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Angiotensin II inhibits osteogenic differentiation of isolated synoviocytes by increasing DKK-1 expression

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ABSTRACT

The renin-angiotensin system contributes to the pathogenesis of rheumatoid arthritis, but that the mechanism is unclear. This study aims to investigate the effect of angiotensin II (Ang II) on osteogenic differentiation of synoviocytes and the underlying mechanism. Ang II was showed to inhibite osteogenic differentiation of synoviocytes, which was mitigated by a Dickkopf-1 (DKK-1) inhibitor. DKK-1 was upregulated by Ang II, which was weakened by the Ang II type 1 receptor (AT1R) blocker, reactive oxygen species (ROS) scavenger, and p38 inhibitor. Ang II increased the levels of AT1R, ROS, and NADPH oxidase (NOX), and the upregulations were mitigated by the AT1R blocker or NOX inhibitor. Furthermore, Ang II activated the p38 pathway, which was blocked by the AT1R blocker, ROS scavenger, or siRNA-MKK3. In brief, these results indicate that Ang II upregulates NOX expression and ROS production via AT1R, activates the MKK3/p38 signaling, and in turn upregulates DKK-1 expression, participating in the inhibition of osteogenic differentiation of synoviocytes.

1. Introduction

Periarticular osteopenia, the hallmark of rheumatoid arthritis (RA) (Coury et al., 2019; Sun et al., 2018), is mainly mediated by osteoclasts in RA, which induces joint deformity and patient morbidity (Tateiwa et al., 2019; Boyce et al., 2018). However, many studies have also detected a decrease in bone formation at sites of focal bone erosion in RA (Diarra et al., 2007; Walsh et al., 2009; Wang et al., 2018a). For example, Diarra et al. (2007) reported impaired local bone formation in both a mouse inflammatory arthritis model and human RA. Walsh et al. (2009) found that the rate of bone formation at the site of bone erosion in RA was reduced due to the inhibition of Wnt signaling. Wang et al. (2018a) also observed decreased bone formation in a rat model of collagen-induced arthritis. These studies have confirmed a decrease in bone formation in RA, but the underlying mechanism of impaired bone formation has not been elucidated

Dickkopf-1 (DKK-1) is a secreted glycoprotein acting as a potent inhibitor of the canonical Wnt/β-catenin signaling that plays a crucial role in bone formation (Glinka et al., 1998). Studies have shown that DKK-1 expression increases in the synovial tissue and acts as an important negative regulator of bone formation in RA animal models and patients (Diarra et al., 2007; Walsh et al., 2009; Choe et al., 2016; de Rooy et al., 2013). Tanida et al. (2013) also observed that etanercept treatment suppresses DDK-1 and promotes osteoblastogenesis. Another study (Diarra et al., 2007) also detected the increase of DKK-1 expression in the synovial tissues from RA patients, TNF-induced arthritis mice and collagen-induced arthritis mice, and identified that inhibition of DKK-1 reversed the bone destruction in those RA animal models.

The renin-angiotensin system (RAS), a well-known hormone system regulating blood pressure and electrolyte homeostasis, has been identified in various organs and tissues, including the synovial tissues (Wang et al., 2018b; Yongtao et al., 2014; Paul et al., 2006; Bader and Ganten, 2008). RAS in synovial tissues was found to take part in the pathogenesis of RA, and inhibition of RAS using angiotensin-converting enzyme (ACE) inhibitors or angiotensin type 1 receptor (AT1R) blocker could mitigate the symptoms of RA in animal models (Silveira et al., 2013; Sagawa et al., 2005). In our recent study, RAS in synovial tissues could increase the expression of DKK-1 in CIA rats (Wang et al., 2018a). However, no research by far has been reported to clarify whether RAS inhibits osteogenic differentiation of isolated synoviocytes and the

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1357-2725/

Table 1
Sequences of primers used.

Target genes	Primer sequences (5'-3')	GenBank no.
DKK-1	F: CCTTGGATGGGTATTCCAGA R: TCCATGAGAGCCTTTTCTCC	NM_012242.4
AT1R	F: TCCAAGATGATTGTCCCA R: CTATCACCACCAAGCTGT	NM_032049.3
P22phox	F: TGGCGGGCGTGTTTGTGT R: CCACGGCGGTCATGTACTTC	NM_000101.4
P67phox	F: AAGCTGTTTGCCTGTGAGGT R: CTGCTTCCAGACACACTCCA	NM_001127651.3
GAPDH	F: AACGGGAAGCTTGTCATC R: ACTCCACGACGTACTCAG	NM_001357943.2

specific role of DKK-1 in this process.

Angiotensin II (Ang II), a primary effector in RAS, plays its functions mainly through its AT1R and AT2R (Kaneko et al., 2011). Increasing evidence has indicated that Ang II induces production of reactive oxygen species (ROS) in osteoblasts, vascular smooth muscle cells and neutrophils by upregulating the activity of NADPH oxidase (NOX) via AT1R (Zhang et al., 2014; Wakui et al., 2013; El Bekay et al., 2003). ROS is considered a significant physiological modulator of mitogen activated protein kinases (MAPKs) signaling pathway which includes extracellular signal-regulated kinase (ERK)1/2, c-jun NH2-terminal kinase (JNK) and p38MAPK, and ROS-mediated MAPKs signaling pathway is involved in various diseases (Wang et al., 2019; Wen et al., 2019). The p38MAPK pathway has been proved to participate in upregulating the expression of DKK-1 in breast cancer, osteotropic prostate cancer and myeloma (Rachner et al., 2015; He et al., 2012; Browne et al., 2016). Furthermore, TNF-α is found to increase the DKK-1 expression in synoviocytes via p38MAPK signaling, and inhibitor of p38MAPK signaling or sirRNA-p38MAPK could mitigate the effect of TNF-α on DKK-1 (Diarra et al., 2007). However, it remains unclarified whether Ang II also induces NOX activation and ROS production that activate the p38MAPK signaling pathway, which in turn upregulates the DKK-1 expression and inhibits the osteogenic differentiation of isolated rheumatoid synoviocytes. The present study was thus motivated, aiming to gain a better understanding of the effect and mechanism of Ang II in the bone formation in RA.

2. Materials and methods

2.1. Cell culture and treatment

Synoviocytes were prepared from synovial tissues of RA patients undergoing knee replacement surgery. All the patients met the revised criteria for RA diagnosis of American College of Rheumatology (Arnett et al., 1988). Informed consent was obtained from each patient and the research protocol was approved by the Ethical Committee of the Affiliated Hospital of Qingdao University, China. Synovial tissues were minced and then digested by type I collagenase (Sigma, USA) in DMEM medium (Gibco BRL, USA) supplemented with 10 % fetal bovine serum (Gibco BRL, USA) and 1 % streptomycin and penicillin (Gibco BRL, USA) at 37 °C. Synoviocytes from passages 3–8 in culture were collected and used in the next experiment. Cells from each patient were used separately in three independent experiments for each of the assays described below. In the following experiments, the synoviocytes were treated with, or without, human Ang II (1 nM - 1 μM, MedChemExpress, USA), AT1R blocker (Olmesartan, 10 μM, MedChemExpress, USA), AT2R blocker (PD123319, 10 μM, Selleck Chemicals, USA), scavenger of free radicals (N-acetylcysteine, NAC, 1 Mm, Selleck Chemicals, USA), NOX inhibitor (diphenyleneiodonium, DPI, 50 μM, Selleck Chemicals, USA), p38MAPK inhibitor (SB203580, 10 μM, Selleck Chemicals, USA), MEK inhibitor (U0126, 5 μM, MedChemExpress, USA), JNK inhibitor (SP600125, 10 μM, Selleck Chemicals, USA) or DKK-1 inhibitor (WAY262611, 5 μM, MedChemExpress, USA) for per-set time periods,

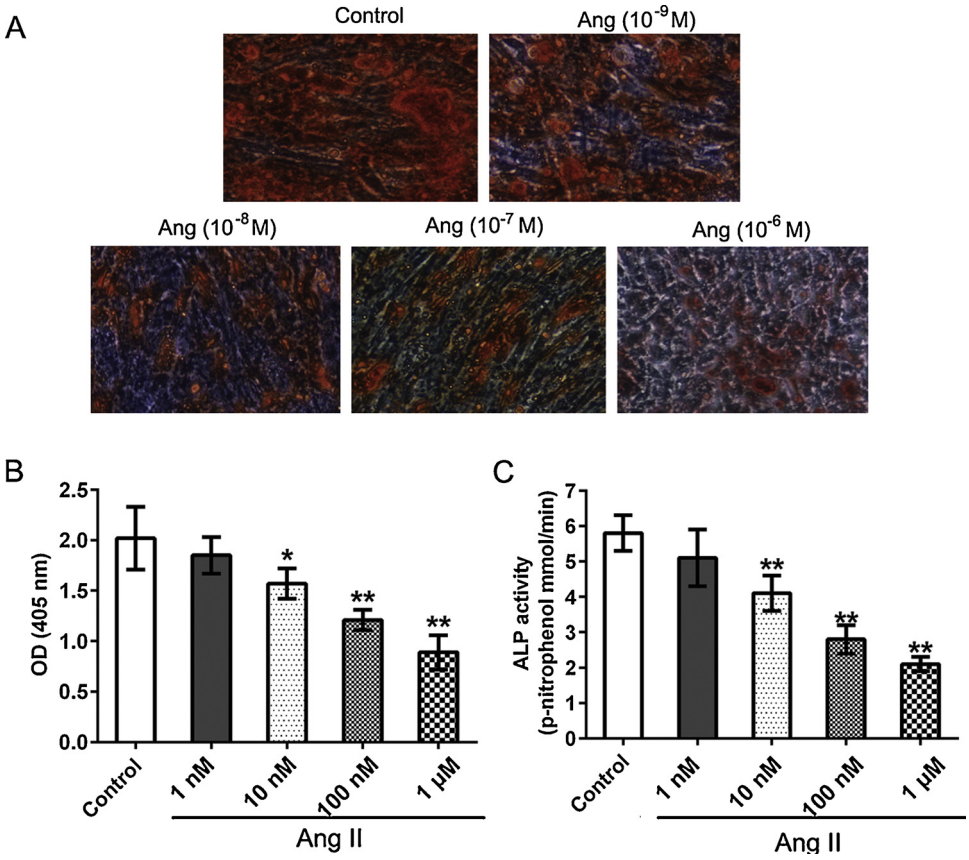

Fig. 1. Ang II inhibited osteogenic differentiation of isolated synoviocytes. Synoviocytes were cultured in osteogenic medium without or with various concentrations of Ang II (1 nM - 1 μM) for 21 days, and the matrix mineralization was then evaluated by alizarin red staining and ALP activity was detected. Data are representative images or expressed as the mean ± SD (n = 3 wells/treatment). The results shown are from cells from one patient, which are representative of results from all three patients. A. Representative alizarin red staining images (magnification x 100). B. Mineralization quantitated from stained mineral deposits. C. ALP activity. *$p < 0.05$ vs. the control group; **$p < 0.01$ vs. the control group (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.).

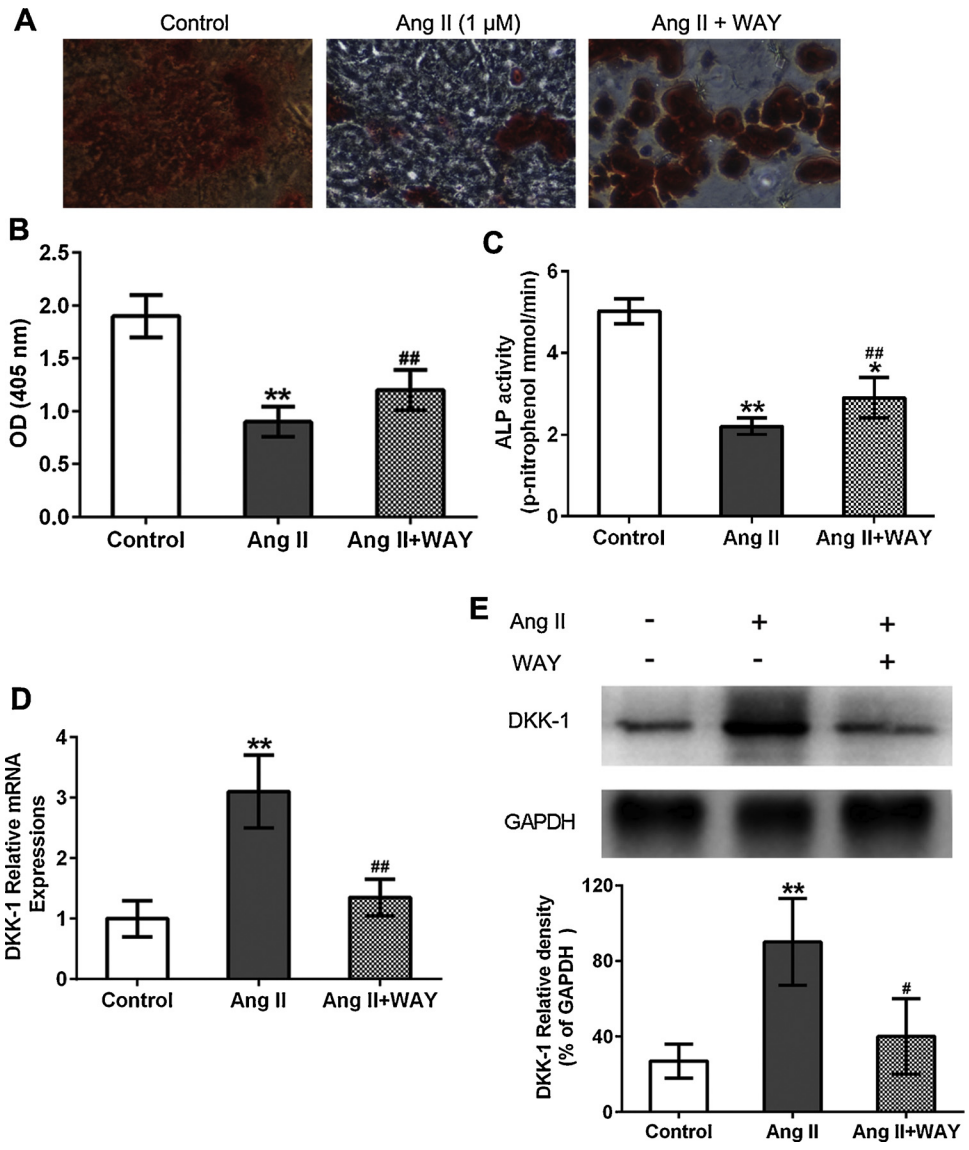

Fig. 2. Ang II inhibited osteogenesis of synoviocytes via DKK-1. Synoviocytes were cultured in osteogenic medium without Ang II, with Ang II (1 μM) alone, or with Ang II plus an inhibitor of DKK-1 (WAY262611, WAY, 5 μM) for 21 days. Then, matrix mineralization was evaluated by alizarin red staining and ALP activity was detected. DKK-1 expression was determined by real-time PCR and Western blot. Data are representative images or expressed as the mean ± SD (n = 3 wells/treatment). The results shown are from cells from one patient, which are representative of results from all three patients. A. Representative alizarin red staining images (magnification x 100). B. Mineralization quantitated from stained mineral deposits. C. ALP activity; D. Real-time PCR of DKK-1 mRNA; E. Western blot analysis of DKK-1 expression. *$p < 0.05$ vs. the control group; **$p < 0.01$ vs. the control group; #$p < 0.05$ vs. the Ang II group; ##$p < 0.01$ vs. the Ang II group (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.).

and then harvested for subsequent analyses. The cell viability in every group was detected by cell Counting Kit-8 assay (Dojindo Laboratories, China).

2.2. Transfection of small interfering RNA (siRNA) in RA synoviocytes

MKK3 and MKK6 in RA synoviocytes were silenced by using a siRNA transfection technique. RA synoviocytes were cultured in 6-well plates (50,000 cells per well) overnight and then transfected with control-specific siRNA (si-Ctr), MKK3-specific siRNA (siRNA-MKK3) and MKK6-specific siRNA (siRNA-MKK6) (Guangzhou RiboBio, China) using siRNA Transfection Reagent (Guangzhou RiboBio, China) for 48 h, according to the manufacturer's protocol. Efficacy of MKK3 and MKK6 silencing was validated by Western-blot.

2.3. Osteogenesis in vitro

For osteogenesis examination, RA synoviocytes were plated in 12-well plates at 5 × 10 (Tateiwa et al., 2019) cell density and cultured in osteogenic base medium containing 100 nM dexamethasone (Sigma, USA), 10 mM β-glycerophosphate (Sigma, USA) and 50 μM ascorbic acid (Sigma, USA) for up to 21 days. The medium was changed every 3 days.

2.4. Alizarin red staining

After the osteogenic induction for 21 days, cells were washed using PBS, fixed with 2.5 % glutaraldehyde for 15 min, washed with PBS again, and then stained with alizarin red (Sigma, USA) at room temperature for 20 min. Images were obtained by a microscope and calcium nodules were detected as red bodies. Alizarin red staining was quantitatively analyzed following a previous study (Zhang and Li, 2018). Briefly, alizarin red was extracted using 10 % acetic acid (Sigma, USA) and neutralized using 10 % Ammonium hydroxide (Sigma, USA), and its optical density was measured at 405 nm.

2.5. Alkaline phosphatase (ALP) activity assay

ALP, a typical marker of osteogenic differentiation, was evaluated quantitatively using a commercially available kit. Briefly, after osteogenic induction of the synoviocytes for 21 days, the protein in cells was extracted using RIPA buffer and determined by a bicinchoninic acid (BCA) protein assay kit (Beyotime, Jiangsu, China). The protein concentration in each sample was uniformed, and the ALP activity was evaluated using an ALP activity assay kit (Beyotime, China) based on the conversion of colorless p-nitrophenyl phosphate to colored p-nitrophenol and then detection of the optical density at 405 nm to

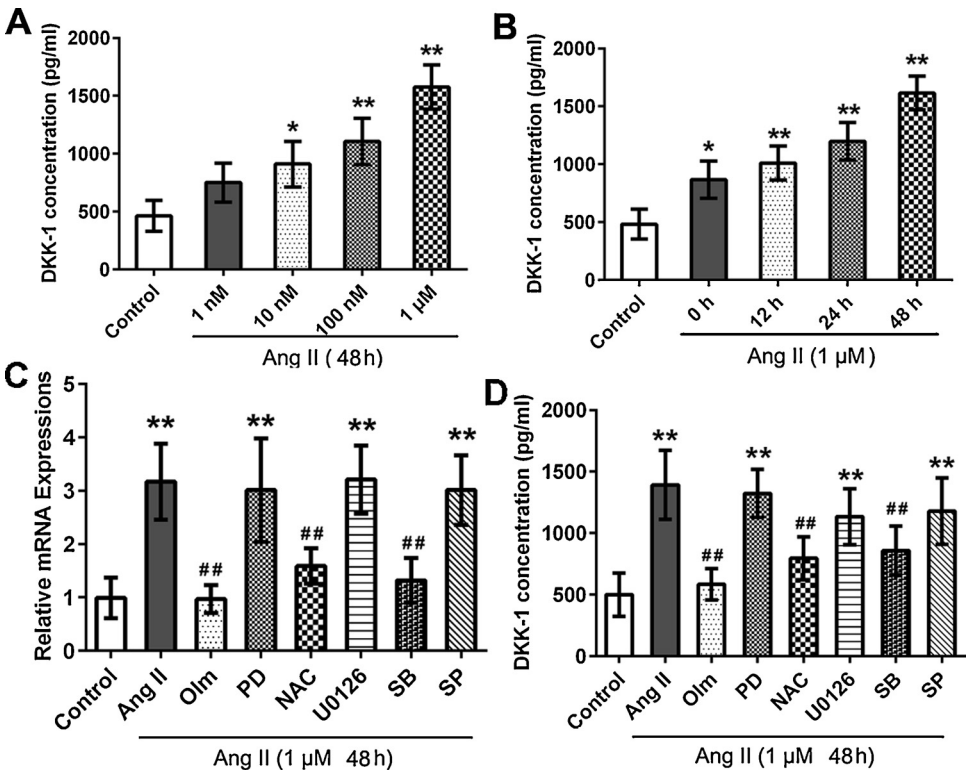

Fig. 3. Ang II increased DKK-1 expression in synoviocytes. Synoviocytes were pretreated in triplicate with, or without, the AT1R antagonist (olmesartan, Olm, 10 μM), AT2R antagonist (PD123319, PD, 10 μM), scavenger of free radicals (Nacetylcysteine, NAC, 1 mM), p38MAPK inhibitor (SB203580, SB, 10 μM), MEK inhibitor (U0126, 5 μM) or JNK inhibitor (SP600125, SP, 10 μM) for 30 min, and then exposed to the indicated concentrations of Ang II for 48 h or to Ang II at 1 μM for the indicated time periods, respectively. The relative levels of the mRNA and protein expressions of DKK-1 in different groups were characterized by real-time PCR and ELISA. Data are expressed as the mean ± SD (n = 3 wells/treatment). The results shown are from cells from one patient, which are representative of results from all three patients. A and B. Dose- and time-dependent effect of Ang II on DKK-1 expression; C and D. The effect of pre-treatment with different inhibitors on Ang II-induced DKK-1 expression. *$p < 0.05$ vs. the control group; **$p < 0.01$ vs. the control group; ##$p < 0.01$ vs. the Ang II group.

calculate the concentration of p-nitrophenol. The ALP activity was expressed as concentration of produced p-nitrophenol per min (mmol/min). All determinations were performed in duplicate.

2.6. Enzyme-linked immunosorbent assay (ELISA)

DKK-1 concentration in the supernatants from human synoviocytes was measured using a commercially available ELISA kit (R&D Systems, Minneapolis, MN, USA) following the manufacturer's instructions. Briefly, the cells were seeded at 10 (Walsh et al., 2009) cells per well and were analyzed in duplicate. The DKK-1 concentration was expressed as pg/ml in culture supernatants.

2.7. Western blot

After treatment, the cells were harvested, lysed in RIPA buffer (Thermo Scientific, USA) on ice and centrifuged at 10,000 × g for 20 min at 4 °C. The protein was denatured at 95 °C for 5 min in loading buffer. Equal amount of cell lysate (15 μl) was loaded on SDS polyacrylamide gels under a constant voltage of 90 V for 120 min and then transferred onto PVDF membranes. The membranes were blocked with 3 % bovine serum albumin or 5 % fat-free milk for 2 h at room temperature and then incubated with rabbit polyclonal antibody anti-human AT1R (Abcam, USA, 1:800), rabbit polyclonal antibody anti-human P22phox (Abcam, USA, 1:1000), rabbit polyclonal antibody anti-human P67phox (Abcam, USA, 1:1000), rabbit monoclonal antibody anti-human p38MAPK (Cell Signaling Technology, USA, 1:1000), rabbit monoclonal antibody anti-human phospho-p38MAPK (Cell Signaling Technology, USA, 1:1000), rabbit polyclonal antibody anti-human MKK3 (Cell Signaling Technology, USA, 1:1000), rabbit polyclonal antibody anti-human MKK6 (Cell Signaling Technology, USA, 1:1000), and rabbit monoclonal antibody anti-human GAPDH (Cell Signaling Technology, USA, 1:2000) overnight at 4 °C, respectively. The blots were washed and then detected with anti-rabbit IgG, HRP-linked Antibody (Cell Signaling Technology, USA, 1:2000). Finally, the bound was visualized using ECL-Plus reagent. The intensity of antibody staining was scanned and analyzed using ImagePro Plus 6.0.

2.8. Real-time polymerase chain reaction (PCR)

Total RNA was prepared with TRIzol reagent (Invitrogen, Carlsbad, USA) from the cultured synoviocytes. The concentration and purity of total RNA were detected using spectrophotometry. RNA was then reversely transcribed into cDNA using the Revert First Strand cDNA Kit (Fermentas, Canada). The targeted gene mRNA level was determined by real-time PCR using the SYBR Green PCR master mix (TaKaRa, Japan). The relative expression of the target genes was normalized to the expression of GAPDH (housekeeping gene) and analyzed by the $2^{-\Delta\Delta Ct}$ method. All the primer sequences are presented in Table 1.

2.9. Reaction oxygen species (ROS) generation

The cultured synoviocytes were seeded in 6-well plates with media and grown until 70 % confluence. ROS in cells was monitored using a Fluorometric Intracellular ROS Kit (MAK142; Sigma, USA). The cells were then collected and washed thrice with PBS. The level of intracellular ROS was detected by fluorescent microscope (Olympus, Japan) and flow cytometry (Partec, Germany). Three repeats were performed.

2.10. Statistical analysis

Statistical analyses were performed using SPSS 17.0 (SPSS Inc., Chicago, IL, USA). All data are presented as the mean ± standard deviation (SD). Differences among groups were analyzed using Kruskal–Wallis test or one-way analysis of variance test; subgroup analysis was performed using the LSD test. A $P < 0.05$ was considered statistically significant for all analyses.

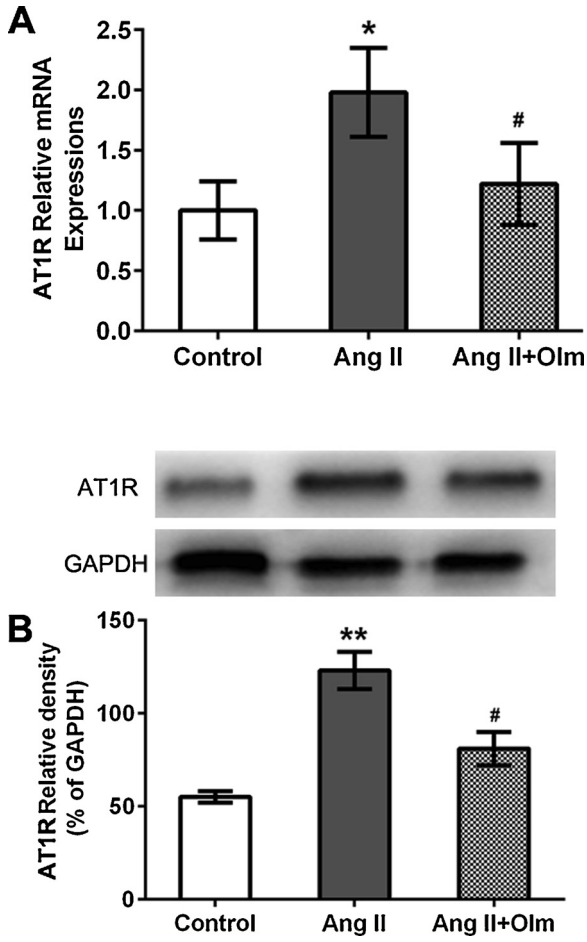

Fig. 4. Effect of Ang II on AT1R expression in synoviocytes. Synoviocytes were pre-treated with, or without, the AT1R antagonist (olmesartan, Olm, 10 μM), for 24 h. The relative levels of the mRNA and protein expressions of AT1R in different groups were detected by real-time PCR and Western blot. Data are representative images or expressed as the mean ± SD (n = 3 wells/treatment). The results shown are from cells from one patient, which are representative of results from all three patients. A. The relative mRNA level of AT1R. B The relative protein level of AT1R. *p < 0.05 vs. the control group; **p < 0.01 vs. the control group; #p < 0.05 vs. the Ang II group.

3. Results

3.1. Ang II inhibited osteogenesis of synoviocytes

Mineralization of the extracellular matrix is one of the key markers of synoviocytes differentiating into osteoblasts. To evaluate the effect of Ang II in this differentiation process, synoviocytes were cultured for 21 days in osteogenic medium either without Ang II (control group) or with various concentrations of Ang II (1 nM - 1 μM). Alizarin red staining was performed to evaluate the level of matrix mineralization. Compared with that in the control group, less matrix mineralization was visually seen under microscope in the Ang II treatment groups (Fig. 1 A). The quantity of mineralization decreased significantly in a dose-dependent manner (Fig. 1B). ALP, the marker of osteogenesis, was also detected. The results demonstrated that Ang II reduced ALP activity in a dose-dependent manner (Fig. 1C).

3.2. Ang II inhibited osteogenesis of synoviocytes via DKK-1

Synoviocytes were cultured for 21 days in osteogenic medium without Ang II, with Ang II (1 μM) alone, or with Ang II plus WAY262611. The results showed that Ang II inhibited matrix mineralization of synoviocytes, which was alleviated by WAY262611 (Fig. 2 A and B). A similar pattern was also detected in ALP activity under different treatment conditions (Fig. 2 C). The previous study has shown that WAY-262611 took effect in synoviocytes by decreasing the expression of DKK-1 (Choe et al., 2016), therefore, we detected the expression of DKK-1 in our study to investigate whether WAY262611 alleviated Ang II-indcued osteogenesis via decreasing the expression of DKK-1 in synoviocytes. Notably, DKK-1 expression at both mRNA and protein levels increased in cells treated with Ang II alone, and the increase was mitigated by the addition of WAY262611 (Fig. 2 D and E). These results suggested the involvement of DKK-1 in the process of Ang II inhibiting the osteogenesis of synoviocytes.

3.3. Ang II induced DKK-1 expression

To understand the mechanism underlying the effect of Ang II on DKK-1, synoviocytes were cultured with various concentrations of Ang II (1 nM 1 μM) for 48 h and the level of DKK-1 expression was determined by ELISA. The results showed that Ang II increased the DKK-1 level in synoviocytes in a dosedependent manner (Fig. 3 A). Subsequently, synoviocytes were treated with Ang II (1 μM) for different lengths of time, and the increase of the DKK-1 level appeared to be timedependent (Fig. 3 B). The effect of Ang II on DKK-1 expression was blocked by pretreatment with olmesartan (an AT1R blocker) but not PD123319 (an AT2R antagonist), suggesting that Ang II enhanced the expression of DKK-1 through its AT1R instead of AT2R (Fig. 3 C and D). In addition, pre-treatment with NAC (a scavenger of free radicals) or SB203580 (a p38MAPK inhibitor), but not U0126 (MEK1/2 inhibitor) or SP600125 (a JNK inhibitor), blocked the Ang II-induced DKK-1 expression (Fig. 3 C and D), implying that the AT1R, ROS and p38MAPK pathways might be involved in the effect of Ang II on DKK-1 expression in synoviocytes.

3.4. Ang II enhanced AT1R expression

Synoviocytes were pre-treated with, or without, olmesartan, an AT1R blocker, and then exposed to Ang II (1 μM) for 24 h. The level of AT1R expression was detected using real-time PCR and Western blot. The results showed that Ang II upregulated the relative level of AT1R mRNA expression, and the upregulation was blocked by olmesartan (Fig. 4A). A similar pattern was also detected for the AT1R protein expression (Fig. 4B).

3.5. Ang II induced ROS production via AT1R and NOX

Synoviocytes were pretreated with or without different inhibitors, including olmesartan, PD123319, DPI, and SB203580, and then exposed to Ang II (1 μM) for 24 h. Intracellular ROS was examined by immunofluorescent assay and flow cytometry. The results of immunofluorescent assay indicated that Ang II enhanced ROS production, which was blocked by the pre-treatment with olmesartan or DPI (Fig. 5A). Similarly, flow cytometry indicated that Ang II significantly increased the level of intracellular ROS, which was abrogated by the pre-treatment with olmesartan or DPI (Fig. 5 B and C). These results suggested that Ang II increased ROS production in synoviocytes by activating AT1R and NOX, but was independent of p38MAPK signaling.

3.6. Ang II enhanced NOX expression

Synoviocytes were pretreated with, or without, olmesartan or PD123319, and then exposed to Ang II (1 μM) for 24 h. The relative levels of p22phox and p67phox, two main subunits of the NOX, were examined using real-time PCR and Western blot. The results showed that Ang II increased the mRNA levels of p22phox and p67phox, which was weakened by olmesartan (Fig. 6 A). A similar pattern of the protein levels of p22phox and p67phox was also detected (Fig. 6 B). These

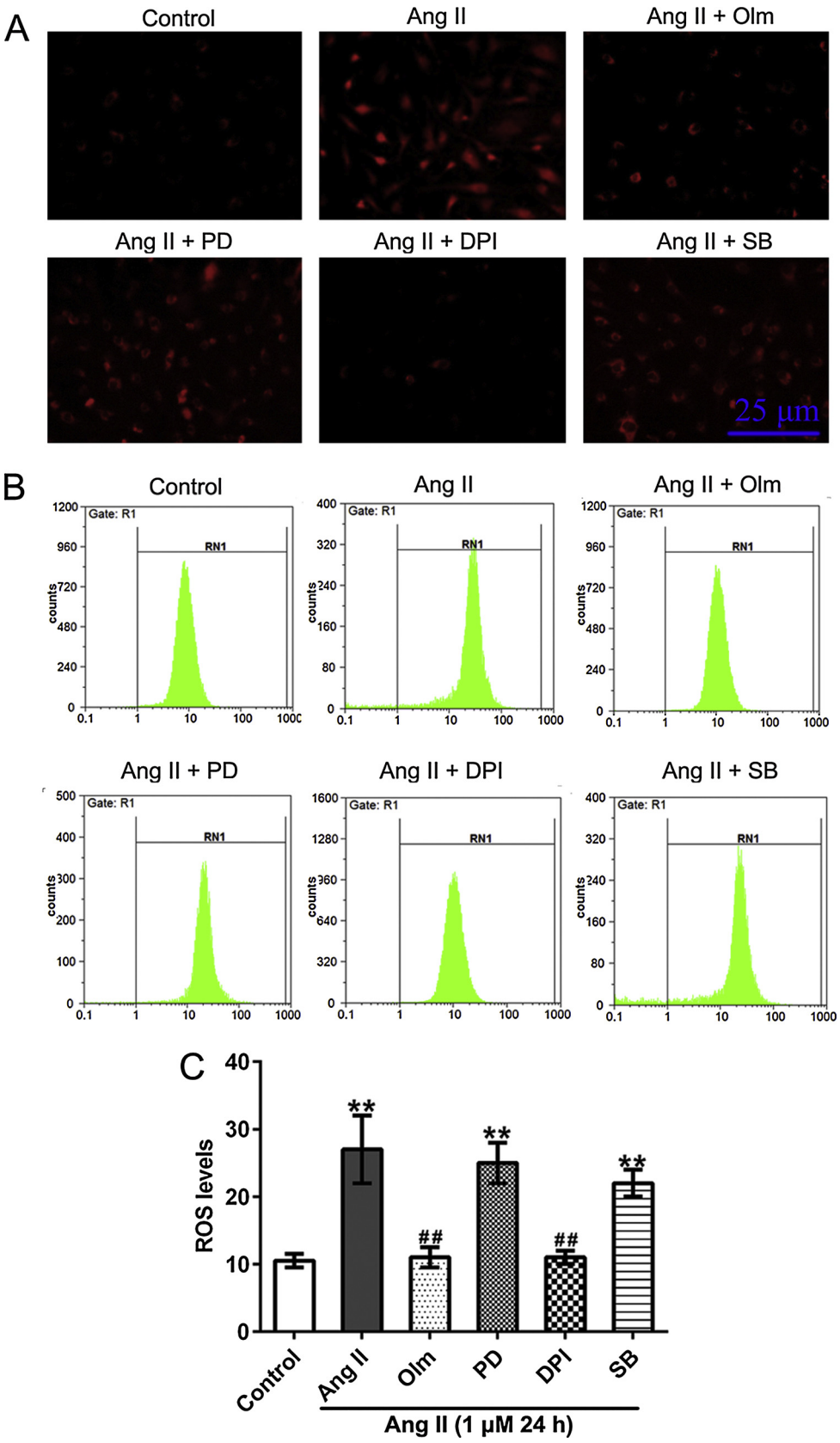

Fig. 5. Effect of Ang II on ROS production in synoviocytes. Synoviocytes were pretreated with or without the AT1R antagonist (olmesartan, Olm, 10 μM), AT2R antagonist (PD123319, PD, 10 μM), scavenger of free radicals (Nacetylcysteine, NAC, 1 mM), NOX inhibitor (diphenyleneiodonium, DPI, 50 μM), or p38MAPK inhibitor (SB203580, SB, 10 μM) for 30 min, and then exposed to Ang II (1 μM) for 24 h. The level of intracellular ROS was determined by fluorescent microscopy and flow cytometry. Data are representative images or expressed as the mean ± SD (n = 3 wells/treatment). The results shown are from cells from one patient, which are representative of results from all three patients. A. Fluorescent microscopy analysis (magnification x 400). B. Flow cytometry analysis. C. Quantitative analysis of the intracellular ROS level. $^{**}p < 0.01$ vs. the control group; $^{\#\#}p < 0.01$ vs. the Ang II group.

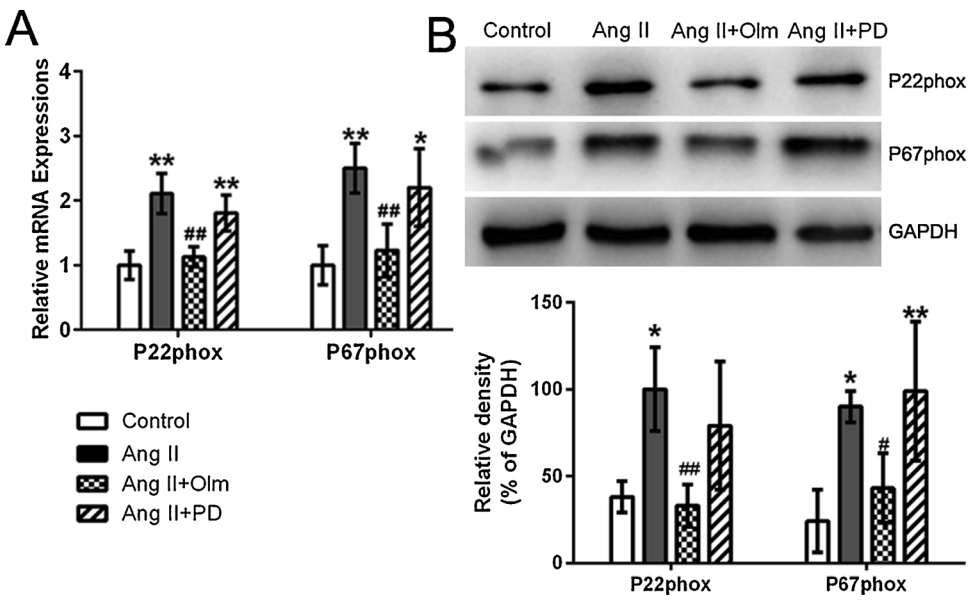

Fig. 6. Effect of Ang II on the expressions of P22phox and P67phox in synoviocytes. Synoviocytes were pre-treated with, or without, the AT1R antagonist (olmesartan, Olm, 10 μM) or AT2R antagonist (PD123319, PD, 10 μM) for 30 min, and then exposed to Ang II (1 μM) for 24 h. The relative mRNA and protein levels of P22phox and P67phox in different groups were detected by real-time PCR and Western blot assays. Data are representative images or expressed as the mean ± SD (n = 3 wells/treatment). The results shown are from cells from one patient, which are representative of results from all three patients. A. The relative mRNA levels of P22phox and P67phox. B. The relative protein levels of P22phox and P67phox. *$p < 0.01$ vs. the control group; **$p < 0.01$ vs. the control group; $^{\#}p < 0.01$ vs. the Ang II group; $^{\#\#}p < 0.01$ vs. the Ang II group.

results suggested that Ang II enhanced the NOX expression.

3.7. MKK3/p38MAPK signaling was involved in Ang II-induced DKK-1 expression

SB203580, an inhibitor of p38MAPK, was found to have blocked the effect of Ang II on DKK-1 expression. However, a further examination revealed that Ang II significantly increased the relative level of p38MAPK phosphorylation, and this effect was mitigated by the pre-treatment with olmesartan or NAC (Fig. 7 A). In addition, our experiment in which siRNA-MKK3 and siRNA-MKK6 were used to inhibit the upstream kinases of p38MAPK (Fig. 7 B) showed that it was MKK3 but not MKK6 that mediated the Ang II-induced activation of p38MAPK signalling (Fig. 7 C). In the further experiment, Ang II-induced DKK-1 expression was inhibited by blocking the p38MAPK signaling using SB203580 or siRNA-MKK3, but not siRNAMKK6 (Fig. 7 D and E). These results suggested that Ang II increased the DKK-1 expression in synoviocytes through the MKK3/p38MAPK pathway.

4. Discussion

Synoviocytes are the predominating cells in the synovium, and play a central role in the stromal environment within RA diseases (Choe et al., 2016; Lavocat et al., 2016). Moreover, synoviocytes from synovial membrane have been proven to differentiate into osteoblasts in vitro (Lavocat et al., 2016; Osta et al., 2015), therefore, we used synoviocytes to investigate the effect of Ang II on osteogenesis in this study. And the primary finding of this study is that Ang II inhibited the osteogenic differentiation of isolated synoviocytes partially by increasing DKK-1 expression. Further investigation into the underlying mechanisms of Ang II-induced upregulation of DKK-1 expression revealed that Ang II enhances DKK-1 generation in synoviocytes via its AT1R through the NOX/ROS/MKK3/p38MAPK pathway.

A number of studies have been conducted investigating the role of Ang II in bone formation. A study in vitro suggests that Ang II might be involved in inhibiting the differentiation of rat calvarial osteoblastic cells through its interaction with AT1R (Hagiwara et al., 1998). An in vivo study also reports the participation of the Ang II/AT1R pathway in glucocorticoid-induced osteoporosis through suppressing bone formation in rabbits (Yongtao et al., 2014). Our recent study also reveals that the active RAS in synovial tissues took part in the pathogenic process of periarticular osteopenia in RA rats, partly via inhibiting bone formation (Wang et al., 2018a). In agreement with these studies, the present research identifies that Ang II inhibited the osteogenic differentiation of isolated synoviocytes in a dose-dependent manner.

DKK-1, an inhibitor of the Wnt signaling pathway, is a cytokine that inhibits bone formation in RA (Diarra et al., 2007; Wang et al., 2018a). Kato et al. (2018) found that glucocorticoids reduced the osteogenic differentiation ability of adipose tissue-derived mesenchymal stem cells by increasing the expression of Dkk-1. Bajada et al. (2009) reported that the reduced capacity of non union stromal cells to form osteoblasts was associated with significantly elevated secretion of Dkk-1. In the present study, the Ang II-induced increase in DKK-1 was mitigated by WAY262611, an inhibitor of DKK-1. Along with this change, the inhibited osteogenesis of synoviocytes was reversed. This indicates that Ang II suppresses the osteogenic differentiation of synoviocytes through increasing the expression of DKK-1.

Ang II plays a broad range of roles through binding to its AT1R and/or AT2R, both of which are identified to exist in synoviocytes (Wang et al., 2018a). In this study, pre-treatment with the AT1R antagonist, olmesartan, dramatically mitigated the enhancing effect of Ang II on DKK-1 expression, indicating that Ang II enhances DKK-1 expression in synoviocytes via AT1R. To our knowledge, this study is the first to investigate the effect of Ang II on DKK-1 expression in vitro.

Ang II may stimulate ROS production and ROS plays a crucial part in Ang II-mediated biological functions (Wang et al., 2018b; El Bekay et al., 2003; Zhang et al., 2019). In this study, pre-treatment with NAC, the scavenger of free radicals, weakened the enhancing effect of Ang II on DKK-1 expression, indicating that Ang II enhances DKK-1 expression in synoviocytes via ROS. NOX is considered as responsible for ROS production in mammalian cells (Zhang et al., 2014; Wen et al., 2019). El Bekay et al. (2003) found that Ang II induced the production of ROS in neutrophils by upregulating the activity of NOX via AT1R. This study identified a similar effect of Ang II on ROS in synoviocytes. Ang II induced ROS production and increased the expressions of P22phox and P67phox, two major subunits of NOX, via AT1R. Whereas, the ROS production was weakened by the treatment with DPI, a NOX inhibitor. These results indicate that Ang II induces NOX expression via AT1R and then promotes ROS production in synoviocytes.

p38MAPK signaling has been found involved in upregulating DKK-1 expression in prostate cancer cells, breast cancer cells, myeloma cells and melanoma cells (Rachner et al., 2015; He et al., 2012; Browne et al., 2016). In this study, pre-treatment with SB203580, an inhibitor of p38MAPK signaling alleviated the effect of Ang II on DKK-1 expression. Ang II enhanced the activation of p38MAPK, which was wenkened by the pretreatment with an AT1R antagonist and the ROS

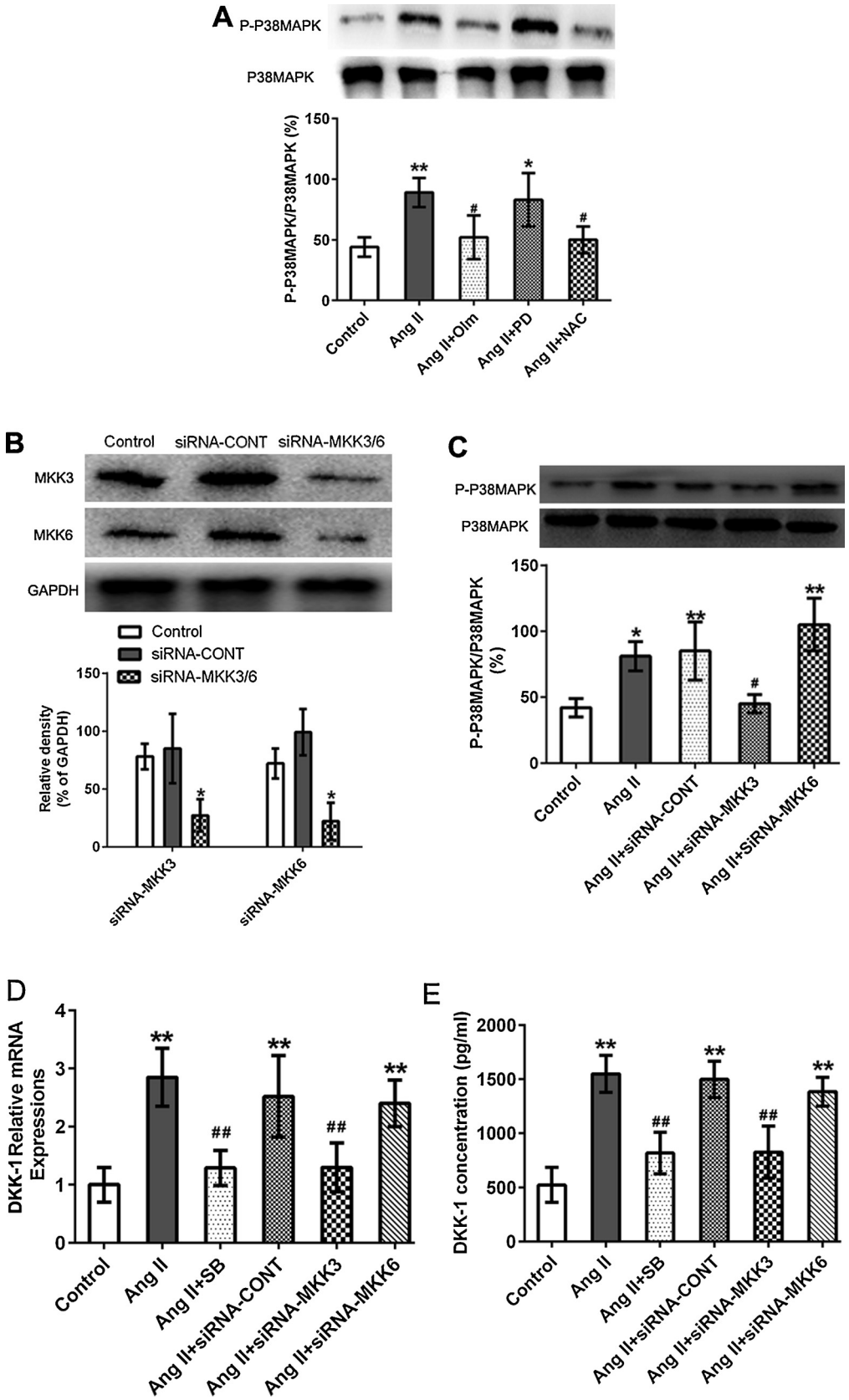

Fig. 7. Involvement of MKK3/p38MAPK in Ang II-induced DKK-1expression in synoviocytes. A. The relative level of p38MAPK phosphorylation. Synoviocytes were pre-treated with, or without, the AT1R antagonist (olmesartan, Olm, 10 μM), AT2R antagonist (PD123319, PD, 10 μM), or scavenger of free radicals (Nacetylcysteine, NAC, 1 mM) for 30 min, and exposed to Ang II (1 μM) for 24 h. The relative level of p38MAPK phosphorylation was determined by Western blot assays. B and C. The relative protein levels of MKK3 and MKK6. Synoviocytes were transfected with control-, MKK3-, or MKK6-specific siRNA for 48 h, and then treated with Ang II for 24 h in triplicate. The relative level of p38MAPK phosphorylation was determined by Western blot assays. D and E. The relative mRNA and protein levels of DKK-1. Synoviocytes were pre-treated with p38MAPK inhibitor (SB203580, SB, 10 μM) for 30 min or transfected with control-, MKK3-, or MKK6-specific siRNA for 48 h, and then exposed to Ang II (1 μM) for 24 h. The relative levels of the mRNA and protein expressions of DKK-1 in different groups were detected by real-time PCR and ELISA. Data are representative images or expressed as the mean ± SD (n = 3 wells/treatment). The results shown are from cells from one patient, which are representative of results from all three patients. *$p < 0.01$ vs. the control group; **$p < 0.01$ vs. the control group; $^{\#}p < 0.01$ vs. the Ang II group; $^{\#\#}p < 0.01$ vs. the Ang II group.

scavenger NAC. These results indicated that Ang II activated p38MAPK signaling via its AT1R and ROS, and in turn increased the expression of DKK-1 in synoviocytes. This finding is in agreement with previous studies in which ROS is considered a significant physiological modulator of p38MAPK signaling (Wang et al., 2019; Wen et al., 2019). The upstream kinases of p38MAPK are MKK3 and MKK6 (Brunet and Pouyssegur, 1997). MKK3/p38MAPK signaling was reported to be the main pathway in TNF-α-induced DKK-1expression in synoviocytes

(Diarra et al., 2007). Similarly, this study also identified that siRNA-MKK3, rather than siRNA-MKK6, mitigated the Ang II-induced phosphorylation of p38MAPK and the expression of DKK-1 in synoviocytes.

In the present study, we found that Ang II up-regulates NOX expression and stimulates ROS production via AT1R, activates the MKK3/p38MAPK signaling, and in turn upregulates DKK-1 expression, participating in inhibition of the osteogenic differentiation of isolated synoviocytes. The findings may provide insights into understanding the role of Ang II in the pathogenesis of RA, and may facilitate the development of novel therapies for this disease.

Authors' contributions

Yongtao Zhang and Huimin Ding contributed equally to this study.

CRediT authorship contribution statement

Yongtao Zhang: Conceptualization, Methodology, Software. **Huimin Ding:** Conceptualization, Validation. **Qichun Song:** Supervision. **Ze Wang:** Formal analysis. **Wanqing Yuan:** Supervision, Data curation. **Yuanzhong Ren:** Investigation, Resources. **Zhiping Zhao:** Investigation, Resources. **Changyao Wang:** Writing - original draft, Writing - review & editing, Project administration, Data curation.

Declaration of Competing Interest

The authors declare no conflicts of interest.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.biocel.2020.105703.

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