Original Article

Tumor necrosis factor receptor-associated factor 6 (TRAF6) inhibition modulates bone loss and matrix metalloproteinase expression levels in collagen-induced rheumatoid arthritis rat

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Background: Rheumatoid arthritis (RA) is a main characterized by persistent synovitis, systemic inflammation, and autoantibodies. Tumor necrosis factor receptor-associated factor 6 (TRAF6) is an E3 ubiquitin ligase and is a crucial cytoplasm signal adaptor that can regulate critical biological processes. This research aims to explore the function of TRAF6 on bone loss and matrix metalloproteinase (MMP) expression in collagen-induced RA rats.

Methods: The RA model in rats (Sprague Dawley rat, 5–6 weeks old, weight 246.88±8.31 g) was set up via using collagen-induced RA. The shRNA-TRAF6 knockdown efficiency was tested using real-time reverse transcription-polymerase chain reaction (qRT-PCR) and western blot, respectively. The rats were divided into four groups: the control group, RA group, RA + shRNA-NC group, and RA + TRAF6-shRNA group. The tartrate-resistant acidic phosphatase (TRAP), hematoxylin and eosin (H&E), and Saffron O staining were employed to test the bone injury. The mRNA and protein expressive of Osteoclast-associated receptor (OSCAR), TRAP, Osterix (OSX), Collagen type I alpha 1 (COL1A1), Distal-less homeobox2 (Dlx2), tissue inhibitor of metalloproteinase (TIMP), matrix metalloproteinase-1(MMP-1), Cyclooxygenase 2 (COX2) and qRT-PCR performed MMP-13 and western blot, respectively.

Results: The mRNA and protein expression levels of TRAF6 were down-regulated in the RA + TRAF6-shRNA group. After the levels of TRAF6 were inhibited, the levels of bone volume/total volume (BV/TV), trabecular bone thickness (Tb.Th), and trabecular bone number (Tb.N) were increased, while the levels of trabecular bone space (Tb.Sp), Osteocalcin and ALP were deceased. The mRNA and protein expression levels of OSCAR, TRAP, MMP-1, COX2, and MMP-13 were reduced obviously in the RA + TRAF6-shRNA group compared with the RA + shRNA-NC group, while the levels of TIMP-1, OSX, CoL1A1, and DLx2 were enhanced obviously.

Conclusions: Inhibition of TRAF6 reduces bone loss and MMP expression levels in collagen-induced RA rat, and supplies an alternative treatment method in RA.

Keywords: tumor necrosis factor receptor-associated factor 6 (TRAF6); rheumatoid arthritis (RA); bone loss; matrix metalloproteinase expression

Submitted Sep 03, 2020. Accepted for publication Oct 28, 2020.
doi: 10.21037/apm-20-1894
View this article at: http://dx.doi.org/10.21037/apm-20-1894

Introduction

Rheumatoid arthritis (RA) is a main characterized by persistent synovitis, systemic inflammation, and autoantibodies. Women, smokers, and those with a family history of the disease are most often affected (1). Methotrexate is the drug of choice to treat RA, sometimes combined with biological agents, including tumor necrosis factor (TNF) inhibitors (2). However, even with these treatment options, including disease-altering anti-rheumatic drugs and available biological agents, many patients still cannot adequately control their disease (3). Radiation synovial replacement, synovectomy, or axial correction with tendon transfer are essential methods for surgical treatment of RA in earlier stages (4). Many recent advancements in pharmacologic therapy have improved RA outcomes significantly, and many further patients can be treated. However, the improvement of RA results is accompanied by substantial financial costs (5). In traditional RA treatment, due to the short biological half-life and poor bioavailability, high doses and frequent doses is require. These anti-RA medications may cause serious side effects on extra-articular tissues. Studies have shown that nanotechnology has become a promising tool for the development of new drug delivery systems for the treatment and diagnosis of intractable diseases such as rheumatoid arthritis (6,7). At present, RNA interference (RNAi) is a powerful endogenous process initiated by short double-stranded RNAs, which results in sequence-specific posttranscriptional gene silencing. The possibility of blocking the expression of any protein carries huge expectations for potential therapeutic applications in a wide range of diseases (8). Therefore, improving the treatment of RA is an integral part of our future research.

Tumor necrosis factor receptor-associated factor 6 (TRAF6) is a significant binding protein in the TRAF family and can regulate the TNF receptor superfamily. The interleukin 1 receptor/Toll-like receptor family, and is considered a vital participant in regulating inflammation and immunity (9-11). TRAF6 is necessary for many biological processes, including the chronic innate immune signaling with myelodysplasia (12), anti-myeloma and anti-bone resorptive (13), tumor angiogenesis (14), and cancer metastasis (15,16). Notably, the overexpression of TRAF6 in the synovium is related to the increase of compensatory bone formation. Therefore, TRAF6 is engaged in the pathogenesis of bone metabolic imbalance via regulating the synovial inflammation of RA (17). Sinomenine suppresses expression levels of differentiation factor 88 (MyD88) and TRAF-6 in RA-Fibroblast-like synoviocytes (FLSs) (18). Tomatidine inhibits osteoclast production and reduces bone loss caused by estrogen deficiency by regulating TRAF6-mediated signal transduction (19). However, down-regulation of TRAF-6 plays the role is not reported in osteoclast, osteoblast, and chondrocyte.

We present the following article in accordance with the ARRIVE reporting checklist (available at http://dx.doi.org/10.21037/apm-20-1894).

Methods

Animals

Sixty Sprague Dawley (SD) rats (5–6 weeks old, weight 246.88±8.31 g) were retrieved from the Shanghai Laboratory Animal Center, Chinese Academy of Sciences (Shanghai, China). All animal care and handling were performed considering the National Institutes of Health (NIH) Guidelines for the Care and Use of Laboratory Animals and were approved by the People's Hospital of Ningxia Hui Autonomous Region. The research was reviewed and approved by the Ethics Committee for Experimental Animal Management and Animal Welfare of People's Hospital of Ningxia Hui Autonomous Region. Rats were housed individually in stainless steel cages with glass water bottles. Rats were held for adaptation for 14 d before collagen-induced RA. Rats were kept in a room under the following conditions: temperature of 23±1 ℃, the humidity level of 50–60%, and the 12 hours light/dark cycle.

RA model rats

Of the sixty rats, 15 rats were randomly selected as the control group, and the remaining 45 rats were selected as the RA group. The RA model in rats was set up via using collagen-induced RA. So rats were divided randomly into four groups: control group, RA group, RA + shRNA-

NC group and RA + shRNA-TRAF6 group. Type II bovine collagen was dissolved in 0.05 mol/L acetic acid at a concentration of 2 mg/mL and then emulsified in an equal volume of complete Freund's adjuvant (CFA; Jon Drex, Washington, USA). Rats were immunized intracutaneously with 200 µL emulsion divided into four points symmetrically along the back. After seven days, a similar amount of bovine collagen type II emulsified into CFA injected intracutaneously as a booster dose (20). The severity of collagen-induced RA was assessed using a scoring system (21). Through this method, we judge whether the RA was set up successfully.

shRNA-mediated TRAF6 knockdown

Following manufacturer's instructions, peripheral blood mononuclear cells (PBMC) were retrieved from the peripheral blood in the RA patients and healthy controls. In short, Ficoll immediately isolated PBMC (TBD Science, Tianjin, China), and then resuspended at a cell concentration of 10^5/mL in RPMI-1640 medium containing 10% fetal bovine serum (FBS). HANBIO Company designed the effective TRAF6-shRNA sequence. The HANBIO Company synthesized the TRAF6-shRNA lentiviral vector (Shanghai, China). An shRNA-NC was used as a control for all experiments. After 24 hours, TRAF6-shRNA and shRNA-NC lentiviral vector were transfected into PBMC with lipofectamine 3000 reagents (Life Technologies Corporation) considering the manufacturer's instructions. The shRNA knockdown efficiency was evaluated using Real-time reverse transcription-polymerase chain reaction (qRT-PCR) and western blot.

qRT-PCR

According to manufacturers' protocol, total RNA was isolated by employing the TRIzol reagent kit (Invitrogen, Beijing, China). The total RNA concentration was measured by adopting the Gene Quant ProRNA/DNA Calcula-tor (Amersham Pharmacia Biotec, UK). The PrimeScript RT reagent Kit (TakaRa, Dalian, China) was used to perform reverse transcription. The 2 SYBR Premix Ex Taq™ II (TakaRa, Dalian, China) was employed to assemble the reaction system of qRT-PCR. The reaction system is carried out in the Bio-Rad CFX-96 (Bio-Rad, CA, USA). GAPDH was used for normalizing. The qRT-PCR data were analyzed using the $2^{-\Delta\Delta Ct}$ method to calculate the relative expression levels of mRNA. Primer sequences were used in this study in *Table 1*.

Western blot assay

The RIPA lysis buffer (Beyotime Institute of Biotechnology, Shanghai, China) was employed to extract proteins in the bone homogenate following the manufacturer's protocol. The BCA Protein Assay Kit were retrieved from Biyuntian Biological Technology Co., Ltd. (Shanghai, China) and were employed to measure the protein concentrations. Primary antibody (*Table 2*) were integrated with the targeted protein with incubation at room temperature for 1 hour. Secondary antibodies conjugated with goat anti-rabbit IgG horseradish peroxidase (HRP)-conjugated secondary antibodies for one hour. The band densities were decided and analyzed with an automatic digital gel image analysis system Bio-Rad CFX-96 (Bio-Rad, CA, USA).

Bone morphometry

Rat femurs were dissected free of soft tissue, fixed in4% paraformaldehyde for 24 hours, and scanned under micro-computed tomography (µCT). The morphometry of trabecular bones was assessed by high-resolution Inveon microtomography (Siemens, Munich, Germany). Parameters included bone volume/total volume (BV/TV), trabecular bone thickness (Tb.Th), trabecular bone number (Tb.N), and trabecular bone space (Tb.Sp).

Histological examination

Bone samples were fixed with 10% neutral buffered formalin. Samples were then decalcified in diethyl-pyrocarbonate treated 0.2 M ethylenediaminetetraacetic acid (EDTA), embedded in paraffin wax, cut into sections (5 µm-thick), and stained with tartrate-resistant acidic phosphatase (TRAP), hematoxylin and eosin (H&E), and Saffron O staining.

Statistical analysis

Each experiment in this study was conducted three times. The data were presented as mean ± SD. Statistical analyses between two groups were conducted using Student's *t*-tests and SPSS 25.0 software (IBM, Armonk, USA). The significance of differences between treatment groups was studied using a one-way analysis of variance (ANOVA). A P

Table 1 Primer sequences

Gene	Forward	Reverse
Tumor necrosis factor receptor-associated factor 6 (TRAF6)	5'-CAG TGG TCG TAT CGT GCT TA-3'	5'-CCT TAT GGT TTC TTG GAG TC-3'
Osteoclast-associated receptor (*OSCAR*)	5'-CCC AGC TTC ATA CCA CCC TA-3'	5'-GAA GAG AAG GGG AGC GAT CT-3'
Triiodothyronine receptor auxiliary protein (*TRAP*)	5'-TCA CCC TGA CCT ATG GTG C-3'	5'-GCC GGA CTC CAA TGT TAA AGC-3'
Osterix (*OSX*)	5'-CCT CTG CGG GAC TCA ACA AC-3'	5'-AGC CCA TTA GTG CTT GTA AAG G-3'
Collagen type I alpha 1 (*COL1A1*)	5'-CCT GGA TGC CAT CAA AGT CT-3'	5'-AAT CCA TCG GTC ATG CTC TC-3'
Distal-less homeobox2 (*Dlx2*)	5'-CTC TGC CTG CCT CAT AAG G-3'	5'-ATC GTA AGA ACA GCG CAA CC-3'
Tissue inhibitor of metalloproteinase (*TIMP*)	5'-CAA CTG CGG AAC GGG CTC TTG-3'	5'-CGG CAG CGT AGG TCT TGG TGA A-3'
Matrix metalloproteinase-1 (*MMP-1*)	5'-CAG ATG GGC ATA TCC CTC TAA GAA-3'	5'-CCA TGA CCA AAT CTA CAG TCC TCAC-3'
Cyclooxygenase 2 (*COX2*)	5'-CAC GCA GGT GGA GAT GAT CTA C-3'	5'-ACT TCC TGG CCC ACA GCA AAC T-3'
MMP-13	5'-CAT GCC AAC AAA TTC CCT GCT GTG GT-3'	5'-TCT CCT CCC TGC ACC TCC AGA TTT-3'

Table 2 Antibody information

Antibody	Antibody information
anti-TRAF6	1:1,000, #8028, Cell Signaling
anti-OSCAR	1:1,000, sc-34233, Santa Cruz Biotechnology
anti-TRAP	1:1,000, #15094, Cell Signaling
anti-OSX	1:1,000, ab209484, abcam
anti-COL1a1	1:1,000, #39952, Cell Signaling
anti-DLx2	1:5,000, ab272902, abcam
anti-TIMP	1:1,000, #8946, Cell Signaling
anti-MMP-1	1:1,000, #54376, Cell Signaling
anti-COX2	1:1,000, #12282, Cell Signaling
anti-MMP13	1:1,000, #69926, Cell Signaling

value of <0.05 was showing statistical significance.

Results

TRAF6 expression in collagen-induced RA rat

Considering previous research, TRAF6 plays a direct role in the proinflammatory effects and proliferation of RA FLSs (22). As shown in *Figure 1*, the mRNA and protein expression levels of TRAF6 were up-regulated, obviously in the RA group compared with the control group (P<0.05). TRAF6 in RA was inhibited using Lentiviral-TRAF6-shRNA transfection. The mRNA and protein expression levels of TRAF6 were down-regulated, obviously in the RA + TRAF6-shRNA group compared with the RA + shRNA-NC group (P<0.05).

Effect of down-regulation of TRAF6 on bone-specific indicators

As shown in *Figure 2*, the levels of BV/TV, Tb.Th and Tb.N were deceased in the RA group compared with the control group, while the levels of Tb.Sp, Osteocalcin, and ALP were increased (P<0.05). However, after the levels of TRAF6 were inhibited, the levels of BV/TV, Tb.Th and Tb.N were increased in the RA + TRAF6-shRNA group compared with the RA + shRNA-NC group (P<0.05), while the levels of Tb.Sp, Osteocalcin, and ALP were decreased (P<0.05). In summary, inhibition of TRAF6 might break the balance between osteoclast and osteoblast production.

Effect of down-regulation of TRAF6 on osteoclast production

As shown in *Figure 3A*, TRAP staining detected osteoclast production. The result showed that the number of osteoclasts was increased significantly in the RA group compared with the control group. However, after the levels of TRAF6 were inhibited, the number of osteoclasts

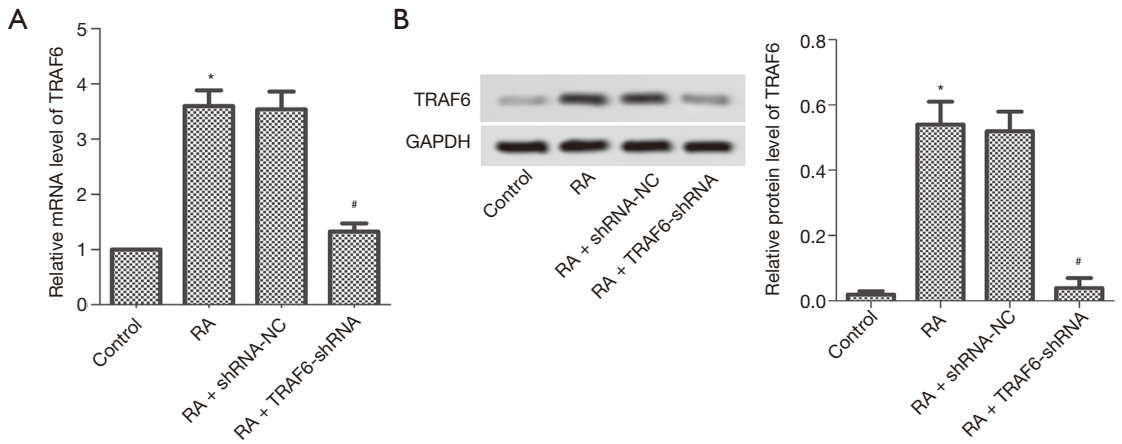

Figure 1 TRAF6 expression in collagen-induced rheumatoid arthritis rat. (A) The mRNA expressive level of TRAF6 was performed by qRT-PCR; (B) the expressive protein levels of TRAF6 were performed by western blot. *, P<0.05 compared with the control group; #, P<0.05 compared with the RA + shRNA-NC group. TRAF6, tumor necrosis factor receptor-associated factor 6; qRT-PCR, real-time reverse transcription-polymerase chain reaction; .

was decreased significantly in the RA + TRAF6-shRNA group compared with the RA + shRNA-NC group. Also, as shown in *Figure 3B,C*, the mRNA and protein expression levels of OSCAR and TRAP were enhanced obviously in the RA group compared with the control group (P<0.05). However, after the levels of TRAF6 were inhibited, the mRNA and protein expression levels of OSCAR and TRAP were reduced in the RA + TRAF6-shRNA group compared with the RA + shRNA-NC group (P<0.05). In summary, inhibition of TRAF6 might suppress osteoclast production.

Effect of down-regulation of TRAF6 on osteoblast production

As shown in *Figure 4A*, HE stains showed that osteoblast loss was observed in the RA group compared with the control group. However, after the levels of TRAF6 were inhibited, HE stains showed that osteoblast loss was recovered in the RA + TRAF6-shRNA group compared with the RA + shRNA-NC group. The osteoblast production was detected by Saffron O staining. The result showed that the number of osteoblasts was suppressed significantly in the RA group compared with the control group. However, after the levels of TRAF6 were inhibited, the number of osteoblasts was elevated significantly in the RA + TRAF6-shRNA group compared with the RA + shRNA-NC group. The contents of cartilage (*Figure 4B*) and trabecular bone (*Figure 4C*) were markedly decreased in the RA group compared with the control group, but were increased after the levels of TRAF6 were inhibited in the RA + TRAF6-shRNA group compared with the RA + shRNA-NC group (P<0.05). The mRNA and protein expression levels of OSX, CoL1A1, and DLx2 were detected by qRT-PCR and western blot (*Figure 4D,E*). The mRNA and protein expression levels of OSX, CoL1A1, and DLx2 were reduced in the RA group compared with the control group (P<0.05). However, after the levels of TRAF6 were inhibited, The mRNA and protein expression levels of OSX, CoL1A1, and DLx2 were enhanced obviously in the RA + TRAF6-shRNA group compared with the RA + shRNA-NC group (P<0.05). In brief, inhibition of TRAF6 might promote osteoblast production.

Effect of down-regulation of TRAF6 on MMPs

As shown in *Figure 5*, the mRNA and protein expression levels of TIMP-1, MMP-1, COX2, and qRT-PCR detected MMP-13 and western blot (*Figure 5A,B*). The mRNA and protein expression levels of TIMP-1 were reduced obviously in the RA group compared with the control group while increasing the mRNA and protein expression levels of MMP-1, COX2, and MMP-13 (P<0.05). However, after the levels of TRAF6 were inhibited, The mRNA and protein expression levels of TIMP-1were enhanced obviously in the RA + TRAF6-shRNA group compared with the RA + shRNA-NC group, while decreasing mRNA and protein

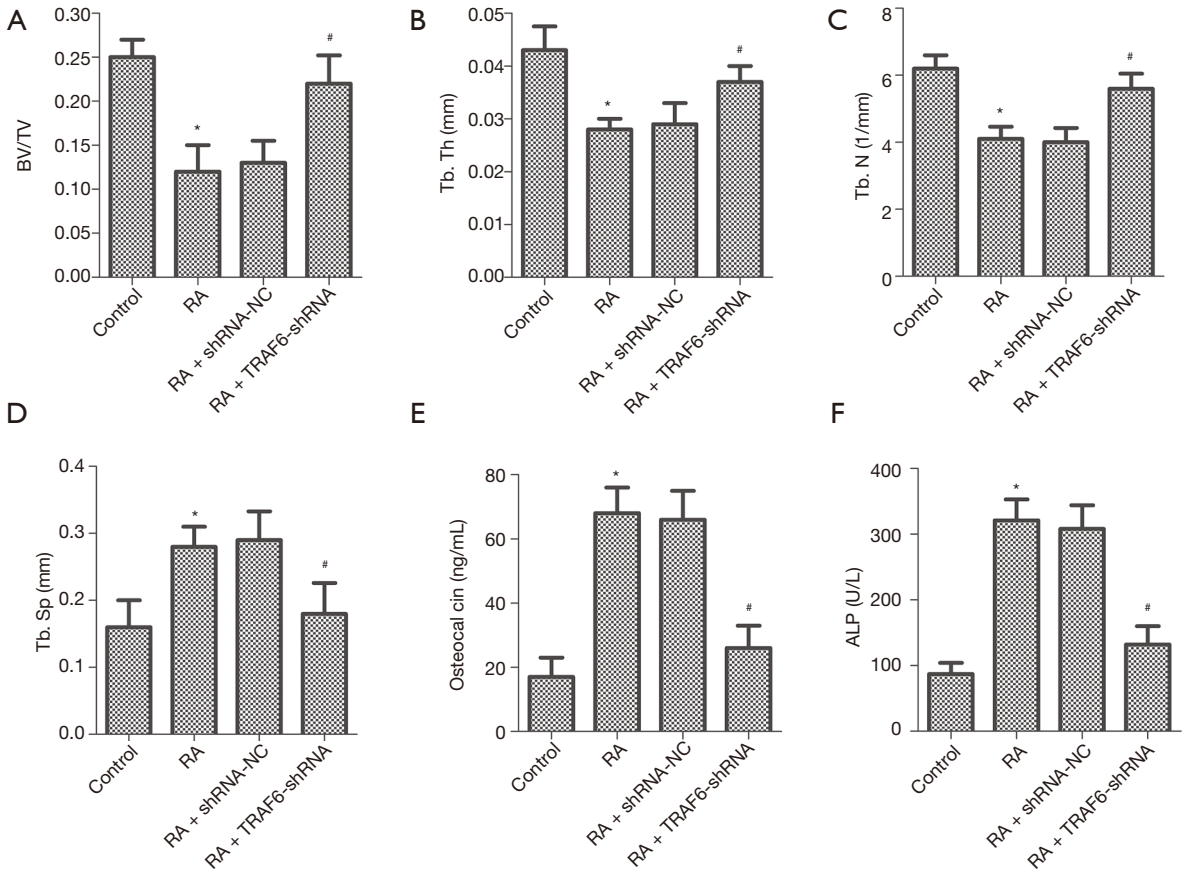

Figure 2 Effect of down-regulation of TRAF6 on bone-specific indicators. The statistical analysis of (A) BV/TV, (B) Tb.Th (mm), (C) Tb.N (mm), (D) Tb.Sp (mm), (E) osteocalcin (ng/mL) and (F) ALP (U/L). *, P<0.05, *vs.* control group; #, P<0.05, *vs.* RA + shRNA-NC group. TRAF6, tumor necrosis factor receptor-associated factor 6; BV/TV, bone volume/total volume; Tb.Th, trabecular bone thickness; Tb.N, trabecular bone number; Tb.Sp, trabecular bone space.

expression levels of TIMP-1, MMP-1, COX2, and MMP-13 (P<0.05). In summary, inhibition of TRAF6 might improve MMPs production.

Discussion

There are three significant players directly responsible for the pathogenesis of rheumatoid joint destruction, including the Synovial mesenchymal cells, matrix metalloproteinases (MMPs), and osteoclasts (23). The BV/TV value reflects the distribution density of trabecular bone and is an important index used to evaluate trabecular bone microstructure. In our research, the levels of BV/TV, Tb.Th and Tb.N were decreased in the RA group, while the levels of Tb.Sp, Osteocalcin, and ALP were increased. However, after the levels of TRAF6 were inhibited, the levels of BV/TV, Tb.Th and Tb.N were increased in the RA + TRAF6-shRNA group compared with the RA + shRNA-NC group, while the levels of Tb.Sp, Osteocalcin, and ALP were decreased. These results suggested that inhibition of TRAF6 broke the balance between osteoclast and osteoblast production.

It is widely known that osteoclasts have a unique ability to destroy bone and play a vital function in homeostatic bone remodeling and arthritic bone erosion. Osteoclasts are immune cells, not only mediating the bone destruction but also are involved in immune response (24). Recently, it was demonstrated that Osteoclasts play a critical role in bone destruction in RA. RA synovial tissue provides a suitable microenvironment for the differentiation of monocyte-macrophage lineage cells into osteoclasts is the main reason. Simultaneously, the formation of osteoclasts is induced by nuclear factor receptor activator kappa B ligand (RANKL) plays a critical regulatory role in the bone destruction of RA (25). Importantly, TRAF6 ubiquitin ligase is necessary

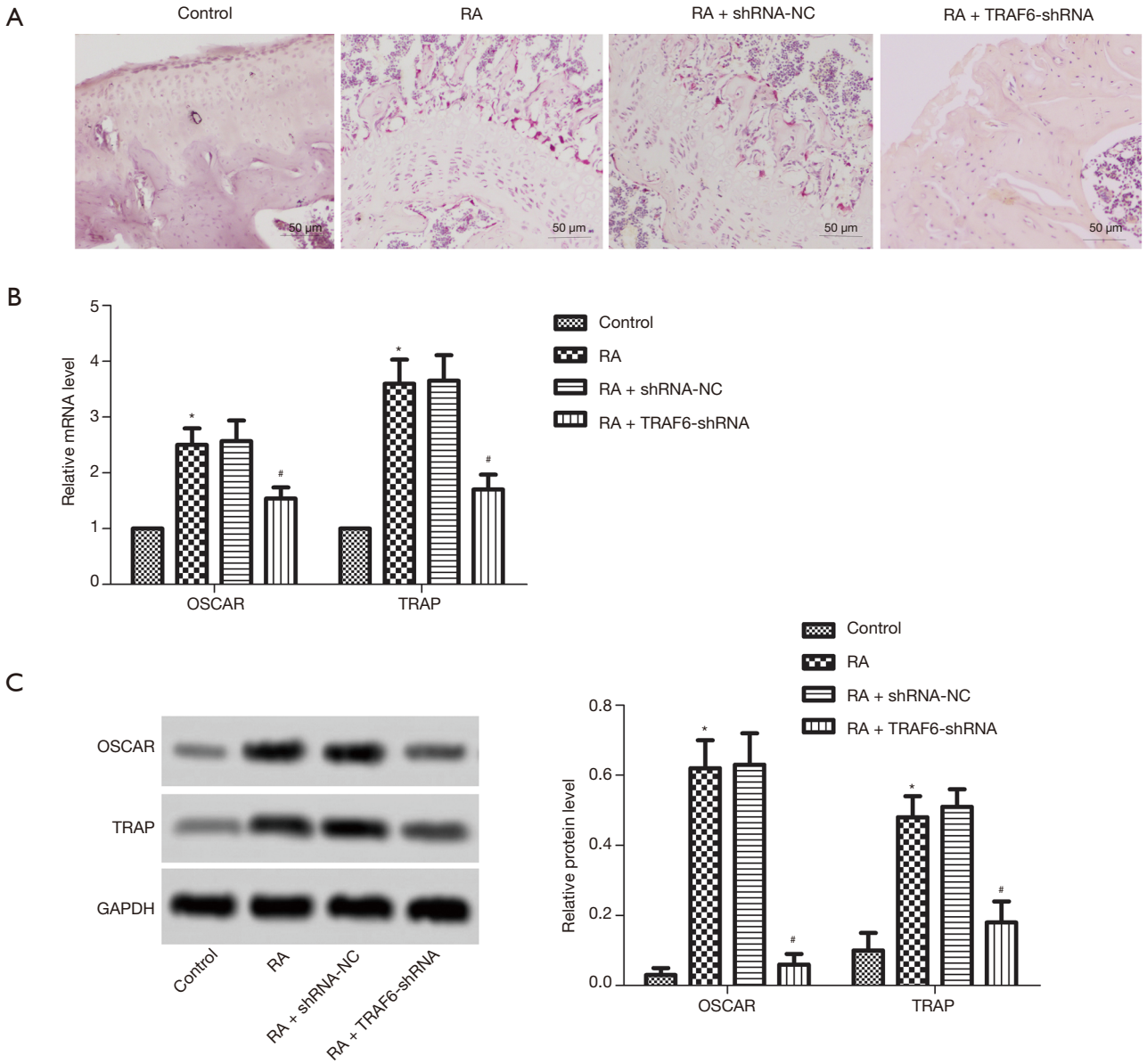

Figure 3 Effect of down-regulation of TRAF6 on osteoclast production. (A) TRAP staining detected the osteoclast production. Magnification 400×; (B) the mRNA expressive levels of OSCAR and TRAP were performed by qRT-PCR; (C) the expressive protein levels of OSCAR and TRAP were performed by western blot. *, P<0.05, *vs.* control group; #, P<0.05, *vs.* RA + shRNA-NC group. TRAF6, tumor necrosis factor receptor-associated factor 6; TRAP, tartrate-resistant acidic phosphatase; OSCAR, osteoclast-associated receptor; qRT-PCR, real-time reverse transcription-polymerase chain reaction.

for RANKL signaling and differentiation of osteoclast (26). Previous research has demonstrated an association between a genetic variant of TRAF6 and low bone mineral density (BMD) among patients with RA (27).

Elevated synovial expression of TRAF6 is related to the severity of synovitis and density of CD68+ cell, and takes part in the pathogenesis of RA synovitis and differentiation of osteoclast (28). In our research, the down-regulation of TRAF6 significantly decreased the number of osteoclasts in TRAP staining. Also, after the levels of TRAF6 were

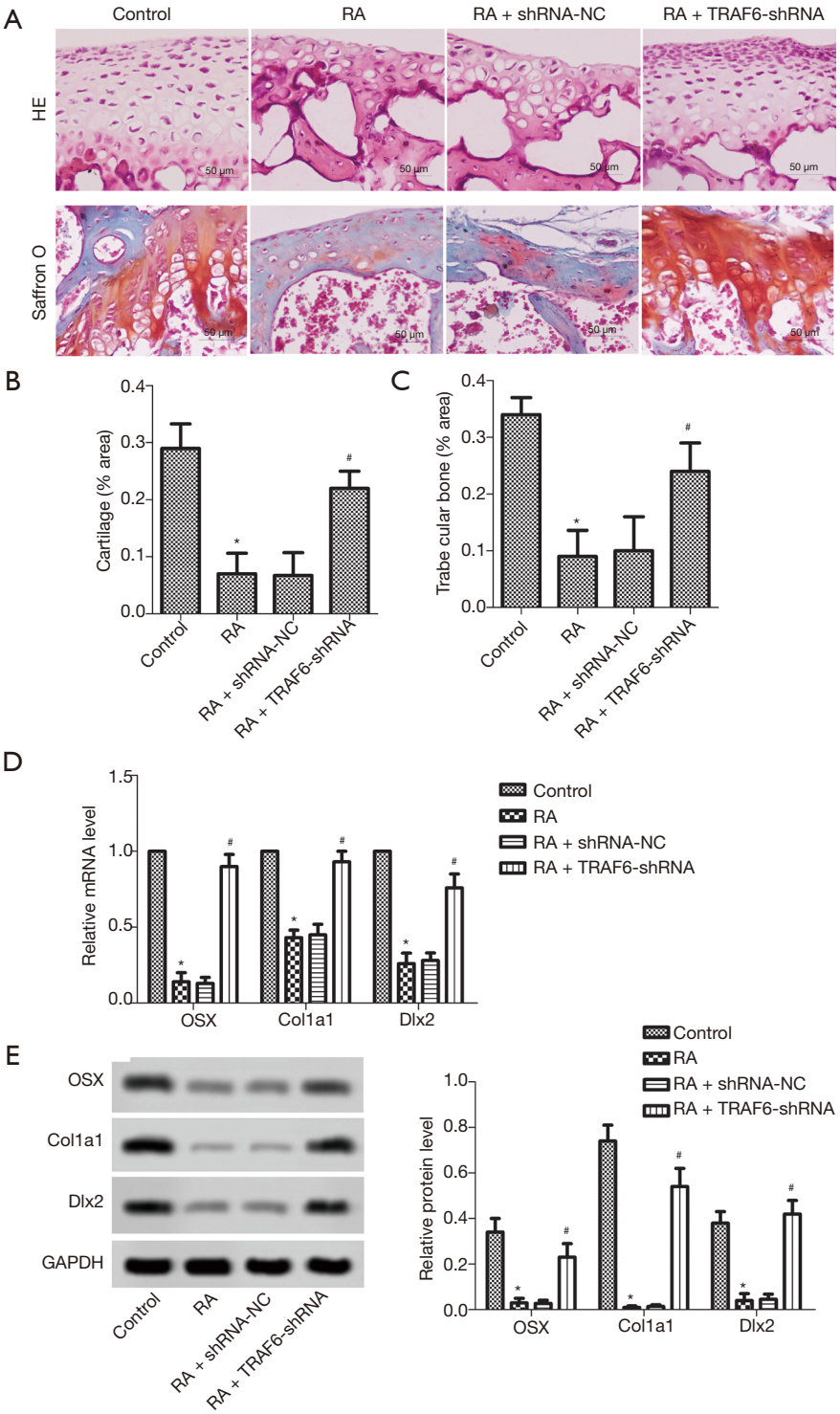

Figure 4 Effect of down-regulation of TRAF6 on osteoblast production. (A) HE stains. The osteoblast production was detected by Saffron O staining. Magnification 400×. The relative area of collagen (B) and trabecular bone (C) were analyzed using Image J software. (D) The mRNA expressive levels of OSX, CoL1A1, and DLx2 were performed by qRT-PCR. (E) The expressive protein levels of OSX, CoL1A1, and DLx2 were performed by western blot. *, P<0.05, *vs.* control group; #, P<0.05, *vs.* RA + shRNA-NC group. TRAF6, tumor necrosis factor receptor-associated factor; OSX, osterix; COL1A1, Collagen type I alpha 1; Dlx2, Distal-less homeobox2; qRT-PCR, real-time reverse transcription-polymerase chain reaction.

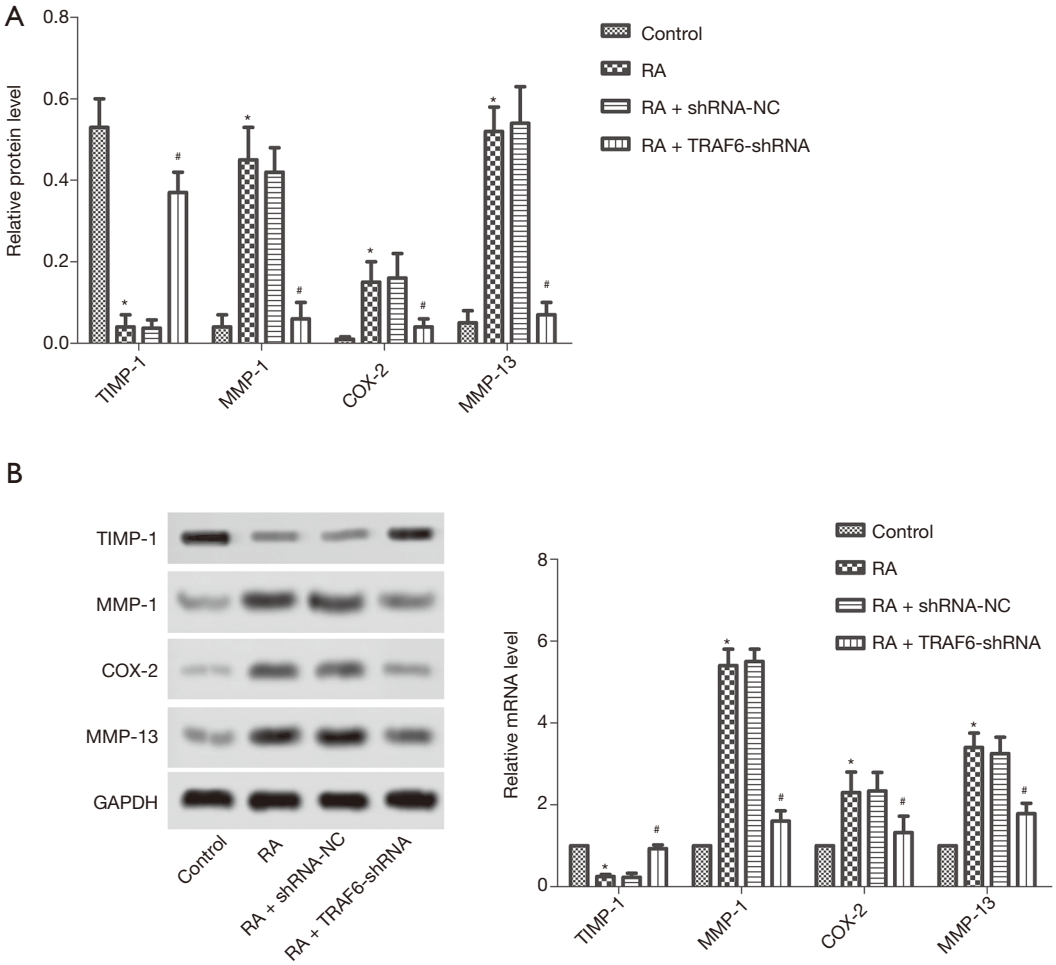

Figure 5 Effect of down-regulation of TRAF6 on MMPs. (A) The mRNA expressive levels of TIMP-1, MMP-1, COX2, and MMP-13 was performed by qRT-PCR; (B) the expressive protein levels of TIMP-1, MMP-1, COX2, and MMP-13 were performed by western blot. *, P<0.05, *vs.* control group; #, P<0.05, *vs.* the RA + shRNA-NC group. TRAF6, tumor necrosis factor receptor-associated factor; MMP, matrix metalloproteinase; qRT-PCR, real-time reverse transcription-polymerase chain reaction.

inhibited, the mRNA and protein expression levels of OSCAR and TRAP were reduced obviously in the RA + TRAF6-shRNA group compared with the RA + shRNA-NC group. Recently, a study has exhibited that OSCAR-collagen signaling in monocytes plays a proinflammatory role and facilitates monocytes differentiation into osteoclasts and bone resorption and contributes to the pathogenesis of RA (29,30). The TRAP isoform 5b is a potential serum marker for osteoclastic activity (31). These results suggested that inhibition of TRAF6 suppressed osteoclast production in the development process of RA.

Bone homeostasis lies with the absorption of bone by osteoclasts and the formation of bone by osteoblasts. This imbalance in the tight coupling process can bring about diseases, including osteoporosis. Together, osteoblasts produce cytokines to regulate the differentiation and formation of osteoclasts. Also, osteoclast progenitor cells can reduce the sensitivity to osteoblast-induced apoptosis through the Fas-Ligand (FasL)/FAS pathway (32). Research has exhibited B cells restrain bone formation in RA by inhibiting osteoblast differentiation (33). The pathogenesis of RA is related to the inhibition of osteoblast differentiation. Restoring the function of osteoblasts plays a vital function in the treatment of RA. Interleukin (IL)-35 stimulates the differentiation of basal and TNF-activated osteoblasts via regulating the Wnt/catenin signaling pathway. Therefore, IL-35 has essential significance in the treatment of RA bone loss drugs and drug applications (34).

OSX is an osteoblast-specific transcription factor, which is essential for osteoblast differentiation and bone formation. It is worth noting that OSX is the upstream regulator of Special AT-rich sequence-binding protein 2 (Satb2) during bone formation (35). CoL1A1-driven transgenic markers are in the osteoblast lineage progression (36). DLX2 can induce Wnt1 transcription and regulate Wnt/catenin signaling pathway to accelerate the osteogenic differentiation of human bone marrow mesenchymal stem cells (hBMSCs) (37). In our research, HE stains showed that osteoblast loss was observed in the RA group. However, after the levels of TRAF6 were inhibited, HE stains showed that osteoblast loss was recovered in the RA + TRAF6-shRNA group compared with the RA + shRNA-NC group. The result showed that the number of osteoblasts was suppressed significantly in the RA group. However, after the levels of TRAF6 were inhibited, the number of osteoblasts was elevated significantly in the RA + TRAF6-shRNA group compared with the RA + shRNA-NC group. The contents of cartilage and trabecular bone were markedly decreased in the RA group, but were increased after the levels of TRAF6 were inhibited in the RA + TRAF6-shRNA group compared with the RA + shRNA-NC group. The mRNA and protein expression levels of OSX, CoL1A1, and DLx2 were reduced in the RA group. However, after the levels of TRAF6 were inhibited, the mRNA and protein expression levels of OSX, CoL1A1, and DLx2 were enhanced obviously in the RA + TRAF6-shRNA group compared with the RA + shRNA-NC group. These results suggested that inhibition of TRAF6 promoted osteoblast production.

Cartilage degradation is put down to metalloproteinases (MPs) belonging to the MMP family; further, integrins and metalloproteinases with thrombospondin type 1 motifs produced by inflamed joint tissues (38). The expression of MMPs was detected in the joint ligaments, tendons, and cartilage tissues. They are known to contribute to the development, remodeling, and maintenance of healthy tissues through their ability to lyse various extracellular matrix substrates. Their role has been extended to cell growth, migration, differentiation, and apoptosis (39). Chondrocytes play a role in the development of RA through TMEM147-mediated NF-κB activation and propose a treatment strategy for RA (40). Previous results have revealed that siTRAF6 can attenuate arthritis in mice with collagen-induced arthritis, as have evidenced by the reduction of serum anti-CII, MMP-1, MMP-3, and MMP-9 and the decreasing of histological damage. It is worth noting the blockade of TRAF6 inhibited the migration and invasion of human RA-FLSs with IL-1β-stimulated (41). The correlation between TIMP-1 baseline levels and peri-articular bone loss over one year suggests that TIMP-1 can be used as a biomarker of peri-articular bone loss in the early stage of RA (42).

Interestingly, RA synovial fibroblasts promote TREM-1 expression in monocytes via COX-2/prostaglandin E2 (PGE2) pathway (43). Kaempferol suppresses synovial fibroblast proliferation and the production of and MMPs, COX-2, and PGE2 in RA (44). In our research, the mRNA and protein expression levels of TIMP-1 were reduced obviously in the RA group, while increasing the mRNA and protein expression levels of MMP-1, COX2, and MMP-13. However, after the levels of TRAF6 were inhibited, The mRNA and protein expression levels of TIMP-1were enhanced obviously in the RA + TRAF6-shRNA group compared with the RA + shRNA-NC group, while decreasing mRNA and protein expression levels of TIMP-1, MMP-1, COX-2 and MMP-13 ($P<0.05$). These results suggested that inhibition of TRAF6 improved MMPs production.

In conclusion, the down-regulation of TRAF6 restored the balance of osteoclasts and osteoblasts and improved the expression of MMP, which plays a vital role in the recovery of RA.

Acknowledgments

Funding: The Ningxia Natural Science Foundation Project supported this work (2020 AAC02035), National Natural Science Foundation of China Regional Science Foundation Project (81660281), Ningxia Hui Autonomous region Department of People's and Social Affairs overseas students innovation and entrepreneurship project (2017).

Footnote

Reporting Checklist: The authors have completed the ARRIVE reporting checklist. Available at http://dx.doi.org/10.21037/apm-20-1894

Data Sharing Statement: Available at http://dx.doi.org/10.21037/apm-20-1894

Conflicts of Interest: All authors have completed the ICMJE

uniform disclosure form (available at http://dx.doi.org/10.21037/apm-20-1894). The authors have no conflicts of interest to declare.

Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. All animal care and handling were performed considering the National Institutes of Health (NIH) Guidelines for the Care and Use of Laboratory Animals and were approved by the People's Hospital of Ningxia Hui Autonomous Region. The research was reviewed and approved by the Ethics Committee for Experimental Animal Management and Animal Welfare of People's Hospital of Ningxia Hui Autonomous Region.

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(English Language Editor: J. Chapnick)

Cite this article as: Guo J, Cao X, Ma X, Hao C, Wu L, Zhang M, Yang Y, Zhao J, Chen K, Yin Z. Tumor necrosis factor receptor-associated factor 6 (TRAF6) inhibition modulates bone loss and matrix metalloproteinase expression levels in collagen-induced rheumatoid arthritis rat. Ann Palliat Med 2020;9(6):4017-4028. doi: 10.21037/apm-20-1894

