

# Aliskiren has chondroprotective efficacy in a rat model of osteoarthritis through suppression of the local renin-angiotensin system

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**Abstract.** The local renin-angiotensin system (RAS) has been reported to have an important role in the pathogenesis and progression of metabolic bone diseases, including osteoarthritis (OA). Aliskiren is the first in a new class of orally effective direct renin inhibitors and is approved for the treatment of hypertension in humans. However, its efficacy in patients with OA is unknown. A rat model of OA was induced to investigate the potential efficacy of aliskiren. Effects of aliskiren on the cartilage structure were detected by safranin O staining and its effects on the widths of the proliferation zone and hypertrophic zone (HZ) of chondrocytes were analyzed by Masson's staining. Tartate-resistant acid phosphatase staining was used to evaluate the effects of aliskiren on osteoclasts in the chondrocytes. Relative histological analyses were performed. Additionally, the expression levels of factors associated with osteoclast differentiation (receptor activator of nuclear factor  $\kappa$ B ligand and osteoprotegerin), articular cartilage destruction [tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) and matrix metalloproteinase 9] and osteoblast differentiation [runt related transcription factor 2 (Runx2)], along with RAS components (renin, renin-receptor, angiotensin type 1 receptor (AT1R), AT2R, angiotensin converting enzyme (ACE) and angiotensin II (Ang II)] were detected in samples from the proximal tibias. Aliskiren did not fully suppress the inflammatory reaction in OA model animals and had marginal regulatory effects on biochemical bone markers induced by OA. However, aliskiren attenuated cartilage destruction, abnormal cartilage cellularity and the expansion of the HZ of chondrocytes, and significantly attenuated the expression of interleukin-1, TNF- $\alpha$ , Runx2 and procollagen type I N-terminal propeptide. These chondroprotective properties were accompanied by reductions in the levels of RAS components (renin, Ang II, ACE and AT1R),

indicating that aliskiren exerts multiple effects of on bone formation, osteoblast differentiation and articular cartilage protection via the RAS. OA activates the local bone RAS, inhibits bone formation and stimulates bone resorption. Aliskiren, a renin inhibitor, demonstrated chondroprotective efficacy in a rat model of OA through suppression of the local RAS.

## Introduction

Osteoarthritis (OA) is the most common form of arthritis, characterized by a progressive loss of articular cartilage, osteophyte formation, and changes within subchondral bones, resulting in debilitating chronic pain in affected individuals (1). Multiple causal factors, including ageing, traumatic injuries, biomechanical factors that affect the joints, abnormal gait biomechanics, and genetic and metabolic elements associated with obesity and inflammation, commonly lead to the development of OA (2-4), which affects ~630 million people worldwide. Recommendations for the non-pharmacological treatment, including weight loss, low-impact exercise and the strengthening of muscles, in addition, non-steroidal anti-inflammatory medicines (NSAIDs), such as cortisone and hyaluronic acid, are commonly used for pain relief, and the effects of NSAIDs are well established (5). Though surgical treatments are suggested, they also have the potential to cause infection and damage to surrounding structures (6,7).

Naturally derived chemicals, synthetic agents and biological molecules have been tested in pre-clinical and clinical studies (8,9). The present study focused on aliskiren, a nonpeptide piperidine designed by molecular modeling of transition-state analogs of angiotensinogen. The drug is a renin-angiotensin system (RAS) inhibitor and act as a potential chondroprotective agent (10). Previously, it has been demonstrated by that the local tissue RAS has an important role in bone metabolism independent of the systemic involvement of the RAS, identified to regulate regeneration, cell growth, apoptosis inflammation and angiogenesis (10). Furthermore, different components of the RAS have been observed to be synthesized and active in osteoblasts and osteoclasts, and also expressed in cartilage cells and bone joints (11-15). For example, Tsukamoto *et al* (16) reported that local RAS components were expressed particularly in the chondrocytes of epiphyseal plates in the tibia and spine.

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Angiotensin II (Ang II) is the main effector molecule in the systemic and local RAS. Ang II, which is produced by cleavage of angiotensin I (Ang I) by angiotensin-converting enzyme (ACE) and binds to angiotensin type 1 and 2 receptors (AT1R and AT2R) to exert its biological effects, has deleterious effects on bones (17). It has been reported that human articular chondrocytes express Ang II receptors (18). Furthermore, Kawahata *et al* (19) investigated the direct effects of Ang II, via Ang II receptors, on differentiation, proliferation and apoptosis of chondrocytes *in vivo*. Notably, the formation of Ang II can be inhibited by aliskiren, as it has been demonstrated to be a direct inhibitor of renin, which hydrolyzes angiotensinogen to Ang I (20,21). A previous study reported that treatment with aliskiren markedly increased bone volume, trabecular bone number, connectivity density and bone mineral density, and reduced trabecular bone separation in ovariectomized mice compared with vehicle-treated mice (17), suggesting that aliskiren may be a useful potential strategy to treat OA. However, the mechanism and exact cause of the effects remain unclear.

The current study aimed to investigate the mechanism of the effects of aliskiren in an animal model of OA. Specific outcomes demonstrated that aliskiren attenuated cartilage destruction, abnormal cartilage cellularity and the expansion of the hypertrophic zone (HZ) in chondrocytes. Additionally, aliskiren significantly attenuated expression of interleukin-1 (IL-1), tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ), runt related transcription factor 2 (Runx2) and procollagen type I N-terminal propeptide (PINP) in the proximal tibias. These chondroprotective properties were associated with reductions of RAS components (renin, renin receptor, Ang II, ACE and AT1R), indicating the multiple effects of aliskiren on bone formation, osteoblast differentiation and articular cartilage protection occur via the local RAS.

## Materials and methods

**Animals.** Wistar rats (n=30; 6-week-old; 140-230 g) were obtained from the Chinese Academy of Medical Sciences (Beijing, China). The animals were housed three per cage in a room with controlled temperature conditions (21-22°C) and lighting (12 h light/dark cycle) with access to sterile food and water. All animal procedures were approved by the Animal Ethics Committee of Soochow University (Suzhou, China).

### *Induction of OA in the rats and treatment with aliskiren.*

Animals were randomly assigned to treatment groups prior to the start of the study. OA was surgically induced in the left knees by transecting the medial collateral ligament and removing the medial meniscus. Animal groups included age-matched sham control (sham), OA placebo (OA group) and OA treated with aliskiren (n=10 in each group). The sham surgery consisted of incision and suture. A total of 3 days after the induction of OA, aliskiren (1.5 mg/kg) was administered daily into an intra-articular space, while solvents only (49.5% polyethylene glycol400 and 0.5% Tween-80 in PBS) were used in the placebo groups for 14 days. Following this, systolic blood pressure (SBP) was measured using the tail-cuff plethysmography method (LE 5001 Pressure Meter; Letica Scientific Instrument, Barcelona, Spain). Rats were anaesthetized and an

injection was conducted to the intercondylar fossa from the patellar tendon in a sagittal plane to avoid potential damage to the loadbearing cartilage surface; rats were subsequently sacrificed.

**Analysis of the physiological and biochemical markers in serum.** The detection of IL-1 (R&D Systems, Inc., Minneapolis, MN, USA; cat. no. SRLB00), IL-6 (R&D Systems, Inc.; cat.no. SR6000B), matrix metalloproteinase 9 (MMP9) activity (R&D Systems, Inc.; cat. no. RMP900) and PINP (Sangon Biotech Co., Ltd., Shanghai, China; cat. no. C506172) were measured using commercially available ELISA kits, according to the manufacturer's instruction. The renin assay was performed as previously described (14).

**Staining and histological analyses.** Tibia samples were decalcified in 10% EDTA for 2 weeks, embedded in paraffin and sectioned at 4  $\mu$ m thickness. Following deparaffinization and rehydration, slides were stained with Weigert's iron hematoxylin solution for 5 min. They were differentiated in 1% acid-alcohol, and stained with 0.02% fast green solution for 3 min at room temperature. They were then rinsed in 1% acetic acid for 5 min, stained in 1% safranin O solution for 30 min and treated with graded ethyl alcohol and xylene at room temperature (22). The sections were imaged using a light optical microscope (Olympus Corporation, Tokyo, Japan). A modified Mankin's histological score (1) [original scoring proposed by Mankin *et al* (23)] was used to score histological injuries of the articular cartilage as follows. The structure was scored on a scale of 0-6 as follows: 0, normal; 1, irregular surface, including fissures into the radial layer; 2, pannus; 3, absence of superficial cartilage layers; 4, slight disorganization (cellular row absent, some small superficial clusters); 5, fissure into the calcified cartilage layer; and 6, disorganization (chaotic structure, clusters, and osteoclasts activity). Joint space width was estimated by measuring the sum of the nearest distance of medial and lateral tibiofemoral joints using X-ray tomosynthesis, as previously described (24). Histological evaluation was performed by two independent experienced researchers who were blinded to the treatment group.

For Masson staining, freshly dissected tibias were dissected and fixed overnight with 4% formaldehyde in PBS (pH7.2), processed and embedded in paraffin. Tibia sections were cut at 3 mm and the sections were stained with Masson, performed as per the manufacturer's instructions (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany). Cellular abnormalities were scored on a scale of 0-3 as follows: 0, normal; 1, hypercellularity, including small superficial clusters; 2, clusters; and 3, hypocellularity. The matrix staining was scored on a scale of 0-4 as follows: 0, normal/slight reduction in staining; 1, staining reduced in the radial layer; 2, staining reduced in the interterritorial matrix; 3, staining present only in the pericellular matrix; and 4, staining absent.

Tartate-resistant acid phosphatase (TRAP) staining was used for the identification of osteoclasts in the metaphysis of tibias according to the manufacturer's directions (Sigma-Aldrich; Merck KGaA).

**Immunohistochemical analyses.** Slides for immunohistochemistry were deparaffinized and rehydrated using a graded

ethanol series. The metaphysis of tibias specimens were depleted of endogenous peroxidase activity by adding methanolic H<sub>2</sub>O<sub>2</sub>, and then blocked with 10% normal goat serum (Epitomics; Abcam, Shanghai, China) for 30 min. Samples were incubated overnight at 4°C with rabbit anti-renin antibody (1:50; Santa Cruz Biotechnology, Inc., Dallas, TX, USA; cat. no. sc-137252). The samples were then incubated for 1 h at room temperature with a biotinylated rabbit anti-mouse secondary antibody (1:200; Vector Laboratories, Burlingame, CA, USA; cat. no. BA-9200). The bound secondary antibody was then amplified using the Elite ABC kit, according to the manufacturer's instructions (Vector Laboratories, Inc.). The antibody-biotin-avidin-peroxidase complex was visualized using 0.02% 3,3'-diaminobenzidine staining for 10 min at room temperature. The sections were mounted onto gelatin-coated slides that were air-dried overnight at room temperature, the coverslips were then mounted using Permount medium (Thermo Fisher Scientific, Inc., Waltham, MA, USA) and imaged using a light optical microscope (Olympus Corporation).

**Reverse transcription-quantitative polymerase chain reaction (RT-qPCR).** Total RNA was isolated from proximal tibias using the TRIzol method (Invitrogen; Thermo Fisher Scientific, Inc.). cDNA was prepared by reverse transcription of single-stranded RNA using the High Capacity cDNA Reverse Transcription kit (Applied Biosystems; Thermo Fisher Scientific, Inc.), according to the manufacturer's instructions. Briefly, 1 or 2 µg of mRNA, 2 µl of RT buffer, 0.8 µl of dNTP mixture, 2 µl of RT random primers, 1 µl of Multi-Scribe reverse transcriptase and 4.2 µl of nuclease-free water were used for each cDNA synthesis. Reactions were incubated in a PCR thermocycler at 25°C for 10 min, 37°C for 2 h and 85°C for 5 min; they were then cooled to 4°C. Following RT, cDNA was stored at -20°C. RT-qPCR was carried out using the SYBR<sup>®</sup> Premix Ex Taq<sup>™</sup> kit (Takara Bio, Inc., Otsu, Japan), according to the manufacturer's instructions. The 20 µl reaction mix consisted of 2 µl 30-fold diluted 1st-strand cDNA, 10 µl 2X SYBR<sup>®</sup> Premix Ex Taq<sup>™</sup>, 0.4 µl 10 µM forward and reverse primer, 0.4 µl 50X ROX Reference Dye and 6.8 µl DEPC-treated water. The primer pairs used in these reactions were as follows: osteoprotegerin (OPG), forward, 5'-GCACATTTGGCCTCCTGCTAATTC-3' and reverse, 5'-ACTCTCGGCATTCACCTTTGGTCCC; receptor activator of nuclear factor κB ligand (RANKL), forward, 5'-CAGCCA TTTGCA CACCTCACCATC-3' and reverse, 5'-TTTCGT GCTCCC TCCTTTCATCAG-3'; Runx2, forward, 5'-GAA CCAAGAAGGCACAGACA and reverse, 5'-AACTGCCTG GGGTCTGAAAA-3'; TNF-α, forward, 5'-CAAGGAGGA GAAGTTCCCAA-3' and reverse, 5'-CGGACTCCGTGATGT CTAAG-3'; carbonic anhydrase II (CAII), forward, 5'-CCA GTTTCACCTTTCACCTG-3' and reverse, 5'-AGGCAGGTC CAATCTTCAA-3'; MMP9, forward, 5'-GCCATTGCTGAT ATCCA-3' and reverse, 5'-GCCTTGCTCTTGGTAGTGA-3'; GAPDH, forward, 5'-ACCACAGTCCATGCCATCAC-3' and reverse, 5'-TCC ACC ACC CTG TTG CTG TA-3'. Reactions were performed in an ABI7300 Real-Time quantitative instrument (Applied Biosystems; Thermo Fisher Scientific, Inc.). The thermocycling conditions were as follows: Initial denaturation at 95°C for 30 sec, followed by 40 cycles of 95°C for 5 sec and 60°C for 31 sec. The expression level of the internal control GAPDH was used as a housekeeping gene, and the comparative

2<sup>-ΔΔC<sub>q</sub></sup> method (25) was used to quantify gene expression levels. The products were then analyzed by electrophoresis on a 1% agarose gel and visualized by staining with ethidium bromide.

**Western blot analysis.** Protein was collected from rat proximal tibias that were lysed in radioimmunoprecipitation buffer (RIPA) containing protease inhibitors at 4°C for 30 min. Cell lysates were prepared with a RIPA lysis buffer kit (Santa Cruz Biotechnology, Inc.), and the protein concentrations were quantified using a Bio-Rad protein assay (Bio-Rad Laboratories, Inc., Hercules, CA, USA). Proteins (30 µg) were separated on 8% SDS-PAGE and transferred to polyvinylidene difluoride membranes (Amersham; GE Healthcare, Chicago, IL, USA). The membranes were blocked in 5% non-fat milk (Merck KGaA) overnight at 4°C. Transferred membranes were then stained with the following primary antibodies: Anti-renin (1:500; Abcam, Cambridge, MA, USA; cat. no. ab180608), anti-AT1R (1:200; R&D Systems, Inc.; cat. no. YB-0,1173), anti-AT2R (1:200; R&D Systems, Inc.; cat. no. YB-0,1110), anti-ACE (1:200; Boster Biological Technology, Pleasanton, CA, USA; cat. no. PB0089), anti-Ang II (1:200; Abcam; cat. no. EPR2931) and anti-β-actin (1:200; Abcam; cat. no. ab8227) overnight at 4°C. Subsequently, protein bands were detected by incubation with a horseradish peroxidase-conjugated secondary antibody (1:1,000; Beijing Zhongshan Golden Bridge Biotechnology Co., Ltd., Beijing, China; cat. no. A50-106P) at room temperature for 1 h. Signals were detected using an enhanced chemiluminescence kit (Wuhan Booute Biotechnology Co., Ltd, Wuhan, China; cat. no. orb90504) and exposed to Kodak X-OMAT film (Kodak, Rochester, NY, USA). Each experiment was performed at least three times and the results were analyzed using Alpha View Analysis Tools (AlphaView SA software, version 3.2.2; ProteinSimple; Bio-Techne, Minneapolis, MN, USA).

**Statistical analysis.** Three or four independent experiments were conducted and data are expressed as the mean ± standard deviation. Statistical differences among multiple independent groups were determined using one-way analysis of variance followed by a Dunnett's post hoc test. For the evaluation of histological scores, non-parametric statistical analysis (Kruskal-Wallis and Mann-Whitney U tests) was conducted. Statistical analyses were performed using SPSS statistical software package standard version 16.0 (SPSS, Inc., Chicago, IL, USA). P<0.05 was considered to indicate a statistically significant difference.

## Results

**Physiological and biochemical markers in serum.** According to detection of the physiological and biochemical markers in serum (Table I), there were no significant differences in the body weight and SBP among the sham, OA and aliskiren groups. The level of renin in serum was increased in OA animals compared with the sham group, and its level was comparable in the sham and aliskiren groups. Additionally, IL-1 and IL-6 levels in OA animals were significantly higher than the sham group, indicating that the inflammatory reactions were induced by OA. Following treatment with

aliskiren, the IL-1 level was decreased compared with the OA group, whereas IL-6 remained at a comparable level to the OA group. PINP, a bone formation and OA biological marker, was correlated with functional impairment (26), thus in the OA and aliskiren group, the PINP concentration exhibited a significant reduction compared with the sham group. Aliskiren did not fully suppress the inflammatory and had marginal upregulatory effects on biochemical bone markers induced by OA; however, the renin and IL-6 concentrations were significantly reduced by aliskiren compared with the OA group.

*Chondroprotective efficacy of aliskiren in OA rat.* In order to evaluate whether aliskiren had a chondroprotective effect on OA, tibias from each of the three treatment groups were isolated and stained, then analyzed microscopically. The safranin O staining results revealed that the chondrocytes were uniformly stained. The layer structures of cartilage and tidemarks were clear in the sham group, indicating the complete cartilage structure. By contrast, the OA-induced group exhibited smaller red-stained areas (Fig. 1A), indicating fissures in the calcified cartilage layer. Consistent with this, the OA-induced group revealed significantly higher staining and structure scores compared with the sham group (Fig. 1B and C). These histomorphological features in the cartilage were significantly reduced in the aliskiren-treatment group compared with the OA group, exhibiting relatively higher red areas and a lower staining score than the OA group, which indicated that aliskiren suppressed cartilage injury induced by OA.

Subsequently, Masson staining was performed to measure the widths of the proliferation zone (PZ) and hypertrophic zone (HZ) of the chondrocyte area in the growth plate. The cellular abnormalities were scored on a scale of 0-3. The results demonstrated that OA induced the expansion of the HZ in the chondrocyte zone of the growth plate, was associated with a decrease in the PZ and an increase in the cell scores (Fig. 1D-F). To some extent, aliskiren reversed the cartilage cell dysfunction induced by OA, exhibiting significantly increased PZ width and reduced HZ widths, along with significantly decreased cell scores compared with the OA group. Taken together the results suggest that aliskiren had chondroprotective efficacy in the OA group by suppressing cartilage injury and reversing cartilage cell dysfunction.

*Aliskiren exhibits no suppressive effects on osteoclasts.* Osteoclasts numbers were detected by TRAP staining (Fig. 2A and B) in the metaphysis of tibias. In addition, the protein expression levels (Fig. 2C and D) of decoy receptor OPG and RANKL, the essential cytokines of osteoclast biology, were also analyzed. The results demonstrated that osteoclast numbers were significantly increased and OPG/RANKL ratio was decreased by OA compared with the sham group. The generation and differentiation of osteoclasts is activated by increased RANKL protein expression level and reduced OPG level; however, no significant differences were in the OPG/RANKL ratio following treatment with aliskiren. The results suggested that aliskiren exhibited no suppressive effects on differentiation and proliferation of osteoclasts.

Table I. Physiological and biochemical markers in serum.

Factor	Sham	OA	Aliskiren
Body weight (g)	25.4±2.4	24.7±2.8	25.1±2.2
SBP (mm Hg)	88.3±5.7	92.7±6.9	84.8±7.5
Renin (ng/ml)	126.5±10.7	195.4±28.2 <sup>a</sup>	141.2±18.7 <sup>b</sup>
IL-1 (pg/ml)	4.74±0.58	9.54±1.61 <sup>a</sup>	6.14±1.27 <sup>b</sup>
IL-6 (ng/ml)	22.6±2.7	55.8±12.3 <sup>a</sup>	61.6±15.8 <sup>a</sup>
PINP (ng/ml)	341.8±19.2	251.4±28.5 <sup>a</sup>	267.7±30.5 <sup>a</sup>

<sup>a</sup>P<0.05 vs. sham; <sup>b</sup>P<0.05 vs. OA. OA, osteoarthritis; IL, interleukin; SBP, systolic blood pressure; PINP, procollagen type I N-terminal propeptide.

*Effects of aliskiren on the expression of Runx2, MMP-9, CAII and TNF- $\alpha$ .* Numerous studies have reported that cartilage damage is associated with increased production of the MMPs, and TNF- $\alpha$  is the predominant catabolic cytokines involved in the destruction of the articular cartilage in OA both *in vitro* and *in vivo* experiments (27-29). Additionally, Runx2 is a transcription factor that promotes chondrocyte maturation and osteoblast differentiation (30), and the expression of CAII is characteristic of the early stage of osteoclast differentiation (31). To assess the effects of aliskiren on OA-associated gene expression, the above factors were detected and analyzed (Fig. 3). Significant increases in the mRNA expressions of MMP-9, CAII and TNF- $\alpha$ , and a significant decrease in Runx2 were observed in the OA group compared with the sham group. Among them, the increased TNF- $\alpha$  level and reduced level Runx2 were significantly abrogated following aliskiren treatment, while MMP-9 and CAII were not changed by aliskiren. To a certain extent, the results indicated that aliskiren attenuated cartilage destruction and osteoblast differentiation inhibition by regulating in gene levels.

*Chondroprotective efficacy of aliskiren in the OA model via suppression of RAS.* The above results indicate that the chondroprotective efficacy of aliskiren in the OA animal model was characterized by suppressed cartilage injury and increased osteoblast differentiation. Aliskiren is an inhibitor of the RAS and the previous study was performed to determine whether the efficacy of aliskiren is dependent on the RAS.

In the current study, the renin level was analyzed by immunohistochemical analyses. The results demonstrated that the renin protein expression was observably induced in the metaphysis of tibias of the OA group compared with sham rat (Fig. 4A). Consistently, the renin mRNA and protein levels were also markedly increased in the OA group compared with the sham group, as demonstrated by RT-qPCR and western blotting, respectively (Fig. 4B and C). Aliskiren, a direct renin inhibitor, reduced renin expression compared with the OA group (Fig. 4). However, the expression of renin-receptor exhibited no differences among the different groups, which is in accordance with a previous study, demonstrating that aliskiren binds to the S3<sup>bp</sup> binding site of renin, which is essential for its activity, and thus reduces plasma renin activity

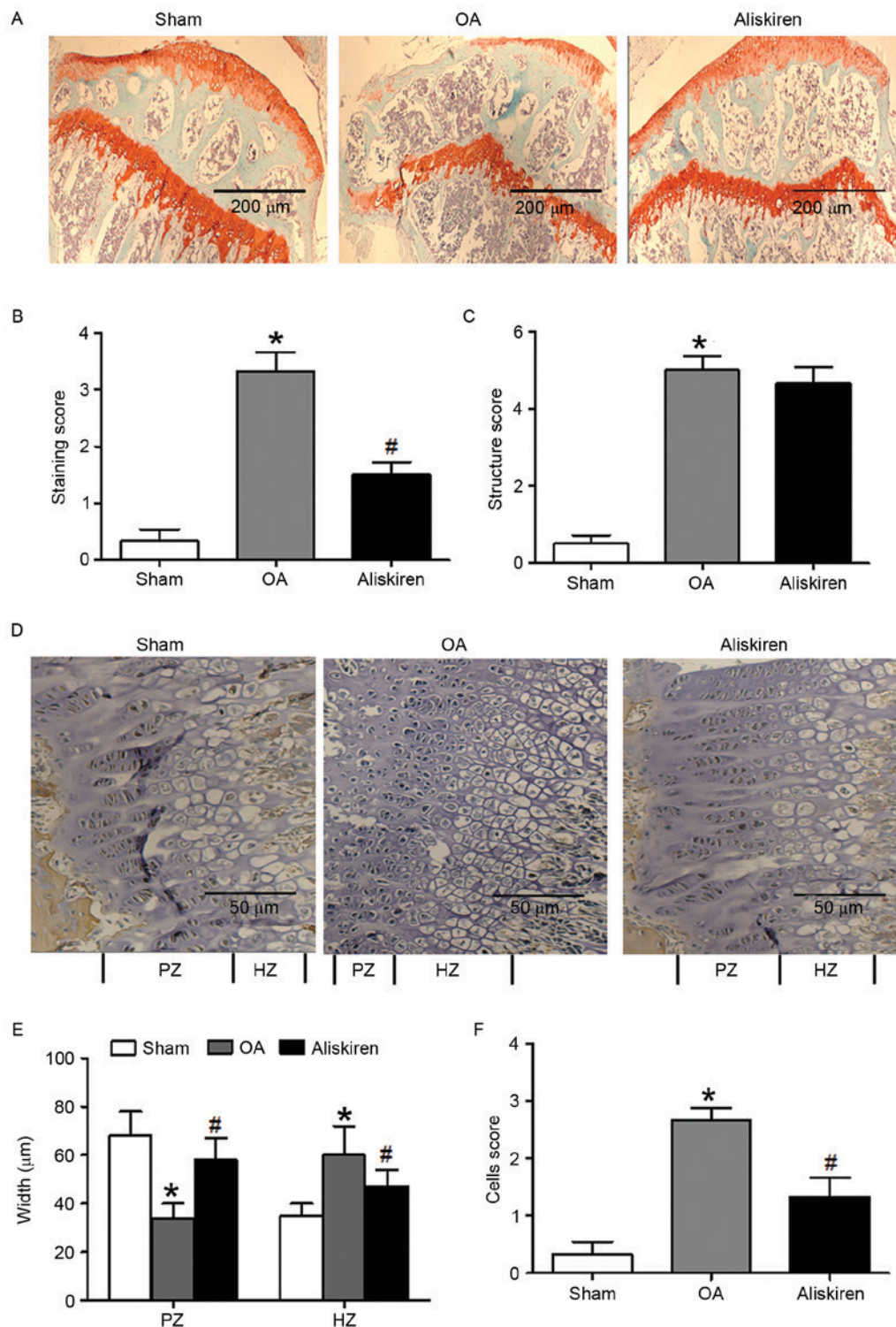


Figure 1. Effects of Aliskiren on the cartilage structure and chondrocytes. The cartilage structure was examined in sham, OA model and OA model with aliskiren treatment animals. (A) Images of the cartilage structure by safranin O staining following treatment with aliskiren. (B) Staining scores of cartilage structure following aliskiren treatment. (C) Structure scores of cartilage structure following treatment with Aliskiren. (D) Images of the PZ and HZ following treatment with a liskiren by Masson staining at growth plate of chondrocytes. (E) Widths of the PZ and HZ at growth plate of chondrocytes following treatment with aliskiren. (F) Cell scores following treatment with aliskiren. Data are expressed as the mean  $\pm$  standard deviation. \* $P < 0.05$  vs. sham; # $P < 0.05$  vs. OA. OA, osteoarthritis; PZ, proliferation zone; HZ, hypertrophic zone.

and suppresses the formation of both Ang I and Ang II. The renin-receptor was not involved in this process (32,33).

In the present study, the mRNA and protein expression levels of Ang II, ACE, AT1R and AT2R were also detected (Fig. 5). The results revealed that Ang II, ACE

and AT1R protein and mRNA levels were significantly increased in the OA group compared with the sham group, whereas AT2R was decreased in OA rat. Notably, these changes were abrogated by aliskiren treatment. The analysis suggests that aliskiren has chondroprotective

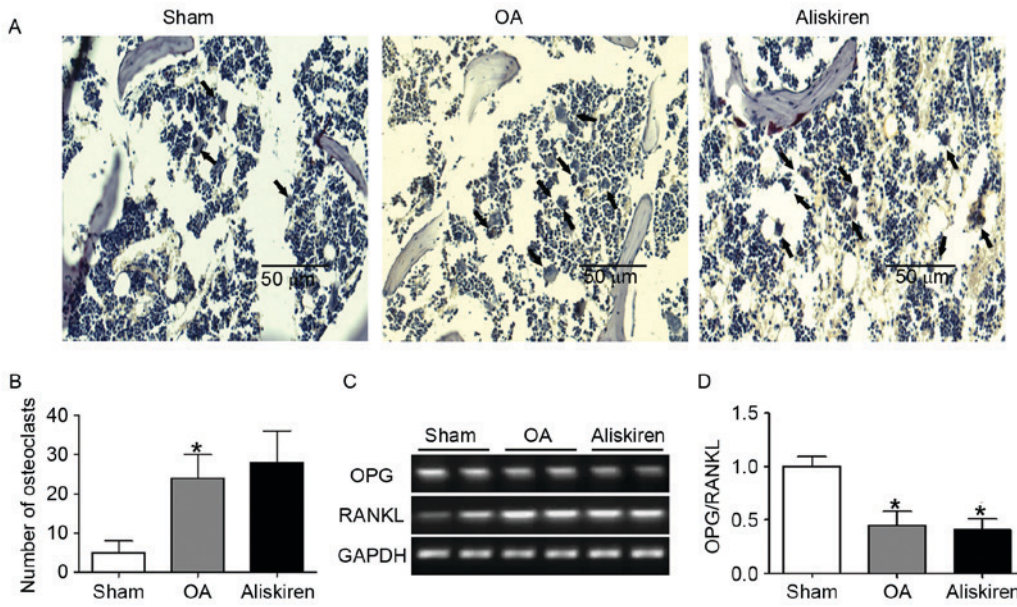


Figure 2. Effects of aliskiren on the osteoclasts in sham, OA and aliskiren-treated animals. (A) TRAP staining images of osteoclasts following aliskiren. (B) Effects of aliskiren on the number of osteoclasts. (C) Agarose gels displaying the effects of aliskiren on the mRNA expression of RANKL and OPG. (D) Reverse transcription-quantitative polymerase chain reaction results demonstrating the effects of aliskiren on the OPG/RANKL ratio of mRNA expression levels. Data are expressed as the mean ± standard deviation. \*P<0.05 vs. sham. OA, osteoarthritis; OPG, osteoprotegerin; RANKL, receptor activator of nuclear factor κB ligand.

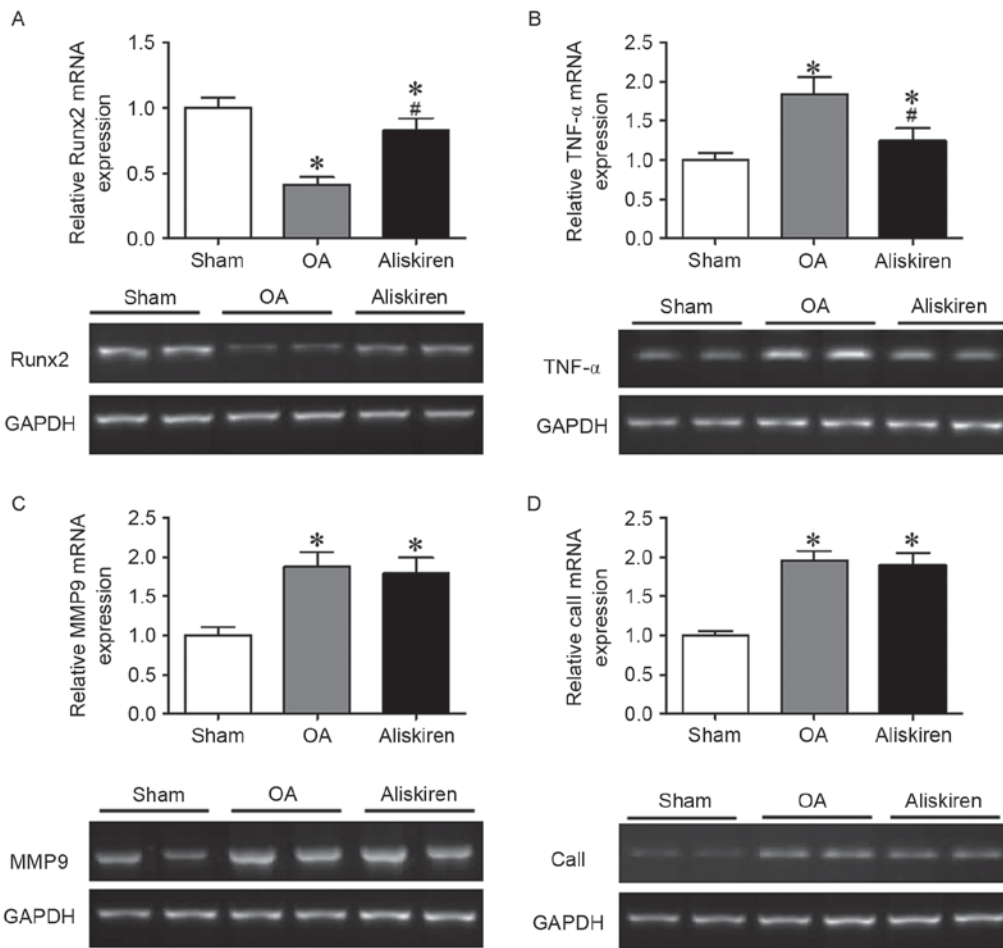


Figure 3. Effects of aliskiren on the mRNA expression levels of Runx2, MMP9, CaII and TNF-α in the sham, OA and aliskiren-treated animals. Effects of aliskiren on the mRNA expression levels of (A) Runx2, (B) TNF-α, (C) MMP9 and (D) CaII. GAPDH was used as the internal control. The results from reverse transcription-quantitative polymerase chain reaction are presented graphically, with the results of the agarose gels shown below. Data are expressed as the mean ± standard deviation. \*P<0.05 vs. sham; #P<0.05 vs. OA. OA, osteoarthritis; Runx2, runt related transcription factor 2; TNF-α, tumor necrosis factor-α; MMP9, matrix metalloproteinase9; CaII, carbonic anhydrase II.

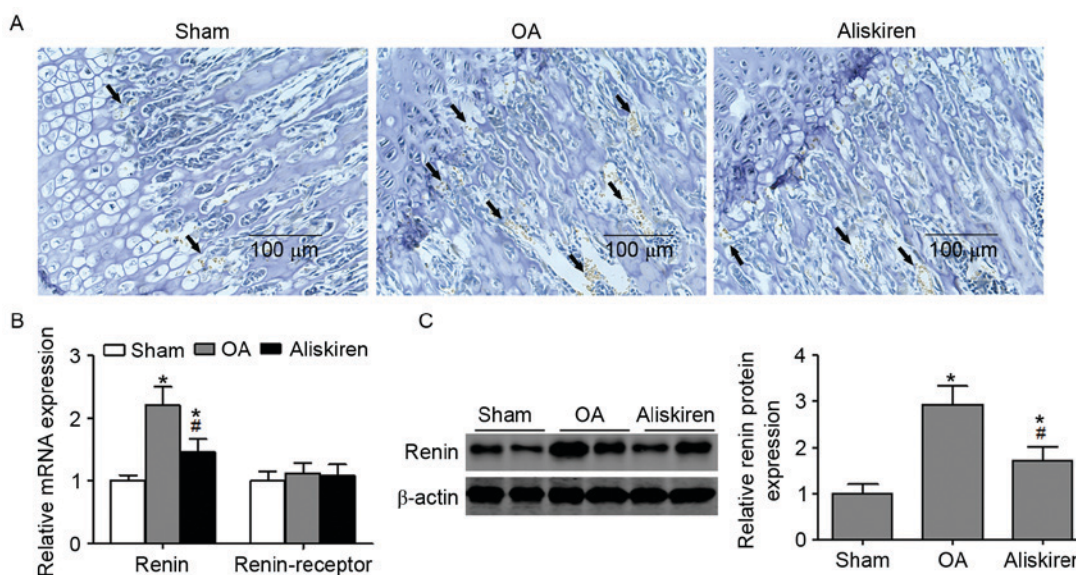


Figure 4. Effects of aliskiren on the level of renin and its receptor in sham, OA and aliskiren-treated animals. (A) Immunohistochemical detection of renin. (B) mRNA expression of renin and renin receptor. (C) Protein expression of renin and renin receptor. Data are expressed as the mean  $\pm$  standard deviation. \* $P < 0.05$  vs. sham; \*\* $P < 0.05$  vs. OA. OA, osteoarthritis.

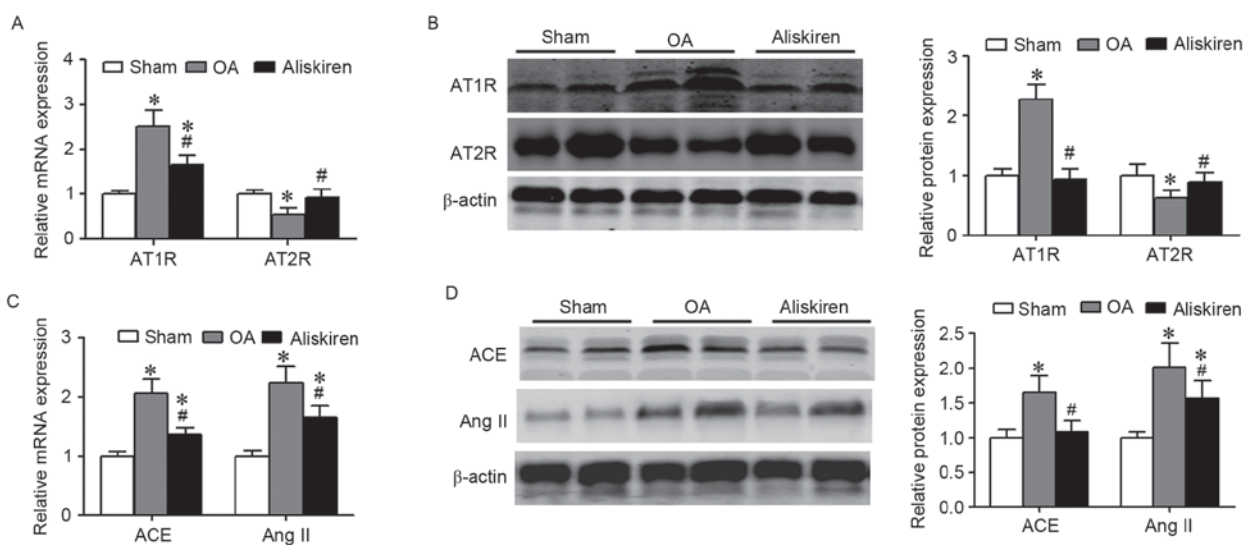


Figure 5. Effects of aliskiren on the expression of AT1R, AT2R, ACE and Ang II in the proximal tibias of sham, OA and aliskiren-treated animals. (A) mRNA and (B) protein expression of AT1R and AT2R. (C) mRNA and (D) protein expression of ACE and Ang II. mRNA and protein levels were measured by reverse transcription-quantitative polymerase chain reaction and western blot analysis, respectively;  $\beta$ -actin was used as the internal control for western blotting. Data are expressed as the mean  $\pm$  standard deviation. \* $P < 0.05$  vs. sham; \*\* $P < 0.05$  vs. OA. OA, osteoarthritis; Ang, angiotensin; ACE, angiotensin-converting enzyme; AT1R, angiotensin type 1 receptor; AT2R, angiotensin type 2 receptor.

efficacy in an OA model and is closely associated with the local RAS.

## Discussion

OA is a disease of unknown etiology that involves degeneration of articular cartilage, limited intra-articular inflammation manifested by synovitis, and changes in the subchondral bone (27). In the OA model used in the present study, these features were partially observed by the incomplete cartilage structure, the abnormal cartilage cellularity and the expansion of the HZ of the chondrocyte zone in the growth plate, along with the significantly increased expression of IL-1 and TNF- $\alpha$ ,

which are the predominant catabolic cytokines involved in the destruction of the articular cartilage in the proximal of tibias during OA. These abnormal properties were associated with activation of RAS components (renin, renin-receptor, Ang II, ACE and AT1R). It was previously hypothesized that Ang II, accompanied by its receptor, act on bone cells via the tissue RAS to regulate osteoclast differentiation and affect bone metabolism (34). The abnormal features observed in the OA model of the current study may also be explained by this hypothesis.

Because the vascular system has an important role in bone remodeling, the effect of the RAS on bone metabolism is considered to be associated with the regulation of blood flow

and directly associated with the local RAS during bone metabolism. It has been previously reported that activation of the local RAS stimulates the expression of osteoclastogenic cytokines in osteoblasts (35). Furthermore, Ang II, the dominant effector peptide of the RAS, reduces the mRNA expression of osteocalcin (a protein specifically expressed during maturation of osteoblastic cells) and decreases the activity of alkaline phosphatase (a marker of osteoblastic differentiation) via its receptor AT1 (36). Therefore, it is reasonable to hypothesize that the chondroprotective benefits of inhibiting the RAS are attributed primarily to reduced level of Ang II and reduced downstream signaling.

ACE inhibitors and AT1R blockers have been in use for 15-20 years and have been beneficial in reducing Ang II activity and associated disorders. However, these medications were reported to cause renin elevation, which has deleterious effects suggesting that may be more beneficial to block renin directly (37). Renin is a circulatory enzyme secreted by the kidneys that acts on angiotensinogen. Renin inhibitors bind to the active site of renin and inhibit its binding to angiotensinogen, which is the rate-determining step of the RAS cascade and, consequently, prevents the formation of Ang I and Ang II (36).

Aliskiren, a renin inhibitor, effectively blocks the generation of active renin and reduced the expression of downstream components of the local RAS in the present study. The results of the current study are supported by studies in non-hypertensive and hypertensive human subjects (38). Among the inhibiting factors, Ang II is a potent stimulator of osteoclastic bone resorption (11), and it promotes the differentiation and activation of osteoclasts indirectly via upregulating the expression of RANKL by binding to different receptors in osteoblasts. Shimizu *et al* (39) reported that Ang II directly induced RANKL expression in osteoblasts through the activation of AT1R. However, another report demonstrated that Ang II induced RANKL expression via AT2R (40). The results of the present study revealed that increased Ang II was accompanied by increased RANKL and AT1R expression, suggesting Ang II may induce RANKL expression through the activation of AT1R. However, the mechanism of how Ang II induces RANKL requires further investigation. A previous study reported that the significant increase in osteoclast activation in the tibia and significant decrease in bone density in an ovariectomy rat model were attenuated by treatment with an ACE inhibitor, imidapril (39). Notably, in the present study, the osteoclast number, OPG/RANKL ratio and CAII expression, which are associated with osteoclast differentiation, were not attenuated by aliskiren.

In the present study, OA decreased the expression of Runx2 and activated the local RAS in the bone of model animals. When the animals were treated with aliskiren, the activation of the local RAS in bone was blocked. Thus, OA may induce decreased expression Runx2 by activating the local RAS in bone. Runx2 is a key mediator involved in controlling osteoblast differentiation. Thus, the inhibition of bone formation in OA may be partially caused by the OA-induced activated RAS leading to decreased Runx2.

Additionally, administration of aliskiren attenuated cartilage destruction, abnormal cartilage cellularity and the expansion of the HZ of the chondrocyte zone of the growth

plate, and significantly attenuated the OA-induced expression of IL-1, TNF- $\alpha$  and PINP in the present study. Aliskiren also reduced the expression of RAS components (renin, Ang II, ACE and AT1R), suggesting that aliskiren has multiple effects on bone formation, osteoblast differentiation and articular cartilage protection via the RAS. Aliskiren directly reduces the renin level, which subsequently decreases the Ang II level and reduces its binding to AT1R, thus, reducing its effects on osteoblasts. Aliskiren may also affect calcium metabolism (34,41). However, in the present study, aliskiren did not fully suppress the inflammatory reaction and had marginal regulatory effects on biochemical bone markers induced by OA. Additionally, its influence osteoblast differentiation was limited.

In conclusion, OA activates the local bone RAS, inhibits bone formation and stimulates bone resorption. Aliskiren, a renin inhibitor, provides chondroprotective efficacy in a rat model of OA through suppression of the local RAS. This research improves the understanding of the pathophysiology of OA and provides a promising option for the treatment of OA. Further research should focus on additional clinical trials to assess the side effects of aliskiren.

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