TIMP-1 suppressed by miR-138 participates in endoplasmic reticulum stress-induced osteoblast apoptosis in osteoporosis

Ting-Bin Yan, Ci Li, Guang-Jun Jiao, Wen-Liang Wu & Hai-Chun Liu

To cite this article: Ting-Bin Yan, Ci Li, Guang-Jun Jiao, Wen-Liang Wu & Hai-Chun Liu (2018): TIMP-1 suppressed by miR-138 participates in endoplasmic reticulum stress-induced osteoblast apoptosis in osteoporosis, Free Radical Research, DOI: 10.1080/10715762.2017.1423070

To link to this article: https://doi.org/10.1080/10715762.2017.1423070

Accepted author version posted online: 02 Jan 2018.
TIMP-1 suppressed by miR-138 participates in endoplasmic reticulum stress-induced osteoblast apoptosis in osteoporosis

Ting-Bin Yan, CiLi, Guang-Jun Jiao, Wen-Liang Wu, Hai-Chun Liu*

1 Department of Orthopedics, Qilu Hospital of Shandong University, Jinan; 250012, China

*Corresponding author: Hai-Chun Liu, MD.
Mailing address: Department of Orthopedics, Qilu Hospital of Shandong University.

Running title: The role of miR-138 in osteoporosis

Abstract

Objective The aim of this study was to investigate the role of miR-138 in osteoporosis and its underlying mechanism.

Methods Hydrogen peroxide (H2O2) was used to induce osteoporotic injury of osteoblasts. The cell viability and apoptosis of MC3T3-E1 cells was assessed using MTT assay and flow cytometry, respectively. The cell transfection was carried out to modulate the expression levels of miR-138 and TIMP-1 in MC3T3-E1 cells. Luciferase reporter gene assay was performed to determine the interaction between miR-138 and TIMP-1 3’UTR.

Results In the present study, H2O2 inhibited osteoblasts growth and induced intracellular endoplasmic reticulum (ER) stress accompanied by high expression of miR-138. We also
confirmed that miR-138 promoted osteoblasts apoptosis in vitro and in vivo. MiR-138 was further indicated to inhibit osteoblast survival via negative regulating TIMP-1 expression. Moreover, the down-regulated TIMP-1 also mediated the ER stress-induced apoptosis of osteoblasts.

**Conclusion** We confirmed that miR-138 and ER stress were induced in osteoporosis and then promoted the apoptosis of osteoblasts, at least in part, through TIMP-1.

**Keywords** Osteoporosis; osteoblast apoptosis; MiR-138; TIMP-1

**Introduction**

Osteoporosis is a common age-related degenerative disease characterized by degenerative changes in bone microstructure and decreased bone density[1]. The dynamic balance between the formation of new bone and the bone absorption is especially important for the maintenance of bone metabolism[2]. Osteoblasts are the main cells in bone formation, which play a major role in the metabolism, growth and repair of bone tissue[3]. The abnormal proliferation, differentiation and apoptosis of osteoblasts also play an important role in the development of osteoporosis. Osteoblast apoptosis was considered to be a critical event in the pathogenesis of osteoporosis[4]. Rats injected with corticosterone exhibited the symptoms of osteoporosis, such as decreased bone density, bone turnover rate and trabecular bone; further examination showed that the apoptosis of vertebral osteoblasts in rats after injection was significantly higher than that in the control group[5].

Endoplasmic reticulum (ER) is the organelle that stores calcium ions, and is also the main site for the synthesis, translation, folding and assembly of cellular proteins[6]. Protein folding was disturbed by cell hypoxia, nutrient deficiency and virus infection, giving rise to the accumulation of unfolded and misfolded proteins in the ER, thereby inducing ER stress[7]. Excessive ER stress destroyed cell homeostasis and induced cell apoptosis. The ER stress-induced apoptosis of osteoblasts has been considered as an important mediator in bone formation and the pathogenesis of osteoporosis[8].

Tissue inhibitor of metalloproteinase-1 (TIMP-1) was identified to involve in bone metabolism, and TIMP-1 over-expressed in osteoblasts increased the bone mineral density and bone mass in mice, but decreased overall bone turnover[9]. Xie et al. reported that suppression of TIMP-1 was contributed to osteoblast apoptosis in vitro[10]. TIMP-1 was decreased in neuronal cells to respond to ER stress during neurodegenerative diseases[11]. Thus we inferred that TIMP-1 was involved in
the pathogenesis of osteoporosis, and the mechanism might be related to osteoblasts apoptosis and ER stress.

MicroRNAs (miRNAs), as a class of non-coding RNA, were indicated to implicate several biological processes and human diseases pathogenesis including osteoporosis[12]. So far, multiple miRNAs have been found to dysregulate in osteoporosis and participate in osteoporosis progression. For example, miR-503 was significantly decreased in the serum of patients with postmenopausal osteoporosis, and the bone resorption of ovariectomy (OVX) mice was inhibited by the antagonomir-induced miR-503 silencing, preventing bone loss[13]. MiR-138 has been demonstrated to significantly inhibit osteogenic differentiation of human stromal stem cells in vivo and in vitro[14,15]. In addition, miR-138 has also been found to damage the proliferation and differentiation of skeletal cells, and to induce skeletal cell apoptosis[16]. The agonomir of miR-138, therefore, is expected to become a new adjuvant therapy for osteoporosis. However, the role of miR-138 in osteoporosis and its concrete mechanism need further study.

Given that the bioinformatics analysis (microrna.org) revealed the possibility of interaction between miR-138 and TIMP-1 3'-UTR, we determined the role of miR-138 in osteoporosis, and whether and how miR-138 participated in osteoporosis through interacting with TIMP-1.

Materials and methods

Cell culture and treatment

The osteoblastic cell line MC3T3-E1 was obtained from American Type Culture Collection (ATCC) and maintained in α-MEM medium (LifeTechnologies) containing 10% fetal bovine serum (FBS, Gibco), 1% streptomycin/penicillin (HyClone) with 5% CO₂ at 37°C. For hydrogen peroxide (H₂O₂) treatment, MC3T3-E1 cells were treated with H₂O₂ added in complete medium for different durations (0, 2, 4, 6 h), and the working concentration of H₂O₂ was 400μM. Forthapsigargin (Tg) or tunicamycin (Tm) exposure, MC3T3-E1 cells were incubated with Tg (10nM, Sigma, United States) or Tm (1μg/ml, Sigma, United States) for 24 h.

Detection of the cell viability and apoptosis

After H₂O₂/Tg treatment or cell transfection, the relative cell viability of MC3T3-E1 cells was assessed using the MTT assay, which is a classic measurement of cell viability and proliferation. Briefly, MC3T3-E1 cells were cultured in 96-well plates (1×10⁴ cells/well) and were then incubated with MTT solution (20μL, 5mg/ml) with 5% CO₂ at 37°C for 4 h. The medium was removed and dimethyl sulfoxide (DMSO) (150μL/well) (Sigma) was added to dissolve formazan crystals. The absorbance was determined at 490 nm wavelength by a microplate reader (Bio-Rad). Each cell group was repeated 5 wells and the experiment was performed in triplicate.

The apoptosis of MC3T3-E1 cells was observed by Annexin V-FITC/PI assay. This assay was performed with Annexin V–FITC/PI kit as described previously[17,18]. After AnnexinV–FITC and PI staining, the cell sample was analyzed on a flow cytometry (BD Biosciences) at emission wavelength of 530 nm and an excitation wavelength of 488 nm.

Western blotting analysis

In this study, western blotting analysis was used to detect the protein levels of the apoptosis-related proteins, cleaved caspase-3, Bax, Bcl-2, ER stress markers CHOP, p-eIF2α/eIF2α, and TIMP-1. The protein of MC3T3-E1 cells was extracted using Protein Extraction Reagent (Thermo Scientific, NO.78501) according to the manufacturer’s instructions. The protein of mouse bone
tissue was extracted using Tissue Protein Extraction Reagent (Thermo Scientific, NO.78510). The concentration of total protein was determined by using BCA Protein Assay Kit (Thermo Scientific, NO.23225) at a Microplate Reader. The total protein (20μg/sample) was separated by SDS-PAGE electrophoresis and then transferred to the nitrocellulose membranes. The membranes were immersed in 5% skim milk for 2 h at room temperature. The proteins on the nitrocellulose membranes were incubated with the primary antibodies, anti-TIMP-1 (1:1000, Cell Signaling Technology), anti-cleaved caspase-3 (1:1000, Santa Cruz), anti-Bax (1:1000, Abcam), anti-Bcl-2 (1:2000, Abcam), anti-CHOP, (1:1000, Abcam), anti-p-eIF2α (1:500, Santa Cruz), anti-eIF2α (1:500, Santa Cruz), and GAPDH (1:2000, Cell Signaling Technology), at 4°C overnight. The proteins were incubated with the horseradish peroxidase-conjugated second antibodies and visualized using ECL reagents (GE Healthcare).

**Quantitative RT-PCR analysis**

Quantitative RT-PCR was used to detect the expression levels of miR-138 (stem loop PCR) and TIMP-1 mRNA and performed as described previously[14]. The total RNA was extracted from MC3T3-E1 cells by TRIzol Reagent (Invitrogen, NO.15596018) and used for analysis of the relative expression of TIMP-1 mRNA. The microRNA Isolation Kit (Sigma, SNC50-1KT) was used for the extraction of total miRNA containing miR-138. The total RNA and miRNA were reverse transcribed to cDNA, and the products were amplified using SYBR qRT-PCR Kit (Clontech, NO.639201). In the present study, the reverse transcription reaction for miR-138 was performed using miRNA First Strand cDNA Synthesis (Stem-loop Method) (Sangon Biotech (Shanghai) Co., Ltd.). GAPDH was used as the reference for TIMP-1, U6 used as the reference for miR-138. The primers of miR-138 and TIMP-1 were provided by Sangon Biotech (Shanghai) Co., Ltd: miR-138, (forward) 5'-GACCCAGATTCCACCATAT-3' and (reverse) 5'-CAGTGCAGGGTCCGAGGT-3'; TIMP-1, (forward) 5'-CCAGAAGTCAACCAGACAT-3' and (reverse) 5'-TTCCAGCAATGCACAACACTCCT-3'; GAPDH, (forward) 5'-GAAGATGGTGATGGGATTTC-3' and (reverse) 5'-GAAGGTGAAGGTCGGAGT-3'; U6, (forward) 5'-AAAGACCTGTACGCCAACAC-3' and (reverse) 5'-TGCATACTCCTGCTTGTGAT-3'.

**Cell transfection**

In the present study, miR-138 expression was modulated by miR-138 mimic, inhibitor, and antagonimir-138, which were purchased from RiboBio Co., Ltd. (Guangzhou, China). The pcDNA-TIMP-1, a plasmid expression vector for TIMP-1, was transfected into MC3T3-E1 cells to increase TIMP-1 expression. The cell transfection assay was performed as described previously[19]. Briefly, MC3T3-E1 cells (4×10^5 cells/well) were seeded in 6-well plates overnight. The cells in each well were incubated with 100nMmiR-138 mimic/inhibitor or pcDNA-TIMP-1 and Lipofectamine 2000 reagent (Invitrogen). The si-TIMP1 is the target sequence for TIMP1 specific siRNA. The si-TIMP1 was designed and synthesized by Santa Cruz Biotechnology, Inc. (Dallas, TX, USA), and its sequences were as follows: 5'-CAAACATCTTCGGCAGTTTAGCCGA-3'. The working concentration of si-TIMP1 was 20nM. The cell transfection was performed prior to H2O2/Tg treatment.

**Luciferase assay**

TIMP-1 3’UTR fragment containing the putative miR-138 binding sites (WT) or site-directed mutation (MUT) was inserted into pGL4 luciferase reporter vector (Life technologies). MC3T3-E1 cells (2×10^5 cells/well) were seeded in 24-well plates and co-transfected with pGL4-TIMP-1-3’UTR (WT/MUT) and miR-138 inhibitor/mimic. Renilla pRL-TK vectors (Promega) were used as the
control. After 48h of transfection, the intracellular luciferase activities were measured using the Luciferase Reporter Assay System (Promega) according to manufacturer's instructions.

**Mice studies**

C57BL/6 mice were purchased from the laboratory animal center of Shandong University. All animal experiment were strictly followed the guidelines for the Care and Use of Laboratory Animals of the National Institute of Health and supported by the Ethics Committee of Qilu Hospital of Shandong University. Twenty-four female C57BL/6 mice aged 6 weeks were randomly divided into four groups: Sham group (n=6), OVX group (n=6), OVX+Con-antagomir group (n=6), OVX+antagomir-138 group (n=6). As indicated above, the mice had sham operation or bilateral ovariectomy (OVX) as described previously [20]. For the administration of miRNA antagomir, Con-antagomir or antagomir-138 (80mg/kg) injected into OVX mice from the tail vein once a day on days 1 to 3 for 3 consecutive weeks. The mice were sacrificed at third weeks after the last injection and bone samples were isolated for detection of CHOP, eIF2α, cleaved caspase-3, Bax and Bcl-2.

**Statistical analysis**

The data in this study was analyzed using Graphpad Prism 6 software (GraphPad, La Jolla, CA), and the results were expressed as mean ± standard deviation (SD). The Student’s t test was applied to analyze the statistical difference between continuous variables. P value <0.05 was considered statistically significant, and ‘***’, ‘##’, ‘&&’ indicates P<0.01.

**Results**

**Effects of H2O2 on the proliferation, apoptosis and endoplasmic reticulum (ER) stress of osteoblasts**

H2O2 (400 μM) exposures significantly reduced the relative cell viability of MC3T3-E1 cells within 6 hin a time dependent manner (Figure 1A). Meanwhile, apoptosis analysis indicated that H2O2 induced the osteoblasts apoptosis in vitro (Figure 1B). It was also revealed that ER stress activator, thapsigargin (Tg, 10 nM) for 24 h could inhibit the proliferation and promote apoptosis of MC3T3-E1 cells (Figure 1A&B). Moreover, both H2O2 and Tg evidently increased the protein expression of pro-apoptotic molecules cleaved caspase-3 and Bax, but inhibited pro-survival protein Bcl-2 in MC3T3-E1 cells (Figure 1C). Furthermore, the ER stress markers CHOP and p-eIF2α protein were induced in osteoblasts by H2O2 or Tg (Figure 1D). We also observed that miR-138 was dramatically increased in MC3T3-E1 cells treated with H2O2 or Tg (Figure 1E). These results indicated that H2O2 could inhibit osteoblasts growth and induce intracellular ER stress accompanied by high expression of miR-138, suggesting that miR-138 might participate in the H2O2-induced growth inhibition on osteoblasts.

**MiR-138 mediated the role of H2O2 in the proliferation and apoptosis of osteoblasts**

To explore the role of miR-138 in the H2O2-induced growth inhibition on osteoblasts, miR-138 inhibitor, the specific inhibitor of miR-138, was transfected into MC3T3-E1 cells to inhibit miR-138 expression. As shown in Figure 2A, miR-138 expression was dramatically decreased in MC3T3-E1 cells transfected with miR-138 inhibitor. Moreover, miR-138 inhibitor largely weakened the cytotoxicity of H2O2, as shown by rescuing osteoblasts viability and inhibiting the cell apoptosis (Figure 2 B&C). In addition, miR-138 inhibitor was also reversed the induction of H2O2 on pro-apoptotic molecules cleaved caspase-3 and Bax/Bcl-2 ratio (Figure 2D). The ER
stress markers CHOP and p-eIF2α protein induced by H\textsubscript{2}O\textsubscript{2} were significantly reduced in MC3T3-E1 cells with miR-138 silencing (Figure 2E).

**MiR-138 negatively regulated TIMP-1 in osteoblasts**

H\textsubscript{2}O\textsubscript{2} treatment significantly decreased the expression of TIMP-1 in MC3T3-E1 cells both at mRNA and protein levels with over time (Figure 3A). The bioinformatics analysis (microrna.org) revealed that miR-138 could combine with TIMP-1 3\textsuperscript{\prime} UTR by the potential binding sites, as shown in Figure 3B. MC3T3-E1 cells were co-transfected with the luciferase reporter plasmids carrying wild type (WT)/site-directed mutant (MUT) of TIMP-1 3\textsuperscript{\prime} UTR and miR-138 mimic/inhibitor. Compared with the control for miR-138 mimic, miR-138 overexpression dramatically reduced the relative luciferase activity in MC3T3-E1 cells with the luciferase reporter plasmids carrying WT of TIMP-1 3\textsuperscript{\prime} UTR, whereas the relative luciferase activity in the cells with the luciferase reporter plasmids carrying MUT of TIMP-1 3\textsuperscript{\prime} UTR was not affected by miR-138 mimic (Figure 3C). Conversely, miR-138 knockdown increased the relative luciferase activity in MC3T3-E1 cells with the luciferase reporter plasmids carrying WT of TIMP-1 3\textsuperscript{\prime} UTR, but had no discernible effect on the relative luciferase activity in the cells with the luciferase reporter plasmids carrying MUT of TIMP-1 3\textsuperscript{\prime} UTR (Figure 3D). Both the mRNA and protein expressions of TIMP-1 in MC3T3-E1 cells were negatively regulated by miR-138 (Figure 3C&D).

**MiR-138 mediated the role of H\textsubscript{2}O\textsubscript{2} in the proliferation and apoptosis of osteoblasts through negative regulating TIMP-1**

MiR-138 silencing was shown to reduce the H\textsubscript{2}O\textsubscript{2}-induced inhibition on osteoblasts viability, but TIMP-1 knockdown could eliminate this effect in large part (Figure 4A). Meanwhile, TIMP-1 knockdown also reversed the inhibition of miR-138 silencing on osteoblasts apoptosis (Figure 4B). Obviously, TIMP-1 was a functional target of miR-138 in osteoblasts proliferation and apoptosis.

**TIMP-1 inhibited the ER stress-induced apoptosis of osteoblasts**

Tg (10 nM) or Tm (1 μg/ml) treatment for 24 h significantly decreased the expression level of TIMP-1 mRNA and protein in MC3T3-E1 cells (Figure 5A&Supplemental Figure 1A). The mRNA level of TIMP-1 was significantly elevated in MC3T3-E1 cells by pcDNA-TIMP-1, a plasmid expression vector for TIMP-1 (Figure 5B). The pcDNA-TIMP-1 also significantly reduced osteoblasts apoptosis induced by Tg or Tm, suggesting that TIMP-1 inhibited the ER stress-induced apoptosis of osteoblasts (Figure 5C&Supplemental Figure 1B). Moreover, TIMP-1 overexpression blocked the induction of Tg or Tm on pro-apoptotic molecules cleaved caspase-3 and Bax/Bcl-2 (Figure 5D&Supplemental Figure 1C).

**MiR-138 silencing alleviated cell apoptosis in osteoporotic mice**

A mouse model of osteoporosis was established based on bilateral oophorectomy (OVX), mouse underwent sham operation (sham) as control. The antagonim-138 was used to inhibit miR-138 expression in vivo and was administrated to OVX mice by intravenous injection. MiR-138 expressions significantly increased in the bone tissues of OVX mice (n=6), whereas antagonim-138 down-regulated the expression level of miR-138 in the bone tissues (n=6) (Figure 6A). TIMP-1 protein expression was decreased in the bone tissues of OVX mice (n=6), but was obviously up-regulated by the antagonim-138 administration in vivo (Figure 6B). Moreover, the apoptosis-related proteins, Caspase-3 activity, cleaved caspase-3 and Bax/Bcl-2 ratio were highly expressed in the bone tissues of OVX mice (n=6), suggesting the appearance of proapoptosis event (Figure 6C&D). However, accompanied by miR-138 silencing, Caspase-3 activity, cleaved caspase-3,
and Bax/Bcl-2 ratio were significantly reduced in the bone tissues of OVX mice (n=6) (Figure 6C&D).

Discussion

The dynamic balance of osteoblasts proliferation and apoptosis is crucial to maintain the number and function of osteoblasts[21]. Under pathological conditions, osteoblasts apoptosis increased abnormally, causing bone loss and exacerbating osteoporosis[3]. Well understanding of the apoptotic mechanism of osteoblasts will provide more choices for the treatment of osteoporosis. MicroRNAs (miRNAs), as the novel regulatory factor in osteoporosis, has caused a great deal of concern in pathological mechanism and clinical treatment[22]. At present, several miRNAs were indicated positive/negative involvement in the context of osteoporosis, such as miR-9, miR-10, miR-148a and miR-214[23,24,25]. MiR-138 has been shown to inhibit osteoblastic differentiation of mesenchymal stem cells, suggesting the important role of it in osteoporosis progression[14,15]. In this study, we further ascertained the role of miR-138 in osteoporosis and its underlying mechanisms.

We observed that miR-138 was increased dramatically in osteoblasts model of osteoporosis and the bone of OVX mice. The dysregulation of miR-138 was associated with the rheumatoid arthritis, esophageal squamous cell carcinoma and prostate cancer[26,27,28]. We also confirmed that miR-138 promoted osteoblasts apoptosis in vitro and in vivo. MiR-138 has been reported to significantly decrease during osteoblast differentiation of human mesenchymal stem cells (hMSCs), and miR-138 has also been approved to play a negative regulation during osteoblast differentiation of hMSCs in vitro and ectopic bone formation in vivo[14]. Moreover, miR-138 inhibited periodontal progenitor differentiation into osteoblasts during periodontal diseases, promoting bone loss[29]. Osteoblast differentiation was also a key event in the arising and developing process of osteoporosis. Obviously, miR-138 was closely related to osteoporosis by regulating osteoblast differentiation. Herein, we further proposed that miR-138 reduced osteoblasts viability and induced cell apoptosis, thereby driving osteoporosis.

TIMP-1 was identified to involve in bone metabolism, and suppression of TIMP-1 was contributed to osteoblast apoptosis in vitro[10]. In the present study, TIMP-1 was decreased in osteoblasts during the apoptosis and ER stress activation. MiR-138 was demonstrated to suppress the proliferation and differentiation of skeletal cells through targeting eukaryotic translation initiation factor 4E binding protein 1 (EIF4EBP1)[16]. Our results indicated that miR-138 inhibited osteoblast survival via negative regulating TIMP-1 expression. Moreover, TIMP-1 was decreased in neuronal cells to respond to ER stress during neurodegenerative diseases[11]. In our study, a significant decrease in TIMP-1 expression was found in osteoblasts with the activation of ER stress, and the down-regulated TIMP-1 mediated the ER stress-induced apoptosis of osteoblasts. Taken together, TIMP-1 has an important role both in the miR-138- and ER stress-induced apoptosis of osteoblasts in osteoporosis.

As a classical matrix metalloproteinase (MMP) inhibitor, TIMP-1 plays its biologic role primarily via inhibition of enzyme activity[30]. Xie et al. reported that TIMP-1 could protect osteoblasts from apoptosis through inhibiting MMP activities[10]. The inadequacy of this study is that it lacks of further analysis for the mechanisms of down-regulated TIMP-1 promoting apoptosis of osteoblasts, which will be one of the key points in the following study.
In summary, this study suggested a novel potential mechanism for accelerating osteoblasts apoptosis in osteoporosis. We confirmed that miR-138 and ER stress were induced in osteoporosis and then promoted the apoptosis of osteoblasts, at least in part, through TIMP-1. Remarkably, miR-138 and ER stress simultaneously induced in osteoporotic osteoblasts, indicating the possible cross-talk between them. This is also the focus of our upcoming research.

Reference


Figure legends

**Figure 1** Effects of H\textsubscript{2}O\textsubscript{2} on the proliferation, apoptosis and endoplasmic reticulum (ER) stress of osteoblasts. MC3T3-E1 cells were treated with H\textsubscript{2}O\textsubscript{2}(400 μM) for different durations (0, 2, 4, 6 h) or treated by ER stress activator, thapsigargin (Tg, 10 nM) for 24 h. (A) The proliferation of MC3T3-E1 cells was assessed using MTT assay. (B) MC3T3-E1 cell apoptosis was measured by double staining with Annexin V-FITC/PI. (C) The apoptosis-related proteins, cleaved caspase-3, Bax, Bcl-2, were determined by western blotting analysis. (D) The protein expressions of ER stress markers CHOP and p-eIF2α were also determined by western blotting analysis. (E) qRT-PCR analysis of the relative expression of miR-138 in MC3T3-E1 cells.** (A-B) The proliferation and apoptosis of osteoblasts. (A) MiR-138 was dramatically decreased in MC3T3-E1 cells by miR-138 inhibitor, the specific inhibitor of miR-138. NC, **the negative control for miR-138 inhibitor.** P<0.01 vs. 0 h.

**Figure 2** MiR-138 mediated the role of H\textsubscript{2}O\textsubscript{2} in the proliferation, apoptosis and ER stress of osteoblasts. (A) MiR-138 was dramatically decreased in MC3T3-E1 cells by miR-138 inhibitor, the specific inhibitor of miR-138. NC, **the negative control for miR-138 inhibitor.** P<0.01 vs. NC. MC3T3-E1 cells were divided into four experimental treatment groups: control, H\textsubscript{2}O\textsubscript{2}(400μM for 4 h), H\textsubscript{2}O\textsubscript{2}+NC, H\textsubscript{2}O\textsubscript{2}+miR-138 inhibitor (50 nM). (B-C) The proliferation and
apoptosis of MC3T3-E1 cells was assessed using MTT assay and Annexin V-FITC/PI double staining method. **P<0.01 vs. control; ## P<0.01 vs. H2O2+NC.

**Figure 3** MiR-138 negatively regulated TIMP-1 in osteoblasts. (A) MC3T3-E1 cells were treated with H2O2 (400 μM) for different durations (0, 2, 4, 6 h), and the expression levels of TIMP-1 mRNA and protein were then determined. **P<0.01 vs. 0 h. (B) The putative binding sites between miR-138 and TIMP-1, and the diagram of the site-directed mutants of TIMP-13'UTR. (C) The effects of miR-138 overexpression on TIMP-1 expression. Luciferase reporter gene assay was performed to identify that there were interaction between miR-138 and TIMP-1 through the potential binding sites. MiR-138 mimic or pre-NC were transfected into MC3T3-E1 cells, and then TIMP-1 expression was determined both at mRNA and protein levels. pre-NC, the scramble-miR control for miR-138 mimics. **P<0.01 vs. pre-NC. (D) TIMP-1 expression changes were subsequently assessed after miR-138 silencing by miR-138 inhibitor transfection. **P<0.01 vs. NC.

**Figure 4** MiR-138 mediated the role of H2O2 in the proliferation and apoptosis of osteoblasts through negative regulating TIMP-1. MC3T3-E1 cells were divided into six experimental treatment groups: control, H2O2 (400 μM for 4 h), H2O2+NC, H2O2+miR-138 inhibitor (50 nM), H2O2+NC+si-NC, H2O2+miR-138 inhibitor+si-TIMP-1. (A) The relative cell viability was measured using MTT assay. (B) MC3T3-E1 cell apoptosis was measured by double staining with Annexin V-FITC/PI. NC, the negative control for miR-138 inhibitor; si-NC, the negative control for si-TIMP-1. **P<0.01 vs. control; ## P<0.01 vs. H2O2+NC; && P<0.01 vs. H2O2+miR-138 inhibitor+si-NC.

**Figure 5** TIMP-1 inhibited the ER stress-induced apoptosis of osteoblasts. (A) MC3T3-E1 cells were treated with Tg (10 nM) for 24 h, and the expression levels of TIMP-1 mRNA and protein were then determined. **P<0.01 vs. Control. (B) The mRNA level of TIMP-1 was significantly elevated in MC3T3-E1 cells by pcDNA-TIMP-1, a plasmid expression vector for TIMP-1. pcDNA, the negative control for pcDNA-TIMP-1. **P<0.01 vs. pcDNA. MC3T3-E1 cells were divided into four experimental treatment groups: control, Tg (10 nM for 24 h), Tg+pcDNA, Tg+pcDNA-TIMP-1. (C) The apoptosis of MC3T3-E1 cells in all experimental treatment groups were assessed. **P<0.01 vs. Control; ## P<0.01 vs. Tg+pcDNA. (D) Western blotting analysis of the protein levels of the apoptosis-related proteins, cleaved caspase-3, Bax, Bcl-2.

**Figure 6** MiR-138 silencing alleviated ER stress and cell apoptosis in osteoporotic mice. A mouse model of osteoporosis was established based on bilateral oophorectomy (OVX), mouse underwent sham operation (sham) as control. The mice (n=24) were randomly divided into four groups: sham (n=6), OVX (n=6), OVX+Con-antagomir (n=6), OVX+antagomir-138 (n=6). The mice were administrated antagomir-138 (80 mg/kg) or Con-antagomir by intravenous injection. (A) Detection of miR-138 expression in bone tissues of all mice. (B-D) The expression changes of TIMP-1 protein, caspase-3 activity, the apoptosis-related proteins, cleaved caspase-3, Bax, Bcl-2, and ER stress markers CHOP, p-eIF2α expression in bone tissues were assessed. Con-antagomir, the antagomir negative control for antagomir-138. **P<0.01 vs. Sham; ## P<0.01 vs. OVX + Con-antagomir.

**Supplemental Figure 1** TIMP-1 inhibited the ER stress-induced apoptosis of osteoblasts. (A) MC3T3-E1 cells were treated by Tm (1 μg/ml) for 24 h, and the expression levels of TIMP-1...
mRNA and protein were then determined. **P<0.01 vs. Control. (B) MC3T3-E1 cells were divided into four experimental treatment groups: control, Tm (1 μg/ml for 24 h), Tm+pcDNA, Tm+pcDNA-TIMP-1. The apoptosis of MC3T3-E1 cells in all experimental treatment groups were assessed. **P<0.01 vs. Control; ##P<0.01 vs. Tm+pcDNA. (C) Western blotting analysis of the protein levels of the apoptosis-related proteins, cleaved caspase-3, Bax, Bcl-2.