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ORIGINAL ARTICLE

Angiopoietin-like 2 upregulation promotes human chondrocyte injury via NF-κB and p38/MAPK signaling pathway

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Abstract

Several cellular and molecular processes participate in the pathologic changes of osteoarthritis (OA). However, the core molecular regulators of these processes are unclear, and no effective treatment for OA disease has been developed so far. ANGPTL2 is well known for its tissue remolding and pro-inflammation properties. However, the role of ANGPTL2 in osteoarthritis (OA) still remains unclear. To explore the expression level of ANGPTL2 in human OA cartilage and investigate the function of ANGPTL2 in human chondrocytes injury, qRT-PCR, western blot and immunohistochemistry were employed to investigate the expression of ANGPTL2 between human OA and normal cartilage samples. Next, human primary chondrocytes were treated with IL-1β to mimic OA progress in vitro, and the expression of ANGPTL2 were tested by qRT-PCR and western blot. Furthermore, the effect of ANGPTL2 in the expression of pro-inflammation cytokines (IL-1β, IL-6), proteolytic enzymes (MMP-1, MMP-13) and component of the cartilage matrix (COL2A1 and aggrecan) in human primary chondrocyte were explored by gain-of-function and loss-of-function methods. Finally, the nuclear factor kappa B (NF-κB) and p38/MAPK signaling pathways were also tested by western blot analysis. In this study, firstly, the expression level of ANGPTL2 was elevated both in human OA cartilage samples and IL-1β stimulated human chondrocytes. Secondly, ANGPTL2 upregulation promotes extracellular matrix (ECM) degradation and inflammation mediator production in human chondrocytes. Finally, ANGPTL2 activated the NF-κB and p38/MAPK signaling pathways via integrin α5β1. This study, for the first time, highlights that ANGPTL2 secreted by human chondrocytes plays a negative role in the pathogenesis of osteoarthritis, and it may be a potential therapeutic target in OA.

Keywords Osteoarthritis · ANGPTL2 · ECM degradation · Inflammation mediators production

Abbreviations

ANGPTL2	Angiopoietin-like 2
NF-κB	Nuclear factor kB
MAPK	Mitogen-activated protein kinase
OA	Osteoarthritis
MMP	Matrix metalloproteinase
ECM	Extracellular matrix
IL	Interleukin

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ITGA5 Integrin α5
ITGB1 Integrin β1

Introduction

Osteoarthritis (OA) is the most prevalent joint disease that causes joint pain and loss of function, which is viewed as a whole organ disease of the knee joint [1]. The pathologic changes occurring in OA joints including degradation of the articular cartilage, formation of osteophytes, thickening of the subchondral bone, inflammation of the synovium, etc. [2]. Several cellular and molecular processes participate in these pathologic changes such as imbalance in catabolism and anabolism in cartilage, hypertrophy and death of chondrocytes and activation of immune cells [2]. However, the core molecular regulators of these processes are unclear. Among the pathologic changes occurred in OA joints, the imbalance in cartilage catabolism and anabolism is one of the crucial factors in degradation of the articular cartilage [2–6]. Several studies have demonstrated that the matrix metalloproteinases (MMPs) family, such as MMP1 and MMP13 promote the excessive cartilage degradation via destroying the cartilage collagen type II network [7–9]. Meanwhile, low-grade, chronic inflammation is also associated with OA progression, and the overexpression of inflammatory cytokines, such as IL-1β, IL-6, TNF-α, have been identified in OA joint fluids and cartilage [1, 10, 11]. Current therapies for OA usually target the symptoms of the disease including pain control, viscosupplementation [12–17]. However, no effective treatment for OA disease has been developed so far, and as the disease continues to advance, joint replacement is often ultimately required to reduce pain and disability [18]. Novel therapeutics are needed to slow or stop the progression that drive OA pathology [19]. Therefore, exploration of the precise molecular mechanisms in the progression of OA to effectively treat OA is needed urgently.

Angiopoietin-like 2 (ANGPTL2) is a circulating protein, belongs to the angiopoietin-like family and contains an N-terminal coiled domain and a C-terminal fibrinogen-like domain (FLD) [20]. ANGPTL2 was first identified as its pro-angiogenic capacity, but it is better acknowledged that it contributes to promote low-grade chronic inflammation, and tissue remodeling [21]. Accumulating data indicated that ANGPTL2 can not only lead to extracellular matrix degradation, also cause inflammatory gene expression (such as IL-1β, IL-6, TNF-α) in chronic inflammation [21–23]. OA was also associated with aging and obesity as previous reported, and abnormal high expression of ANGPTL2 is often associated with aging, obesity, chronic hypoxia and so on [24, 25]. Therefore, these observations elicited our hypothesis that ANGPTL2 might contribute to the pathologic progression of OA.

To validate this, we set out to investigate differences in the expression of ANGPTLT2 in normal and OA human cartilage and found that ANGPTLT2 is highly expressed in human OA cartilage and IL-1β stimulated human primary chondrocytes. Further study suggested that ANGPTLT2 contributed to extracellular matrix (ECM) degradation and inflammation factors production via integrin α5β1/p38 MAPK and NF-κB signaling, and in doing so, defined the function of ANGPTLT2 in OA progression.

Materials and methods

Collection of human cartilage samples

Normal human cartilage samples were collected from 12 donors undergoing total hip replacement operation because of the femoral neck fracture (12 for total RNA and protein extraction, and 4 of them for immunohistochemical analysis). Osteoarthritic human cartilage samples were obtained from knee joints of 22 OA patients after total knee arthroplasty (4 for immunohistochemical analysis, 12 for total RNA and protein extraction and 6 for primary chondrocyte isolation). Patients' information is shown in Supplementary Table 1. For total RNA and protein extraction, the cartilage samples were cut into 1-mm^3 pieces, repeated grinding after adding liquid nitrogen three times and kept frozen at − 80 °C until used.

All cartilage samples were obtained from the First Affiliated Hospital of Anhui Medical University. A written informed consent was obtained from all the patients about the study. This research was approved by the Ethics Committee of the First Affiliated Hospital of Anhui Medical University.

Human primary chondrocyte extraction and culture

Cartilage samples were shaved off from intact, non-fibrillated areas of the articular surface and cut with scissors as smaller as possible. After digested in 0.25% trypsin–EDTA solution (Beyotime, China) for 30 min at 37 °C, the cartilage pieces were digested again in 0.4% collagenase II (Sigma, USA) for 24 h at 37 °C in a humidified atmosphere under 5% CO2 in air. Cells were cultured in growth media (DMEM/F12 1:1, Hyclone, USA) containing 10% fetal bovine serum (CLARK, USA) and 1% penicillin/streptomycin (penicillin–streptomycin solution, MRC, American). All cells were maintained at 37 °C in a humidified atmosphere under 5% CO2 in air and used at the end of second passage.

ANGPTL2 knockdown by siRNA

The cells were inoculated into a six-well plate the day before transfection (18–24 h) to allow the cell density to reach about 50–60% on the second day. Before performing the transfection procedure, each well was replaced with 2 ml of fresh medium (serum and antibiotic free). For cells in each well to be transfected, Opti-MEM (Sigma, USA) was added into two clean sterile centrifuge tubes (125 µl/tube), then 100 pmol siRNA or siNC was added to one of the tubes and 5 µl of Lipo6000TM transfection reagent (Beyotime, China) was mixed in another tube gently. After standing at room temperature for 5 min, the culture medium containing the siRNA was gently added into the culture medium containing Lipo6000TM transfection reagent. After incubating at room temperature for 20 min, the Lipo6000–siRNA mixture was added dropwise to the well. To achieve the highest transfection efficiency, cells were replaced with fresh complete cultures after 5 h of transfection. Cultured for 24 h after transfection, cells were stimulated or not with 10 ng/ml IL-1β and cultured for 24 h to isolate mRNA or proteins (siANGPTL2: sense 5′-gcaaggguuugggaacauutt-3′, antisense 5-aauguucccaaacccuugctt-3′; negative control: sense 5′- uucuccgaacgugucacgutt-3′, antisense 5′-acgugacacguucggagaatt-3′).

RNA isolation, reverse transcription and real-time PCR

For total RNAs isolated from cartilage samples (repeated grinding after adding liquid nitrogen as described), Column Cartilage RNAout (TIANDZ, CHINA) was used according to the manufacturer's instructions. For chondrocytes (cultured in a six-well plate) RNA extraction, TRIzol Reagent (Invitrogen, USA) was used according to the introductions. RNA concentration was measured by spectrophotometric analysis with a NANODROP 2000c (Thermo, USA). Reverse transcription was performed using a PrimeScript RT reagent Kit (TaKaRa, Japan) on a Mastercycler nexus gradient (Eppendorf, German) and real-time PCR was performed using a SYBR Premix Ex Taq II (TaKaRa, Japan) on an Agilent Technologies Stratagene Mx3000P (USA). GAPDH was used as a housekeeping control. Results were calculated using the relative quantitative method ($2^{-\Delta\Delta CT}$). Primer sequences are shown in Supplementary Table 2.

Western blotting analysis

Cartilage samples (repeated grinding after adding liquid nitrogen as described) and chondrocyte were lysed in RIPA Lysis Buffer (Beyotime, China) supplemented with PMSF (Beyotime, China) and phosphatase inhibitor (Beyotime, China). Proteins were separated on 10% or 12% SDS-PAGE gels and transferred to PVDF membranes (Immobilon-P Transfer Membrane 0.45 µm, Millipore, USA). After blocked with 5% skimmed milk powder in TBS-T, membranes were incubated with primary antibodies at 4 °C overnight, followed by incubation with HRP-conjugated secondary antibodies and imaged using the BeyoECL Moon (Beyotime, China) on Tanon 4500SF imaging system (China). All antibodies are shown in Supplementary Table 3.

Immunohistochemistry

Cartilage samples were fixed in 4% paraformaldehyde (Biosharp, China) for 24 h at 4 °C, decalcified in EDTA for 8 weeks at room temperature. After dehydration, the samples were embedded in paraffin and cut into sections. Then the paraffin sections were deparaffinized, rehydrated and heated for antigen retrieval. For inhibiting endogenous peroxidases, the sections were treated in 3% H_2O_2 for 10 min. After blocking with 5% normal goat serum for 2 h, the sections were incubated in the primary antibody overnight at 4 °C, followed by incubation with HRP-conjugated secondary antibodies for 2 h. The signal was developed with DAB (ZSGB-BIO, China) and nuclei were counterstained with hematoxylin. The sections were photographed by confocal laser scanning microscope (LSM880 + airyscan, Germany).

Statistical analysis

All the results of qRT-PCR reported were harvested from at least three independent experiments and each experimental data is the mean of three technical repeats. The Shapiro–Wilk test was performed to evaluate normal distribution of date and the Levene's test was performed for homogeneity of variance. To compare two groups, the independent-samples *t* test was performed. To compare four groups, the data were analyzed using either one-way analysis of variance (ANOVA) followed by Bonferroni test (equal variances) or Welch test followed by Games-Howell (equal variances not assumed). SPSS software (IBM Corporation) was used for date analyses.

Results

ANGPTL2 is upregulated in human OA cartilage and IL-1β-stimulated human primary chondrocytes

To explore the association of ANGPTL2 with OA pathogenesis, the expression levels of ANGPTL2 were first examined in human OA cartilages and IL-1β treated human primary chondrocytes. Identification of human primary chondrocytes was shown in SF.1A–D. As shown in Fig. 1A–C, western blot and Q-PCR assays revealed that

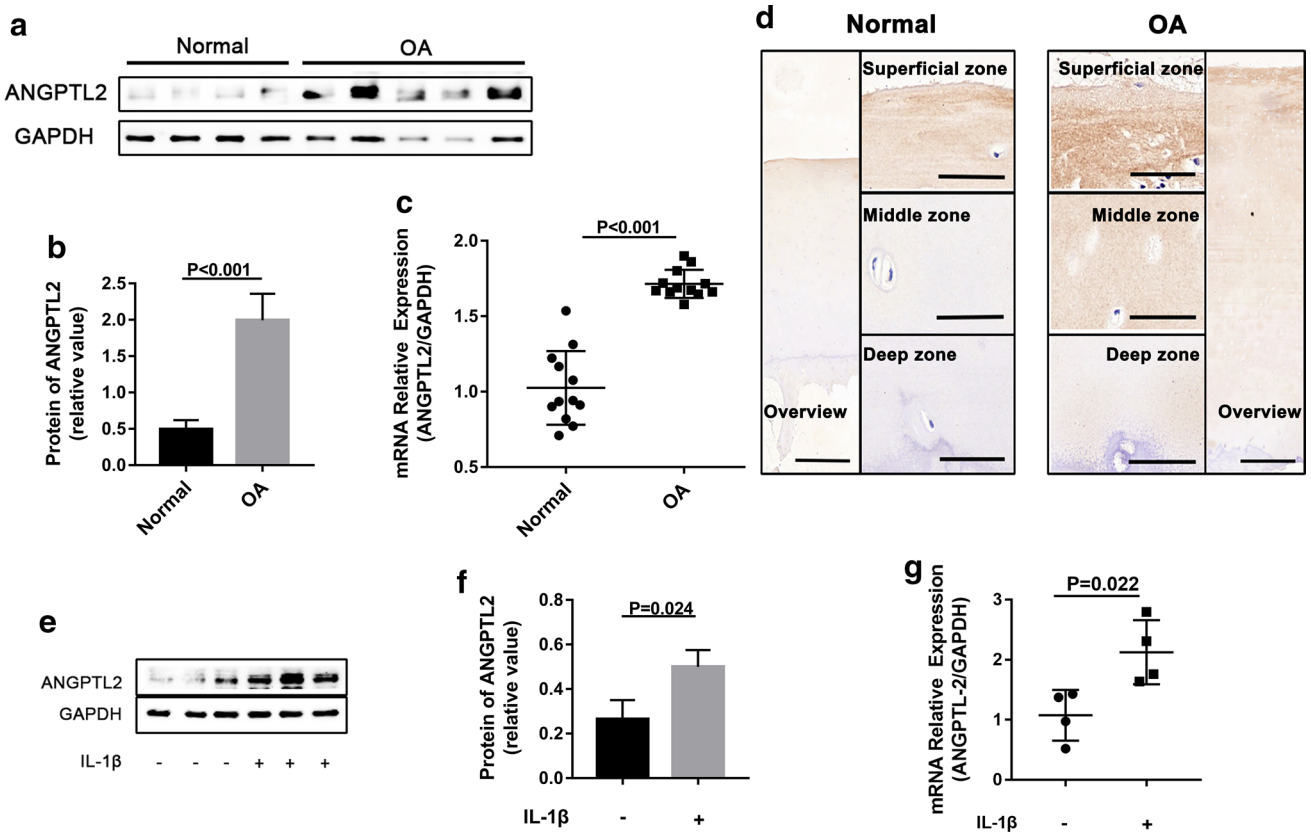

Fig. 1 ANGPTL2 is upregulated in human OA cartilage and IL-1β stimulated human primary chondrocytes. A Western blots analysis for the protein expression of ANGPTL2 isolated from human normal (n = 4) and OA (n = 5) cartilage samples, and B quantification of protein bands measured by Image J software. C qRT-PCR analysis for the mRNA expression levels of ANGPTL2 isolated from human normal (n = 12) and OA (n = 12) cartilages samples normalized to GAPDH expression as a control. D Immunohistochemical staining for ANGPTL2 on human normal and OA cartilage sections. E Western blots analysis for the protein expression of ANGPTL2 isolated from human chondrocytes stimulated with IL-1β (10 ng/ml) or not after 24 (n = 3) and F quantification of protein bands, measured by Image J software. G qRT-PCR analysis for the mRNA expression levels of ANGPTL2 isolated from human primary chondrocytes stimulated with IL-1β (10 ng/ml) or not after 24 (n = 4) and normalized to GAPDH expression as a control. F Results are expressed as mean ± SD, independent-samples *t* test. Scale bars (D, overview) = 500 μm. Scale bars (D, superficial, middle, deep zone) = 50 μm

the expression level of ANGPTL2 were markedly elevated in human OA cartilages (n = 12) compared with normal cartilage (n = 12). Consistent with these results, immunohistochemical staining (Fig. 1D) revealed that ANGPTL2 protein levels were markedly elevated in OA cartilages. IL-1β has been widely used to stimulate chondrocytes to induce osteoarthritis-like phenotype in vitro [26–28]. Therefore, we investigated the expression of ANGPTL2 in IL-1β treated human primary chondrocytes and found that the expression level of ANGPTL2 was significantly upregulated in human primary chondrocytes under stimulated with IL-1β stimulation (Fig. 1E–G). Together, these results suggested that ANGPTL2 was associated with OA pathogenesis.

Effects of ANGPTL2 on MMPs and COL2A1 production in human primary chondrocytes

MMP-1 and MMP-13 are important catabolic factor for extracellular matrix degradation in OA. Interestingly, MMPs expression has been reported to be increased by ANGPTL2 in osteosarcoma [23]. However, it remains unknown whether ANGPTL2 influence MMPs expression in OA pathogenesis. In this study, ANGPTL2 was silenced by siRNA (SF. 2AB). Our results indicated that treatment of chondrocytes with IL-1β could markedly enhance the expression of MMP-1 and MMP-13, whereas their expression level in mRNA and protein were significantly inhibited when ANGPTL2 was silenced in human primary chondrocytes (Fig. 2. A–C). On

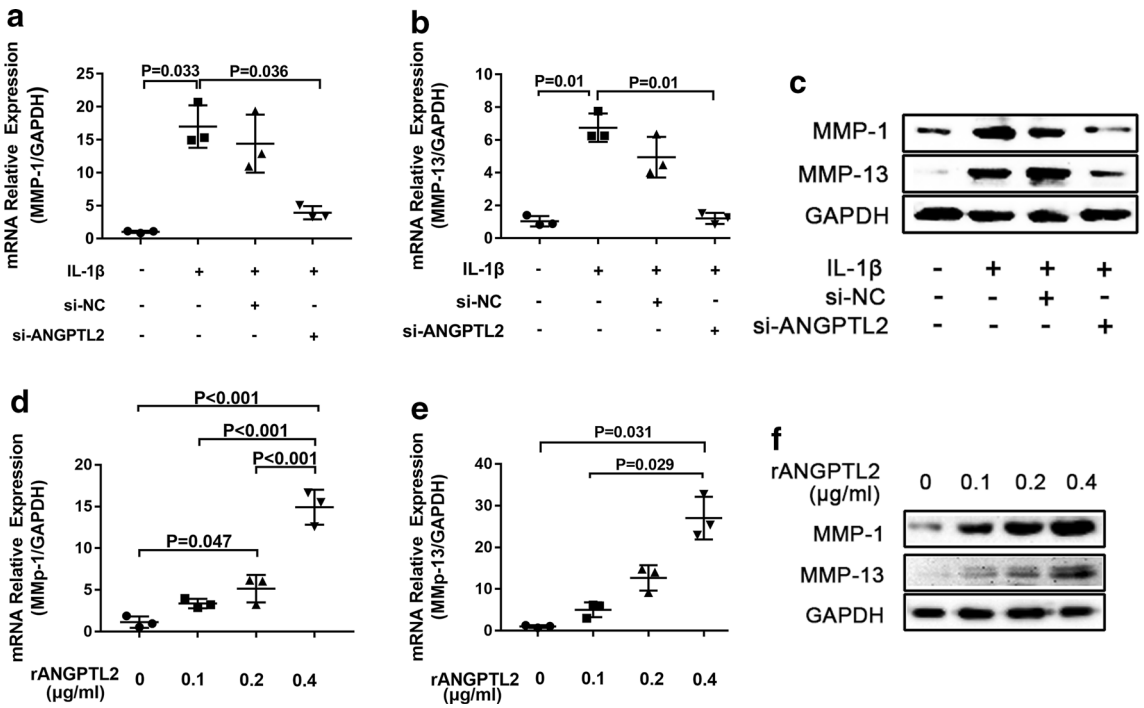

Fig. 2 Effects of ANGPTL2 on MMP-1 and MMP-13 production in human primary chondrocytes. A–C Silencing of ANGPTL2 reduced the expression of MMP-1 and MMP-13. Human chondrocytes were divided into four groups: Control, IL-1β (10 ng/mL), IL-1β (10 ng/ml) + siNC, IL-1β (10 ng/mL) + siANGPTL2. Chondrocytes were pretreated with siNC or siANGPTL2 for 24 h followed by stimulation with IL-1β (10 ng/ml) for 24 h, and then, RNA or protein was extracted from chondrocytes. A, B qRT-PCR analysis for the mRNA expression levels of MMP-1 and MMP-13. C Western blots analysis for the protein expression of MMP-1 and MMP-13. **D–F** rhANGPTL2 increased the expression of MMP-1 and MMP-13. Human primary chondrocytes were treated with a serial dosage (0,0.1,0.2,0.4 μg/ml) of rhANGPTL2 for 24 h. D, E qRT-PCR analysis for the mRNA expression levels of MMP-1 and MMP-13. F Western blots analysis for the protein expression of MMP-1 and MMP-13. Results are expressed as mean ± SD. E Analysis of variance (ANOVA) followed by Bonferroni test (equal variances). A, B, D Welch test followed by Games-Howell test (equal variances not assumed)

the contrary, rhANGPTL2 (PROSPEC, Israel) could also promote MMP-1 and MMP-13 production in a dose dependent manner (Fig. 2D–F). COL2A1 and aggrecan, secreted by chondrocytes, are important components of the cartilage matrix. Our results indicated that treatment of chondrocytes with IL-1β could markedly inhibited the expression of COL2A1 and aggrecan, and only COL2A1 expression level in mRNA and protein were significantly enhanced when ANGPTL2 was silenced in human primary chondrocytes (SF.3A–C). On the contrary, rhANGPTL2 (PROSPEC, Israel) could also suppressed COL2A1 production in a dose dependent manner and there was no significant change in aggrecan expression (SF.3D–F). In addition, as shown in SF 4, there was no significant change in the expression of caspase-3, a key enzyme related to cell apoptosis, when knocking down ANGPTL2 in human primary chondrocytes, knocking down of ANGPTL2 may not affect the apoptosis of human primary chondrocytes. All these suggested that ANGPTL2 upregulation contributed to extracellular matrix degradation by increasing the expression of MMP-1 and MMP-13 and decreasing COL2A1 in human chondrocytes.

Effects of ANGPTL2 on IL-1β and IL-6 production in human primary chondrocytes

Low-grade, chronic inflammation has a central role in the pathogenesis of OA. Interestingly, ANGPTL2 is also acknowledged for its pro-inflammatory properties. However, the pro-inflammatory properties of ANGPLT2 remain unknown in OA pathogenesis. IL-1β-stimulation of human chondrocytes resulted in a marked up-regulation of the mRNA and protein levels of IL-1β and IL-6, but knockdown of ANGPLT2 resulted in significant inhibition of expression of IL-1β and IL-6 (Fig. 3A–C). Additionally, treatment of chondrocytes with rhANGPTL2 can markedly promote IL-1β and IL-6 production (Fig. 3D–F). These results suggested that ANGPTL2 might exert potent pro-inflammatory effects through increasing the expression of IL-1β and IL-6 in chondrocytes.

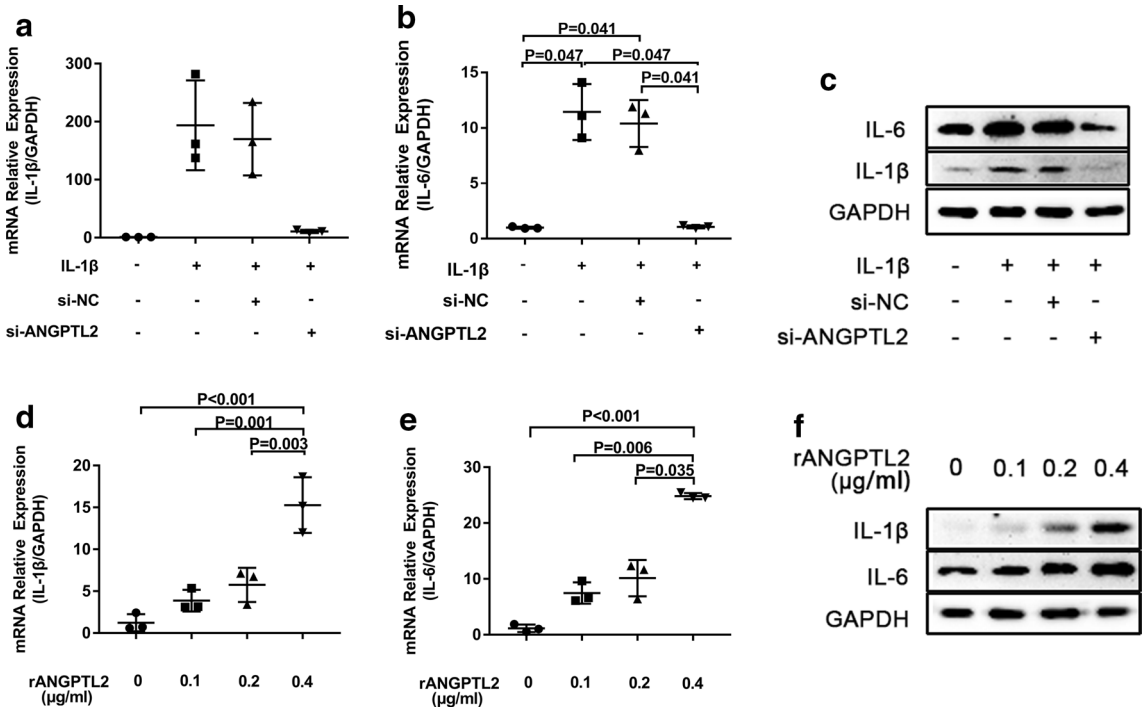

Fig. 3 Effects of ANGPTL2 on IL-1β and IL-6 production in human primary chondrocytes. A–C Silencing of ANGPTL2 reduced the expression of MMP-1 and MMP-13. Human primary chondrocytes were divided into four groups: Control, IL-1β (10 ng/mL), IL-1β (10 ng/mL) + siNC, IL-1β (10 ng/ml) + siANGPTL2. Chondrocytes were pretreated with siNC or siANGPTL2 for 24 h followed by stimulated with IL-1β (10 ng/ml) for 24 h, and then, RNA or protein was extracted from chondrocytes. A, B qRT-PCR analysis for the mRNA expression levels of IL-1β and IL-6. C Western blots analysis for the protein expression of IL-1β and IL-6. **D–F** rhANGPTL2 increased the expression of IL-1β and IL-6. Human primary chondrocytes were treated with a serial dosage (0,0.1,0.2,0.4 μg/ml) of rhANGPTL2 for 24 h. D, E qRT-PCR analysis for the mRNA expression levels of IL-1β and IL-6. F Western blot analysis for the protein expression of IL-1β and IL-6. Results are expressed as mean ± SD. D Analysis of variance (ANOVA) followed by Bonferroni test (equal variances). A, B, E Welch test followed by Games-Howell (equal variances not assumed)

ANGPTL2 activates NF-κB and p38/MAPK signaling via integrin α5β1 in human primary chondrocytes

Much research in recent years has indicated that ANGPTL2 can influence the p38 MAPK and NF-κB signaling pathways, and both pathways play an important role in mediating the expression of MMPs and inflammatory mediators in OA progression [23, 24, 29, 30]. To investigate the molecular mechanisms responsible for the observed chondrocyte injury of ANGPTL2, we determined whether ANGPTL2 promotes chondrocyte injury through the activation of p38 MAPK and NF-κB signaling. Results in Fig. 4A–C illustrate that stimulation of chondrocytes with IL-1β activated the phosphorylation of p38 and p65. Surprisingly, and silencing ANGPTL2 in chondrocytes can suppress the p38 and p65 phosphorylation levels. In addition, we found that rhANGPTL2 (0.1, 0.2, 0.4 μg/ml) could also enhance the p38 MAPK and p65 phosphorylation levels (Fig. 4.D–F). It was reported that integrin α5β1 functions as ANGPTL2 receptors in ATDC and other cells [23, 25, 30]. To investigate the relationship between integrin α5β1 and ANGPTL2, we first examined the expression of ITGA5 (integrin α5) and ITGB (integrin β1) in normal and OA human cartilage and chondrocyte (treated with IL-1β) [30]. Our results indicated that ITGA5 (integrin α5) and ITGB (integrin β1) are highly expressed in human OA cartilage and IL-1β stimulated human primary chondrocytes (Fig. 5.A–D). ATN-161 is an antagonist of integrin α5β1 and has been widely used in integrin α5β1 blocking studies [31–33]. Our results indicated inhibition of integrin α5β1 with ATN-161(100 μg/ml) significantly antagonizes the phosphorylation levels of p38 and p65 in primary chondrocyte treated with rhANGPTL2 (0.4 μg/ml) (Fig. 5. E–G).

These data suggested that high expression of ANGPTL2 in OA progression exerts its function by regulating p38 MAPK and NF-κB signaling via integrin α5β1.

Discussion

OA has been recognized as a degenerative disease of cartilage in elderly population, which damages the entire joint structure and leads to chronic pain and joint disability [2, 34–38]. In recent decades, accumulating research has identified various risk factors for the incidence of OA. However,

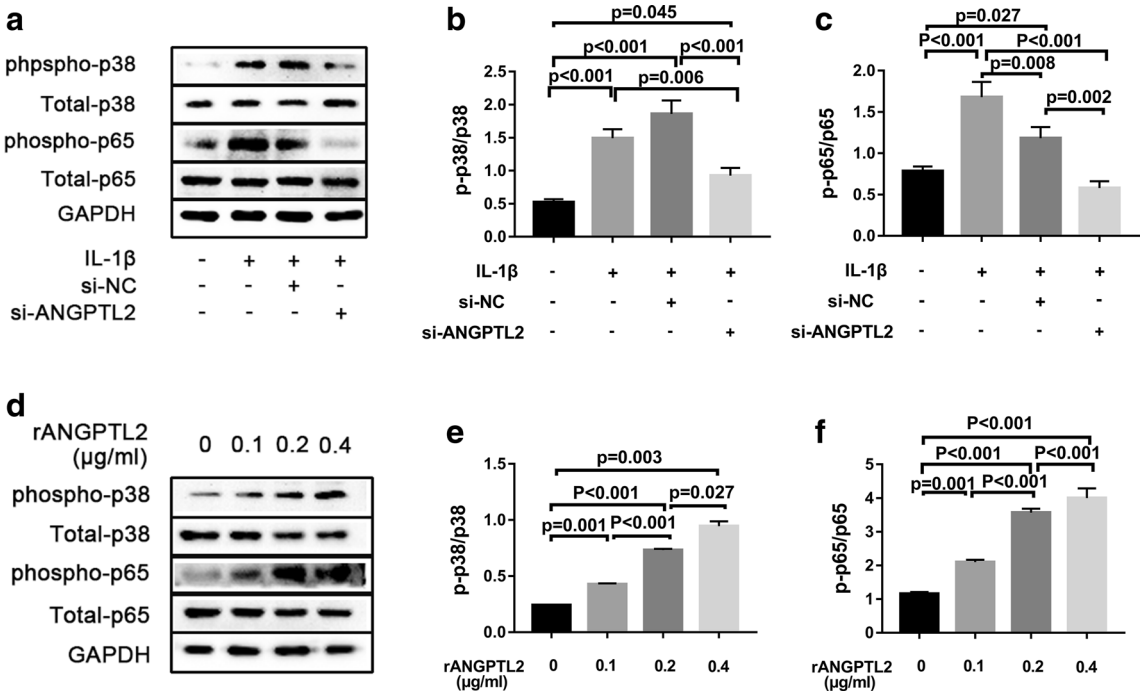

Fig. 4 Effects of ANGPTL2 on the NF-κB and p38/MAPK signaling pathways. A–C Silencing of ANGPTL2 reduced the activation of NF-κB and p38/MAPK signaling pathways. Human primary chondrocytes were divided into four groups: Control, IL-1β (10 ng/mL), IL-1β (10 ng/mL) + siNC, IL-1β (10 ng/mL) + siANGPTL2. Chondrocytes were pretreated with siNC or siANGPTL2 for 24 h followed by stimulated with IL-1β (10 ng/ml) for 24 h, and then, protein was extracted from chondrocytes. A Western blots analysis for the protein expression of p65, phosphorylated p65, p38 and phosphorylated p38. B The ratios between phosphorylated p38 and p38. C The ratios between phosphorylated p65 and p65. Quantification of protein bands measured by Image J software. Results are expressed as mean ± SD. One-way analysis of variance (ANOVA) followed by Bonferroni test (equal variances). D–F rhANGPTL2 increased the activation of NF-κB and p38/MAPK signaling pathways. Human primary chondrocytes were treated with a serial dosages (0,0.1,0.2,0.4 μg/ml) of rhANGPTL2 for 24 h. D Western blots analysis for the protein expression of p65, phosphorylated p65, p38 and phosphorylated p38 extracted from chondrocytes. E The ratios between phosphorylated p38 and p38. F The ratios between phosphorylated p65 and p65. Quantification of protein bands measured by Image J software. Results are expressed as mean ± SD. One-way analysis of variance (ANOVA) followed by Bonferroni test (equal variances)

to date, there is no curative treatment for OA. Therefore, clarifying the relative molecular regulators in the progression of OA may contribute to improve therapeutic effect, even cure it. In this study, we observed the obvious elevation of ANGPTL2 in human OA cartilage samples. Furthermore, IL-1β, a key contributor to OA development, also promoted the expression of ANGPTL2. Therefore, these results suggested that ANGPTL2 plays a potential crucial role of ANGPTL2 in the progression of OA.

The destruction of articular cartilage plays an important role in OA development. COL2A1 and aggrecan, secreted by chondrocytes, are important components of the cartilage matrix. In normal context, the cartilage matrix synthesis and degradation are maintained in a dynamic balance in articular cartilage. However, the homeostasis will be broken under OA pathologic condition. It is well acknowledged that matrix-degrading genes, such as MMP-1, MMP-3, MMP-13, play an important role in physiological turnover of OA cartilage by degrading the extracellular matrix (ECM) molecules [5, 10, 39, 40]. In addition, ANGPTL2 enhances tumor cell invasion by increasing expression and activity of MMPs in osteosarcoma [23]. Therefore, to examine the function of ANGPTL2 in OA progression, we investigated its role in chondrocyte metabolic dysfunction. Similar to other studies, IL-1β treatment promoted the expression of MMP-1 and MMP-13 production. Intriguingly, ANGPTL2 inhibition could counteract the production of MMP-1 and MMP-13 upon IL-1β stimulation. In addition, MMP-1, MMP-13 were also increased in chondrocyte treated with rhANGPTL2. And besides, we also found that knockdown of ANGPTL2 increased COL2A1 expression and chondrocyte treated with rhANGPTL2 inhibited COL2A1 expression. Accordingly, these results suggest that ANGPTL2 suppression may ameliorate OA pathologic progression by inhibiting the extracellular matrix degradation.

Accumulating studies indicate that low-grade chronic inflammation plays also a pivotal role in OA pathogenesis by releasing various inflammatory cytokines and mediators [1, 11, 41]. Among these cytokines, IL-1β, IL-6 and TNF-α, have been extensively studied in OA, and exerts a

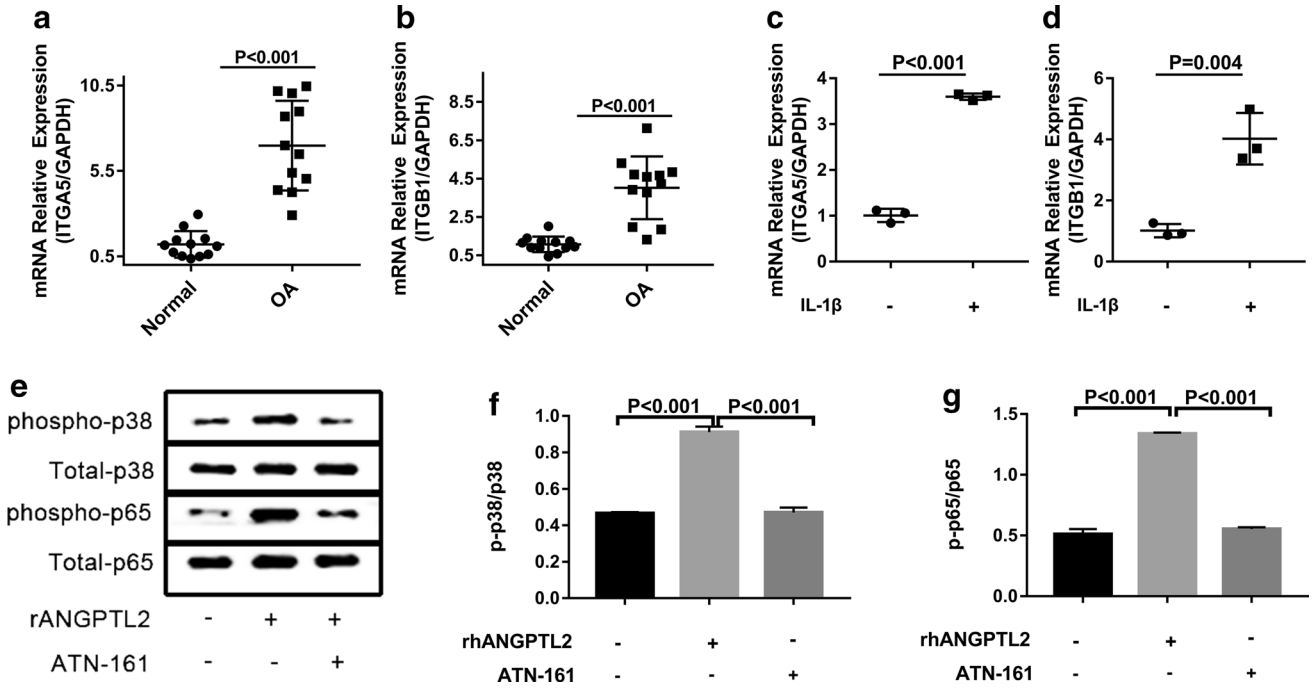

Fig. 5 ANGPTL2 activates NF-κB and p38/MAPK signaling via integrin α5β1. A, B qRT-PCR analysis for the mRNA expression levels of ITGA5 and ITGB1 isolated from human normal (n = 12) and OA (n = 12) cartilages samples normalized to GAPDH expression as a control. C, D qRT-PCR analysis for the mRNA expression levels of ITGA5 and ITGB1 isolated from human primary chondrocytes stimulated with IL-1β (10 ng/ml) or not after 24 (n = 4) and normalized to GAPDH expression as a control. E, F, G Human chondrocytes were divided into three groups: control, rhANGPTL2 (0.4 μg/ml), rhANGPTL2 (0.4 μg/ml) +ATN-161 (100 μg/ml). Chondrocytes were pretreated with ATN-161 (100 μg/ml) for 24 h followed by stimulated with rhANGPTl2 for 24 h, and then, protein was extracted from chondrocytes. Western blots analysis was used to evaluate the expression of p65, phosphorylated p65, p38 and phosphorylated p38. F The ratios between phosphorylated p38 and p38. G The ratios between phosphorylated p65 and p65. Quantification of protein bands measured by Image J software. Results are expressed as mean ± SD. A, B, C, D Independent-samples *t* test. F, G One-way analysis of variance (ANOVA) followed by Bonferroni test (equal variances)

significant role in OA development. IL-1β stimulation in chondrocytes can increase the expression of MMPs and inflammatory cytokines that further exacerbate inflammatory response and promote OA progression. An increasing body of evidence has shown that inhibition of chondrocyte inflammation was a promising therapeutic strategy in OA. Previews studies have shown that ANGPTL2 can lead to inflammatory response in the context of obesity and diabetes. Here, our data revealed that blocking ANGPTL2 expression could significantly antagonize the IL-1β-triggered inflammatory genes expression including IL-1β and IL-6. Intriguingly, rhANGPTL2 treatment promoted inflammatory response by elevating the releasing of IL-1β and IL-6.

The mechanism of response to OA progression is complicated, and several signal pathways are involved, such as NF-κB pathway and p38 MAPK pathway [23, 24, 29, 30]. The inhibitors of NF-κB proteins (IκB) phosphorylation is induced by IL-1β stimulation, results in the phosphorylation of p65 and activated p65 is translocated from the cytoplasm to the nucleus. Consequently, the expression of catabolic enzymes and inflammatory mediators is triggered in the nucleus [42, 43]. In addition, p38 MAPK pathway is implicated in multiple pathologic activities, like tumor metastasis and some inflammatory diseases, but mass evidence has shown that p38 MAPK pathway plays an important role in OA pathogenesis and progression. Moreover, IL-1β treatment in chondrocytes can enhance the phosphorylation of p38. It was also reported this pathway can trigger the expression of inflammation-related genes and catabolic enzymes, and consequent cartilage destruction, including IL-6, MMP-1 and MMP-13. Integrin α5β1, composed of one α5- and one β1-subunit, reportedly functions as ANGPTL2 receptor in ATDC and other cells. In this study, our data indicated that IL-1β-stimulation of human chondrocytes resulted in a marked up-regulation of the expression levels of p-p65, p-p38, ITGA5 and ITGB1, however, knockdown of ANGPLT2 resulted in significant inhibition of expression of p-p65, p-p38. Additionally, treatment of chondrocytes with rhANGPTL2 can markedly promote p-p65 and p-p38 production and inhibition of integrin α5β1 with ATN-161 significantly antagonize the phosphorylation levels of p38 and p65 in primary chondrocyte treated with rhANGPTL2. These results suggested that ANGPTL2 might promote chondrocyte injury through

the NF-κB and p38 MAPK signaling pathways via integrin α5β1.

Both articular cartilage and epiphyseal cartilage belong to hyaline cartilage, but they are different in structure and function. In epiphyseal cartilage, endochondral osteogenesis is a progressive process involved in bone formation and growth. Undifferentiated mesenchymal stem cells initially condensate and differentiate into immature chondrocytes (early-phase differentiation), which then undergo successive maturation steps to differentiation become hypertrophic chondrocytes (late-phase differentiation) [44]. In a previous study, ATDC cells (chondrogenic cell line, not chondrocyte) were treated with insulin to mimic progress chondrogenic differentiation in vitro. Knockdown of ANGPLT2 resulted in significant inhibition of expression of chondrocyte marker (COL2A1 and aggrecan) in early-phase differentiation and the hypertrophic chondrocyte marker (Col10a1, MMP-13) in late-phase differentiation (in our study, we also observed that knockdown of ANGPLT2 decreased the expression of MMP-13 in vitro OA model). In ANGPTL2 knockout mice, they observed a decrease both in size and quantity of hypertrophic chondrocytes. And the expression level of the hypertrophic chondrocyte marker (Col10a1, MMP-13) was also inhibited. By contrast, apoptosis chondrocyte in the hypertrophic zone increased in ANGPTL2 knockout mice [30]. All these reveled that ANGPTL2 promote chondrogenic differentiation (mesenchymal stem cells into chondrocytes and chondrocytes into hypertrophic chondrocytes) and ultimately cartilage ossification in epiphyseal cartilage during bone growth. Different from epiphyseal cartilage, it is widely accepted in the textbooks that articular cartilage in adult consists of only terminal differentiated chondrocyte and lacks mesenchymal stem cells. In addition, both hypertrophy of articular chondrocytes and ossification of cartilage are important pathogenesis of OA [45, 46]. On the whole, ANGPTL2 is not a protective protein of cartilage which eventually converts epiphyseal cartilage into bone, promoting chronic inflammation of articular cartilage and degradation of cartilage matrix.

In conclusion, this study is the first research showing that ANGPTL2 secreted by human chondrocytes plays a negative role in the pathogenesis of osteoarthritis, and it may be a potential therapeutic target in osteoarthritis.

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Author contributions Zongsheng Yin, Wei He and Jiegou Xu designed the study. Wenshan Shan, Zhenfei Ding, Guanjun Cui performed in vitro experiments. Chao Chen, Wei Huang, Wei Lu and Fuen Liu detected the expression level of ANGPTL2 in OA cartilage samples. Sha Luo participated in the supplementary experiment. Wenshan Shan wrote the paper.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no competing interests.

Ethics approval All cartilage samples were obtained from the First Affiliated Hospital of Anhui Medical University. This research was approved by the Ethics Committee of the First Affiliated Hospital of Anhui Medical University.

Informed consent All patients obtained a written informed consent about the study.

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