

## EXPERIMENTAL STUDY

# AT1/2R affects the proliferation and apoptosis of chondrocytes induced by oxygen-glucose deprivation

Cai HQ<sup>1</sup>, Miao MY<sup>1</sup>, Zhang WL<sup>1</sup>

Department of Orthopaedics, Shanghai Children's Medical Center, Shanghai Jiaotong University School of Medicine, Shanghai, China. [wzhangzwl@126.com](mailto:wzhangzwl@126.com)

**ABSTRACT**

**AIM:** Investigate how AT1/2R affected the proliferation and apoptosis of chondrocytes induced by oxygen-glucose deprivation.

**METHODS:** The proliferation and apoptosis of ATDC5 cells was detected by CCK-8 assay and flow cytometry analysis. The expression of Bax, Bcl-2, caspase-3, cleaved-caspase-3, AT1R, AT2R and HIF-1 was determined by Western blot analysis. The collagen II expression was detected by ELISA assay.

**RESULTS:** Increased ratio of AT1R to AT2R induced by Ang II suppressed the proliferation of oxygen-glucose deprivation ATDC5 cells. Telmisartan, as AT1R inhibitor, promoted the proliferation and inhibited the apoptosis of ATDC5 cells and oxygen-glucose deprivation ATDC5 cells. The collagen II expression either intracellular or cellular supernatant was decreased after Ang II treatment, which was reversed by telmisartan. And, telmisartan reduced the AT1R expression while increased the AT2R expression in ATDC5 cells and oxygen-glucose deprivation ATDC5 cells.

**CONCLUSIONS:** Ang II caused an increased ratio of AT1R to AT2R, which suppressed the proliferation of oxygen-glucose deprivation ATDC5 cells. Furthermore, telmisartan caused a decrease of AT1R and increase of AT2R, which promoted the proliferation and inhibited the apoptosis of oxygen-glucose deprivation ATDC5 cells. This new finding could provide a new insight into the treatment of osteoarthritis (Fig. 4, Ref. 19). Text in PDF [www.elis.sk](http://www.elis.sk)

**KEY WORDS:** angiotensin II type 1/2 receptor, chondrocytes, proliferation, apoptosis, oxygen-glucose deprivation.

**Introduction**

Osteoarthritis (OA) is a chronic bone and joint disease caused by articular cartilage degeneration and hyperosteogeny and gonarthrosis are most common. Knee osteoarthritis (KOA) is characterized by articular cartilage degeneration, osteoporosis and synovitis (1–3). With the increasing incidence of OA, it has become an increasingly concerning social problem. At present, there is no effective radical cure for OA, which mainly focuses on the control of symptoms and improvement of joint function.

Studies indicated that OA was correlated with hypertension (4–6). Renin-angiotensin system (RAS) plays a key role in the regulation of cardiovascular system. RAS regulates the blood

pressure and fluid balance to maintain homeostasis. Angiotensin II (Ang II) is an important effective factor in RAS system and Ang II combines with Angiotensin II type 1/2 receptor (AT1/2R) to exert the corresponding physiological effects. The abnormal increase of AT1R activity has an important relationship with vascular remodeling, diabetic nephropathy, atherosclerosis and inflammation (7). However, stimulation of AT2R has physiological effects opposite to stimulation of AT1R, such as vasodilation, inhibition of inflammatory response, anti-fibrosis, and promotion of neuronal repair and regeneration (8–11). Study showed that Ang II, AT1R, AT2R iNOS and TGM2 were significantly increased in rheumatoid arthritis (RA), but AT1R was more significantly increased than AT2R (12). The ratio of AT1R to AT2R was significantly increased which has broken the original balance of AT1R/AT2R. In OA, increased expression of Ang II, AT1R and AT2R also exists (13–15). Therefore, we speculated that the changed expression of Ang II, AT1R and AT2R may lead to occurrence of OA.

The hypoxic environment is also closely related to OA, and the effect and mechanism of anoxia on OA have been discovered and confirmed by continuous research (16). However, there is still no direct evidence about above mechanisms for the effect of anoxia on chondrocytes. Therefore, this study was aimed to investigate how AT1/2R expression affected proliferation and apoptosis of chondrocytes induced by oxygen-glucose deprivation.

<sup>1</sup>Department of Orthopaedics, Shanghai Children's Medical Center, Shanghai Jiaotong University School of Medicine, Shanghai, China  
H.Q. Cai and M.Y. Miao contributed equally.

**Address for correspondence:** W.L. Zhang, Department of Orthopaedics, Shanghai Children's Medical Center, Shanghai Jiaotong University School of Medicine, No. 1678 Dongfang Road, Shanghai 200127, P.R. China.

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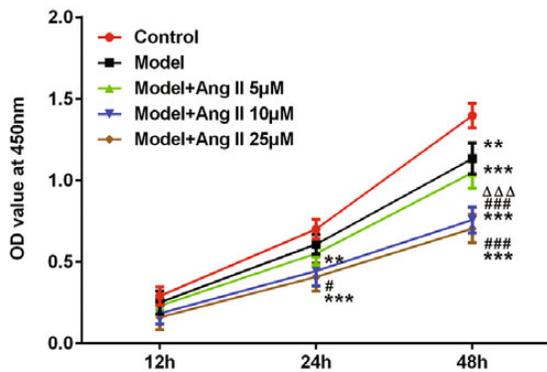


Fig. 1. Ang II affects the proliferation of oxygen-glucose deprivation chondrocytes. \*\*  $p < 0.01$  and \*\*\*  $p < 0.001$  vs control group. ####  $p < 0.001$  vs model group.  $\Delta\Delta\Delta$   $p < 0.001$  vs model+Ang II 5  $\mu$ M group.

## Materials and methods

### Model of oxygen-glucose deprivation chondrocytes.

Chondrocytes (ATDC5 cells) were purchased from Nanjing Cobioer Biosciences Co., Ltd. ATDC5 cells were cultured in DMEM/F12 medium (containing 10% fetal bovine serum and 1% penicillin-streptomycin). When cell fusion reached 90%, chondrocyte inducing conditional media was added for further incubation. All cells were placed in a 37 °C and 5% CO<sub>2</sub> incubator for culture. ATDC5 cells were divided into control group, model group and model+Ang II group. In the model group, the serum-containing medium was removed by washing the cells with PBS for three times. Then, ATDC5 cells were treated with sugar-deficient solution and incubated in 95% N<sub>2</sub> and 5% CO<sub>2</sub> incubator for 6 h. In model+Ang II groups, oxygen-glucose deprivation ATDC5 cells were separately treated with 5, 10 and 25  $\mu$ M Ang II and 24 h later, 5, 10 and 25  $\mu$ M Ang II was separately added to cells for incubation for another 48 h.

### Telmisartan treatment.

The oxygen-glucose deprivation ATDC5 cells were treated with 10  $\mu$ M telmisartan (Telm) for 1 h and 10  $\mu$ M Ang II for 24 h. Then, 10  $\mu$ M Ang II was added to cells for incubation of another 48 h.

### CCK-8 assay.

ATDC5 cells ( $1 \times 10^4$ /well) were inoculated in 96-well plates. After cells were treated with different concentrations of Ang II, 10  $\mu$ L CCK8 solution was added to each well, and the culture plate was incubated in an incubator for 3 h. The absorbance (OD) value at 450 nm was determined by a microplate reader.

### Western blot analysis.

ATDC5 cells in different treatment groups were added with RIPA lysate to extract the proteins. The concentration of extracted proteins was determined according to the instructions of the BCA protein detection kit. The protein samples of the same concentration were subjected to SDS-PAGE electrophoresis. The membrane was treated with 5% non-fat milk powder for 2 h. The primary antibodies (anti-Bax, anti-Bcl-2, anti-caspase-3, anti-cleaved-caspase-3, anti-AT1R, anti-AT2R and anti-HIF-1) were added to the membrane which was blocked overnight at 4 °C. After washing with TBST, a HRP-coupled secondary antibody was added to the membrane which was incubated at room temperature for 1 h. The protein density was quantitatively analyzed by Image J software and GAPDH was used as an internal control. The expression level of the target protein was reflected by the ratio of the gray value of the target protein band to GAPDH band.

### Flow cytometry analysis.

ATDC5 cells in different treatment groups were collected and centrifuged at 1000 g for 5 min. After the supernatant was discarded, 195  $\mu$ L Annexin v-FITC solution was added to suspend the cells and then 5  $\mu$ L Annexin v-FITC solution was added and the cells were mixed gently. 10  $\mu$ L propidium iodide (PI) was added to mixed cells, which was incubated at room temperature in dark for 15 min. The sample was placed on FACSCalibur flow cytometer to detect cell apoptosis.

### ELISA assay.

The ATDC5 cells in control group, control+Ang II group and control+Ang II+Telm group were selected and centrifuged to separate the cells and supernatant. The level of Collagen II in cells and supernatant was detected by ELISA assay according to the kit instructions.

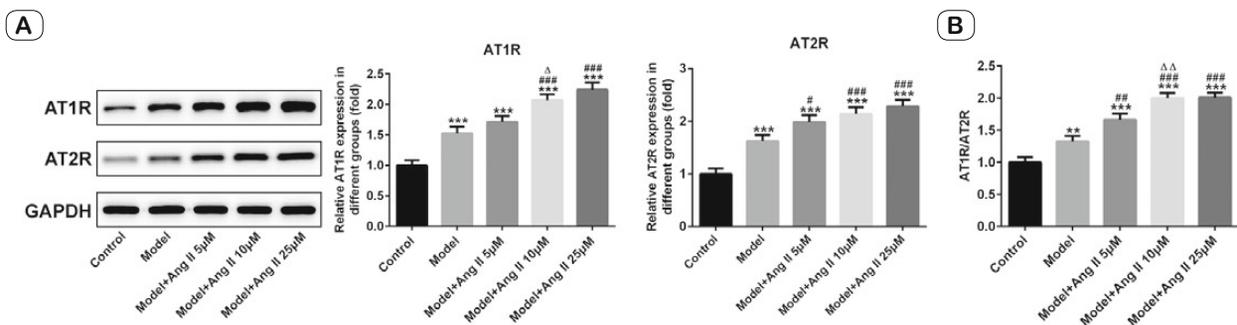


Fig. 2. Ang II affects the expression of AT1/2R. (A) The expression of AT1R and AT2R in ATDC5 cells and oxygen-glucose deprivation ATDC5 cells was detected by Western blot analysis. \*\*\*  $p < 0.001$  vs Control group. ####  $p < 0.001$  vs model group.  $\Delta$   $p < 0.05$  vs model+Ang II 5  $\mu$ M group. (B) The ratio of AT1R to AT2R in ATDC5 cells and oxygen-glucose deprivation ATDC5 cells. \*\*  $p < 0.01$  and \*\*\*  $p < 0.001$  vs control group. #  $p < 0.01$  and ####  $p < 0.001$  vs model group.  $\Delta\Delta$   $p < 0.01$  vs model+Ang II 5  $\mu$ M group.

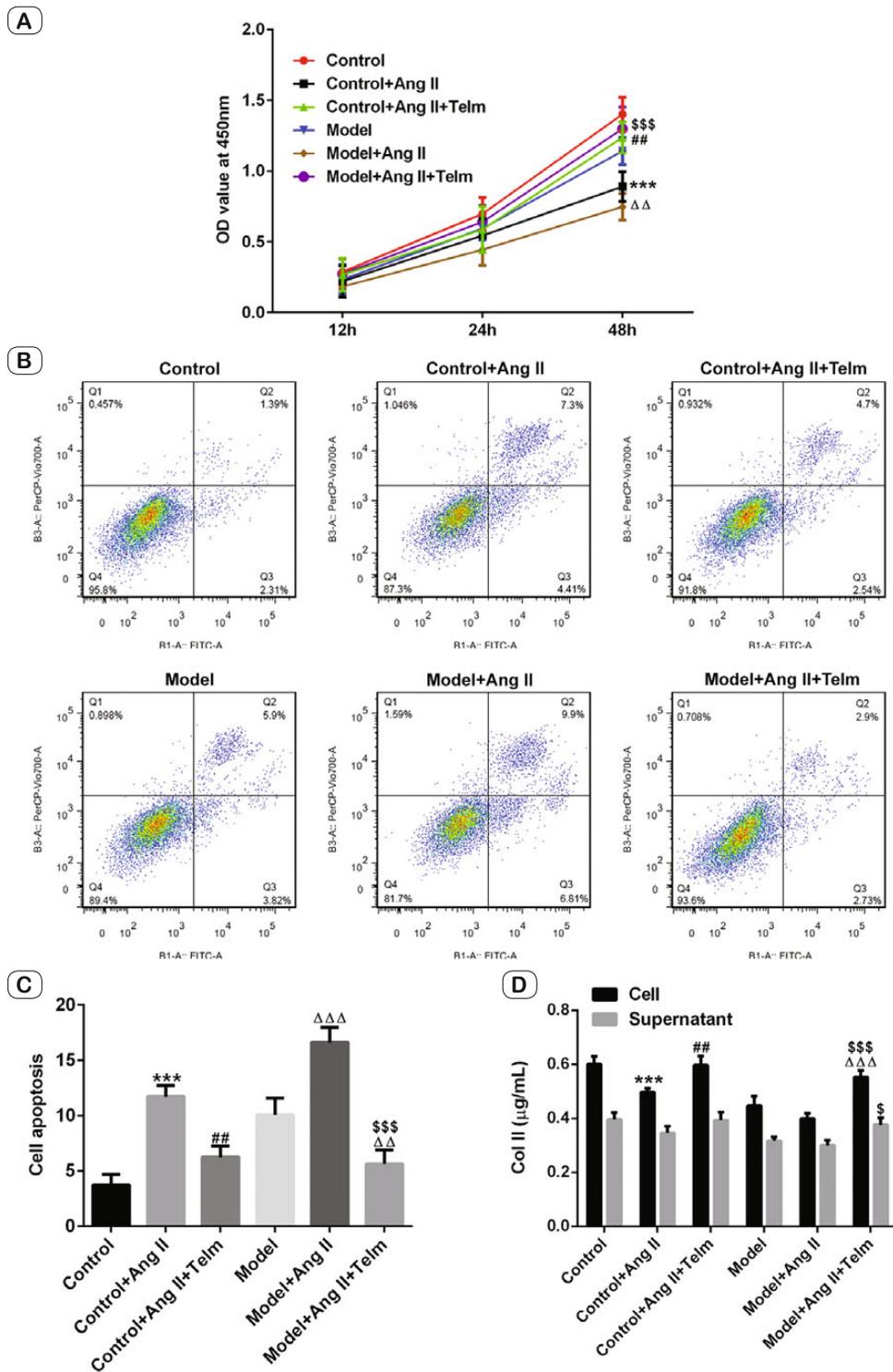


Fig. 3. Telmisartan affects the proliferation, apoptosis and collagen II expression of oxygen-glucose deprivation chondrocytes. (A) The proliferation of ATDC5 cells and oxygen-glucose deprivation ATDC5 cells was detected by CCK-8 assay after treatment with telmisartan (Telm). \*\*\* p < 0.001 vs control group. ## p < 0.01 vs Control+Ang II group. ΔΔ p < 0.01 vs model group. \$\$\$ p < 0.001 vs model+Ang II group. (B/C) The apoptosis of ATDC5 cells and oxygen-glucose deprivation ATDC5 cells was detected by flow cytometry analysis after treatment of telmisartan (Telm). \*\*\* p < 0.001 vs control group. ## p < 0.01 vs control+Ang II group. ΔΔ p < 0.01 and ΔΔΔ p < 0.001 vs model group. \$\$\$ p < 0.001 vs model+Ang II group. (D) The collagen II expression in ATDC5 cells and oxygen-glucose deprivation ATDC5 cells and supernatant after treatment of telmisartan (Telm) was analyzed by ELISA assay. \*\*\* p < 0.001 vs control group. ## p < 0.01 vs control+Ang II group. ΔΔΔ p < 0.001 vs model group. \$ p < 0.05 and \$\$\$ p < 0.001 vs model+Ang II group.

### Statistical analysis.

SPSS 19.0 software was used for statistical analysis of all the data. Measurement data were expressed as mean  $\pm$  standard deviation. One-way analysis of variance was used for comparison among multiple groups, and LSD-t method was used for multiple comparisons among groups.  $p < 0.05$  was considered statistically significant.

## Results

### Ang II affects the proliferation of oxygen-glucose deprivation chondrocytes

After ATDC5 cells were treated with sugar-deficient solution and incubated in 95%  $N_2$  and 5%  $CO_2$  incubator, the proliferation activity was gradually decreased from 12 h to 48 h. Moreover, Ang II treatment further inhibited the cell proliferation activity. The inhibition effect of 25  $\mu M$  Ang II treatment on cell proliferation activity was not obvious with that of 10  $\mu M$  Ang II treatment (Fig. 1).

### Ang II affects the expression of AT1/2R

The expression of AT1/2R was increased in oxygen-glucose deprivation ATDC5 cells (Fig. 2A). Furthermore, Ang II treatment promoted the expression of AT1/2R in oxygen-glucose deprivation

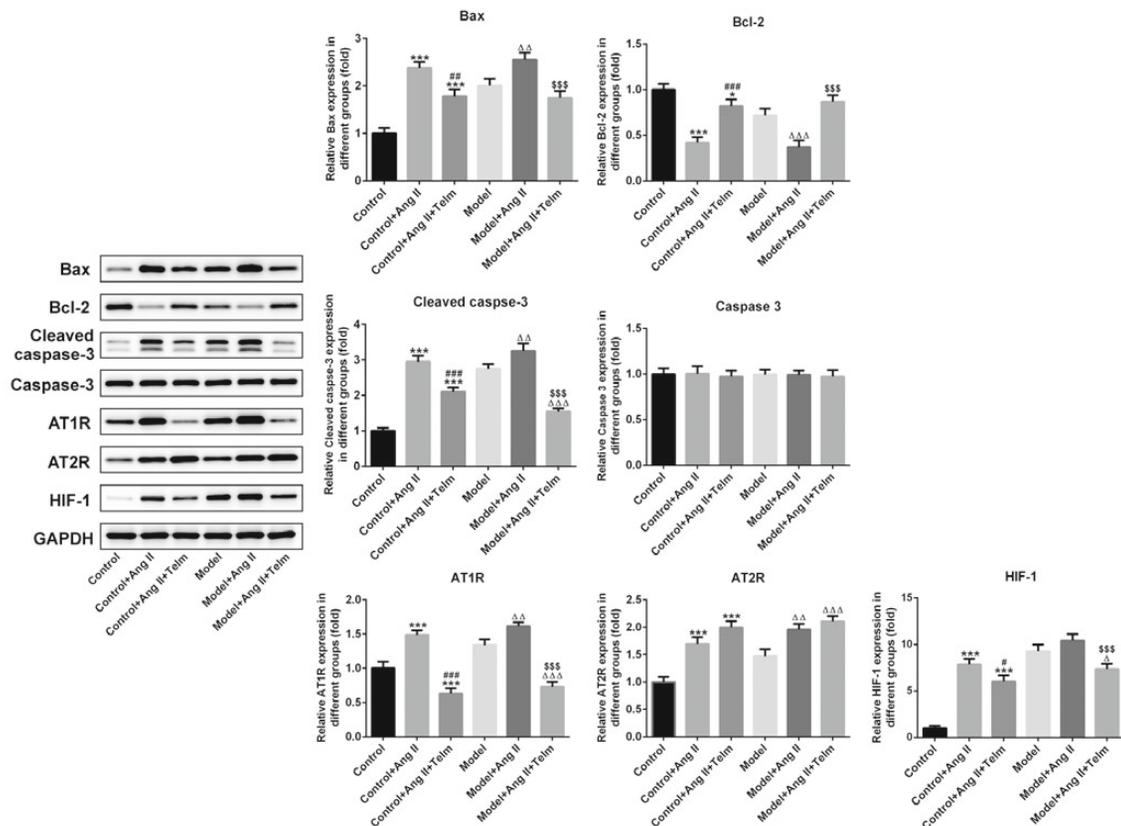
ATDC5 cells and the promotion effect of 25  $\mu M$  Ang II treatment was not obvious with that of 10  $\mu M$  Ang II treatment. As shown in Figure 2B, the ratio of AT1R to AT2R was increased and Ang II treatment further up-regulated the ratio. The effect of 25  $\mu M$  Ang II treatment on ATDC5 cells was not obvious with 10  $\mu M$  Ang II treatment.

### Telmisartan affects the proliferation, apoptosis and collagen II expression of oxygen-glucose deprivation chondrocytes

As shown in Figure 3A, Ang II treatment suppressed the proliferation activity of ATDC5 cells and oxygen-glucose deprivation ATDC5 cells and telmisartan could improve the proliferation activity of those cells. As shown in Figures 3B and 3C, the cell apoptosis was enhanced either in control+Ang II group or model+Ang II group while telmisartan decreased apoptosis of ATDC5 cells and oxygen-glucose deprivation ATDC5 cells. The collagen II expression in Ang II treatment group was decreased while telmisartan improved the collagen II expression in either intracellular or cellular supernatant (Fig. 3D).

### Telmisartan affects the expression of apoptosis-related proteins, AT1/2R and HIF-1

The expression of Bax, c-caspase-3 and HIF-1 was up-regulated and Bcl-2 expression was down-regulated either in control+Ang



**Fig. 4.** Telmisartan affects the expression of apoptosis-related proteins, AT1/2R and HIF-1. The expression of Bax, Bcl-2, Cleaved-caspase-3, Caspase-3, AT1R, AT2R and HIF-1 in ATDC5 cells and oxygen-glucose deprivation ATDC5 cells after treatment of telmisartan (Telm). \*  $p < 0.05$  and \*\*\*  $p < 0.001$  vs control group. #  $p < 0.05$ , ##  $p < 0.01$  and ###  $p < 0.001$  vs control+Ang II group. Δ  $p < 0.05$ , ΔΔ  $p < 0.01$  and ΔΔΔ  $p < 0.001$  vs model group. sss  $p < 0.001$  vs model+Ang II group.

II group or model+Ang II group. Ang II treatment promoted the AT1/2R expression in ATDC5 cells and oxygen-glucose deprivation ATDC5 cells. And, telmisartan reduced the AT1R expression while further increased the AT2R expression in ATDC5 cells and oxygen-glucose deprivation ATDC5 cells (Fig. 4).

## Discussion

Here, how AT1/2R expression affected the chondrocytes disintegration induced by oxygen-glucose deprivation was explored. It was shown that increased ratio of AT1R to AT2R suppressed the proliferation of oxygen-glucose deprivation ATDC5 cells which was promoted by the decreased ratio of AT1R to AT2R.

Ang II has two receptor subtypes called AT1R and AT2R, and they were considered to be involved in the progression of RA (17–18). Studies indicated that expression of Ang II, AT1R and AT2R was increased in OA (13–15). In this study, model of oxygen-glucose deprivation chondrocytes simulating the OA model in vitro was constructed. The expression of AT1R and AT2R was also increased in oxygen-glucose deprivation chondrocytes and ratio of AT1R to AT2R was also increased.

AT2R expression was increased in spleen and synovial tissue of adjuvant arthritis (AA) rats and intra-articular injection of AT2R agonist could reduce the infiltration of macrophages and T lymphocytes in synovium to inhibit the abnormal proliferation of synovio-cytes. Stimulation of AT2R could antagonize the high activity and strong chemotactic ability of peripheral monocytes mediated by IL-1 and inhibition of AT1R signal transduction played a protective role in the course of RA. The imbalance of AT1R/AT2R may be involved in synovitis, thus promoting the disease progression (18–19). In this study, telmisartan, as AT1R inhibitor, inhibited the AT1R expression and up-regulated the AT2R expression. Telmisartan promoted the proliferation and inhibited the apoptosis of ATDC5 cells and oxygen-glucose deprivation ATDC5 cells and improved the synthesis and secretion of collagen II in ATDC5 cells and oxygen-glucose deprivation ATDC5 cells by inhibiting AT1R and stimulating AT2R. The above findings were consistent with the functions of AT1R and AT2R in arthritis demonstrated in the present study.

## Conclusion

Generally, Ang II suppressed the proliferation of oxygen-glucose deprivation ATDC5 cells by increasing AT1/2R expression and ratio of AT1R to AT2R. Down-regulation of AT1R and up-regulation of AT2R caused by telmisartan promoted the proliferation and inhibited the apoptosis of oxygen-glucose deprivation ATDC5 cells. Our findings revealed the underlying mechanism of AT1/2R expression for OA development, which contributes to exploration of effective therapeutic targets for OA.

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