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Original Article

USP1 Inhibits NF-κB/NLRP3 Induced Pyroptosis through TRAF6 in Osteoblastic MC3T3-E1 Cells

Dengshuo Sun[1], Yi Peng[2], Shengyang Ge[3], Qiang Fu[1]

[1]Department of Orthopedics, Shanghai General Hospital, Shanghai Jiaotong University, China;
[2]Department of Orthopedics, Zhongshan Hospital, Fudan University, China;
[3]Department of Urology, Huashan Hospital, Fudan University, China

Abstract

Objectives: Deubiquitinase Ubiquitin Specific Protease 1 (USP1) is essential for bone formation, but how USP1 regulates bone formation in response to oxidative stress remains unclear. In this study, we aim to investigate the biological function of USP1 in osteoblastic MC3T3-E1 cells. **Methods**: Hydrogen peroxide (H_2O_2) as an oxidative reagent was used to trigger osteoblastic MC3T3-E1 cellular damage. Flow cytometry was used to evaluate ROS production, apoptosis, and pyroptosis. Real-time PCR and western bolt assay were used to detect the mRNA and protein levels of USP1. Moreover, coimmunoprecipitation was used to validate the relationship between USP1 and TRAF6. **Results**: We demonstrated that USP1 was significantly decreased in MC3T3-E1 cells after H_2O_2 treatment. Overexpressing USP1 restored H_2O_2-decreased alkaline phosphatase activity and reactive oxygen species production. USP1 overexpression inhibited cytokine release and NLP3 inflammasome activation, which was mediated by NF-κB. Overexpressing USP1 prevented NF-κB translocation. USP1 formed a complex with TRAF6, inhibiting TRAF6 ubiquitination. **Conclusion**: USP1 exhibits protective role in MC3T3-E1 cells by suppressing NF-κB-NLRP3 mediated pyroptosis in response to H_2O_2. The involvement of USP1 and TRAF6 in NLRP3 inflammasome signaling suggests a future therapeutic potential to improve clinical symptoms in osteoporosis.

Keywords: NF-κB, NLRP3, Pyroptosis, TRAF6, USP1

Introduction

Bone mass is maintained through a delicate balance between bone formation and resorption by osteoblasts and osteoclasts, respectively[1]. Abnormal bone remodeling process results in pathological conditions, including osteoporosis. Osteoporosis is characterized by low bone mass and microarchitectural deterioration of bone tissue[2]. Osteoporosis is linked to increased fragility, often leading to a reduced quality of life, and hip and vertebral fractures are associated with decreased life expectancy[3]. Major risk factors of osteoporosis are aging, gender, race, family history, and body size[2]. Although many signaling pathways toward osteoporosis have been reported, an effective treatment is still lacking.

Oxidative stress, resulting in reactive oxygen species (ROS) accumulation, is the leading cause of osteoblast cell damage and death[4,5]. Hydrogen peroxide (H_2O_2), an oxidative reagent, has previously been used to cause oxidative stress in osteoblast cells[6], and H_2O_2 has become widely used to study osteoporosis pathogenesis in cell cultures. ROS trigger NLR family pyrin domain containing 3 (NLRP3) signaling cascade[7], leading to pyroptosis. Pyroptosis is a pro-inflammatory programmed cell death in response to infection and cellular damages, distinct from other forms of cell death[8]. Pyroptosis is involved in various inflammatory diseases and is mediated by inflammasomes as well as the activation of caspase-1 to trigger downstream signaling[8]. NLRP3 and pro-caspase-1, together with the nucleotide-biding oligomerization domain and leucine-rich repeat-containing proteins, form inflammasomes[9]. Inflammasomes recruit pro-caspase-1 and cleave it into active caspase-1[9]. Working as a protease, caspase-1 activates IL-1β, IL-18, and gasdermin D (GSDMD), transforming these molecules into mature forms. The NLRP3 inflammasome is essential for defending against infections,

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Corresponding author: Dr. Qiang Fu, Department of Orthopedics, Shanghai General Hospital, Shanghai Jiaotong University, 85 Wujin Road, Shanghai 200080, China
E-mail: johson_fu@126.com • 12301010005@fudan.edu.cn
ORCID: 0000-0002-5168-4604

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and contributes to the pathogenesis of inflammatory disorders, including autoinflammatory diseases and Alzheimer's disease[10].

Recently, activation of the NLRP3 inflammasome has been associated with postmenopausal osteoporosis. As an anti-inflammatory hormone, melatonin inhibits estrogen deficiency-triggered osteoporosis through suppression of NLRP3 inflammasomes *in vivo*[11]. NF-κB is required for the activation of NLRP3 inflammasome[7,12]. Ubiquitination is crucial for NLRP3 inflammasome activation through the regulation of NF-κB[7]. Ubiquitin Specific Protease-7 (USP7) suppresses NF-κB ubiquitination[13]. USP4, USP20, and USP2a stabilize TRAF6, an E3 ubiquitin ligase, through deubiquitination, terminating NF-κB-triggered transcriptional responses[14-16]. USP1 is required for regular bone development[17]. However, the way that USP1 controls inflammatory responses in osteoporosis has not been explored.

Thus, we aim to study the molecular interactions between USP1, NF-κB, and TRAF6 in NLRP3 inflammasome-induced osteoporosis pathogenesis. We hypothesized that USP1 would interact with TRAF6 to regulate NLP3 and NF-κB signaling cascades to contribute to inflammatory activation in osteoblastic cells.

Materials and methods

Cell culture and H_2O_2 treatment

MC3T3-E1 cells were purchased from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China). Cells were cultured in minimum essential medium (α-MEM) (Hyclone, Logan, UT, USA) in the presence of 10 % fetal bovine serum (Gibco, Grand Island, NY, USA) at 37°C with 5% CO_2. Cells were cultured overnight before 24 h treatment of H_2O_2 (0.1, 0.2, 0.4 and 0.6 mM).

Lentivirus preparation

Short hairpin RNA (shRNA) oligos targeting mouse *Usp1* (Table S1) were cloned into AgeI- and EcoRI-digested pLKO.1 (Addgene, Cambridge, MA, USA). Lentiviral particles were produced in 293T cells with psPAX2 and pMD2.G.

Alkaline phosphatase (ALP) assay

The activity of ALP was measured using the ALP activity kit (Jiancheng, Nanjing, China). Protein concentration was determined with a BCA assay kit (Thermo Fisher Scientific, Waltham, MA, USA). ALP activity was calculated as U/g (units per gram). Experiments were performed in triplicates.

Quantitative real-time PCR

RNA was extracted in TRIzol (Thermo Fisher Scientific, Waltham, MA, USA). cDNA was synthesized using a RevertAid First Strand cDNA synthesis kit (Thermo Fisher Scientific, Waltham, MA, USA) after DNase I treatment. Real-time PCR was performed using an ABI 7300 instrument (Applied Biosystems, Foster City, CA, USA) with a SYBR Green PCR kit (Thermo Fisher Scientific, Waltham, MA, USA). Expression was normalized to GAPDH. Primers are listed in Table S2.

Preparation of total lysates, cytosolic and nuclear fractions, and western blotting

Lysates were homogenized in a radioimmunoprecipitation buffer in the presence of the proteinase inhibitor (Beyotime, Shanghai, China). Cytosolic and nuclear fractions were extracted using NE-PER™ Nuclear and Cytoplasmic Extraction Reagents (Thermo Fisher Scientific, Waltham, MA, USA). Western blotting was performed as previously published[18]. In brief, membranes were incubated with primary antibodies (Table S3) overnight at 4°C. Quantification was performed using Image J software (http://rsb.info.nih.gov/ij/, Bethesda, MD, USA).

Evaluation of ROS production, apoptosis and pyroptosis using flow cytometry

After washing with ice-cold PBS, cells were incubated with 2',7'-Dichlorodihydrofluorescein diacetate (DCFH-DA) (10 μM, Beyotime, Shanghai, China) for 20 min at 37°C. Cells were stained with either annexin V-fluorescein isothiocyanate (FITC) apoptosis detection kits (KeyGEN Biotech, Nanjing, China), or caspase-1 (#9122, Bio-Rad Laboratories, Hercules, CA, USA) and propidium iodide (PI) (P3566, Thermo Fisher Scientific, Waltham, MA, USA). Cell pyroptosis (positive for caspase-1and PI) was analyzed using flow cytometry (BD Biosciences, Franklin Lakes, NJ, USA).

IL-1β and IL-18 measurement

The media content of IL-1β and IL-18 was measured by an Enzyme-linked immunosorbent assay (ELISA) kit (Jiancheng, Nanjing, China) according to the instruction of the manufacturer.

Coimmunoprecipitation (co-IP)

Cell lysates were incubated with anti-USP1 (PA5-44962, Thermo Fisher Scientific, Waltham, MA, USA), anti-TRAF6 (Ab33915, Abcam, Cambridge, UK), or control IgG (Santa Cruz Biotechnology, Dallas, TX, USA) for 60 min at 4°C. The lysates were mixed with protein A/G-agarose for 3 h at 4°C. Precipitates were washed three times before western blot analysis was performed.

Statistical analysis

All statistical analyses were performed using the GraphPad Prism 7.0 software (San Diego, CA, USA). All values were presented as mean±SD. Group difference between more than two groups was compared using ANOVA with Sidak's test, and the Student's t-test was used to compare the difference between two groups. Statistical significance was set at $p<0.05$.

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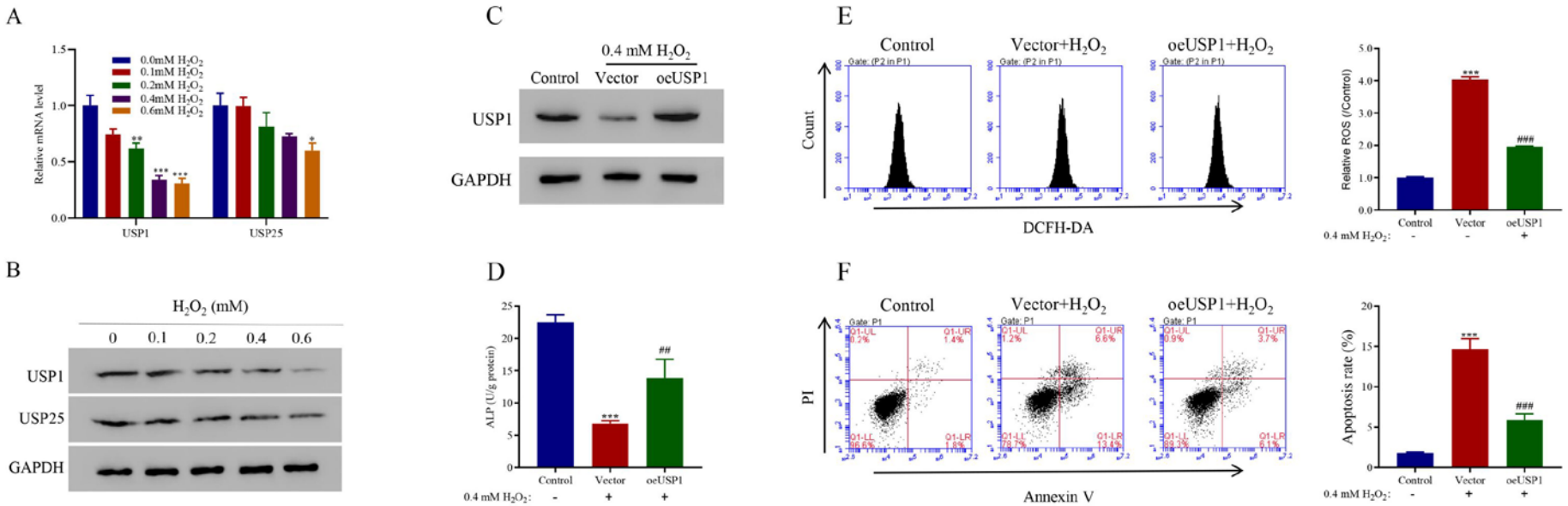

Figure 1. H_2O_2 administration reduced USP1 expression in MC3T3-E1 cells. MC3T3-E1 cells were incubated in the presence of 0.1, 0.2, 0.4, or 0.6 mM H_2O_2 for 24 h. (A) qRT-PCR examined USP1 and USP25 mRNA levels. (B) Western blot analysis for the protein levels of USP1 and USP25. **P<0.01, ***P<0.001 vs 0; ##P<0.01 vs 0.1 mM; $P<0.05 vs 0.2 mM. (C) Western blot analysis for the protein levels of USP1. (D) Quantification of ALP activity. (E) Quantification of ROS levels using flow cytometry. (F). USP1 overexpression suppressed apoptosis. ***P<0.001 vs Control; ##P<0.01, ### P<0.001 vs Vector+H_2O_2.

Results

USP1 was decreased in osteoporosis individuals and H_2O_2 administration reduced USP1 expression in MC3T3-E1 cells.

To define the expression changes in the Ubiquitin Specific Protease (USP) family in osteoporosis, we first compared USP family mRNA levels from control mesenchymal stem cells to patients with osteoporosis, using the GSE35956 dataset. We found that only USP1 and USP25 were decreased in the individuals affected by osteoporosis (Figure S1). We chose to investigate the roles of USP1 in the current study. To further evaluate USP1 function during osteoporosis, we examined USP1 changes at both transcriptional and translational levels in response to H_2O_2. H_2O_2 significantly decreased USP1 content in a concentration-dependent manner in MC3T3-E1 cells (Figure 1A, B). USP25 did not exhibit similar changes (Figure 1A, B), suggesting that the observed responses were USP1-specific and that the higher concentration of H_2O_2 was not cell-toxic.

USP1 overexpression reversed H_2O_2-induced decrease in ALP activity, ROS production, and the increase in cell apoptosis.

Next, we overexpressed USP1 (Figure 1C) and investigated the resulting biological effects. We also verified the overexpressed viruses (Figure S2A). First, we measured ALP activity, a key osteoblast differentiation biomarker. H_2O_2 reduced ALP levels, but overexpressing USP1 attenuated these changes (Figure 1D). Hydrogen peroxide (H_2O_2) mediates cell apoptosis, and it is commonly used in MC3T3-E1 cells to study osteoporosis[19]. Next, we measured USP1 overexpression in H_2O_2-induced ROS production. Consistent with previously published work, we observed that H_2O_2

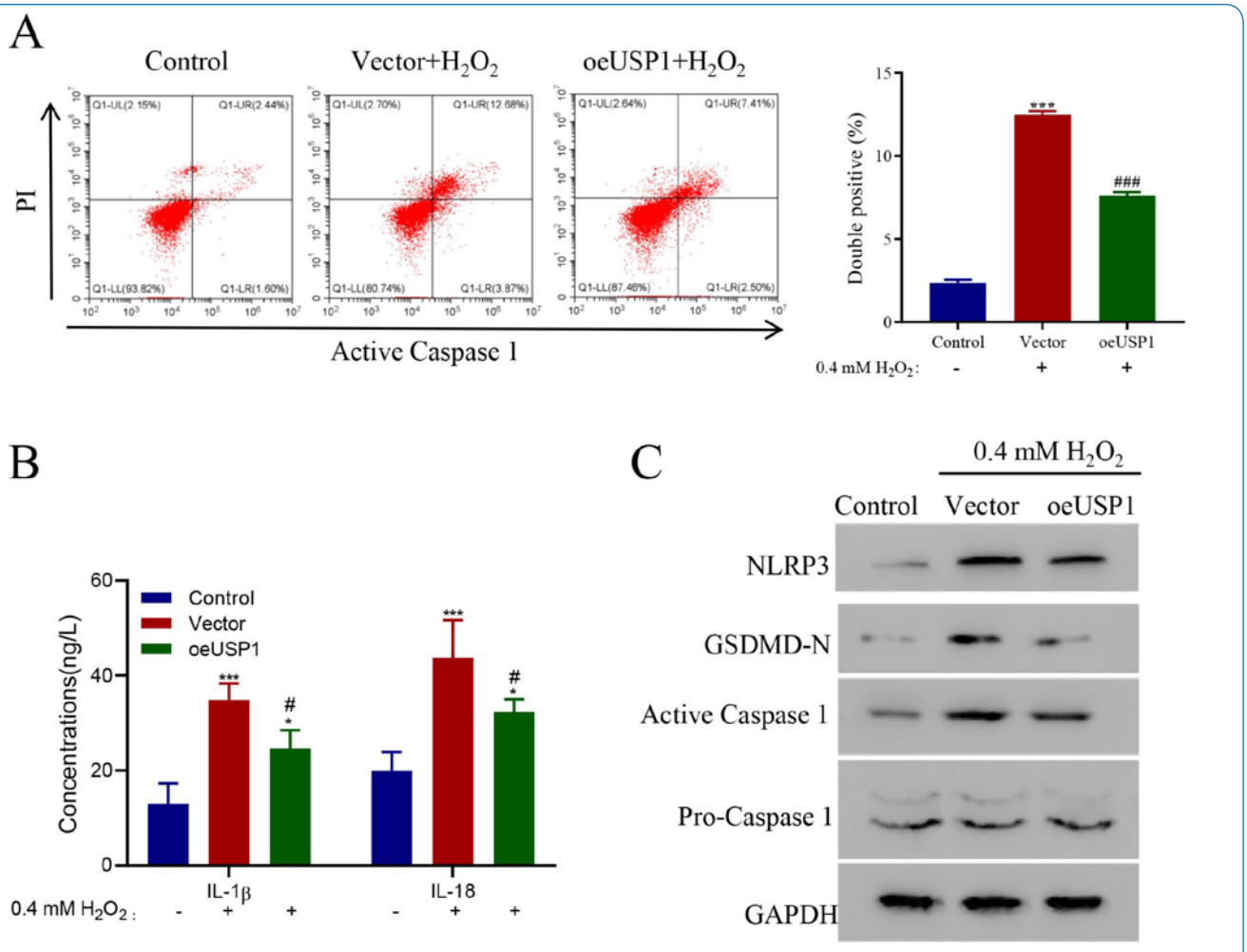

Figure 2. USP1 overexpression dampened H_2O_2-induced pyroptosis. 24 h after USP1 viral infection, MC3T3-E1 cells were treated with 0.4 mM H_2O_2 for another 24 h. (A) Quantification of caspase-1 and propidium iodide positive cells using flow cytometry. (B) Quantification of IL-1β and IL-18 content in supernatant using ELISA. (C) Western blot analysis for the protein levels of NLRP3, GSDMD-N, caspase-1, and pro-caspase-1. **P<0.01, ***P<0.001 vs Control; ##P<0.01, ### P<0.001 vs Vector+H_2O_2.

triggered significant ROS accumulation, but USP1 blocked H_2O_2-induced ROS increase (Figure 1E). In addition, we investigated the effects on apoptosis. As a commonly used apoptosis inducer in various cell types, H_2O_2 treatment caused an increase in Annexin-positive cells, and USP1 overexpression suppressed this change (Figure 1F).

USP1 overexpression dampened H_2O_2-induced pyroptosis

Since apoptosis was affected by USP1 levels, we wondered whether USP1 could regulate pyroptosis. Through observing another pyroptotic marker, caspase-1, we found that overexpressing USP1 abolished H_2O_2-triggered pyroptosis (Figure 2A). We then measured IL-1β and IL-18 content using ELISA. H_2O_2 dramatically elevated both cytokines, while overexpressing USP1 significantly reduced this elevation (Figure 2B). Next, we examined the protein levels of several pyroptotic markers using western blotting. USP1 suppressed H_2O_2-induced elevation in NLRP3, GSDMD-N, and active caspase-1 (Figure 2C). Notably, pro-caspase-1 levels did not change (Figure 2C), suggesting that the NLRP-3-pyroptosis pathway is activated after H_2O_2 administration, and USP1 inhibits pyroptosis activation.

USP1 effects were mediated by NF-κB signaling.

Next, we explored the signaling pathways underlying USP1 in pyroptosis. It is well-established that NF-κB signaling plays a key role in activating pyroptosis. We found that H_2O_2 activated NF-κB, causing nuclear translocation from the cytoplasm, and USP1 inhibited NF-κB translocation (Figure 3A). To confirm the results of USP1 overexpression, we induced loss of function using USP1 shRNA. We designed 3 different shRNAs and chose shUSP1-1 for further analysis (Figure S2B). Knocking down USP1 decreased ALP content, and, in the presence of pyrrolidine dithiocarbamate (PDTC),

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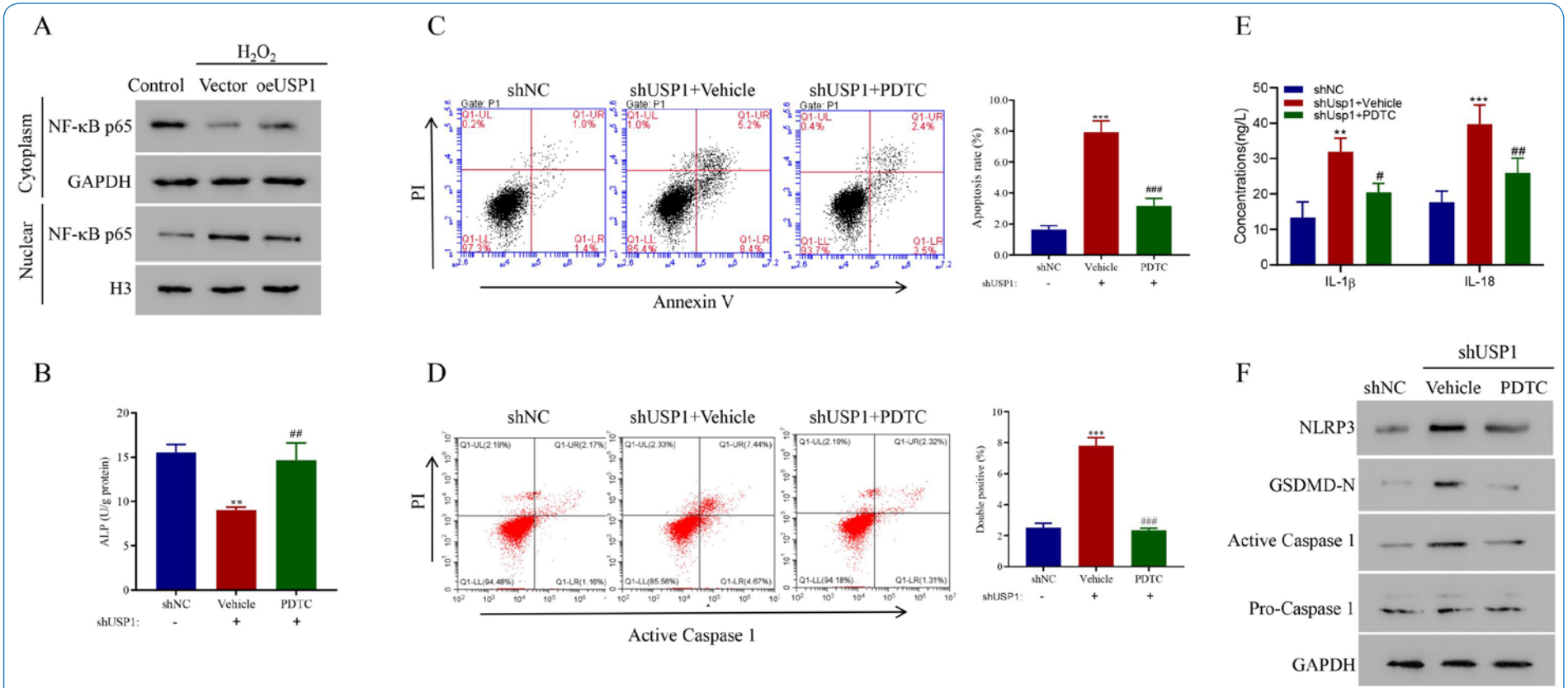

Figure 3. Effects of USP1 knockdown on MC3T3-E1 cells. USP1 effects were mediated by NF-κB signaling. (A) 24 h after infection, MC3T3-E1 cells were treated with 0.4 mM for another 24 h. Western blot analysis for the protein levels of NF-κB p65 in cytoplasm and nuclei. (B-F) 24 h after USP1 viral infection, MC3T3-E1 cells were treated with 10 μmol/l PDTC (NF-κB inhibitor) for another 24 h. (B) Quantification of ALP activity. (C) Quantification of Annexin V positive cells using flow cytometry. (D) Quantification of caspase-1 positive cells using flow cytometry. (E) Quantification of IL-1β and IL-18 content in supernatant using ELISA. (F) Western blot analysis for the protein levels of NLRP3, GSDMD-N, caspase-1, and pro-caspase-1.

an NF-κB inhibitor, the ALP change was reversed and comparable to the control (Figure 3B). This result suggested that NF-κB is involved in USP1 signaling. Consistent with our USP1 overexpression results, knocking down USP1, using either Annexin V (Figure 3C) or active caspase-1 (Figure 3D), promoted pyroptosis. PDTC administration prevented USP1 knock-down-promoted apoptosis (Figure 3C-3D). In addition, PDTC blocked USP1 knock-down-induced IL-1β and IL-18 elevation (Figure 3E). Lastly, we examined pyroptotic markers when USP1 was suppressed. Consistent with the USP1 overexpression data, USP1 shRNA activated NLRP3, GSDMD-N, and active caspase-1 (Figure 3F). PDTC eliminated USP1-caused elevation in pyroptotic markers (Figure 3F). These results suggest that NF-κB acts downstream for USP1.

USP1 stabilized TRAF6 to regulate NF-κB signaling.

Finally, we searched for the downstream target of USP1. We found that USP1 was

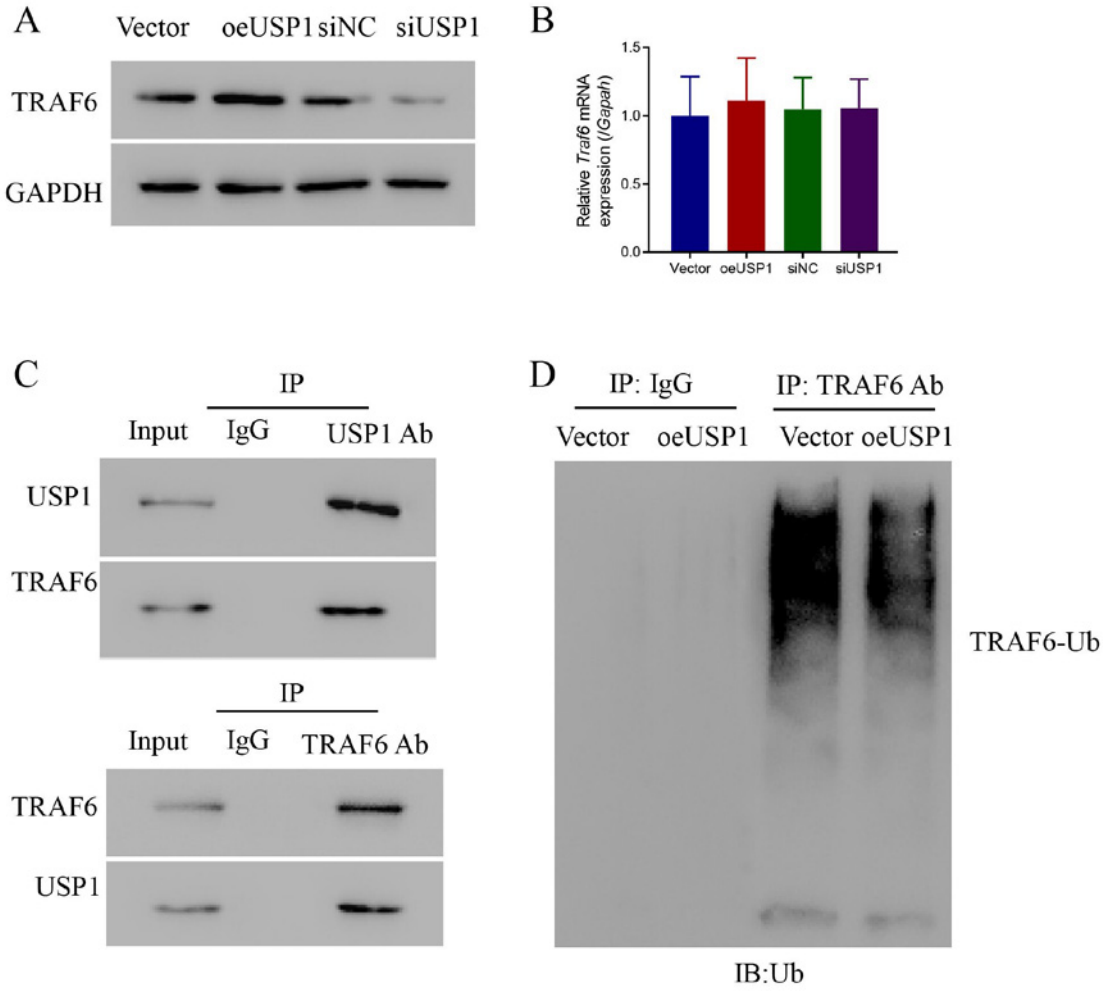

Figure 4. USP1stabilized TRAF6 to regulate NF-κB signaling in MC3T3-E1 cells. (A) Western blot analysis for the protein levels of TRAF6 when USP1 was overexpressed or knocked down. (B) Quantification of TRAF6 mRNA levels when UPS1 was overexpressed or knocked down. (C) USP1 formed a complex with TRAF6, co-immunoprecipitation analysis. (D) USP1 overexpression inhibited TRAF6 ubiquitination, co-immunoprecipitation analysis and blotting with ubiquitin antibody.

negatively correlated with TRAF6 levels (Figure 4A), and that this alteration occurred at the translational level but not at the transcriptional level (Figure 4B). We used Co-Immunoprecipitation (Co-IP) to examine USP1's interaction with TRAF6. We immunoprecipitated with USP1 and blotted with TRAF6, or immunoprecipitated with TRAF6 and blotted with USP1. We found that there was an interaction between USP1 and TRAF6 (Figure 4C). Ubiquitination is essential for TRAF6's stability. Our result showed that overexpressing USP1 remarkably attenuated TRAF6 ubiquitination (Figure 4D), suggesting that USP1 stabilizes TRAF6 by decreasing TRAF6 ubiquitination, therefore regulating NF-κB signaling.

Discussion

The strength and integrity of our bones depends on maintaining a delicate balance between bone formation through osteoblasts and bone resorption through osteoclasts. The dysregulation of bone remodeling leads to osteoporosis. ROS increases oxidative stress and contributes to osteoporosis 6. Gaining a better understanding of the molecular regulation underlying osteoporosis, by studying H_2O_2-induced damage in cell cultures, can provide a future clinical strategy. In this study, we identified that USP1 suppresses NLP3 inflammasomes through TRAF6 ubiquitination, and operates through NF-κB signaling in response to oxidative exposure.

Aging and decreased levels of estrogen cause the accumulation of oxidative stress and deficient antioxidants, major contributing factors to the development of osteoporosis[20,21]. The disrupted antioxidant defense barrier results in bone loss through lipid peroxidation, osteoblast apoptosis, and bone resorption[22]. Our data confirm that H_2O_2-induced osteoblast dysfunction is caused by oxidative

damage, resulting in reduced ALP and elevated cytokine production[6]. Therefore, H_2O_2 administration in MC3T3-E1 cells can be widely used to study osteoblastic processes including pathological disorders, such as osteoporosis.

NLRP3 is critical not only for inflammatory disorders but also in metabolic diseases, and can be activated by various molecular pathways[23]. Recently, it has been observed that the NLRP3 inflammasome is required for bone resorption and osteoclast differentiation[24]. However, molecular regulation had not been well-studied. Our results demonstrate that these inflammatory responses are triggered during oxidant accumulation and the NLRP3 inflammasome is activated, which leads to the loss of osteoblasts, consistent with previous studies[11,25].

TRAF6 is well-recognized as the therapeutic target for osteoporosis to suppress osteoclast function[26,27]. Our data show that USP1 decreases TRAF6 ubiquitination. This suggests that USP1 may serve as a future clinical target in osteoporosis. Although it is still unclear how USP1 alters TRAF6 ubiquitination, since TRAP6 inhibits RANK and MAPK cascades in osteoblast activation[26], UPS1 may regulate RANK and MAPK signaling.

It has been previously reported that USP1 is the key protein in osteosarcoma, the most common primary bone tumor, and that USP1 is essential for osteogenesis[28,29]. However, USP1's roles in osteoporosis are completely unknown. Previous studies have reported that another of USP1's family members, USP7, regulates the balance between ubiquitination of deubiquitinating of NF-κB for optimal transcription, suggesting complex layers of regulation[13]. We are the first to identify the connection between USP1 and TRAP6, and the involvement of NF-κB signaling in osteoblast injuries. Future studies will focus on: 1) *In vivo* validation of USP1 and NLRP3 regulation, and the generation of cytokines in osteoporosis 2) How ubiquitination activities regulate NF-κB transcriptional responses and other NF-κB target genes.

Conclusion

Our results indicate that USP1 is beneficial to MC3T3-E1 cells that have sustained H_2O_2-induced cell damage. In addition, it seems that USP1 acts on TRAF6 by suppressing TRAF6 ubiquitination to control NF-κB signaling. In the future, USP1 may serve as a therapeutic target to treat osteoporosis in clinical settings.

Authors' contributions

DS conceived and designed the study, and drafted the manuscript. YP and SG collected, analyzed, and interpreted the experimental data. QF revised the manuscript for important intellectual content. All authors read and approved the final manuscript.

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Supplementary Figures.

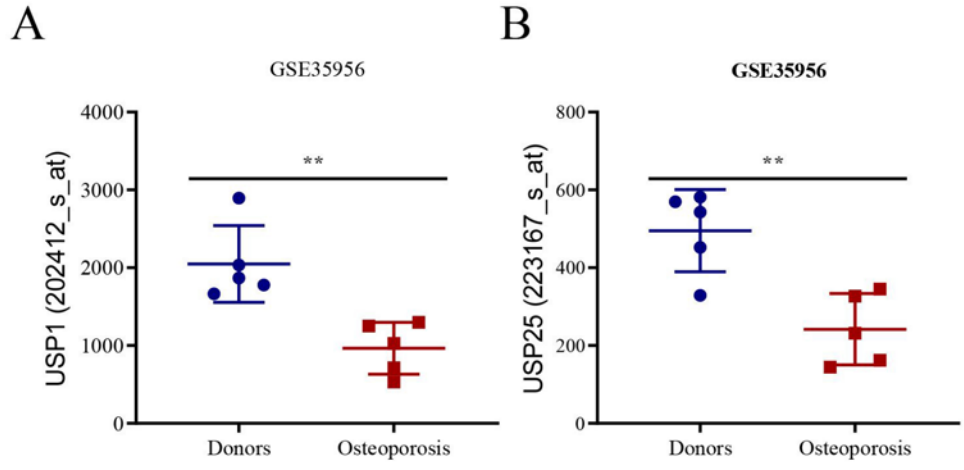

Figure S1. The levels of USP1 and USP25 in the control and the patients with osteoporosis. Using the GSE35956 dataset, the levels of USP1 (A) and USP25 (B) were compared between control mesenchymal stem cells and patients with osteoporosis.

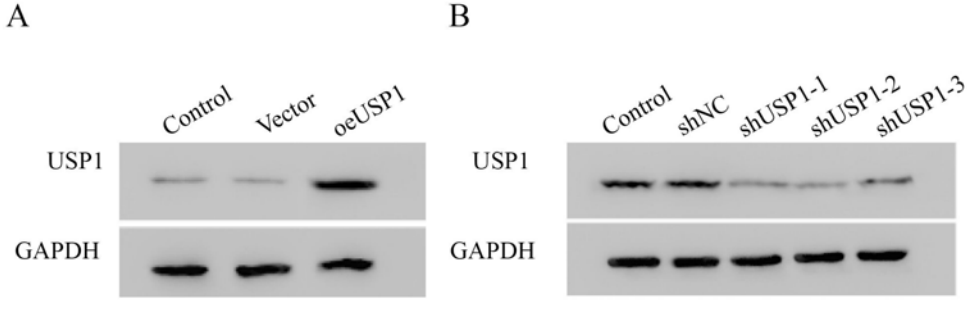

Figure S2. The protein level of USP1.

Supplementary Tables.

Table S1. Target sequences of mouse *Usp1* shRNAs.

shRNA	Target sequence
shUsp1-1	5' GCTTCCTTCAATAAGTTAA 3'
shUsp1-2	5' GAAGAGAGAGAACTTGTTA 3'
shUsp1-3	5' CGGCAAGGTTGAAGAACAA 3'

Table S2. Primer sequences for real-time PCR.

Gene	Forward primer	Reverse primer
Usp1	5' TGAGGAATACGGAGGACG 3'	5' GAATGCTGGGTTGCTTAG 3'
Usp25	5' GGAGGAGACAGGCTATTACCA 3'	5' TCAAGGCAATCGCTCTCTGAA 3'
Traf6	5' CTACATTTGGAAGATTGGC 3'	5' AGTGCTTCAGACTGGTCG 3'
Gapdh	5' TCAACGGCACAGTCAAGG3'	5' GCAGAAGGGGCGGAGATG 3'

Table S3. Antibody list.

Primary antibody	Company	Catalog No.
USP1	Proteintech	14346-1-AP
USP25	Abcam	Ab187156
GSDMD-N	Abcam	Ab255603
Active Caspase-1	Proteintech	22915-1-AP
Pro-Caspase-1	Abcam	Ab179515
TRAF6	Abcam	Ab137452
GAPDH	Proteintech	60004-1-1G
NF-κB	Abcam	Ab16502
H3	Abcam	Ab1791
Ubiquitin	Abcam	Ab7780
GAPDH	Cell Signaling Technology	#5174