


MiR-128-3p Post-Transcriptionally Inhibits WISPI to Suppress Apoptosis and Inflammation in Human Articular Chondrocytes via the PI3K/AKT/NF- κ B Signaling Pathway

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Abstract

In osteoarthritis (OA), the synthesis and decomposition of the extracellular matrix (ECM) are imbalanced. High expression levels of Wnt1-inducible signaling pathway protein 1 (WISPI) promote the synthesis of matrix metalloproteinases and induce the degradation of cartilage, which aggravates the OA. The aim of this study was to explore the role of miR-128-3p in the development of OA. In the present study, the expression of WISPI and miR-128-3p in osteoarthritic tissues and chondrocytes was detected using quantitative reverse transcription PCR (RT-qPCR) and Western blotting. Then we predicted that WISPI might be a potential target gene of miR-128-3p by TargetScan and verified using luciferase reporter gene assay. The effect of miR-128-3p or WISPI on chondrocytes was evaluated by cell proliferation assay, apoptosis, and caspase-3 activity assay. To further reveal the molecular mechanisms of miR-128-3p in osteoarthritic development, the degradation of chondrocyte matrix and production of proinflammatory cytokines in osteoarthritic chondrocyte model were detected by ELISA. To mimic the osteoarthritic microenvironment in vitro studies, chondrocytes were stimulated with interleukin (IL)-1 β , and then we found that the expression of miR-128-3p was downregulated. Overexpression of WISPI inhibited the proliferation of chondrocytes, which induced apoptosis, degradation of chondrocyte matrix, production of proinflammatory cytokines, and activated the phosphatidylinositol-3-kinase (PI3K)/protein kinase B (Akt)/nuclear factor kappa-light-chain-enhancer of activated B cells (NF- κ B) pathway. Then, we identified that miR-128-3p was a negative regulator of WISPI by directly targeting its 3'-untranslated region (UTR). Moreover, the PI3K allosteric activator 740 Y-P abolished the inhibition of miR-128-3p in apoptosis, degradation of chondrocyte matrix, and inflammation. Our results showed that miR-128-3p targets WISPI to regulate chondrocyte proliferation, apoptosis, degradation of chondrocyte matrix, and production of proinflammatory cytokines via the PI3K/Akt/NF- κ B pathway, which plays a suppressed role in OA.

Keywords

osteoarthritis, chondrocyte, microRNA-128-3p, WISPI, PI3K/Akt/NF- κ B pathway, apoptosis, ECM, inflammation

Introduction

With the changes in the way of life and work, and the aging of the social population, degenerative diseases of bones and joints have become high-risk diseases, and one of the representatives is osteoarthritis (OA). OA seriously affects patients' quality of life, increases family burden, and causes significant social and economic losses¹. In recent years, it is believed that the degeneration and wear of articular cartilage is a key pathologic change in OA².

There are many factors that can cause OA, and its molecular mechanism is quite complex. The specific mechanism of OA is still not fully understood, which mainly involves

many cell types, cytokines, extracellular matrix, gene expression, and signaling pathways. In OA, an imbalance in the synthesis and decomposition of extracellular matrix

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(ECM) leads to the destruction of articular cartilage and loss of function. For example, matrix metalloproteinase (MMP), ADAMTS-4/5³, interleukin-1 (IL-1), and tumor necrosis factor- α (TNF- α) induce cell apoptosis and matrix degradation, meanwhile, they activate p38MAPK signal transduction pathway, IL-1 pathway, and Wnt/ β -catenin pathway. The imbalance of metabolism of ECM, such as type II collagen, proteoglycan, and gelatin, leads to the disorder of chondrocytes' external environment and induces cell apoptosis. Then the apoptosis of chondrocytes leads to further reduction of ECM synthesis, thus forming a vicious circle.

Abnormal Wnt signaling pathway is one of the important factors leading to the formation of OA. Wnt1-inducible signaling pathway protein 1 (WISP1) is a member of the connective tissue growth factor family and is the product of the Wnt/ β -catenin signaling pathway⁴. Recent studies have shown that WISP1 has the effect of promoting articular cartilage destruction⁵. WISP1 is a cysteine-rich secreted protein polypeptide, which is synthesized and secreted by a variety of cells, such as fibroblasts, smooth muscle cells, endothelial cells, and myocardium cells, and presents in various tissues and organs. WISP1 can mediate cell adhesion, promote ECM synthesis, stimulate cell migration, and regulate complex biological processes. It also participates in angiogenesis and tumorigenesis^{6,7}. WISP1 is hardly expressed in normal cartilage and synovium, but it is abnormally increased in degenerated cartilage and synovium (such as OA), and promotes the breakdown of collagen and proteoglycan by inducing the secretion of MMPs and aggrecanases, leading to further exacerbation of OA⁸.

MicroRNAs are a class of endogenous, noncoding single-stranded small RNAs of 19–24 nucleotides in length that regulate gene expression at the post-transcriptional level and participate in a series of important processes in the life processes⁹. They are widely presented in animal and plant cells, and they can inhibit the expression of target genes by degrading mRNA or inhibiting the translation of mRNA. They play a key regulatory role in biological processes such as cell proliferation, differentiation, apoptosis, and development¹⁰. MiR-128-3p has been studied in many diseases, such as lung cancer¹¹, liver cancer¹², and rheumatoid arthritis¹³. Previous studies report that miR-128-3p is frequently downregulated in hepatocellular carcinoma (HCC) and significantly inhibits proliferation of HCC cells by inducing G1 arrest¹⁴. Cai et al. found that miR-128-3p induces cells antiapoptotic effects and inhibits caspase 3 activation. Overexpression of miR-128-3p accelerates the proliferation of non-small cell lung carcinoma (NSCLC) cells in non-small cell lung cancer¹⁵. What's more, miR-128-3p can significantly promote the inflammatory response of rheumatoid arthritis¹³. However, the role of miR-128-3p in OA remains unclear.

Here, to mimic the osteoarthritic microenvironment in vitro studies, osteoarthritic chondrocytes were stimulated with IL-1 β (10 ng/ml). We demonstrated that the expression of WISP1 is upregulated in OA and it aggravates OA. MiR-128-3p can directly target WISP1 and decrease the

expression of WISP1. We further found that WISP1 mediated cell proliferation, apoptosis, and ECM degradation through the phosphatidylinositol-3-kinase (PI3K)/protein kinase (Akt)/ nuclear factor kappa-light-chain-enhancer of activated B cells (NF- κ B) pathway. These data indicated that miR-128-3p directly targets WISP1 to inhibit chondrocyte apoptosis and ECM degradation by inhibiting the PI3K/Akt/NF- κ B pathway. This result may provide a new target for the treatment of OA.

Materials and Methods

Patients' Tissue Samples

Osteoarthritic cartilage tissues were collected from 15 patients (age 64.2 ± 5.7 years) who underwent total knee arthroplasty. Normal tissues were obtained from 15 knee joint trauma patients. The study was approved by the ethics committee of Huaihe Hospital of Henan University (Kaifeng, China), and informed consent was obtained from all patients.

Cell Culture

Human chondrocytes C28/I2 were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS; GIBCO, Carlsbad, CA, USA), 100 U/ml penicillin and 100 mg/ml streptomycin, and cultured in a humidified incubator at 37°C under 5% carbon dioxide. Primary chondrocytes were used for research and stimulated with IL-1 β (10 ng/ml) in serum-free medium for 24 h.

Cell Transfection

Primary human osteoarthritic chondrocytes were plated in a six-well plate, at about 70% confluence, transfected with miR-128-3p mimic, inhibitor, or their negative control using Amaxa Nucleofactor System and the Chondrocyte Transfection kit according to the manufacturer's protocol (Lonza, Walkersville, MD, USA). In order to observe the role of WISP1 in OA, we performed gain-of-function and loss-of-function experiments in chondrocytes. For overexpression, the coding sequence (CDS) of WISP1 was cloned into the vector pcDNA3.1, and the obtained expression vector or empty vector was transfected into chondrocytes, respectively. The WISP1-siRNA sequence is 5'-CCAGGTCC-TATGGATTAAT-3' and control siRNA sequence is 5'-CCACCTTATAGGTTGGAAT-3'. siRNAs were synthesized at TAKARA Bio Inc. company and transfected into primary chondrocytes using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA). The co-transfection of miR-128-3p mimic and pcDNA-WISP1 was conducted using Lipofectamine 2000. The cells were cultured for 48 h and then stimulated with or without IL-1 β (10 ng/ml) in serum-free medium for 24 h.

Table 1. Primer Sequences Used in Quantitative PCR.

Gene	Forward primer (5'–3')	Reverse primer (5'–3')
miR-128-3p	GGTCACAGTGAACCGGTC	GTGCAGGGTCCGAGGT
WISP1	AGGTATGGCAGAGGTGCAAG	GTGTGTGTAGGCAGGGAGTG
β -actin	AGTGTGACGTTGACATCCGT	GCACTCAGTAACAGTCCGC
U6 RNA	CTCGCTTCGGCAGCAC	AACGCTTCACGAATTTGCGT

RNA Extraction and Quantitative Reverse Transcription-PCR (RT-qPCR)

Total RNA was extracted from the tissues and cells using Trizol reagent (Life Technologies, California, USA) and OD260 nm values were determined for quantification. Equal amounts of RNA were then reversely transcribed into cDNA with the PrimeScript RT reagent Kit (Takara, Dalian, China), and used as a template for RT-qPCR. The gene expressions were determined using the SYBR Premix DimerEraser Kit (Takara); β -actin used as an internal reference. PCR program was conducted on a real-time PCR System (ABI7500; Applied Biosystems, Waltham, MA, USA) under the following conditions: 95°C for 1 min followed by 35 cycles of 95°C for 20 s, then 56°C for 10 s, and 72°C for 15 s. The relative expression levels were calculated using the $2^{-\Delta\Delta CT}$ method. Primer sequences are listed in Table 1.

Western Blot Analysis

Chondrocytes were washed twice with phosphate-buffered saline (PBS) and lysed using radioimmunoprecipitation assay lysis buffer (CW Biotech, Beijing, China), and the protein concentrations were measured by a bicinchoninic acid protein assay kit (Thermo Fisher Scientific Inc., Waltham, USA). An equal amount of total protein was then separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to polyvinylidene difluoride membranes using β -actin as an endogenous control. The membranes were blocked with 5% skim milk for 2 h at room temperature and then incubated overnight at 4°C with primary antibodies, including anti-WISP1 antibody, anti-PI3K antibody, anti-p-PI3K antibody, anti-p65 antibody, anti-p-p65 antibody, anti- β -actin antibody (Abcam, Cambridge, UK). After washing three times with tris buffered saline with Tween-20, the membranes were incubated with the secondary antibody for 1 h at room temperature. Protein bands were visualized using the ECL system, and the optical density of the protein bands was quantified using Image J software.

Cell Proliferation Assay

The effect of miR-128-3p and WISP1 on chondrocyte proliferation was determined using the cell counting kit-8 (CCK-8) assay (Dojindo, Kumamoto, Japan). After 24, 36, 48, 60, and 72 h of transfection, the supernatant was discarded and 10 μ l of CCK-8 was added to each well. After

incubated at 37°C for 2 h, the absorbance at 450 nm was recorded.

Apoptosis and Caspase-3 Activity Assay

Primary osteoarthritic chondrocytes were washed with pre-cooled PBS, fixed in 4% paraformaldehyde for 15 min, and stained with 1% 4',6-diamidino-2-phenylindole (DAPI). After removing DAPI, the cells were washed with PBS and observed under a fluorescence microscope. Cells with agglomerated or fragmented nuclei were considered to be apoptotic cells. To verify whether miR-128-3p induced caspase-3 activity, the assay was performed using a caspase-3 colorimetric assay kit (Abcam, Cambridge, UK) essentially as described by the manufacturer of the kit. Luminescence was measured in BioTek microplate reader (BioTek, Winooski, VT, USA).

ELISA

Twenty-four hours after the transfection of chondrocytes, the cells were treated with IL-1 β (10 ng/ml) at 37°C for 24 h and then centrifuged at 10,000 rpm for 5 min at 4°C. The supernatant was used to detect the secretion of MMP3, MMP9, collagen-II, collagen-X, ADAMTS-5, IL-6, IL-17, and TNF- α by ELISA.

Luciferase Reporter Gene Analysis

The potential binding sites of miR-128-3p and WISP1 were predicted using the online database. To further identify the potential relationship, we performed a luciferase reporter gene assay. The wild-type and mutant 3'-UTR sequences of WISP1 were cloned into the pGL3 promoter vector containing the luciferase reporter gene, respectively. HEK-293 T cells were seeded in 24-well plates, and when grown to approximately 70% confluences, co-transfected with luciferase plasmid and miR-128-3p mimic or NC-mimic using Lipofectamine 2000. After 24 h of transfection, cells were harvested and assayed using Luciferase Reporter Gene Assay Kit (Promega, Madison, WI, USA); the luciferase reporter gene activity was normalized to Renilla luciferase activity.

Statistical Analysis

Using SPSS (version 23.0) software, the measurement data were expressed as mean \pm standard deviation (SD), and

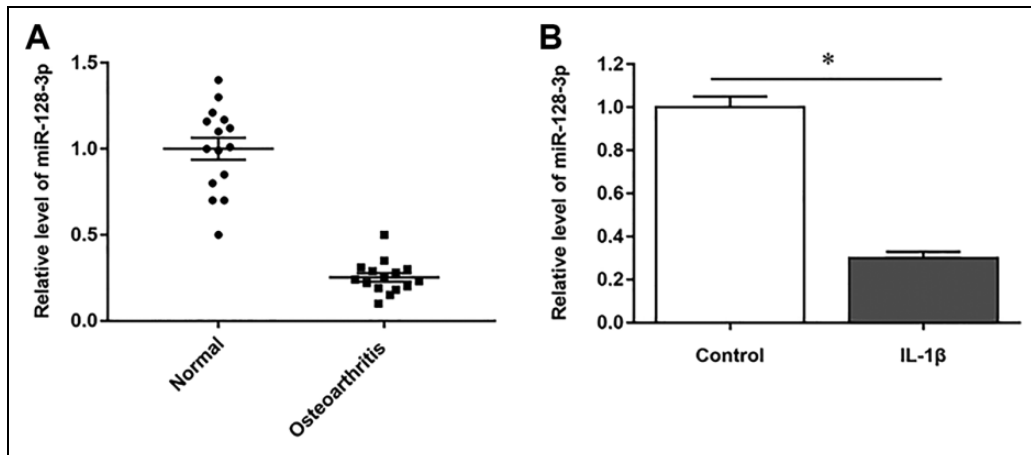


Fig. 1. The expression pattern of miR-128-3p and WISPI in clinical samples of OA. (A) MiR-128-3p relative expression in osteoarthritic cartilage tissues was analyzed by RT-qPCR. (B) The expression level of WISPI in osteoarthritic cartilage tissues was tested by RT-qPCR and normalized to β -actin expression. (C, D) The expression of WISPI was measured using Western blotting and the results were analyzed using Image J. ** $P < 0.01$. OA, osteoarthritis; RT-qPCR, quantitative reverse transcription PCR; WISPI, Wnt1-inducible signaling pathway protein 1.

then one-way analysis of variance and *t*-test were performed. All experiments were repeated at least three times. The difference was statistically significant with $P < 0.05$.

Results

WISPI is Upregulated Whereas miR-128-3p is Downregulated in Clinical Samples

To learn the expression of miR-128-3p and WISPI in OA, the cartilage tissues of clinical osteoarthritic patients were detected. As presented in Fig. 1, the expression of miR-128-3p was significantly decreased (Fig. 1A), while WISPI gene expression and protein levels were increased in osteoarthritic cartilage tissues than that in normal tissues (Fig. 1B–D).

MiR-128-3p Promotes Proliferation and Inhibits Apoptosis of Chondrocytes

To further study the effect of miR-128-3p on the biological function of chondrocytes and its mechanism, we transfected miR-128-3p mimic or inhibitor to chondrocytes, and then its transfection efficiency was detected by RT-qPCR; the results were shown in Fig. 2A. After 48 h of transfection, the expression level of miR-128-3p in chondrocytes was significantly higher in the miR-128-3p mimic group than in the mimic-NC group, which laid the foundation for subsequent experiments. To further verify the relationship between miR-128-3p and WISPI, we detected the expression level of WISPI in chondrocytes by RT-qPCR and Western blotting. We found that the expression level of WISPI in chondrocytes transfected with miR-128-3p mimic was significantly downregulated; it was negatively correlated with the expression of miR-128-3p (Fig. 2B–D). This suggested that overexpression of miR-128-3p might inhibit the expression of

WISPI in OA patients. To better understand the role of miR-128-3p on chondrocyte proliferation and apoptosis, we used CCK-8 methods to detect the proliferative capacity of chondrocytes. The results showed that the proliferative capacity of chondrocytes in the miR-128-3p mimic group was significantly promoted ($P < 0.05$) compared with the NC group (Fig. 2E). We further examined whether the ability of apoptosis was affected after overexpression of miR-128-3p. The results were shown in Fig. 2F; the number of apoptotic cells in the miR-128-3p group was significantly lower than that in the NC group ($P < 0.05$). In addition, compared with its control, miR-128-3p indeed suppressed caspase-3 activation (Fig. 2G). And we could observe the opposite effect on chondrocytes transfected with miR-128-3p inhibitor. The above results showed that overexpression of miR-128-3p promoted proliferation and inhibited apoptosis of chondrocytes.

MiR-128-3p Inhibits the Degradation of Chondrocyte Matrix and the Production of Proinflammatory Cytokines Induced by IL-1 β

To explore the effects of miR-128-3p on the chondrocyte matrix degradation and proinflammatory cytokine production, the chondrocytes were transfected with miR-128-3p mimic or inhibitor and transfected mimic-NC or inhibitor-NC as negative control, then the cells were washed twice with PBS after 24 h, and stimulated with IL-1 β (10 ng/ml) in serum-free medium for 24 h. First, we found that IL-1 β could inhibit miR-128-3p expression in chondrocytes (Fig. 3A). And it was observed that the concentration of MMP3, MMP9, collagen-X, and ADAMTS-5 were significantly inhibited by miR-128-3p mimic, while the collagen-II was induced (Fig. 3B–F). In addition, the cytokines, such as IL-6,

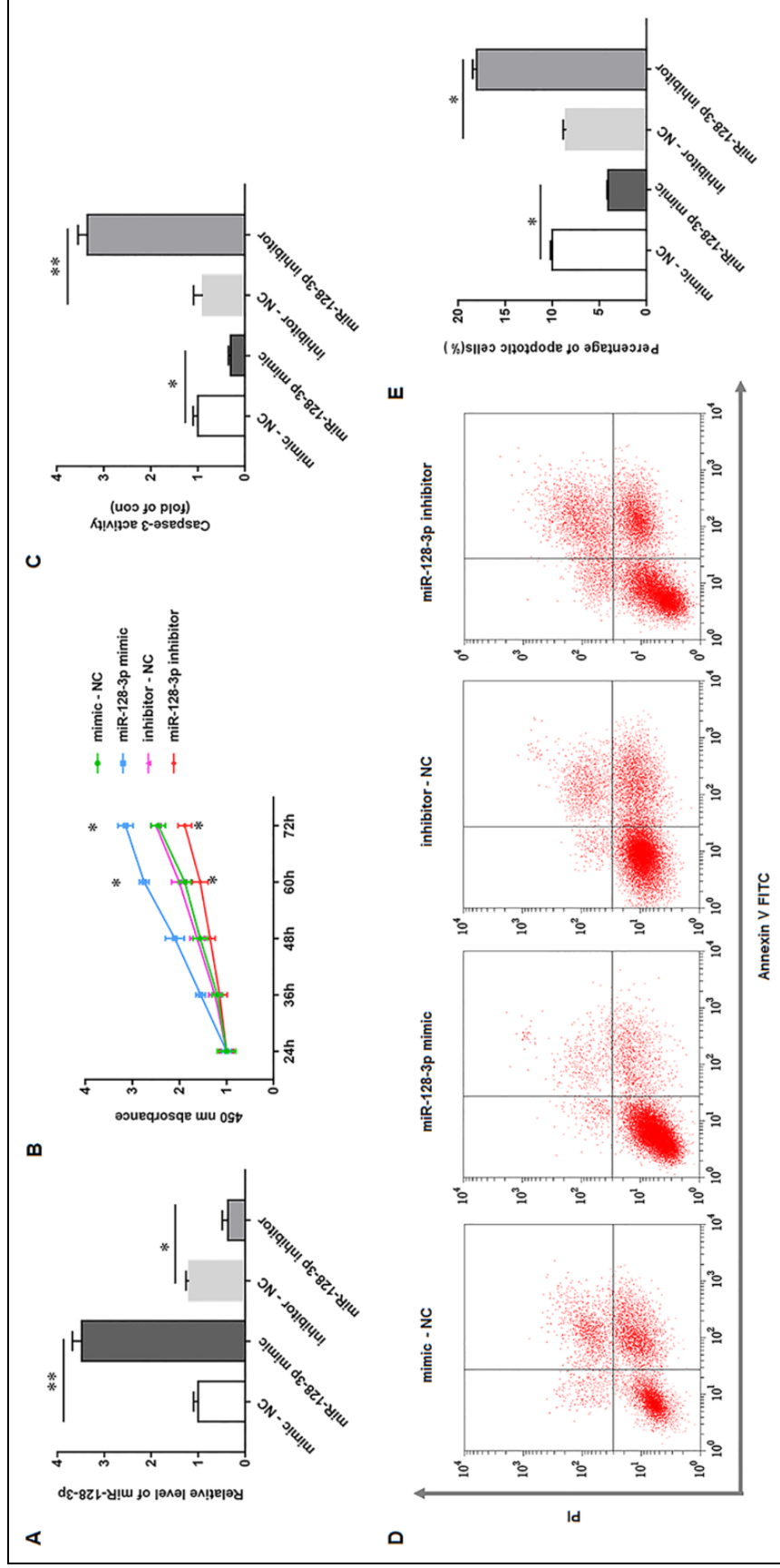


Fig. 2. MiR-128-3p regulates the proliferation and apoptosis of chondrocytes. Chondrocytes were transfected with miR-128-3p mimic, inhibitor, or NC for 48 h. (A, B) MiR-128-3p and WISP1 relative expression in chondrocytes was analyzed by RT-qPCR after transfection. The expression of WISP1 was inversely related to miR-128-3p expression. (C, D) The expression of WISP1 was determined by Western blotting after transfection, and the densitometry results were shown in right. (E) The proliferative capability of chondrocytes was evaluated using cell counting kit-8 assay after transfection. The number of apoptosis cells was counted after transfection. (F) The caspase-3 activity was defined by caspase-3 colorimetric assay kit. Data were shown as mean \pm standard deviation of one representative experiment. Similar results were obtained in three independent experiments. *, $P < 0.05$ versus NC; **, $P < 0.01$ versus NC; RT-qPCR, quantitative reverse transcription PCR; WISP1, Wnt1-inducible signaling pathway protein 1

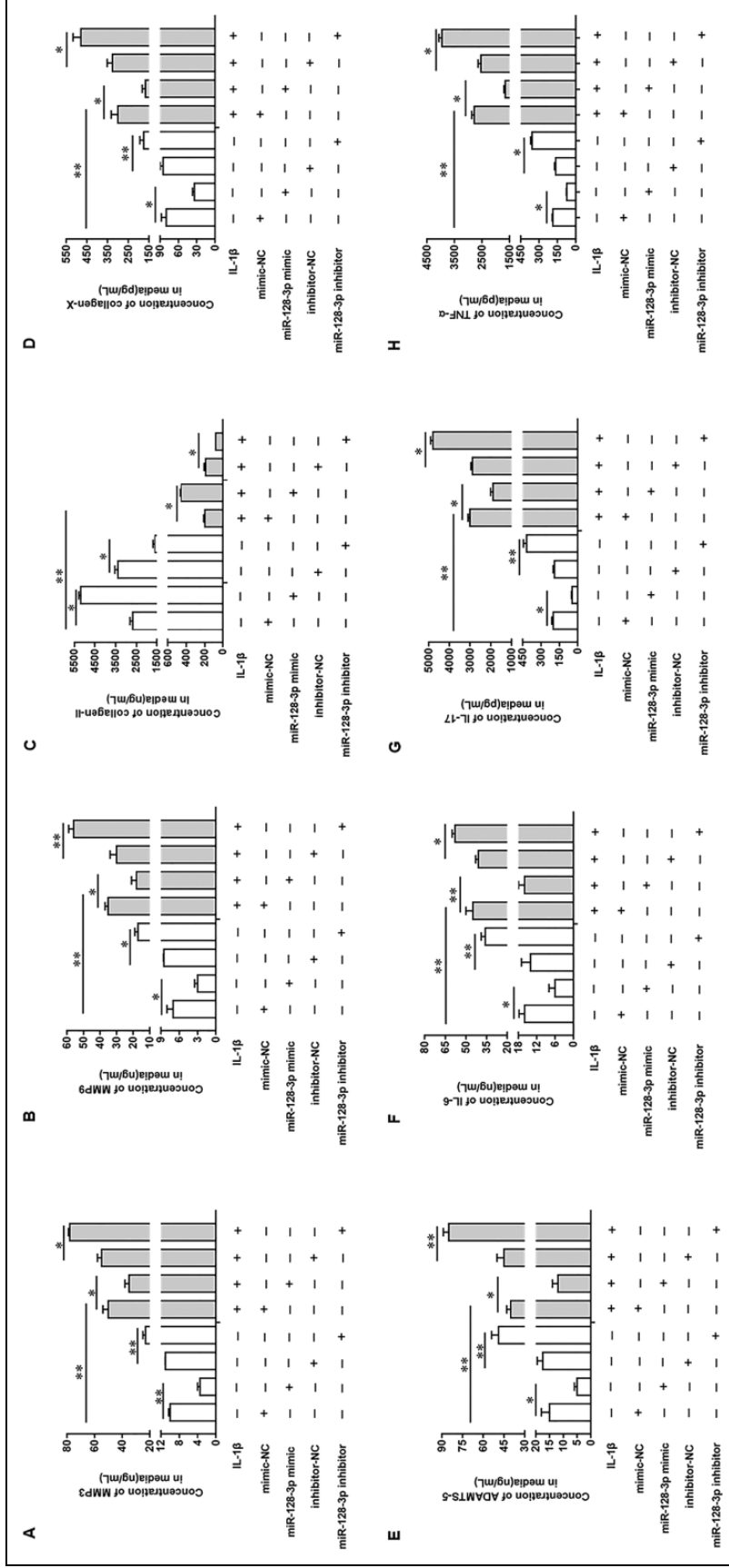


Fig. 3. miR-128-3p inhibits the degradation of the chondrocyte matrix and the production of proinflammatory cytokines. (A) The expression of miR-128-3p in chondrocytes stimulated with or without IL-1β was tested by RT-qPCR. (B–I) The chondrocytes were transfected with miR-128-3p mimic or inhibitor, and transfected mimic-NC or inhibitor-NC as negative control for 24 h, then stimulated with or without IL-1β (10 ng/ml) in serum-free medium for 24 h. Then the levels of MMP3, MMP9, collagen-II, collagen-X, ADAMTS-5, IL-6, IL-17, and TNF-α were determined using ELISA. Results were obtained from three independent experiments. *, $P < 0.05$ versus NC; **, $P < 0.01$ versus NC. IL, interleukin; MMP, matrix metalloproteinases; NC, negative control; RT-qPCR, quantitative reverse transcription PCR; TNF-α, tumor necrosis factor- α .

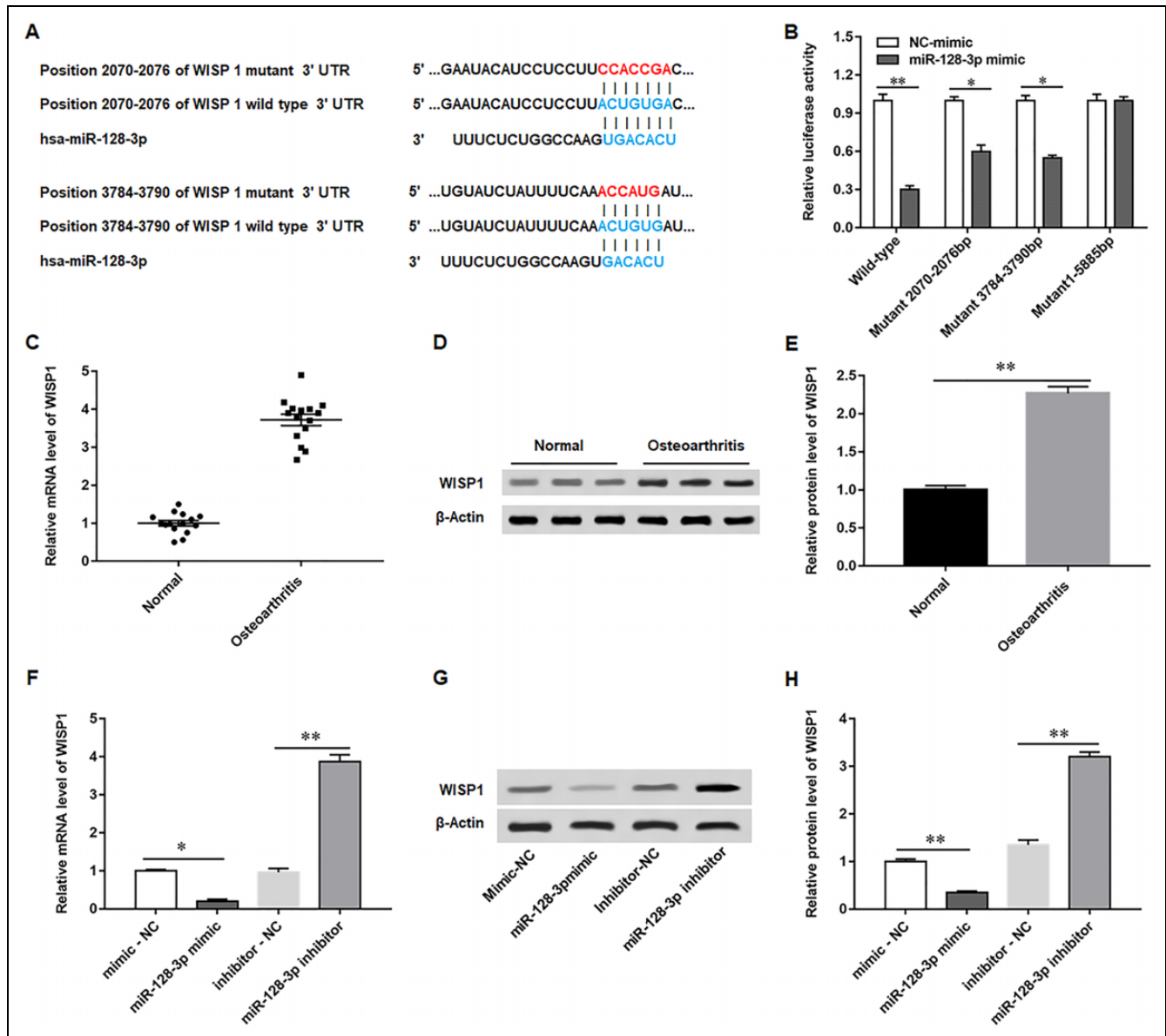


Fig. 4. MiR-128-3p directly targets WISP1. (A) The conserved target sites of miR-128-3p binding to WISP1 3'-UTR were shown. (B) Chondrocytes were co-transfected with pGL3-WISP1 3'-UTR-WT or pGL3-WISP1 3'-UTR-MUT and NC-mimic or miR-128-3p mimic. Firefly and Renilla luciferase activities were determined. *, $P < 0.05$ versus NC. NC, negative control; UTR, untranslated region; WISP1, Wnt1-inducible signaling pathway protein 1.

IL-17, and TNF- α , were markedly lower in miR-128-3p mimic rather than in control (Fig. 3G–I). And these changes were significant with or without IL-1 β stimulation. Taken together, overexpression of miR-128-3p reduced the matrix degradation as well as the inflammatory response.

MiR-128-3p Targets the “Seed Sequence” in the 3'-UTR of WISP1 mRNA

We next investigated the mechanisms of miR-128-3p regulating the chondrocyte behavior of OA. The binding sites

of miR-128-3p and WISP1 were predicted by TargetScan; there were two targeting sites of miR-128-3p binding to WISP1 3'-UTR (Fig. 4A). To test whether WISP1 is directly targeted by miR-128-3p, we built wild-type and mutant-type 3'-UTR of WISP1, and then the luciferase reporter plasmid containing 3'-UTR of WISP1 was co-transfected with miR-128-3p mimic or NC-mimic. The results of luciferase activity assays were shown in Fig. 4B; compared with the NC group, the miR-128-3p group significantly suppressed the luciferase activity of WISP1 wild-type 3'-UTR but did not influence the luciferase activity of the mutants. It could be seen that miR-128-3p directly

targets the WISP1 3'-UTR to inhibit WISP1 expression in osteoarthritic chondrocytes.

WISP1 Reverses the Effect of miR-128-3p on OA

In order to validate the correlation between WISP1 and miR-128-3p, chondrocytes were co-transfected with miR-128-3p and pcDNA-WISP1. As shown in Fig. 5, WISP1 downregulated the expression of miR-128-3p (Fig. 5A). Furthermore, miR-128-3p mimic significantly decreased the expression of WISP1, while pcDNA-WISP1 upregulated the expression of WISP1, indicating that miR-128-3p reversely regulated the expression of WISP1 (Fig. 5B–D). Then, we examined the effects of WISP1 and miR-128-3p on cell proliferation, apoptosis, and ECM degradation. Treatment with WISP1 attenuated the miR-128-3p-induced proliferative capacity of chondrocytes (Fig. 5E) and promoted cell apoptosis, activity of caspase-3, and the secretion of MMP3 (Fig. 5F–H). Others have previously demonstrated that WISP1 is a potent activator of PI3K and Akt, two critical players in cell survival and growth, and TNF- α can translocate p65 to activate the NF- κ B signaling pathway¹⁶. We investigated whether miR-128-3p performs function via the PI3K/Akt/NF- κ B pathway by targeting WISP1 and whether WISP1 can reverse this phenomenon. We analyzed the status of this pathway by measuring the phosphorylation of PI3K and p65. Results showed that treated with WISP1 significantly induced the phosphorylation of PI3K and p65, while miR-128-3p mimic suppressed the phosphorylation (Fig. 5I–K). Co-transfection miR-128-3p with WISP1 partially rescued the changes of cell proliferation, apoptosis, ECM degradation, and the PI3K/Akt/NF- κ B pathway induced by WISP1 overexpression.

Knockdown of WISP1 Inhibits Chondrocyte Apoptosis and ECM Degradation

To investigate the effect of WISP1 on chondrocyte apoptosis, WISP1 was knocked down by siRNA. The results are shown in Fig. 6A–C; with or without stimulation of IL-1 β , the siRNA significantly decreased the expression of WISP1 both in mRNA and protein levels. As shown in Fig. 6D–F, the knockdown of WISP1 suppressed the phosphorylation levels of PI3K and p65. It was shown that si-WISP1 decreased the activation of downstream signaling pathways in OA. Then we found that the apoptosis rate of si-WISP1 chondrocytes was significantly decreased than the siRNA-NC group (Fig. 6G). Furthermore, knockdown of WISP1 decreased the expression of MMP3 in chondrocytes (Fig. 6H). It can be seen that silencing WISP1 can inhibit chondrocyte apoptosis and the secretion of MMPs.

Activation of the PI3K/Akt/NF- κ B Could Antagonize the Effect of miR-128-3p on ECM Degradation, Proliferation, and Apoptosis in Chondrocytes

To further confirm that miR-128-3p regulates chondrocyte proliferation, apoptosis, and ECM degradation by suppressing the expression of WISP1 and the activation of the PI3K/Akt/NF- κ B pathway, we treated chondrocytes with the PI3K allosteric activator 740 Y-P to activate the PI3K/Akt/NF- κ B pathway. The results showed that miR-128-3p mimic significantly promoted the expression of miR-128-3p and inhibited the expression of WISP1 in chondrocytes with or without 740 Y-P treatment (Fig. 7A–D). The chondrocytes transfected with miR-128-3p mimic decreased the expression of p-PI3K and p-p65 while treated with 740 Y-P induced the phosphorylation of PI3K and p65 (Fig. 7E–G). In addition, we detected the downstream target molecules of NF- κ B by ELISA and found that pretreatment of 740 Y-P induced the secretion of MMP3 and inhibited the secretion of collagen-II in miR-128-3p mimic-transfected cells (Fig. 7H, I). Accordingly, 740 Y-P treatment significantly decreased the proliferative capacity of chondrocytes induced by miR-128-3p mimic (Fig. 7J). Additionally, 740 Y-P treatment increased apoptosis and caspase-3 activity in miR-128-3p mimic-transfected cells (Fig. 7K, L). Taken together, miR-128-3p inhibited the PI3K/Akt/NF- κ B pathway by silencing WISP1, thereby regulating the proliferation, apoptosis, and ECM degradation of chondrocytes.

Discussion

Human OA is an inflammatory disease of the synovial joint and is the leading cause of disability in the adult population worldwide. Recent studies have shown that WISP1 has the effect of promoting articular cartilage destruction; it can promote the breakdown of collagen and proteoglycan by inducing the secretion of MMPs and aggrecanases. In this study, we found that WISP1 expression was increased in osteoarthritic cartilage. Furthermore, WISP1 was a direct target of miR-128-3p, which regulates the PI3K/Akt/NF- κ B pathway to suppress cell apoptosis in OA.

There are many studies showing that miR-128 plays an important role in the development of diseases. In primary breast tumors, the expression of miR-128 was significantly decreased. Overexpression of miR-128 could decrease cell viability, increase apoptosis and DNA damage, and reduce chemotherapeutic resistance¹⁷. MiR-128 was downregulated in gastric cancer, and overexpression of miR-128 inhibited migration, invasion, and proliferation of gastric cancer cells by targeting Bmi-1¹⁸. In contrast, miR-128-3p inhibited apoptosis in lung cancer¹¹. And in NSCLC, miR-128-3p inhibited apoptosis and caspase-3 activation and promoted proliferation and survival of NSCLC cells¹⁵. In addition, overexpression of miR-128 enhanced the expression of genes involved in inflammation, proinflammatory cytokines,

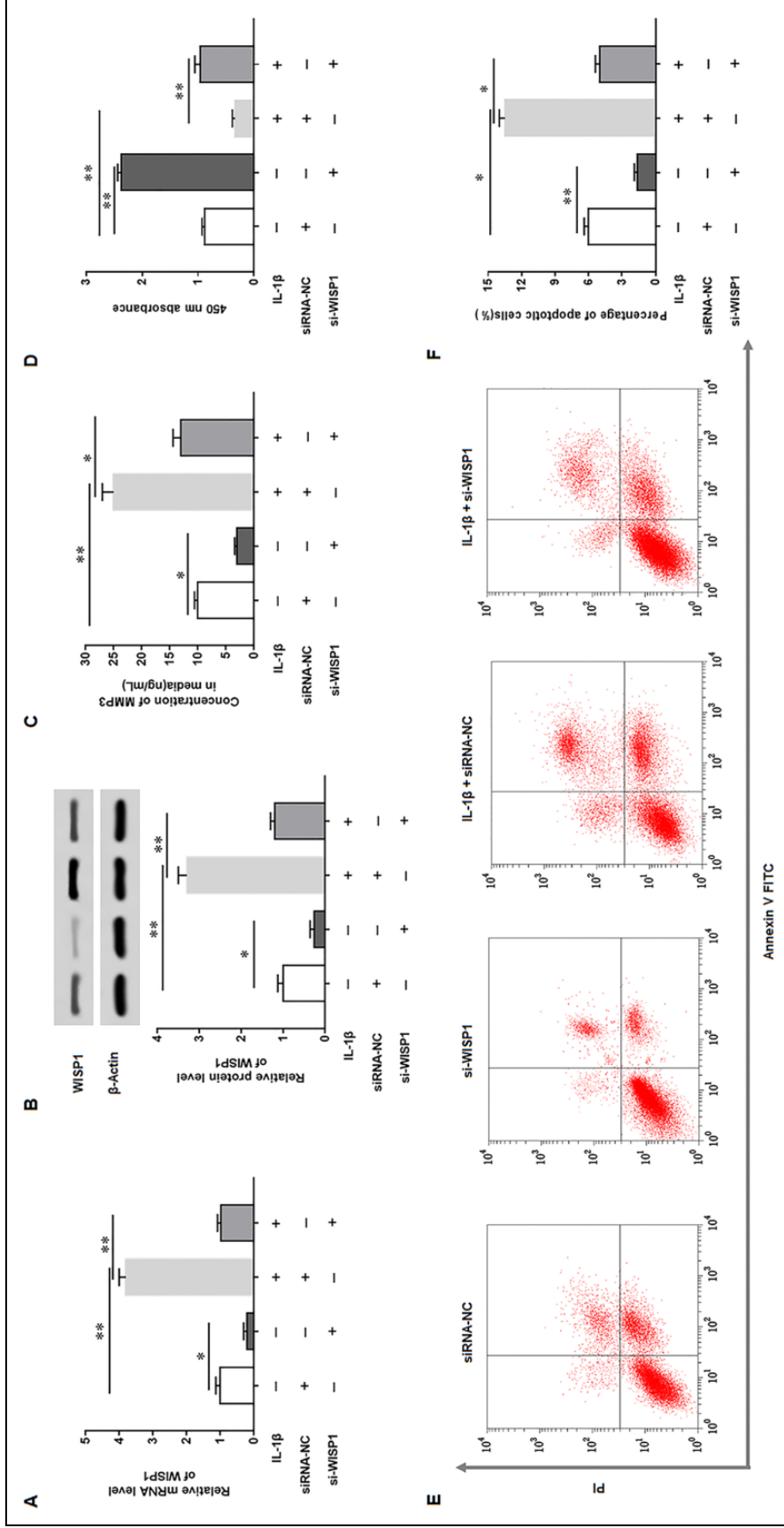


Fig. 5. WISP1 reverses the effect of miR-128-3p on OA. Chondrocytes were transfected with mimic-NC or miR-128-3p mimic together with either the pcDNA3.1 or pcDNA-WISP1. (A, B) Relative MiR-128-3p and WISP1 mRNA expression levels in chondrocytes were analyzed by RT-qPCR after transfection for 48 h. (C, D) WISP1 expression was examined using Western blotting and quantified by densitometry. Cell proliferation (E) and percentage of apoptotic cells (F) were measured by cell counting kit-8 assay and 4',6-diamidino-2-phenylindole staining, respectively. (G) The caspase-3 activity was defined by caspase-3 colorimetric assay kit. (H) The secretion of MMP3 was determined using ELISA. (I-K) The total and phosphorylated PI3K and p65 protein expressions were analyzed by Western blotting, then the p-PI3K/PI3K and p-p65/p65 were quantified by densitometry. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$. MMP, matrix metalloproteinases; NC, negative control; OA, osteoarthritis; PI3K, phosphatidylinositol-3-kinase; RT-qPCR, quantitative reverse transcription PCR; WISP1, Wnt1-inducible signaling pathway protein 1.

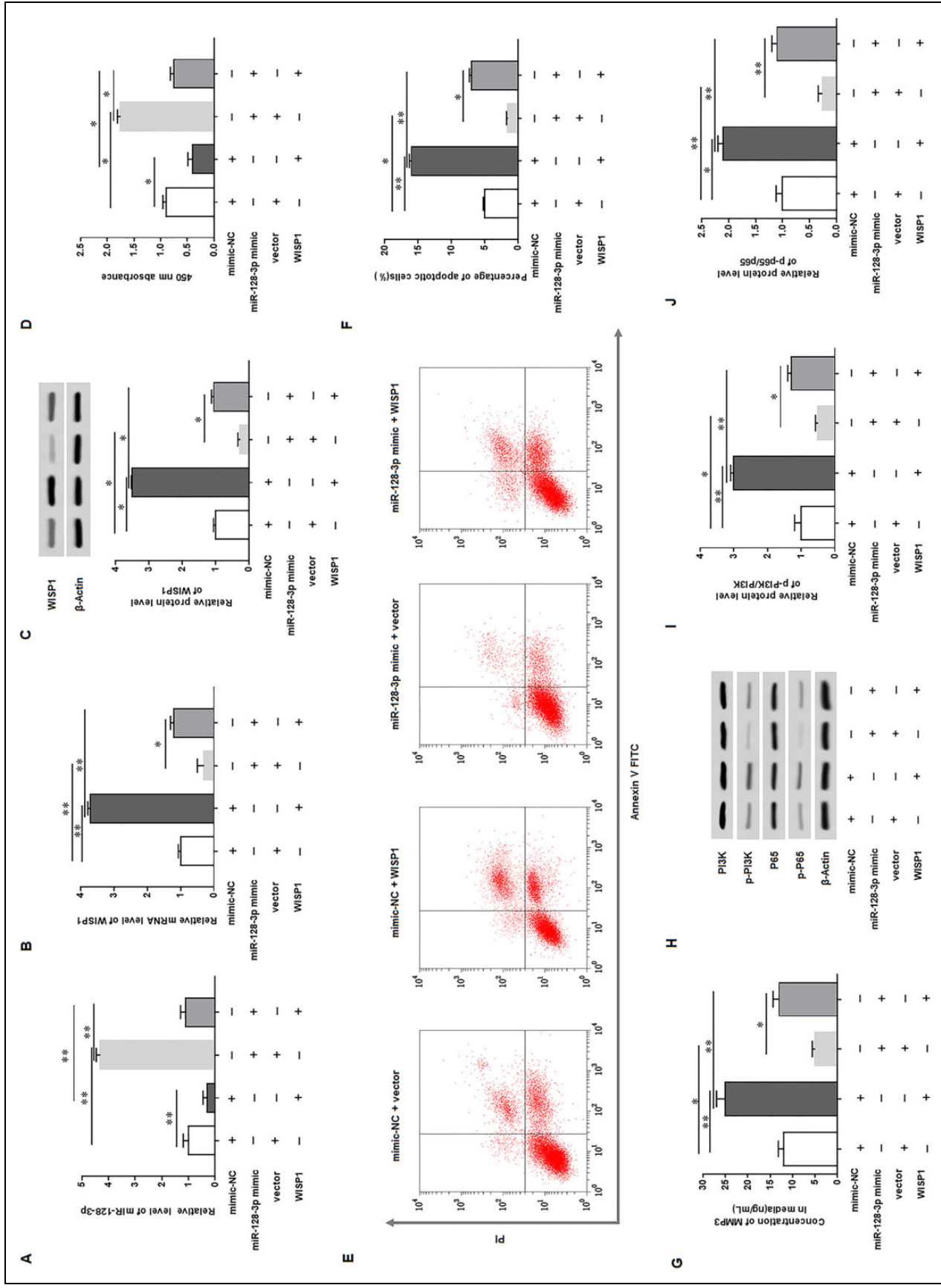


Fig. 6. Knockdown of WISP1 inhibits chondrocyte apoptosis and ECM degradation. Chondrocytes were transfected with WISP1-siRNA (si-WISP1) and its negative control (siRNA-NC), respectively. After cultured for 24 h, stimulated with or without IL-1 β (10 ng/ml) in serum-free medium for 24 h. The mRNA (A) and protein (B, C) expression levels of WISP1 were detected by RT-qPCR and Western blotting. (D-F) Protein levels of PI3K and p65 in chondrocytes were analyzed by Western blotting after transfection with siRNA-NC or si-WISP1 for 48 h. (G) The percentage of apoptotic cells was detected by 4',6-diamidino-2-phenylindole staining. (H) The concentration of MMP3 was detected by ELISA. Data were shown as mean \pm standard deviation of one representative experiment, similar results were obtained in three independent experiments. * $P < 0.05$ versus NC; ** $P < 0.01$ versus NC. Akt, protein kinase B; ECM, extracellular matrix; IL, interleukin; MMP, matrix metalloproteinases; PI3K, phosphatidylinositol-3-kinase; NF- κ B, nuclear factor kappa-light-chain-enhancer of activated B cells; RT-qPCR, quantitative reverse transcription PCR; WISP1, Wnt1-inducible signaling pathway protein 1.

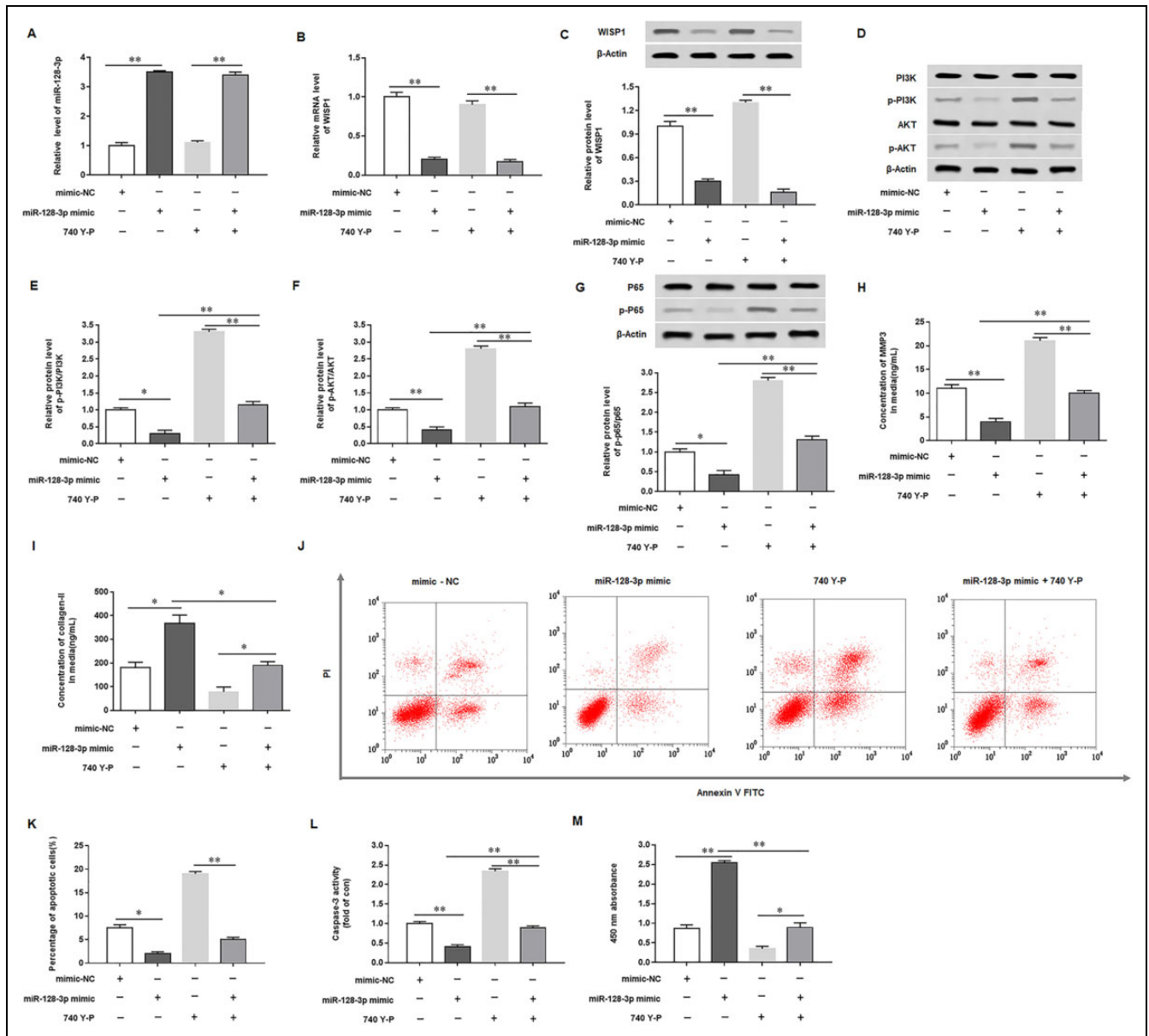


Fig. 7. Activation of the PI3K/Akt/ NF- κ B could antagonize the effect of miR-128-3p on ECM degradation, proliferation, and apoptosis in chondrocytes. Chondrocytes were transfected with miR-128-3p mimic or mimic-NC and then treated with the PI3K allosteric activator 740 Y-P to activate the PI3K/Akt/NF- κ B pathway. (A, B) The expression of miR-128-3p and WISP1 was analyzed by RT-qPCR. (C, D) The protein levels of WISP1 were determined by Western blotting and quantified by densitometry. (E–G) PI3K and p-PI3K, p65, and p-p65 protein expressions were analyzed by Western blotting and quantified by densitometry. p-PI3K/PI3K and p-p65/p65 ratio in chondrocytes transfected with miR-128-3p mimic and treated with 740 Y-P was significantly higher than that transfected with miR-128-3p. (H, I) The levels of MMP3 and collagen-II were determined using ELISA. (J) Cell proliferation was measured by cell counting kit-8 assay. (K) Percentage of apoptotic cells was measured by 4',6-diamidino-2-phenylindole staining. (L) The caspase-3 activity was defined by caspase-3 colorimetric assay kit. *, $P < 0.05$; **, $P < 0.01$. Akt, protein kinase B; ECM, extracellular matrix; MMP, matrix metalloproteinases; PI3K, phosphatidylinositol-3-kinase; NF- κ B, nuclear factor kappa-light-chain-enhancer of activated B cells; RT-qPCR, quantitative reverse transcription PCR; WISP1, Wnt1-inducible signaling pathway protein 1.

and fibrosis in normal rat kidney cells¹⁹. Downregulation of miR-128-3p significantly inhibited the inflammatory response of rheumatoid arthritis by targeting TNFAIP3 to inhibit the activity of the NF- κ B pathway¹³. Considering the role of miR-128 in disease progression is controversial, we

investigated the role of miR-128 in the development of OA. In the present study, we found that the expression of miR-128-3p was significantly decreased in OA. And overexpression of miR-128-3p could promote proliferation and inhibit apoptosis and ECM degradation of chondrocytes.

OA is characterized by articular cartilage damage and cartilage degeneration. Studies have shown that MMPs play a leading role in cartilage degeneration, and the digestion of type II collagen in articular cartilage is also strong³. Overexpression of Wnts and WISP1 in human chondrocytes can significantly increase the expression of type X collagen and decrease the expression of type II collagen²⁰. WISP1 stimulation of human OA synovium can increase the expression of MMP2/3/9/13 and ADAMTS-4/5^{6,7,21}. WISP1 can affect the differentiation of cartilage precursor cells into chondrocytes and also affect the phenotype of chondrocytes²². In synovial fibroblasts with OA, WISP1 promotes the release of IL-6 by activating $\alpha 5\beta 5$ integrin, PI3 K, protein kinase B, and κ light chain enhancer^{23,24}. WISP1 has been reported to activate PI3K-Akt signaling in synovial fibroblast cells²⁵. The serine/threonine kinase Akt is activated by the lipidic product of PI3K^{26,27}. After PI3 K activation, Akt accumulates at its activation site²⁸. Phosphorylation of Akt is promoted by PDK-1 and PDK-2; it affects cell growth, cell cycle entry, and cell survival²⁹. Then it regulates signaling pathways and induces the expression of NF- κ B family; it has been reported that Akt serine/threonine kinase is involved in the induction of NF- κ B^{30,31}. Previous studies have shown that NF- κ B can regulate the production of inflammatory mediators, which is positively correlated with the development of OA^{22,23}. Although the number of new miRNA/target interactions identified continues to increase, its role particularly those affecting signaling pathways in OA remains largely unknown. We hypothesized that WISP1 might be a potential target gene of miR-128-3p and then predicted the binding sites of miR-128-3p and WISP1 by TargetScan and verified this prediction using luciferase reporter gene assay. And we found that miR-128-3p inhibited the phosphorylation of PI3 K and p65, whereas WISP1 overexpression has the reverse effect. Furthermore, the activation of the PI3K/Akt/NF- κ B pathway abolished the suppression of apoptosis and MMP production due to miR-128-3p overexpression in OA. Taken together, miR-128-3p downregulated the expression of WISP1 in chondrocytes, inhibited the activation of the PI3K/Akt/NF- κ B pathway, and regulated chondrocyte behavior of OA.

In conclusion, miR-128-3p can reduce apoptosis, ECM degradation, and inflammation in human osteoarthritic articular chondrocytes via the PI3K/Akt/NF- κ B pathway, which may be related to its targeting of WISP1. These findings may provide a new research direction for clinical targeted therapy of OA.

Ethical Approval

This study was approved by the Ethics Committee at Huaihe Hospital of Henan University.

Statement of Human and Animal Rights

All procedures in this study were conducted in accordance with Huaihe Hospital of Henan University of Ethics Committee's (Approval number: 2020027) approved protocols.

Statement of Informed Consent

Written informed consent was obtained from the patients for their anonymized information to be published in this article.


Declaration of Conflicting Interests

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