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Long non-coding RNA highly up-regulated in liver cancer protects tumor necrosis factor-alpha-induced inflammatory injury by down-regulation of microRNA-101 in ATDC5 cells

Peigang Chu[a], Qiang Wang[b], Zongru Wang[c], Chunzheng Gao[a,*]

[a] The Second Hospital of Shandong University, Jinan 250000, China
[b] Department of Joint Surgery, Taian City Central Hospital, Taian 271000, China
[c] Second Department of Orthopedics, The First People's Hospital of Taian, Taian 271000, China

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ABSTRACT

Background: Osteoarthritis (OA) is a familiar joint degenerative disease. Long non-coding RNAs (lncRNAs) play vital roles in the pathogenesis of OA. Nevertheless, the regulatory impacts of lncRNA highly up-regulated in liver cancer (lncRNA-HULC) on OA remain dimness. The study tried to probe the protective effect of HULC on ATDC5 cells against tumor necrosis factor-alpha (TNF-α)-induced inflammatory injury.

Methods: Relative expression levels of pro-inflammatory cytokines (IL-6, IL-8 and MCP-1) and HULC in OA cartilage tissues and normal cartilage tissues were determined by RT-qPCR. TNF-α induced inflammatory injury model in ATDC5 cells was constructed, and the biological functions of HULC overexpression or suppression in TNF-α-injured ATDC5 cells were assessed. The relevancy between miR-101 and HULC was investigated by utilizing bioinformatics prediction, luciferase reporter assay, RNA pull-down and immunoprecipitation. MiR-101 mimic and inhibitor were transfected into ATDC5 cells, and its regulatory effect on TNF-α-injured ATDC5 cells was examined. Further, NF-κB and MAPK signaling pathways were finally detected by western blot.

Results: Enhancement of IL-6, IL-8 and MCP-1 were observated in OA cartilage tissues, but repression of HULC was discovered in OA cartilage tissues. HULC expression was decreased by TNF-α treatment, and overexpressed HULC significantly relieved TNF-α-induced ATDC5 cells injury. Additionally, miR-101 was mutual repressed with HULC, and overexpressed miR-101 reversed the protective effect of HULC in TNF-α-injured ATDC5 cells. Besides, HULC blocked NF-κB and MAPK pathways via repression of miR-101.

Conclusions: The discoveries testified that HULC protected ATDC5 cells against TNF-α-induced inflammatory injury by repression of miR-101.

1. Introduction

Osteoarthritis (OA), also known as degenerative arthritis, is considered as a non-inflammatory arthritis [1]. The occurrence of OA is based on cartilage degeneration, which may include bone hyperplasia and joint swelling [2,3]. The high risk factors for causing OA are mainly associated with age, joint trauma, obesity, and inherited factor [4]. The clinical manifestations of OA include joint pain, stiffness and joint deformity [5,6]. Recently, the managements of OA include lifestyle changes, physical measures, pain medication and surgery [7,8]. Importantly, artificial joint replacement has become the most effective way to recover joint function and improve the quality of life in patients with advanced OA [9]. However, in the early stage of OA, it is difficult to diagnose because of lacking the specific symptoms or signs. Therefore, the novel diagnostic and therapeutic biomarker is urgently necessary for early OA.

Long non-coding RNAs (lncRNAs) are a type of non-coding RNA molecules with > 200 nucleotides. The abnormal expression of lncRNAs has been reported to play a crucial role in regulating various inflammatory diseases, including OA [10,11].

A expression profile assay disclosed that 4067 enhancement of lncRNAs and 2231 repression of lncRNAs were discovered in OA group, which hinted that lncRNAs might play a momentous role in pathogenesis of OA and might be latent biomarkers or therapic targets of OA [12]. Recent study demonstrated that lncRNA acted as an important regulator in cartilage injury, which could promote chondrocyte extracellular matrix degradation in OA [13]. One study from Li et al. reported that lncRNA cartilage injury-related (lncRNA-CIR) could

* Corresponding author at: The Second Hospital of Shandong University, No.247 Beiyuan Street, Jinan 250000, China.
E-mail address: gaochunzheng123@sina.com (C. Gao).

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Table 1
The primer sequences for RT-qPCR.

Gene name	Sequences
IL-6	Forward: 5′-CTGCAAGAGACTTCCATCCAG-3′
	Reverse: 5′-AGTGGTATAGACAGGTCTGTTGG-3′
IL-8	Forward: 5′-ACCACACTGCGCCAACACAGAAAT-3′
	Reverse: 5′-TCCAGACAGAGCTCTCTTCCATCAGA-3′
MCP-1	Forward: 5′-GCATCCACGTGTTGGCTCA-3′
	Reverse: 5′-CTCCAGCCTACTCATTGGGATCA-3′
HULC	Forward: 5′-ATCTGCAAGCCAGGAAGAGTC-3′
	Reverse: 5′-CTTGCTTGATGCTTTGGTCTGT-3′
β-actin	Forward: 5′-CGTGCGTGACATCAAAGAGAA-3′
	Reverse: 5′-TGGATGCCACAGGATTCCCAT-3′
Rno-miR-101	Forward: 5′-ACACTCCAGCTGGGTACAGTACTGTGATA-3′
	Reverse: 5′-TGGTGTCGTGGAGTCG-3′
U6	Forward: 5′-CTCGCTTCGGCAGCACA-3′
	Reverse: 5′-AACGCTTCACGAATTTGCGT-3′

promote chondrocyte extracellular matrix degradation in OA through acting as a sponge for microRNA (miR)-27b [14]. Further, another interesting research from Zhang et al. revealed that lncRNA ubiquitin-fold modifier conjugating enzyme 1 (lncRNA-UFC1) could promote cell proliferation and reduce apoptosis of chondrocyte in OA by regulation of miR-34a [15]. LncRNA highly up-regulated in liver cancer (lncRNA-HULC) is an important lncRNA with a wide variety functions, which has been reported to be associated with the development of diverse cancers [16,17]. Additionally, emerging evidence stated that down-regulated HULC might be related to chondrocyte death during OA pathogenesis [18]. Nevertheless, the functions of HULC in OA are still indistinct. Further studies are still needed for exploring this matter. The influence of HULC on OA has aroused our strong research interest.

The current study aimed to investigate the regulatory effect of HULC on OA. ATDC5 is a murine chondrocyte cell line, which frequently-used to construct an inflammatory injury model in OA. Therefore, we utilized tumor necrosis factor-alpha (TNF-α) to dispose ATDC5 cells to construct an inflammatory injury model to mimic OA processes. The functions of HULC in cell proliferation, apoptosis and pro-inflammatory cytokines were then determined in TNF-α-treated ATDC5 cells. Related signaling pathways of nuclear factor-kappa B (NF-κB) and p38 mitogen-activated protein kinase (p38MAPK) were examined to uncover the underlying mechanism. The study might provide a novel therapeutic biomarker for early diagnosis and treatment of OA.

2. Materials and methods

2.1. Clinical specimens

OA cartilage tissues were obtained from the patients following total knee replacement surgery and the normal cartilage tissues were obtained from the neck of femur fracture patients (n = 20, 10 females and 10 males; age 54 ± 5 years). These patients were attained from The Second Hospital of Shandong University (Jinan, China) from November 2015 to December 2017. Informed consents from every patient were obtained, and the present study was approved by the Medical Ethics Committee of the The Second Hospital of Shandong University.

2.2. Cell culture and treatment

ATDC5 cells were purchased from RIKEN Cell Bank (Tsukuba, Japan). Human primary chondrocyte cells were isolated based on the previous description of Khair et al. [19]. ATDC5 and the primary chondrocyte cells were cultured in Dulbecco's modified Eagle's medium/Ham's F12 (DMEM/F12, Life Technologies, Carlsbad, CA, USA) containing with 5% fetal bovine serum (FBS, Life Technologies) 100 U/mL penicillin and 100 μg/mL streptomycin in a humidified

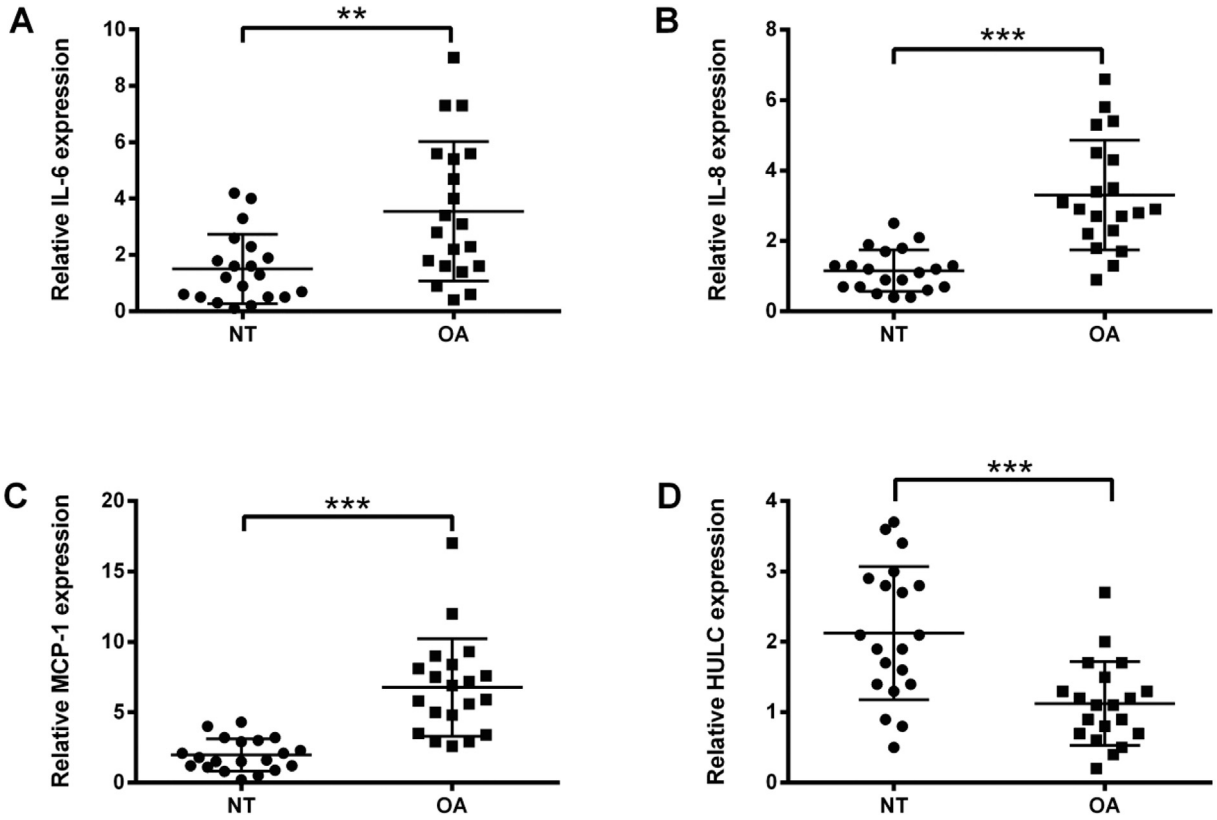

Fig. 1. HULC expression was down-regulated in cartilage tissues from OA patients.
The cartilage tissues and normal cartilage tissues (NT) were obtained from 20 OA patients. Relative expression levels of (A-C) pro-inflammatory cytokines (IL-6, IL-8 and MCP-1) and (D) HULC were determined by using RT-qPCR in NT and OA groups. Data are presented as the mean ± SD. $^{**}P < 0.01$, $^{***}P < 0.001$.

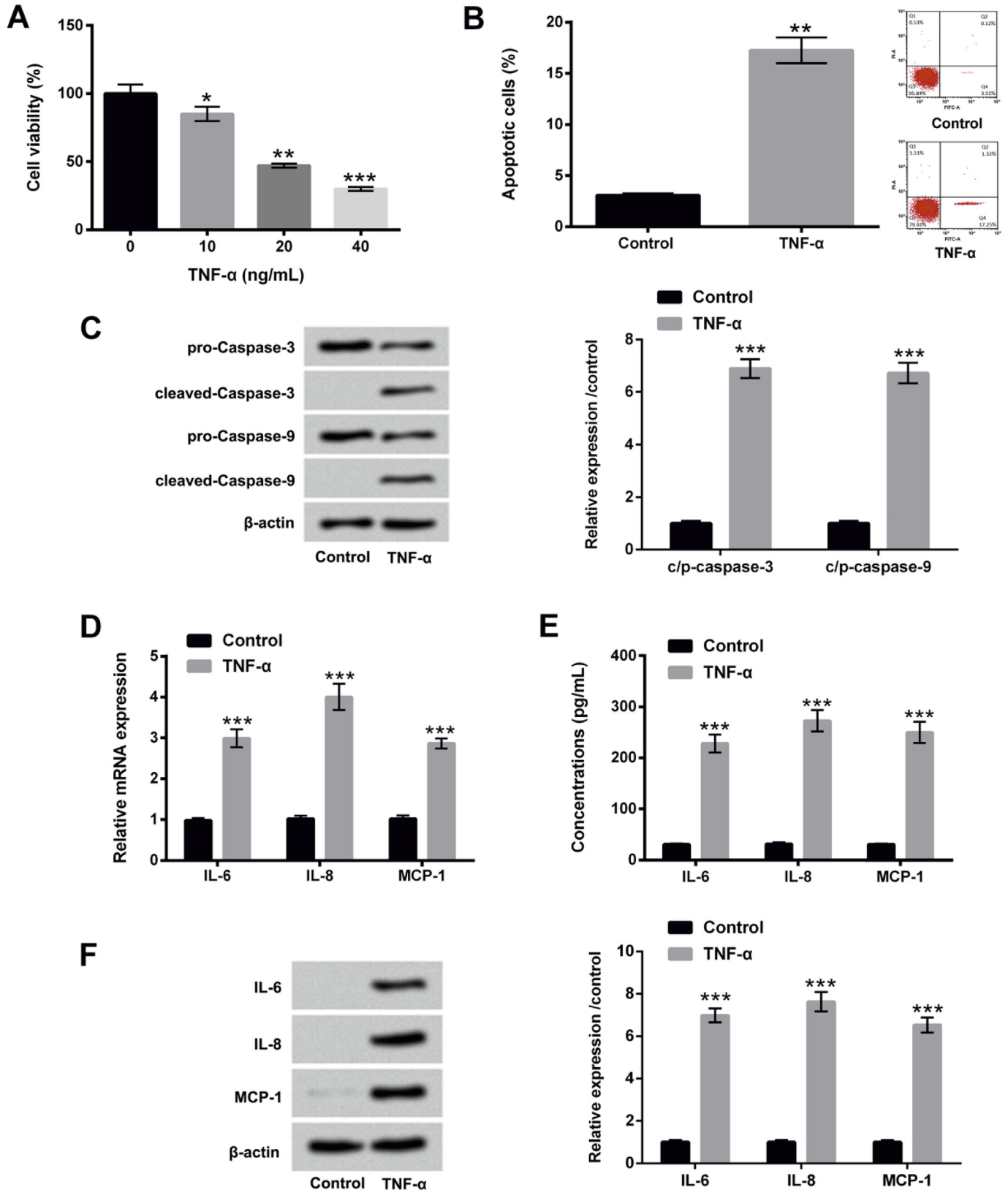

Fig. 2. TNF-α induced ATDC5 cells inflammatory injury.
(A) Different doses of TNF-α (0, 10, 20 and 40 ng/mL) were used to treat ATDC5 cells, cell viability was assessed by CCK-8 assay; ATDC5 cells were then treated with 20 ng/mL TNF-α, (B) cell apoptosis, (C) apoptosis-associated factors (pro/cleaved-Caspase-3/-9) were examined by flow cytometry and western blot; (D) the mRNA expression, (E) the secretions and (F) the protein levels of IL-6, IL-8 and MCP-1 were detected by RT-qPCR, ELISA and western blot assays. Data are presented as the mean ± SD of three independent experiments. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

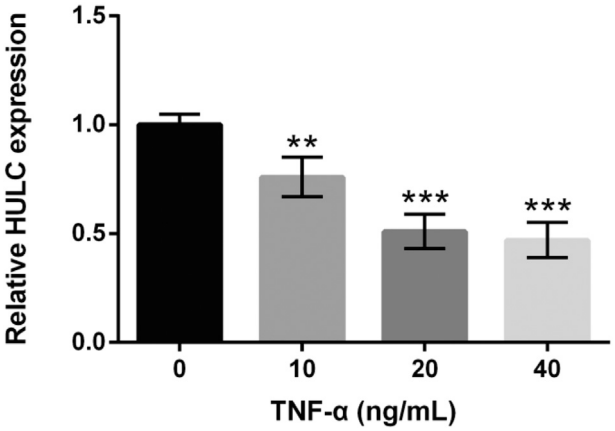

Fig. 3. HULC expression was down-regulated in TNF-α-treated ATDC5 cells. Different concentrations of TNF-α (0, 10, 20 and 40 ng/mL) were utilized to dispose ATDC5 cells, and HULC expression was estimated by RT-qPCR assay. Data are presented as the mean ± SD of three independent experiments. $^{**}P < 0.01$, $^{***}P < 0.001$.

incubator with 5% CO_2 at 37 °C. Once cells were attained 70–80% confluent, they were split by using 0.25% trypsin (Ameresco, Framingham, MA, USA). These cells were then treated with different concentrations of TNF-α (0, 10, 20 and 40 ng/mL, Sigma-Aldrich, St. Louis, MO, USA) for 24 h.

2.3. Cell transfection

Short-hairpin RNA (shRNA) directed against HULC and a non-targeting sequence were ligated into the U6/GFP/Neo plasmid (GenePharma, Shanghai, China), respectively, and they were referred as to sh-HULC and sh-NC. The full-length of HULC sequences were constructed in pcDNA3.1, and it was referred as to pc-HULC. The empty pcDNA-3.1 plasmid was used as its control. MiR-101 mimic, inhibitor and the negative controls (NC mimic or inhibitor) were synthesized (Life Technologies). The Lipofectamine 3000 reagent (Life Technologies) was used for these cells transfection according to the manufacturer's instructions. After transfection for 48 h, cells were harvested for the following experiments.

2.4. Cell viability assay

Cell Counting Kit-8 (CCK-8, Dojindo Molecular Technologies, Gaithersburg, MD) assay was performed to examine the viability of ATDC5 cells after treatment with TNF-α. Briefly, cells were collected and adjusted the cell concentration to 5×10^3 cells/well. After incubation with different concentrations of TNF-α for 24 h in 96-well plate at 37 °C in humidified 95% air and 5% CO_2. Then, 10 μL of CCK-8 solution was added to the each well of culture plate, and cells were incubated for another 1 h at 37 °C. The absorbance was subsequently measured at 450 nm using a Microplate Reader (Bio-Rad, Hercules, CA, USA).

2.5. Apoptosis assay

Annexin V-Phycoerythrin (PE) cell apoptosis detection kit (Beyotime Biotechnology, Shanghai, China) was used to determine the percentage of ATDC5 apoptotic cells. After treatment, the cells were washed and re-suspended with phosphate buffered saline (PBS, Sigma-Aldrich). The re-suspended cells (5×10^4cells/well) were centrifuged for 5 min, and 195 μL Annexin V-PE binging buffer was then added to these cells to re-suspend cells again. After this, 5 μL Annexin V-PE was added to the flow tube, and stained these cells for 15 min at the room temperature in the dark. Cell apoptosis were then immediately examined by using flow cytometry assay (Beckman Coulter, Fullerton, CA, USA).

2.6. Enzyme-linked immunosorbent assay (ELISA)

Culture supernatants from different treatment groups were collected from 24-well plates. Then, the concentrations of pro-inflammatory cytokines of interleukin-6 (IL-6), interleukin-8 (IL-8) and monocyte chemoattractant protein-1 (MCP-1) were measured by using corresponding ELISA kits (R&D Systems, Abingdon, UK) according to the kits manufacturer.

2.7. Bioinformatics prediction and luciferase reporter assay

The latent binding sites between mmu-miR-101 and HULC was predicated by TargetScan (http://www.targetscan.org/) and microRNA database (http://www.microrna.org/). The putative miR-101 target binding sequence in HULC and its mutant of the binding sites were synthesized and cloned in the pmirGLO luciferase vector (Promega, Madison, WI, USA). Then, ATDC5 cells were fostered in 96-well plates, meanwhile co-transfected with wild-type or mutated pmirGLO-HULC and miR-101 mimic or NC mimic. The luciferase activity was evaluated by utilizing the dual luciferase reporter assay (Promega).

2.8. Biotinylated RNA pull-down assay for miR-101 in vitro

ATDC5 cells were transfected with biotinylated miR-101 (Bio-miR-101-WT), miR-101 Mut (Bio-miR-101-Mut) and negative control of miR-101 (Bio-NC) (GenePharma, Shanghai, China). After 48 h transfection, 50 μL samples were divided into equal parts, and the remaining lysates were cultivated with Dynabeads M-280 Streptavidin (Invitrogen, CA, USA) on the basis of the specification. The beads administrated method referred to Wang et al.'s study [20]. Ultimately, Trizol reagent (Life Technologies) was utilized to purify the bound RNAs for real-time quantitative PCR (RT-qPCR) analysis.

2.9. Biotinylated RNA pull-down assay for HULC in vitro

The biotinylated DNA probe complementary to HULC was compounded and dissolved in binding and washing buffer. Afterward, the solutions were incubated with Dynabeads M-280 Streptavidin (Invitrogen) at indoor temperature for 10 min to generate probe-coated beads on the basis of the specification. Subsequently, ATDC5 cell lysates were co-cultivated with the probe-coated beads, and the RNA complexes bound to these beads were eluted and extracted by exploiting Trizol reagent (Life Technologies) for RT-qPCR analysis.

2.10. RNA immunoprecipitation

ATDC5 cells were dissolved by employing a complete RNA lysis buffer comprising protease inhibitor and RNase inhibitor from an EZ-Magna RIP RNA-binding protein immunoprecipitation kit (Millipore, Billerica, MA, USA). RNA immunoprecipitation was executed on the basis of the specification. Subsequently, 100 μL cell lysate was cultivated with RIP immunoprecipitation buffer encompassing magnetic beads conjugated with human anti-Argonaute2 (Ago2) antibody (ab32381, Abcam, Cambridge, UK), and NC normal mouse IgG (ab190475). Samples were cultivated with proteinase K buffer, meanwhile immunoprecipitated RNA was isolated. The concentration of RNA was appraised by a NanoDrop (Thermo Scientific) and the RNA quality was assessed via utilizing a bioanalyser (Agilent, Santa Clara, CA, USA). Furthermore, the purified RNA was subjected to RT-qPCR analysis.

2.11. RT-qPCR assay

Total RNA was extracted from treated cells by using Trizol reagent

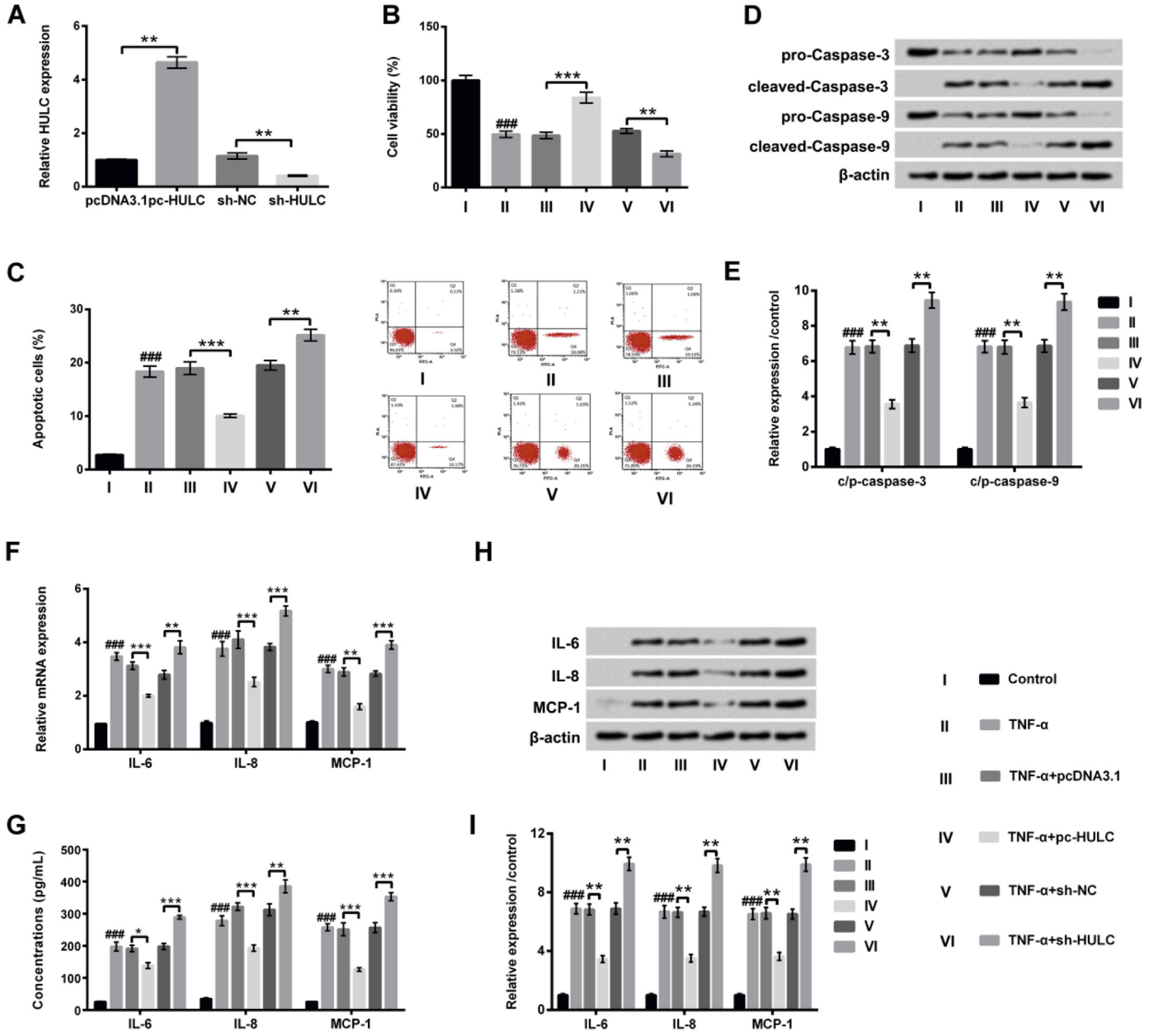

Fig. 4. HULC alleviated TNF-α-induced ATDC5 cells inflammatory injury.
Expression vectors of pc-HULC, sh-HULC and their corresponding controls were transfected into ATDC5 cells. (A) Relative expression level of HULC was determined by RT-qPCR; Transfected cells were treated with 20 ng/mL TNF-α, and then (B) cell viability, (C) apoptosis, and (D and E) apoptosis-associated factors (pro/cleaved-Caspase-3/-9) were determined by CCK-8, flow cytometry and western blot assays; (F) the mRNA expression, (G) the secretions and (H and I) the protein levels of IL-6, IL-8 and MCP-1 were examined by RT-qPCR, ELISA and western blot assays. Data are presented as the mean ± SD of three independent experiments. ###$P < 0.001$: TNF-α vs Control group; *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$: TNF-α + pc-HULC vs TNF-α + pcDNA3.1 or TNF-α + sh-HULC vs TNF-α + sh-NC.

(Life Technologies) based on the manufacturer's instructions. First Strand cDNA Synthesis Kit (TaKaRa, Otsu, Shiga, Japan) was used to synthesize the cDNA. The expression levels of IL-6, IL-8, MCP-1 and HULC were analyzed by using One Step SYBR® PrimeScript®PLUS RT-RNA PCR Kit (TaKaRa Biotechnology, Dalian, China). The Taqman MicroRNA Reverse Transcription Kit and Taqman Universal Master Mix II with the TaqMan MicroRNA Assay (Applied Biosystems, Foster City, CA, USA) were performed to determine the relative expression level of miR-101. β-actin and U6 (Applied Biosystems) were used as control groups for normalizing the expression levels of IL-6, IL-8, MCP-1, HULC and miR-101. Data were calculated by the $2^{-\Delta\Delta Ct}$ method [21]. The special primer sequences utilized in RT-qPCR were presented in Table 1.

2.12. Western blot assay

The cells from different treatment groups were lysed in RIPA lysis buffer (Beyotime Biotechnology) supplemented with protease inhibitors (Roche, Basle, Switzerland). The BCA™ Protein Assay Kit (Pierce, Appleton, WI, USA) was used for quantifying the concentration of the protein in the supernatant. Subsequently, the protein samples were electrophoresed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), and transferred to PVDF membranes. The membranes were then placed in 5% bovine serum albumin (BSA) in

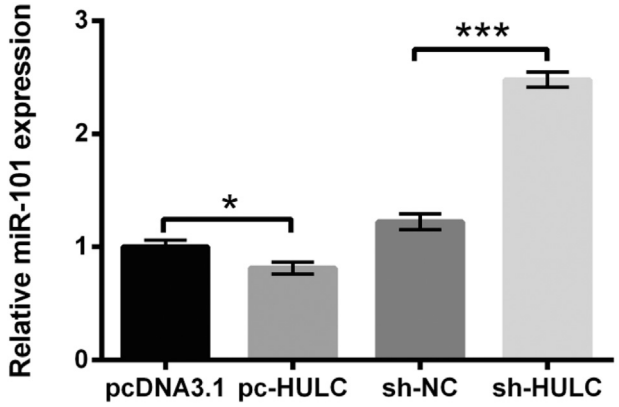

Fig. 5. MiR-101 expression was down-regulated by HULC overexpression in ATDC5 cells.
Expression vectors of pc-HULC, sh-HULC and their corresponding controls were transfected into ATDC5 cells. Relative expression level of miR-101 in these transfected cells was determined by RT-qPCR; *$P < 0.05$, ***$P < 0.001$.

Tris-buffered saline with Tween 20 (TBST), and shocked for 1 h at room temperature. After washing with TBST for three times, the blocked membranes were incubated with primary antibodies at 4 °C overnight. The following primary antibodies were used: pro-Caspase-3 (ab32150), cleaved-Caspase-3 (ab2302), pro-Caspase-9 (ab138412), cleaved-Caspase-9 (ab2324), IL-6 (ab6627), IL-8 (ab110727), MCP-1 (ab151538), t-IκBα (ab178846), phosphorylated (p)-IκBα (ab133462), t-p65 (ab16502), p-p65 (ab76302), t-p38MAPK (ab31828), p-p38MAPK (ab47363) and β-actin (ab8227, Abcam). Afterward, the second antibody of goat anti-rabbit IgG conjugated with horseradish peroxidase (ab205718, Abcam) was used to incubate with the membranes for 1 h at room temperature. Finally, the specific bindings were visualized by ECL Western blotting reagent (GE Healthcare, Braunschweig, Germany). The intensity of the bands were quantified by using ImageJ software (Bio-Rad).

2.13. Statistical analysis

Data from the multiple experiments are presented as the mean ± standard deviation (SD). Statistical analyses were performed by using Graphpad 6.0 statistical software (GraphPad, San Diego, CA, USA). The *P*-values were calculated by using student's *t*-test (comparison between two groups), one-way ANOVA with Sidak's multiple comparisons test or two-way ANOVA with Tukey's multiple comparisons test (comparison of multiple groups). *P*-value < 0.05 was considered to be a statistically significant result.

3. Results

3.1. HULC expression was down-regulated in cartilage tissues from OA patients

The OA cartilage tissues and normal cartilage tissues (NT) from twenty patients were used in the experiment, and relative expression levels of IL-6, IL-8, MCP-1 and HULC were examined by using RT-qPCR assay. The results displayed that the expression levels of IL-6, IL-8 and MCP-1 were significantly up-regulated in cartilage tissues from OA patients compared with that in NT group ($P < 0.01$ or $P < 0.001$, Fig. 1A–C). Interesting results showed that the expression level of HULC was significantly down-regulated in OA cartilage tissue compared with that in NT group ($P < 0.001$, Fig. 1D). These data seems to illustrate that HULC might play a crucial role in the development of OA.

3.2. TNF-α induced ATDC5 cells inflammatory injury

We next constructed a TNF-α induced ATDC5 cells inflammatory injury model to confirm the regulatory effect of HULC on OA. The different concentrations of TNF-α (0, 10, 20 and 40 ng/mL) were used to treat ATDC5 cells, and cell viability, apoptosis and pro-inflammatory cytokines were determined in these treated cells. In Fig. 2A, the results showed that cell viability was decreased by TNF-α treatment at the concentrations of 10 ($P < 0.05$), 20 ($P < 0.01$) and 40 ng/mL ($P < 0.001$). Because of the IC50 of TNF-α was approximately showed at the concentration of 20 ng/mL, therefore, we selected 20 ng/mL TNF-α to dispose ATDC5 cells in the next researches.

Afterward, 20 ng/mL TNF-α was selected for the following experiments. Flow cytometry and western blot results revealed that TNF-α treatment significantly induced cell apoptosis ($P < 0.01$, Fig. 2B), as well as increased cleaved-Caspase-3 and cleaved-Caspase-9 expression in ATDC5 cells ($P < 0.001$, Fig. 2C). Additionally, in Fig. 2D and E, we observed that the mRNA and the concentrations of IL-6, IL-8 and MCP-1 were remarkably increased in TNF-α-treated cells compared with that in control group ($P < 0.001$). Besides, the protein levels of IL-6, IL-8 and MCP-1 were also increased by TNF-α treatment ($P < 0.001$, Fig. 2F). These data indicated that TNF-α induced ATDC5 cells inflammatory injury model was successfully constructed.

3.3. HULC alleviated TNF-α induced chondrocyte cells inflammatory injury

We used the different concentrations of TNF-α (0, 10, 20 and 40 ng/mL) to stimulate ATDC5 cells, and relative HULC expression in these cells was determined by using RT-qPCR. Results showed that HULC expression was significantly decreased by TNF-α at the concentrations of 10 ng/mL ($P < 0.01$), 20 and 40 ng/mL ($P < 0.001$, Fig. 3). pc-HULC, sh-HULC and their corresponding controls were then transfected into ATDC5 cells to change HULC expression. In Fig. 4A, we observed that HULC expression was significantly up-regulated in pc-HULC-transfected cells ($P < 0.01$), and down-regulated in sh-HULC-transfected cells ($P < 0.01$). To further study the effect of HULC on TNF-α-injured ATDC5 cells, cell viability, apoptosis and pro-inflammatory cytokines were examined again. The results showed that HULC overexpression significantly increased cell viability ($P < 0.001$, Fig. 4B), reduced apoptosis ($P < 0.001$, Fig. 4C), down-regulated cleaved-Caspase-3/-9 ($P < 0.01$, Fig. 4D and E), as well as inhibited IL-6, IL-8 and MCP-1 expression, secretions and protein levels ($P < 0.05$, $P < 0.01$, or $P < 0.001$, Fig. 4F–I) in TNF-α-treated ATDC5 cells. However, the effect of HULC suppression on TNF-α-injured ATDC5 cells was contrary to that of HULC overexpression ($P < 0.01$ or $P < 0.001$, Fig. 4B–I). Likewise, the similar results were presented in human chondrocyte cells ($P < 0.01$ or $P < 0.001$, Supplementary Fig. 1A–I). According to these results, we concluded that HULC could alleviate TNF-α-induced chondrocyte cells inflammatory injury.

3.4. HULC exhibited its function via sponging miR-101

MiR-101 is one of the most important miRNAs, which has been reported to play a crucial role in different diseases [22,23]. In the current study, we speculated that miR-101 might participate in regulating the effect of HULC on TNF-α induced ATDC5 cells inflammatory injury. We firstly examined the relative expression of miR-101 in pc-HULC and sh-HULC-transfected cells. Results showed that miR-101 expression level was obviously down-regulated by HULC overexpression compared with its control group ($P < 0.05$, Fig. 5). But, miR-101 expression level was significantly up-regulated by HULC suppression ($P < 0.001$, Fig. 5). Similarly, we also discovered the repression of miR-101 in pc-HULC-transfected human primary chondrocyte cells ($P < 0.01$), and the enhancement of miR-101 in sh-HULC-transfected human primary chondrocyte cells ($P < 0.001$, Supplementary Fig. 2). We next further explored the relevance of HULC and

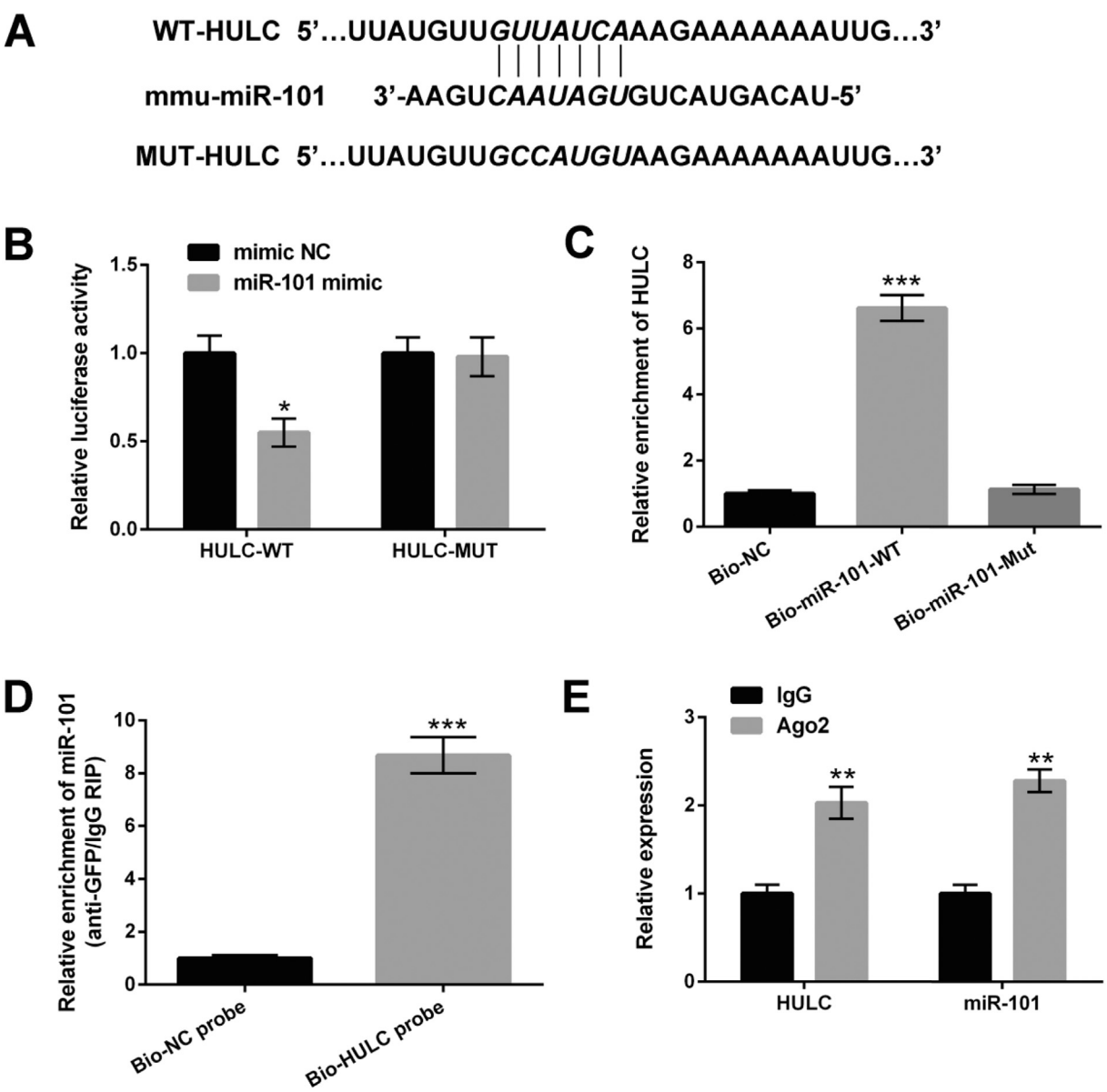

Fig. 6. The mutual inhibition between HULC and miR-101.
(A) The binding site of miR-101 within HULC was predicted by TargetScan and microRNA database. (B) The relevance of HULC and miR-101 was estimated by utilizing dual luciferase reporter assay. (C) The enrichment of HULC was determined by utilizing RT-qPCR in the sample pulled down by biotinylated miR-101. (D) The enrichment of miR-101 was determined by exploiting RT-qPCR in the sample pulled down by biotinylated HULC probe. (E) The reciprocal suppression between HULC and miR-101 was evaluated by RNA immunoprecipitation exploiting antibody against Ago2. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.

miR-101. The binging site between WT-HULC and mmu-miR-101 was showed in Fig. 6A. Additionally, dual luciferase reporter assay disclosed that overexpressed miR-101 restrained the luciferase activity of HULC-WT group ($P < 0.05$). But, there was no evident change in HULC-MUT group (Fig. 6B).

Subsequently, we exploited bio-avidin pull-down system to probe whether miR-101 could pull down HULC. RT-qPCR assay disclosed that the enrichment of HULC was increased in ATDC5 cells with Bio-miR-101-WT transfection ($P < 0.001$), hinting that HULC was pulled down by miR-101 (Fig. 6C). Moreover, the inverse pull-down assay was carried to appraise whether HULC could pull down miR-101 via utilizing a bio-labeled specific HULC probe. As displayed in Fig. 6D, the enrichment of miR-101 was evidently enhanced in ATDC5 cells with Bio-HULC probe ($P < 0.001$), also indicating that miR-101 was pulled down by HULC. Besides, RNA immunoprecipitation was executed by exploiting antibody against Ago2 to further probe the reciprocal suppression between HULC and miR-101. We discovered that the expression levels of HULC and miR-101 were both up-regulated in ATDC5 cells with Ago2 ($P < 0.01$, Fig. 6E), indicating that HULC and miR-101 were both pulled down in ATDC5 cells with Ago2. All above mentioned results certified that HULC exhibited its function might by sponging miR-101 in ATDC5 cells. There might be mutual inhibition between HULC and miR-101.

3.5. HULC alleviated TNF-α induced ATDC5 cells inflammatory injury via mediating miR-101 expression

Next, miR-101 mimic and NC expression vectors were transfected into ATDC5 cells, and RT-qPCR assay revealed that miR-101 expression was markedly up-regulated in miR-101 mimic-transfected cells ($P < 0.001$, Fig. 7A). The subsequent experimental results showed that overexpression of miR-101 significantly reversed the effect of HULC overexpression on cell viability, apoptosis and pro-inflammatory cytokines (IL-6, IL-8 and MCP-1) in TNF-α-treated ATDC5 cells ($P < 0.05$,

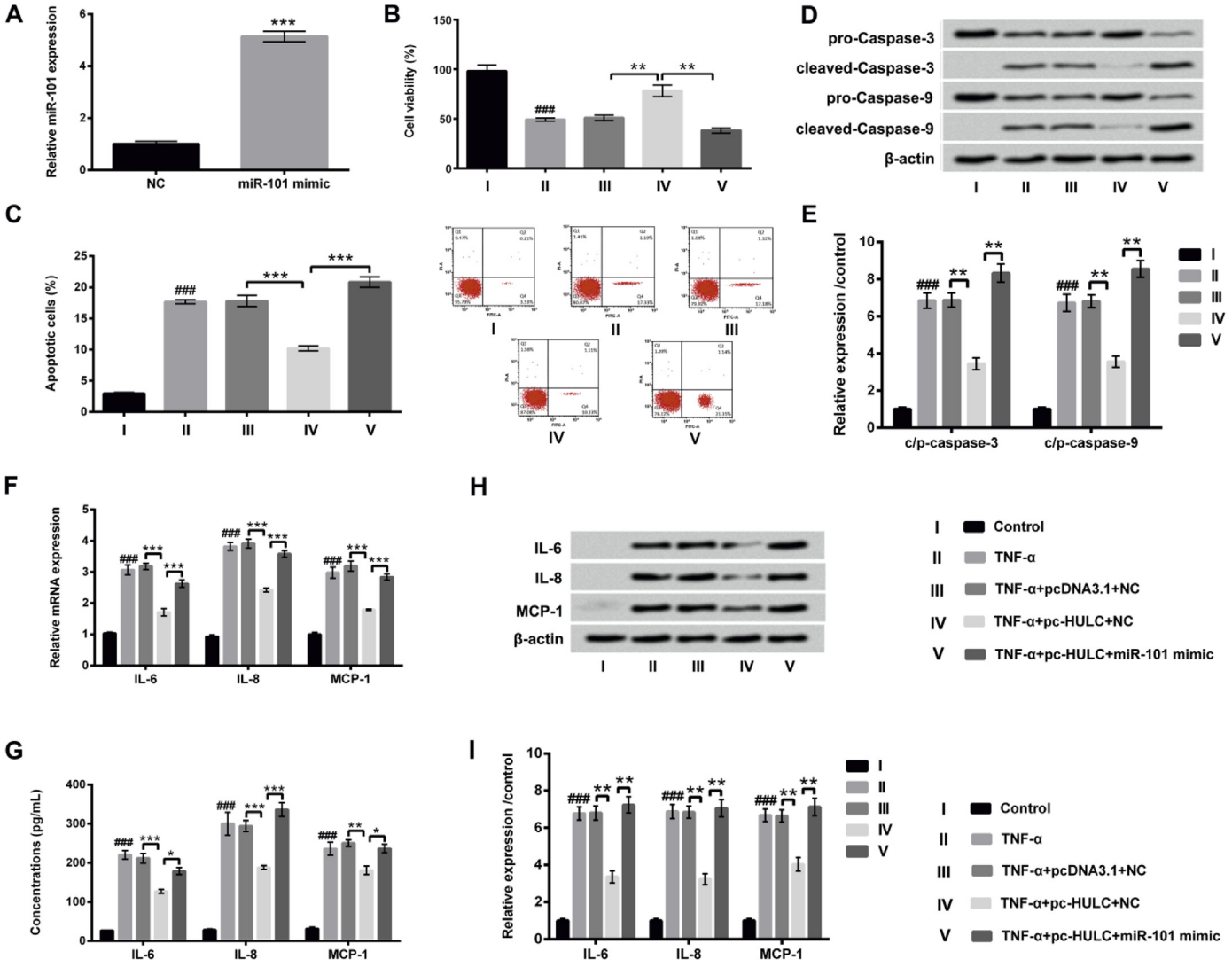

Fig. 7. MiR-101 inhibited the protective effect of HULC on TNF-α-injured ATDC5 cells.
Expression vectors of miR-101 mimic and its negative control were transfected into ATDC5 cells. (A) Relative expression level of miR-101 in these transfected cells was determined by RT-qPCR; Subsequently, ATDC5 cells were transfected with pc-HULC or co-transfected with pc-HULC and miR-101 mimic. After treatment with 20 ng/mL TNF-α, (B) cell viability, (C) apoptosis, and (D and E) apoptosis-associated factors (pro/cleaved-Caspase-3/-9) were determined by CCK-8, flow cytometry and western blot assays; (F) the mRNA expression, (G) the secretions and (H and I) the protein levels of IL-6, IL-8 and MCP-1 were examined by RT-qPCR, ELISA and western blot assays. Data are presented as the mean ± SD of three independent experiments. ###$P < 0.001$: TNF-α vs Control group; *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$: TNF-α + pc-HULC + NC vs TNF-α + pcDNA3.1 + NC or TNF-α + pc-HULC + miR-101 mimic vs TNF-α + pc-HULC + NC.

$P < 0.01$ or $P < 0.001$, Fig. 7B–I). These data indicated that miR-101 inhibited the protective effect of HULC on TNF-α-injured ATDC5 cells. Nevertheless, after transfection with miR-101 inhibitor, we discovered that repression of miR-101 was evidently enhanced cell viability, reduced apoptosis, declined cleaved-Caspase-3 and cleaved-Caspase-9 in TNF-disposed ATDC5 cells (all $P < 0.01$, Supplementary Fig. 3A–D). Additionally, the mRNA expression, the sections and the protein levels of IL-6, IL-8 and MCP-1 were all restrained by suppressed miR-101 in TNF-disposed ATDC5 cells ($P < 0.05$ or $P < 0.01$, Supplementary Fig. 3E–H). These above mentioned data hinted that miR-101 inhibition exerted the protective impacts in TNF-disposed ATDC5 cells.

3.6. HULC inhibited the activations of NF-κB and p38MAPK signaling pathways by down-regulation of miR-101

Finally, we explored the effect of HULC on NF-κB and MAPK signaling pathways in TNF-α-treated ATDC5 cells. Results showed that TNF-α disposition notably up-regulated phosphorylated IκBα, p65, p38MAPK, ERK1/2 and JNK1/2 (all $P < 0.001$, Fig. 8A–F). Overexpression of HULC was found to down-regulate phosphorylated IκBα ($P < 0.001$), p65 ($P < 0.01$), p38MAPK ($P < 0.01$), ERK1/2 ($P < 0.01$) and JNK1/2 ($P < 0.01$) in TNF-α-treated ATDC5 cells (Fig. 8A–F). However, overexpressed miR-101 reversed the inhibitory effect of HULC overexpression on phosphorylated IκBα, p65, p38MAPK, ERK1/2 and JNK1/2 ($P < 0.01$ or $P < 0.001$, Fig. 8A–F). There was no obvious change of t-IκBα, t-p65, t-p38MAPK, t-ERK1/2 and t-JNK1/2 in the diverse administration group. Above data indicated that HULC could block NF-κB and p38MAPK signaling pathways in TNF-α-treated ATDC5 cells by down-regulation of miR-101.

4. Discussion

OA is the most common joint disease, especially endangering the health of the elderly [24]. A previous study reports that lncRNAs are associated with OA pathogenesis, and have significant impact on controlling OA progression [25]. Nevertheless, limited research reported

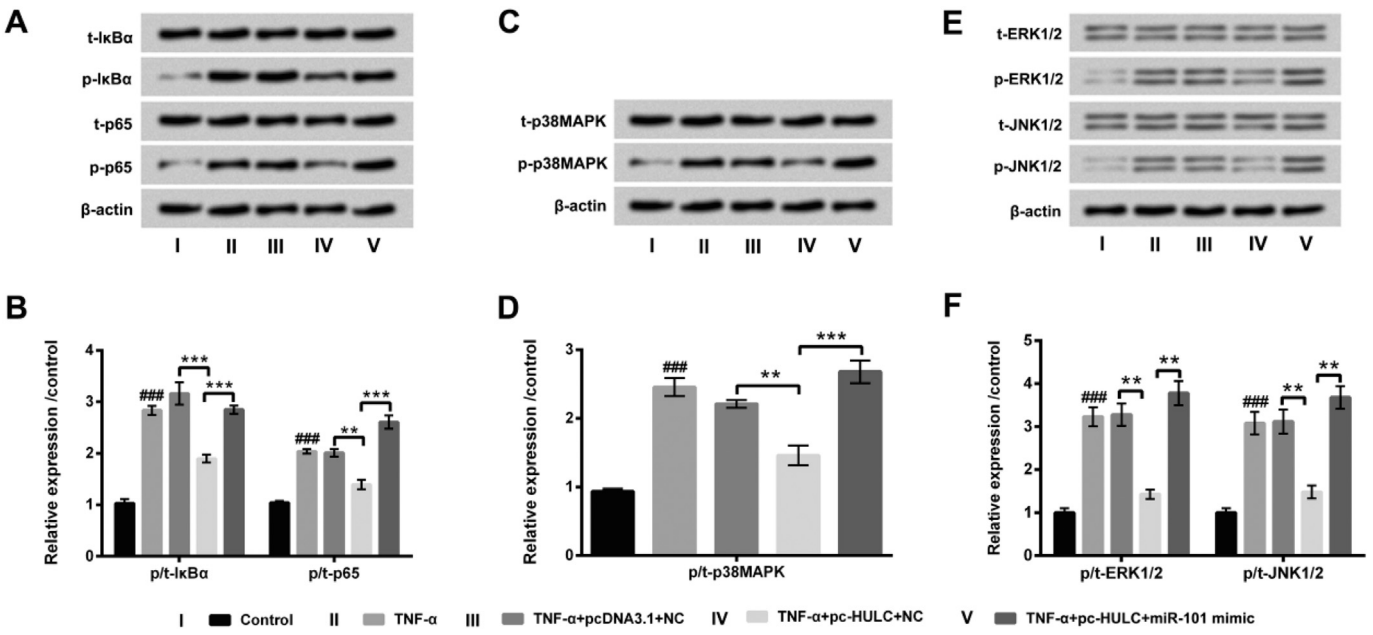

Fig. 8. HULC blocked NF-κB and MAPK signaling pathways by down-regulation of miR-101
ATDC5 cells were transfected with pc-HULC or co-transfected with pc-HULC and miR-101 mimic. After treatment with 20 ng/mL TNF-α, (A and B) the protein levels of p/t-IκBα, p/t-p65 and (C and D) p/t-p38MAPK were examined by western blot assay. (E and F) p/t-ERK1/2 and p/t-JNK1/2 were estimated by western blot assay. Data are presented as the mean ± SD of three independent experiments. ###$P < 0.001$: TNF-α vs Control group; **$P < 0.01$, ***$P < 0.001$: TNF-α + pc-HULC + NC vs TNF-α + pcDNA3.1 + NC or TNF-α + pc-HULC + miR-101 mimic vs TNF-α + pc-HULC + NC.

the effect of HULC on OA. In the present study, we probed the protective impacts of HULC on TNF-α-induced inflammatory injury in ATDC5 cells. Results firstly revealed that HULC expression was restrained in OA cartilage tissues. Then, we discovered that HULC mitigated TNF-α-induced inflammatory injury in ATDC5 cells. Further experimental results showed that miR-101 was mutual repressed with HULC, and overexpressed miR-101 reversed the protective impacts of HULC on TNF-α-injured ATDC5 cells. Finally, we found that HULC inhibited the activations of NF-κB and MAPK pathways through sponging miR-101.

Many inflammatory cytokines, such as TNF-α, IL-1β, IL-6 and IL-8, play major roles in inflammatory response. TNF-α is the earliest and most important inflammatory mediator in the process of inflammation. It can activate neutrophils and lymphocytes, increase the permeability of vascular endothelial cells, regulate the metabolic activity of other tissues and promote the synthesis and release of other cytokines. Recently, TNF-α-evoked cell inflammation model has been employed in many researches. For example, Chen et al. utilized TNF-α to dispose ATDC5 cells and investigated the effect of TNF-α on cell apoptosis, autophagy and cartilage degradation [26]. Additionally, Kim et al. constructed TNF-α-induced cell inflammation model in HT-29 cells, and discovered that Lactobacillus plantarum lipoteichoic acid could alleviate TNF-α-induced inflammation in the HT-29 intestinal epithelial cell line [27]. Therefore, we also built TNF-α-induced cell inflammation model in ATDC5 cells. Moreover, we examined cell viability, apoptosis and IL-6, IL-8 and MCP-1 expression and concentrations in ATDC5 cells after TNF-α exposure.

In clinic, the early diagnosis of OA is rather difficult and even requires therapeutic diagnosis or surgery. The traditional diagnostic method for OA is mainly measured the secretions of inflammatory cytokines to monitor arthritis, however, these inflammatory cytokines can't reflect the extent of articular cartilage damage [28]. Recent studies have found that a variety of lncRNAs is abnormally expressed in OA, which might be participated in regulating the pathogenesis of OA [11,25]. Study from Chang et al. confirmed that eighteen lncRNAs were differentially expressed in injured joints of post-traumatic OA [29]. HULC is an important lncRNA, which has been proven to be a novel biomarker for the detection of different cancers [30,31]. However, whether HULC was an effective biomarker for OA is still unclear. In our study, we examined the relative expression of pro-inflammatory cytokines (IL-6, IL-8 and MCP-1) and HULC in OA cartilage tissues, and results showed that IL-6, IL-8 and MCP-1 expression levels were significantly increased, and HULC expression was decreased in in OA cartilage tissues. These data indicated that HULC might be a novel biomarker and associated with OA pathogenesis.

HULC has been widely reported to play a vital role in different diseases, including inflammatory diseases [32,33]. Study from Zhao et al. demonstrated that HULC could promote ultraviolet radiation b (UVB)-induced HaCaT cells injury by up-regulating BNIP3 [33]. However, based on our best knowledge, the effect of HULC on TNF-α-induced ATDC5 cells injury remains uninvestigated. In the subsequent experiments, we constructed an inflammatory injury model induced by TNF-α in ATDC5 cells to mimic OA pathogenesis. We used the model to further explore the effect of HULC on OA. The results showed that HULC significantly alleviated TNF-α-induced inflammatory injury in ATDC5 cells, indicating the protective effect of HULC on TNF-α-injured ATDC5 cells. Our study is similar with the previous study from Sun et al. revealed the protective effect of lncRNA RP11-445H22.4 on LPS-injured in ATDC5 cells [34].

Increasing evidence uncovered the effect of lncRNA on various biological processes might be achieved by regulating several miRNAs [35]. In OA, Song et al. reported that lncRNA growth arrest-specific 5 (GAS5) contributed to the pathogenesis of OA by down-regulation of miR-21 [36]. Li et al. found that lncRNA plasmacytoma variant translocation 1 (PVT1) promoted cell apoptosis by down-regulating miR-488-3p in OA chondrocytes [37]. Wang et al. testified that repression of lncRNA maternally expressed gene 3 (MEG3) could weaken lipopolysaccharide (LPS)-evoked inflammatory injury via enhancement of miR-203 in ATDC5 cells [38]. MiR-101 is a well-known tumor-suppressor miRNA, which also has been reported to play an important role in OA [39,40]. Recent study stated that miR-101 inhibition could prevent cartilage degradation via mediating extracellular matrix-related genes in a rat model of OA [41]. However, whether miR-101 is involved in regulation of HULC-protective ATDC5 cells remain unclear. Our results

revealed that HULC and miR-101 possessed a mutual inhibitory effect, moreover, miR-101 restrained the protective impacts of HULC against TNF-α-induced inflammatory injury in ATDC5 cells.

NF-κB and MAPK pathways have been considered to be major regulators of inflammation, which can mediate the productions of pro-inflammatory cytokines implicated in OA [42]. Evidence has been proven that NF-κB is a main catabolic pathway in OA joints, which triggers various gene expressions implicating in cartilage destruction and synovial membrane inflammation [43]. Other evidence showed that inhibition of MAPK signaling pathway could suppress cell apoptosis and down-regulated pro-inflammatory cytokines expression in OA chondrocytes [44]. Based on above studies, we explored the effect of HULC and miR-101 on NF-κB and MAPK signaling pathways. Data revealed that HULC inhibited the activations of NF-κB and MAPK (p38MAPK, ERK1/2 and JNK1/2) signaling pathways by regulating miR-101 expression. The results might be underlying mechanism of HULC alleviating TNF-α-induced inflammatory injury in ATDC5 cells.

5. Conclusions

Taken together, the results suggested that HULC protected ATDC5 cells against TNF-α-induced inflammatory injury by sponging miR-101 via inhibiting the activations of NF-κB and MAPK signaling pathways. These findings indicated that HULC might be an important regulator in the pathogenesis of OA, as well as it might be a novel biomarker for the diagnosis and treatment of OA.

Declarations of interest

None.

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Authorship

Conceives and designed the experiments: Chunzheng Gao and Peigang Chu. Performed the experiments: Peigang Chu and Qiang Wang. Analyzed the data: Peigang Chu and Zongru Wang. Wrote the paper: Chunzheng Gao.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.intimp.2019.04.004.

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