrA family of proteases: implications for protein composition and cell fate, Mol. Cell 10 (3) (2002) 443–455.
[14] X.Y. Li, G.F. Ban, B. Al-Shameri, X. He, D.Z. Liang, W.X. Chen, High-temperature requirement protein A1 regulates odontoblastic differentiation of dental pulp cells via the transforming growth factor beta 1/smad signaling pathway, J. Endod. 44 (5) (2018) 765–772.
[15] Z. Shen, F. Sun, Y. Lu, L. Yuan, S. Ge, Q. Gong, H. Shi, High temperature requirement A3 attenuates hypoxia/reoxygenation induced injury in H9C2 cells via suppressing inflammatory responses, Eur. J. Pharmacol. 928 (2022) 175114.
[16] S. Glanz, A. Mirsaidi, C. López-Fagundo, G. Filliat, A.N. Tiaden, P.J. Richards, Loss-of-Function of HtrA1 abrogates all-trans retinoic acid-induced osteogenic differentiation of mouse adipose-derived stromal cells through deficiencies in p70S6K activation, Stem Cell. Dev. 25 (9) (2016) 687–698.
[17] A.N. Tiaden, M. Breiden, A. Mirsaidi, F.A. Weber, G. Bahrenberg, S. Glanz, P. Cinelli, M. Ehrmann, P.J. Richards, 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 (Dayton) 30 (10) (2012) 2271–2282.
[18] G.Y. Nie, Y. Li, H. Minoura, L. Batten, J.K. Findlay, L.A. Salamonsen, A novel serine protease of the mammalian HtrA family is up-regulated in mouse uterus coinciding with placentation, Mol. Hum. Reprod. 9 (5) (2003) 279–290.
[19] M. Bowden, A.E. Drummond, L.A. Salamonsen, J.K. Findlay, G. Nie, Evolutionary conservation of mammalian HTRA3 and its developmental regulation in the rat ovary, J. Exp. Zool. B Mol. Dev. Evol. 312 (7) (2009) 701–713.
[20] H. Newman, Y.V. Shih, S. Varghese, Resolution of inflammation in bone regeneration: from understandings to therapeutic applications, Biomaterials 277 (2021) 121114.
[21] F. Loi, L.A. Córdova, J. Pajarinen, T.H. Lin, Z. Yao, S.B. Goodman, Inflammation, fracture and bone repair, Bone 86 (2016) 119–130.
[22] T. Wenta, D. Zurawa-Janicka, M. Rychlowski, M. Jarzab, P. Glaza, A. Lipinska, K. Bienkowska-Szewczyk, A. Herman-Antosiewicz, J. Skorko-Glonek, B. Lipinska,

HtrA3 is a cellular partner of cytoskeleton proteins and TCP1α chaperonin, J. Proteonomics 177 (2018) 88–111.
[23] H.K. Kleinman, G.R. Martin, Matrigel: basement membrane matrix with biological activity, Semin. Cancer Biol. 15 (5) (2005) 378–386.
[24] R. Zaidel-Bar, S. Itzkovitz, A. Ma'Ayan, R. Iyengar, B. Geiger, Functional atlas of the integrin adhesome, Nat. Cell Biol. 9 (8) (2007) 858–867. Nat Cell Biol, 9, 858-867.
[25] Z. Sun, S.S. Guo, R. Fässler, Integrin-mediated mechanotransduction, JCB (J. Cell Biol.) 215 (4) (2016) 445–456.
[26] S. Dupont, L. Morsut, M. Aragona, E. Enzo, S. Giulitti, M. Cordenonsi, F. Zanconato, J. Le Digabel, M. Forcato, S. Bicciato, N. Elvassore, S. Piccolo, Role of YAP/TAZ in mechanotransduction, Nature 474 (7350) (2011) 179–183.
[27] Y. Guo, F. Mei, Y. Huang, S. Ma, Y. Wei, X. Zhang, M. Xu, Y. He, B.C. Heng, L. Chen, X. Deng, Matrix stiffness modulates tip cell formation through the p-PXN-Rac1-YAP signaling axis, Bioact. Mater. 7 (2022) 364–376.
[28] H. Liu, D. Li, Y. Zhang, M. Li, Inflammation, mesenchymal stem cells and bone regeneration, Histochem. Cell Biol. 149 (4) (2018) 393–404.
[29] P.M. Mountziaris, P.P. Spicer, F.K. Kasper, A.G. Mikos, Harnessing and modulating inflammation in strategies for bone regeneration, Tissue Eng. B Rev. 17 (6) (2011) 393–402.
[30] L. Wang, J.Y. Luo, B. Li, X.Y. Tian, L.J. Chen, Y. Huang, J. Liu, D. Deng, C.W. Lau, S. Wan, D. Ai, K.K. Mak, K.K. Tong, K.M. Kwan, N. Wang, J.J. Chiu, Y. Zhu, Y. Huang, Integrin-YAP/TAZ-JNK cascade mediates atheroprotective effect of unidirectional shear flow, Nature 540 (7634) (2016) 579–582.
[31] S.J. Morrison, D.T. Scadden, The bone marrow niche for haematopoietic stem cells, Nature 505 (7483) (2014) 327–334.
[32] M. Ullah, D.D. Liu, A.S. Thakor, Mesenchymal stromal cell homing: mechanisms and strategies for improvement, iScience 15 (2019) 421–438.
[33] T. Takaku, D. Malide, J. Chen, R.T. Calado, S. Kajigaya, N.S. Young, Hematopoiesis in 3 dimensions: human and murine bone marrow architecture visualized by confocal microscopy, Blood 116 (15) (2010) e41–e55.
[34] A. Malara, C. Gruppi, M. Currao, A.J.B. Balduini, Megakaryocytes contribute to the establishment of the bone marrow environment by expressing extracellular matrix proteins, 3695-3695 122 (21) (2013).
[35] D.L. Kraitchman, M. Tatsumi, W.D. Gilson, T. Ishimori, D. Kedziorek, P. Walczak, W.P. Segars, H.H. Chen, D. Fritzges, I. Izbudak, R.G. Young, M. Marcelino, M. F. Pittenger, M. Solaiyappan, R.C. Boston, B.M. Tsui, R.L. Wahl, J.W. Bulte, Dynamic imaging of allogeneic mesenchymal stem cells trafficking to myocardial infarction, Circulation 112 (10) (2005) 1451–1461.
[36] D. Evangelista-Leite, A.C.O. Carreira, M.Y. Nishiyama, S.E. Gilpin, M.A. Miglino, The molecular mechanisms of extracellular matrix-derived hydrogel therapy in idiopathic pulmonary fibrosis models, Biomaterials 302 (2023) 122338.
[37] G.W. Plant, T.V. Chirila, A.R. Harvey, Implantation of collagen IV/poly(2-hydroxyethyl methacrylate) hydrogels containing Schwann cells into the lesioned rat optic tract, Cell Transplant. 7 (4) (1998) 381–391.
[38] P. Kim, J. Park, D.J. Lee, S. Mizuno, M. Shinohara, C.P. Hong, Y. Jeong, R. Yun, H. Park, S. Park, Mast4 determines the cell fate of MSCs for bone and cartilage development, Nat. Commun. 13 (1) (2022) 3960.
[39] Q. Chen, P. Shou, C. Zheng, M. Jiang, G. Cao, Q. Yang, J. Cao, N. Xie, T. Velletri, X. Zhang, C. Xu, L. Zhang, H. Yang, J. Hou, Y. Wang, Y. Shi, Fate decision of mesenchymal stem cells: adipocytes or osteoblasts? Cell Death Differ. 23 (7) (2016) 1128–1139.
[40] L.C. Gerstenfeld, T.J. Cho, T. Kon, T. Aizawa, A. Tsay, J. Fitch, G.L. Barnes, D. T. Graves, T.A. Einhorn, Impaired fracture healing in the absence of TNF-alpha signaling: the role of TNF-alpha in endochondral cartilage resorption, J. Bone Miner. Res. 18 (9) (2003) 1584–1592.
[41] L.C. Gerstenfeld, T.J. Cho, T. Kon, T. Aizawa, J. Cruceta, B.D. Graves, T.A. Einhorn, Impaired intramembranous bone formation during bone repair in the absence of tumor necrosis factor-alpha signaling, Cells Tissues Organs 169 (3) (2001) 285–294.
[42] P.V. Giannoudis, D. Hak, D. Sanders, E. Donohoe, T. Tosounidis, C. Bahney, Inflammation, bone healing, and anti-inflammatory drugs: an update, J. Orthop. Traumatol. 29 (Suppl 12) (2015) S6–S9.
[43] C.S. Bahney, R.L. Zondervan, P. Allison, A. Theologis, J.W. Ashley, J. Ahn, T. Miclau, R.S. Marcucio, K.D. Hankenson, Cellular biology of fracture healing, J. Orthop. Res. 37 (1) (2019) 35–50.