

Original Article

The role of miR-211-5p in the viability, apoptosis, and migration of chondrocytes in osteoarthritis

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Abstract: The aim of this study was to investigate the expression of miR-211-5p in osteoarthritis (OA) and to explore potential molecular mechanisms for the same. qRT-PCR was used to detect the expression of miR-211-5p in IL-1 β -stimulated chondrocytes. Subsequent to cell transfection, the impact of the miR-211-5p mimic on viability, apoptosis, and migration of chondrocytes was determined using the Cell Counting Kit-8, flow cytometry, and the Transwell assay, respectively. Additionally, the expression profiles of Bcl-2, Bax, cleaved Caspase-3, and pro-Caspase-3 were also determined by qRT-PCR and western blot. The target gene of miR-211-5p was predicted and validated using the luciferase reporter assay. The changes in the expression levels of the NF- κ B pathway proteins after cell transfection were detected by the western blot. The expression of miR-211-5p was observed to be down-regulated in IL-1 β -stimulated chondrocytes. An overexpression of miR-211-5p was seen to promote chondrocytes viability, inhibit apoptosis, and increase the migratory capability of the chondrocytes. ADAMTS5 was identified as a target gene for miR-211-5p and it was determined that miR-211-5p is a negative regulator for the same. It was also determined that the impact of miR-211-5p on chondrocyte viability, apoptosis, and migration is mediated through the regulation of ADAMTS5 expression and the suppression of the NF- κ B pathway. Our study demonstrates that miR-211-5p plays an important role in the pathogenesis of OA. The overexpression of miR-211-5p promotes viability and migration while inhibiting apoptosis of chondrocytes by negatively regulating the expression of ADAMTS5 and suppressing the NF- κ B pathway.

Keywords: Osteoarthritis, chondrocyte, miR-211-5p, ADAMTS5, NF- κ B pathway

Introduction

Osteoarthritis (OA) is a degenerative joint disease in which the repair and degradation of articular cartilage are imbalanced; it is characterized by the breakdown of joint cartilage and underlying bone [1]. The most common symptoms of this disease are joint pain and stiffness [2]. Approximately, 18% of all females and 10% of all males over the age of 60 years are thought to be afflicted with this condition [3]. It has been predicted that the global incidence of OA will continue to rise at a very fast rate. Despite considerable research efforts, currently there are no preventative or therapeutic measures available for treating this disease; this is largely due to the fact that there is no reliable method to diagnose OA prior to the development of radiological evidence [4].

MicroRNAs (miRNAs) are a group of small non-coding RNAs which bind to the 3'-untranslated regions (UTR) of target mRNAs leading to either mRNA destabilization or translational inhibition [5, 6]. Evidence has shown that miRNAs play an important role in multiple cellular and biological process [7]. Additionally, it is known that some miRNAs exhibit a tissue-specific or developmental stage-specific expression pattern and have been reported to be involved in both cancer initiation as well as progression [8, 9]. Several previous studies have reported that miRNAs, such as miR-34a and miR-210, play an important role in chondrogenesis and OA [10-12]. A recent study reported that the expression miR-211-5p was down-regulated in OA and that the overexpression of the same had the potential to rescue abnormal osteoarthritic subchondral bone osteoblast gene expression

[13]. However, the molecular mechanism underlying this phenomenon is still unclear and remains to be elucidated.

In the present study, we detected the expression of miR-211-5p in chondrocytes and investigated the effects of its abnormal expression on proliferation, apoptosis, and migration of chondrocytes. The findings of this study have the potential to lead to the identification of novel therapeutic targets for OA.

Materials and methods

Animals

Sprague-Dawley rats (weighing 200-250 g) were housed under standard diurnal conditions at 22-24°C and fed on a standard commercial diet along with ad libitum access to water. All animals were treated in accordance with the guidelines laid down by the Institutional Animal Care and Use Committee. This study was approved by the Ethics Committee for Experimental Animals, Huzhou Central Hospital.

Cell culture and cell transfection

Articular chondrocytes were isolated from the knee joints of rats as per protocol described in a previous study [14]. Briefly, articular cartilage tissues were cut into small pieces and digested with 0.2% trypsin for 30 min followed by the digestion with 0.2% Type II collagenase for 2 h. The released cells were cultured in DMEM/F12 medium supplemented with antibiotics and 10% fetal bovine serum (FBS). The primary cells were maintained in a monolayer culture until they reached confluence. Thereafter, the chondrocytes were transfected either with the miR-211-5p mimic, pcDNA-ADAMTS5, or the corresponding controls, using Lipofectamine 2000 (Invitrogen). After 12 h of transfection, 10 ng/mL IL-1 β (R&D systems Inc., USA) and/or 10 μ mol/L pyrrolidine dithiocarbamate (PDTC) (Sigma, St. Louise, CA, USA) (inhibitor of NF- κ B) was added to the culture medium.

Cell viability assessment

Chondrocytes were cultured in 96-well culture plates at a density of 5×10^3 cells/well and allowed to adhere to the substratum overnight. The miR-211-5p mimic or the corresponding control was then transfected into the cells and

the chondrocyte culture was treated with IL- β . The viable cell count was determined at 24 and 48 h using the Cell Counting Kit-8 (Dojindo Laboratory, Japan). Briefly, a 10% working solution composed of 2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulphophenyl)-2H-tetrazolium monosodium salt (WST-8) was added to each well followed by a 4 h incubation at 37°C. Absorbance at 450 nm was measured using a SPECTRAmax190 microplate reader (Molecular Devices, Sunnyvale, CA, USA). Cell viability was expressed in terms of fold change with respect to the control.

Apoptosis assay

After 24 h of transfection, the cells were stained either with 0.05 mg/mL of 4,6-diamidino-2-phenylindole (DAPI) or propidium iodide (PI) (Sigma, USA) in phosphate-buffered saline (PBS) for 15 min at room temperature and then visualized using a Leica DMR microscope (Bensheim, Germany). A FACS Calibur (Becton Dickinson, New Jersey, USA) was used to compute the percentage of apoptotic cells. The apoptotic cells were identified based on PI staining of the fragmented DNA.

Cell migration

A modified two-chamber migration assay with a pore size of 8 μ m was used to analyze cell migration. The chondrocytes were suspended in 200 μ L of serum-free medium and seeded onto the upper compartment of a 24-well Transwell culture chamber. The complete medium (600 μ L) was then added to the lower compartment, and the whole was then incubated for 12 h at 37°C. After fixing with methanol, the cells remaining on the upper surface of the filter were removed with a cotton swab. The traversed cells present on the lower side of the filter were stained with 0.1% crystal violet and counted.

Quantitative reverse transcription PCR

Total RNA was extracted from cells using Trizol reagent (Invitrogen, Carlsbad, CA, USA). For cDNA synthesis, 500 ng oligo (dT) (Promega, Madison, USA) or miRNA-specific primers (Invitrogen, USA) were added to 1 μ g RNA. The samples were incubated with 5 μ L of 5 \times first-strand buffer, 20 U RNasin (Takara, DaLian China), 2 μ L 5 mM dNTP, 1 μ L M-MLV reverse

Table 1. Primers used for targets amplification

Name	Forward primer (5'-3')	Reverse primer (5'-3')
GAPDH	AACGTGTCAGTOGTGGACCTG	AGT GGGTGTGCTGTGGAAGT
Bcl-2	CTGGTGGACAACATCGCTCTG	GGTCTGCTGACCTCACTTGTG
Bax	CTGAGCTGACCTTGGAGC	GACTCCAGCCACAAAGATG
Cleaved-caspase 3	TGGAGGCTGACTTCCTGTATGCTT	ACGGGATCTGTTTCTTTGCGTGG
Pro-caspase 3	TACTGTTTAAAGGGCTCGTTAAGCGG	TTAGTGGTGGTGGTGGTGGTGGAGAGTGAAGTACATCTCTTTGGT
ADAMTS5	GCTAGGCGACAAGGACAAGAG	CGCAGGCAGCTTCTTGGTCAG

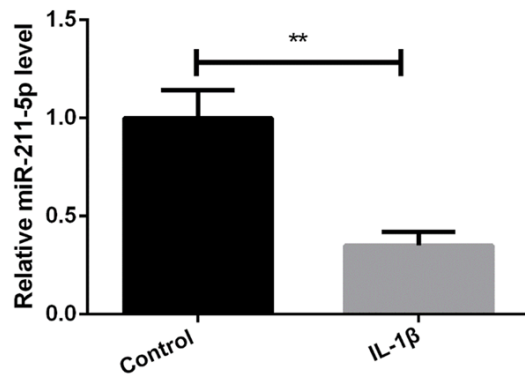


Figure 1. The relative expression level of miR-211-5p in IL-1 β -stimulated chondrocytes detected by qRT-PCR. **P < 0.01 compared to the control group.

transcriptase (Promega, USA) and distilled water (total volume of 25 μ L) for 10 min at 65°C. The qPCR reaction mixture was composed of 12.5 μ L 2 \times SYBR green PCR mix, 1 μ L cDNA template, and 0.3 μ M each of gene-specific forward and reverse primers (in a final volume of 25 μ L). The gene expression levels were calculated by relative quantification using either GAPDH or U6 snRNA as the endogenous reference genes. Primer pairs are shown in **Table 1**.

Western blot

The protein was extracted using the RIA lysis buffer system (Beyotime Biotechnology, Shanghai, China). The quantification of the isolated protein was done using the BCA™ Protein Assay Kit (Pierce, Rockford, IL, USA). The western blot system was established using a Bio-Rad Bis-Tris Gel system and the manufacturer's instructions were followed for the same. Primary antibodies (Santa Cruz, USA) were prepared in 5% blocking buffer at a dilution of 1:1000 and incubated with the membrane for 14-16 h at 4°C. This was followed by the incubation with a horseradish peroxidase-

labeled secondary antibody (Santa Cruz Biotechnology: 1:1000, Santa Cruz, CA, USA) for 1 hour at room temperature. GAPDH antibody was purchased from Sigma-Aldrich, St Louis, MO, USA. After rinsing, the polyvinylidene -difluoride (PVDF) membrane that carried the blots and antibodies was transferred to the Bio-Rad ChemiDoc™ XRS system and 200 μ L of Immobilon Western Chemiluminescent HRP Substrate (Millipore, USA) was added. The intensity of the bands was quantified using Image Lab™ Software (Bio-Rad, Shanghai, China).

Vector construction and luciferase reporter assay

For constructing a luciferase reporter, the 3'UTR fragment of ADAMTS9 or ADAMTS5 containing putative binding sites for miR-211-5p was inserted downstream of the firefly luciferase gene in the pGL3 vector. The mutant 3'UTR carrying mutated sequences within the complementary site for miR-211-5p was generated using the fusion PCR method. The chondrocytes were co-transfected with miR-211-5p as well as the luciferase reporter containing either wild-type or mutant 3'UTR of the target gene; pRL-TK was used as a control vector. After 48 h of transfection, luciferase activity was determined using the Dual Luciferase Assay kit (Promega). Relative luciferase activity was calculated as a ratio of the raw firefly luciferase activity and the Renilla luciferase activity.

Statistical analysis

All experiments were repeated three times. The results of multiple experiments are presented as mean \pm SD. All statistical analyses were performed using the SPSS 19.0 software. P-values were calculated using one-way analysis of variance (ANOVA) and <0.05 were considered to indicate statistically significant results.

Role of miR-211-5p in osteoarthritis

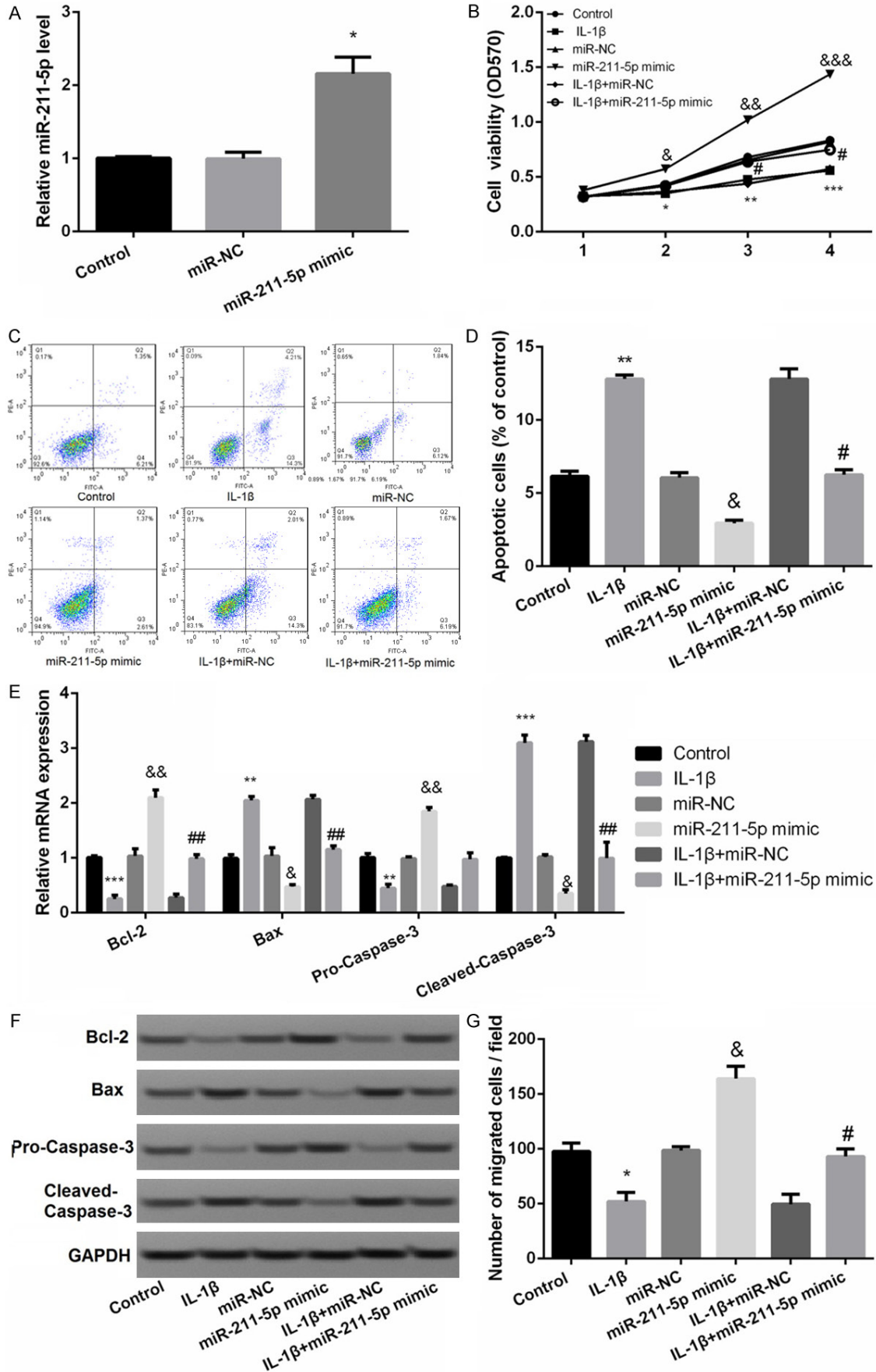


Figure 2. A: The relative expression level of miR-211-5p after the transfection of chondrocytes with miR-211-5p mimic detected by qRT-PCR; B: The effect of miR-211-5p mimic on chondrocyte viability detected by Cell Counting Kit-8; C and D: The effect of miR-211-5p mimic on the apoptosis of chondrocyte detected by flow cytometry; E and F: The relative expression levels of apoptosis-associated proteins after cell transfection assayed by qRT-PCR and western blot; G: The effect of miR-211-5p on cell migration detected by Transwell assay. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ compared to the control group; & $P < 0.05$, && $P < 0.01$, &&& $P < 0.001$ compared to the miR-NC group; # $P < 0.05$, ## $P < 0.01$ compared to the IL-1 β +miR-NC group.

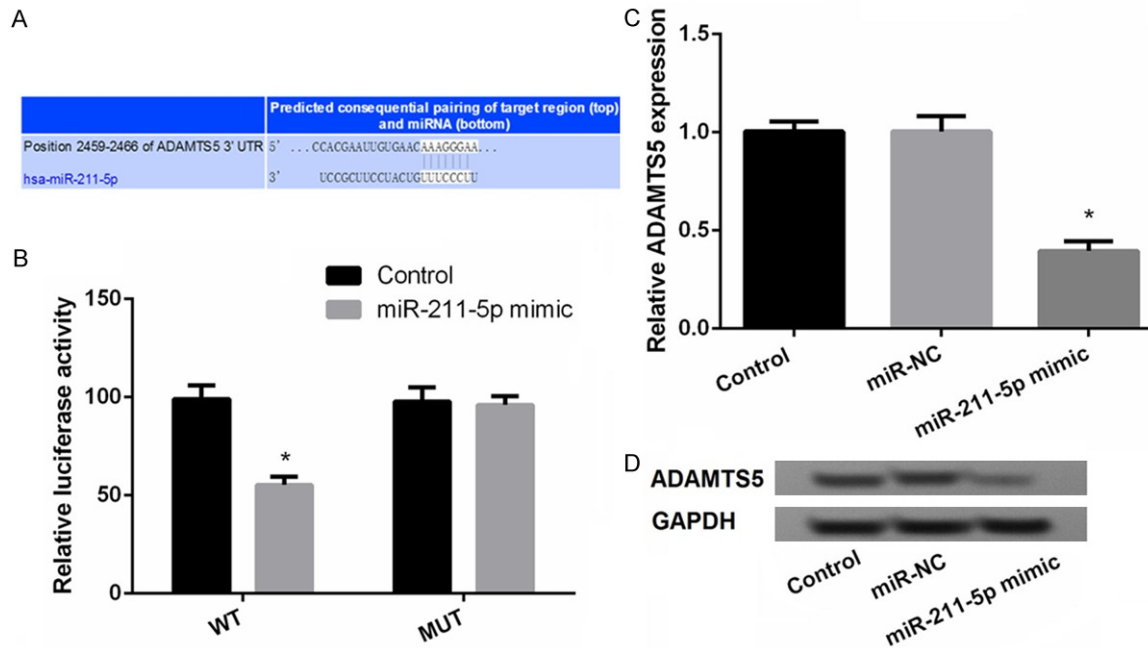


Figure 3. A: The gene sequences of ADAMTS5 regulated by miR-211-5p; B: The relative luciferase activities in wild-type ADAMTS5 3'-UTR and mutant ADAMTS5 3'-UTR in the transfected cells; C and D: The relative expression level of ADAMTS5 in the transfected cells detected by RT-PCR and western blot. * $P < 0.05$ compared to the control group.

Results

Expression of miR-211-5p induced by IL-1 β

To investigate the effect of miR-211-5p, rat primary chondrocytes were incubated with 10 ng/mL IL-1 β in order to simulate OA. The expression level of miR-211-5p was observed to be significantly down-regulated after IL-1 β treatment as compared to control ($P < 0.01$) (Figure 1), which is suggestive of the fact that miR-211-5p may be associated with the pathogenesis of OA.

Effect of miR-211-5p overexpression on cell viability, apoptosis, and migration

In an attempt to analyze the effect of miR-211-5p on OA, chondrocytes were transfected with a miR-211-5p mimic. qRT-PCR revealed that the miR-211-5p mimic significantly up-regulat-

ed the expression of miR-34a ($P < 0.05$) (Figure 2A).

Subsequent to the cell transfection, the viability, apoptosis, and migration of chondrocytes were analyzed. As shown in Figure 2B, the administration of IL-1 β alone caused significant inhibition of chondrocyte viability as compared to the control group, while the miR-211-5p mimic was seen to significantly promote chondrocyte viability compared to the miR-NC group. Further study revealed that the miR-211-5p mimic had the potential to reverse the inhibitory effect of IL-1 β on chondrocyte viability.

The results of the apoptosis assay showed that IL-1 β significantly promoted the apoptosis of chondrocytes ($P < 0.01$) while the miR-211-5p mimic significantly inhibited the same ($P < 0.05$). Moreover, IL-1 β , in combination with the miR-211-5p mimic, was observed to reverse the

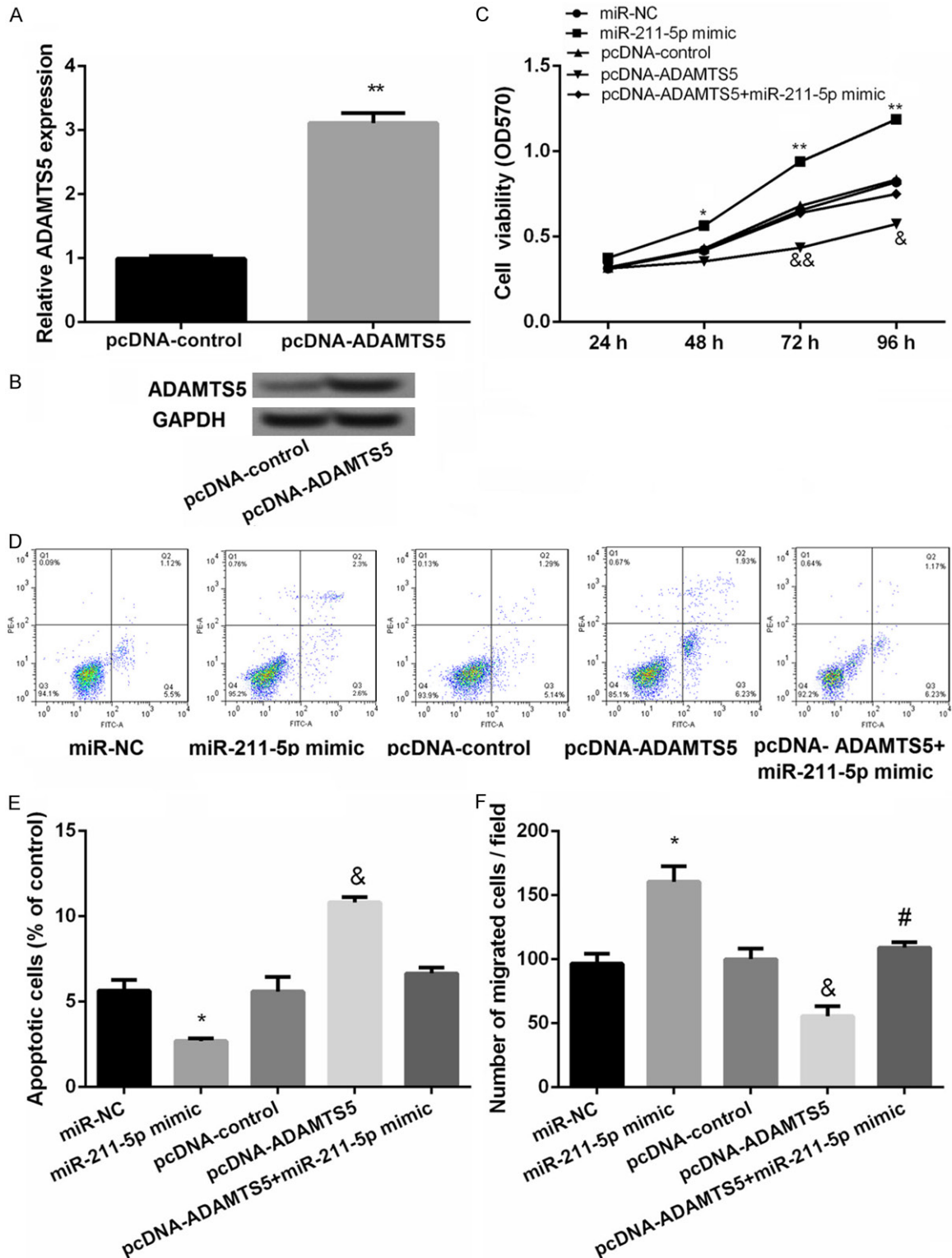


Figure 4. A and B: The relative expression levels of ADAMTS5 after transfection detected by RT-PCR and western blot; C-F: The effect of ADAMTS5 overexpression on viability, apoptosis, and migration of the chondrocytes detected by Cell Counting Kit-8, flow cytometry, and Transwell assay, respectively. * $P < 0.05$, ** $P < 0.01$ compared to the control group; & $P < 0.05$, && $P < 0.01$ compared to the pcDNA-control group; # $P < 0.05$, ## $P < 0.01$ compared to the pcDNA-ADAMTS5 group.

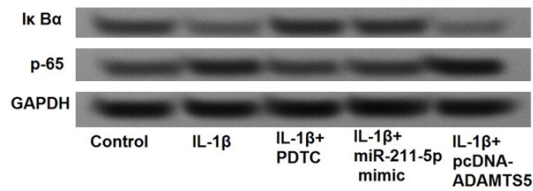


Figure 5. The relative expression levels of IκBα and p65 in chondrocyte detected by western blot.

effect of IL-1β on apoptosis ($P < 0.05$) (Figure 2C and 2D). Our study also investigated the expression profiles of Bax, Bcl-2, cleaved-Caspase-3 and pro-Caspase-3. As presented in Figure 2E and 2F, IL-1β significantly promoted the expression of both Bax as well as cleaved-Caspase-3, while causing a downregulation of the expressions of Bcl-2 and pro-Caspase-3 genes. Interestingly, the miR-211-5p mimic was observed to prevent significantly the IL-1β-mediated up-regulation of Bax and cleaved-Caspase-3 expression as well as the Bcl-2 and pro-Caspase-3 downregulation ($P < 0.001$).

The results of the migration assay revealed that IL-1β inhibited the migration of chondrocytes ($P < 0.05$) while the miR-211-5p mimic significantly increased migration; the mimic was also observed to counteract the inhibitory effect of IL-1β on chondrocyte migration ($P < 0.05$) (Figure 2G).

Prediction and analysis of the effects of miR-211-5p on ADAMTS5

Based on the sequence analysis, it was deduced that ADAMTS5 may be a potential target gene for miR-211-5p (Figure 3A). In order to verify that ADAMTS5 was a direct target of miR-211-5p, a dual-luciferase reporter system was employed. The result of the assay showed that the miR-211-5p mimic could downregulate the luciferase activity of the reporter ($P < 0.05$). A mutation in the 3'-UTR of the ADAMTS5 was observed to free the gene from regulation by miR-211-5p (Figure 3B), which is suggestive of the fact that the site of mutation coincides with the exact site for regulation by miR-211-5p. The results also showed that ADAMTS5 expression could be detected in the chondrocytes that had been transfected with the miR-211-5p mimic. As shown in Figure 3C and 3D, a negative correlation between ADAMTS5 and miR-211-5p expression was found ($P < 0.05$) which implies

that miR-211-5p may negatively regulate the expression of ADAMTS5.

Effects of ADAMTS5 overexpression on cell viability, apoptosis, and migration

To further investigate the role of ADAMTS5 in OA, we transfected chondrocytes with pcDNA-ADAMTS5. As shown in Figure 4A and 4B, ADAMTS5 expression increased significantly after transfection ($P < 0.01$). Subsequently, we evaluated the effects of ADAMTS5 overexpression on cell viability, apoptosis, and migration. The results showed that ADAMTS5 overexpression has the potential to reduce cell viability as well as reverse the effect of the miR-211-5p mimic on the same (Figure 4C). Additionally, ADAMTS5 overexpression promoted apoptosis (Figure 4D and 4E) and significantly inhibited the migration of chondrocytes (Figure 4F) ($P < 0.05$). Moreover, the overexpression of ADAMTS5 reversed the effect of the miR-211-5p mimic on apoptosis and migration of chondrocytes. Taken together, it can be claimed that miR-211-5p can affect cell viability, apoptosis, and migration by negatively regulating ADAMTS5.

miR-211-5p protected chondrocytes through inhibiting NF-κB pathway

To verify if the NF-κB signaling pathway is involved in the protective effect of miR-211-5p, we used PDTC, an inhibitor of NF-κB, in the study. As shown in Figure 5, p65 induction and IκBα reduction proved that IL-1β can activate the NF-κB signaling pathway. This activation was inhibited by the presence of PDTC. The regulation of p65 and IκBα caused by IL-1β was inhibited by the miR-211-5p mimic and promoted by pc-DNA-ADAMTS5, which suggests that miR-211-5p protected chondrocytes from the effects of IL-1β by inhibiting the activation of the NF-κB signaling pathway.

Discussion

In recent times, miRNAs have attracted a great deal of attention on account of the important roles they play in human diseases. They are also increasingly being viewed as potential targets for therapeutic intervention in the future. In the present study, we investigated the expression of miR-211-5p in OA chondrocytes that had been induced by IL-1β. Our results con-

firmed that the expression of miR-211-5p was downregulated in IL-1 β -stimulated chondrocytes. The overexpression of miR-211-5p was seen to promote chondrocyte viability while inhibiting apoptosis and increasing their migratory ability. Further analysis revealed that ADAMTS5 was a target gene for miR-211-5p and was negatively regulated by it. Additionally, we also determined that the effects of miR-211-5p on chondrocyte viability, apoptosis, and migration were achieved by regulating ADAMTS5 expression while suppressing the NF- κ B pathway.

Chondrocytes are crucial for maintaining the dynamic equilibrium between the production of the extracellular matrix and its enzymatic degradation, and the loss of this balance facilitates catabolic events which result in the loss of articular cartilage in OA. Therefore, it can be concluded that chondrocyte viability is essential for maintaining the integrity of articular cartilage [15]. Recently, it was revealed that chondrocyte apoptosis is associated with OA pathogenesis. In OA patients, an increase in the incidence of empty lacunae in the cartilage is directly linked to apoptosis [16, 17]. In this study, we investigated the impact of miR-211-5p on the viability and apoptosis of chondrocytes and discovered that miR-211-5p overexpression strongly promoted the viability and inhibited apoptosis of IL-1 β -stimulated chondrocytes. Additionally, we also analyzed the expression patterns of apoptosis-associated proteins such as Bcl-2, Bax, pro-Caspase-3, and cleaved-Caspase-3. The results showed that the miR-211-5p mimic significantly increased the expressions of Bax and cleaved Caspase-3 while decreasing the expression of Bcl-2 and pro-Caspase-3. This observation was in accordance with the result obtained by flow cytometry. Interestingly, our results mirror previous studies wherein it was reported that miR-211-5p regulated cell proliferation as well as cell survival [18, 19].

Chondrocyte migration is also known to play a key role in OA. It is hypothesized that chondrocytes start migrating from the articular margin, pass the superficial zone and come to rest in the transitional and radial zone; the dysfunction of this process may lead to changes in matrix composition and clustering of chondrocytes as observed in OA [20]. The present study

found that the miR-211-5p mimic could increase the migration ability of chondrocytes. These observations suggest towards the important role of miR-211-5p in the pathogenesis of OA.

It is well established that aggrecan, which is the major proteoglycan found in cartilage, endows cartilage with its unique capacity to resist compression and bear load. In the case of OA patients, aggrecan is observed to be degraded by an aggrecanase belonging to the ADAMTS family of proteinases. ADAMTS is a disintegrin and metalloproteinase that contains thrombospondin motifs [21]. ADAMTS family includes several members that are known to influence the development and progression of arthritis. Among these members, ADAMTS-5 has received a great deal of attention in the pathology of OA largely due to the exemplary efficiency of its aggrecanase [22]. Interestingly, the present study found that ADAMTS5 is a target gene of miR-211-5p and that the overexpression could reduce the viability and migration while promoting apoptosis of chondrocytes. This suggests that the effects of miR-211-5p on chondrocytes can be exerted via regulating the expression of ADAMTS5.

NF- κ B plays a critical role in the regulation of the immune response and inappropriate regulation of this pathway is implicated in the pathogenesis of several diseases including OA [23]. NF- κ B pathway is known to mediate several critical events in the inflammatory response by chondrocytes resulting in progressive extracellular matrix damage and cartilage destruction [24]. In a study conducted by Romanblas [23] the authors have demonstrated that stimulation of articular chondrocytes by IL-1 β results in potent activation of the NF- κ B pathway. In parallel to the aforementioned studies, ours also suggested that the NF- κ B pathway is activated by IL-1 β . However, the miR-211-5p mimic was observed to inhibit this pathway indicating that miR-211-5p may exert a protective effect on chondrocytes by shielding them from IL-1 β and inhibiting the activation of the NF- κ B pathway.

In conclusion, our study demonstrated that miR-211-5p plays an important role in the pathogenesis of OA. The overexpression of miR-211-5p promotes viability and migration and inhibits the apoptosis of chondrocytes by nega-

tively regulating ADAMTS5 expression and suppressing the NF- κ B pathway. Therefore, overexpression of miR-211-5p may have potential therapeutic value for OA.

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Disclosure of conflict of interest

None.

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