

ORIGINAL ARTICLE

MicroRNA-21 controls the development of osteoarthritis by targeting GDF-5 in chondrocytes

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Osteoarthritis is a common cause of functional deterioration in older adults and is an immense burden on the aging population. Altered chondrogenesis is the most important pathophysiological process involved in the development of osteoarthritis. However, the molecular mechanism underlying the regulation of chondrogenesis in patients with osteoarthritis requires further elucidation, particularly with respect to the role of microRNAs. MiR-21 expression in cartilage specimens was examined in 10 patients with knee osteoarthritis and 10 traumatic amputees. The effect of miR-21 on chondrogenesis was also investigated in a chondrocyte cell line. The effect of miR-21 on the expression of growth differentiation factor 5 (GDF-5) was further assessed by luciferase reporter assay and western blot. We found that endogenous miR-21 is upregulated in osteoarthritis patients, and overexpression of miR-21 could attenuate the process of chondrogenesis. Furthermore, we identified GDF-5 as the direct target of miR-21 during the regulation of chondrogenesis. Our data suggest that miR-21 has an important role in the pathogenesis of osteoarthritis and is a potential therapeutic target.

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INTRODUCTION

Osteoarthritis is a widely prevalent disease characterized by the progressive destruction of cartilage and is responsible for a majority of the health and economic burden in the elderly population worldwide.¹ Chondrocytes are the only cells in cartilage and are responsible for the synthesis and turnover of extracellular matrix, which is crucial for joint function.² Chondrogenesis occurs as a result of chondrocyte cell condensation, proliferation and differentiation, the dysregulation of which would eventually lead to osteoarthritis.³ However, the underlying molecular mechanisms regulating chondrogenesis during osteoarthritis development are still poorly understood.

MicroRNAs (miRNAs) comprise a class of small (≈ 22 nucleotides), non-coding RNAs that negatively regulate gene expression by controlling complementary target mRNAs and prohibiting their translation into functional proteins.⁴ A growing body of evidence indicates that miRNAs are involved in the control of various basic cell functions. MiRNAs are crucial for all cellular processes and also have an important role in the process of chondrogenesis and

cartilage remodeling.^{2,5,6} As a result, aberrant miRNA expression profiles have been demonstrated to be associated with osteoarthritis development.^{7–10} Previous studies have demonstrated a signature of 16 miRNAs that distinguishes normal from osteoarthritic cartilage tissue, with 9 miRNAs significantly upregulated and 7 miRNAs downregulated in osteoarthritis tissue compared with normal controls.⁸

MiR-21 has been highlighted due to its importance in tumor progression and metastasis, specifically in the process of cell proliferation and differentiation, which is vital to chondrogenesis and cartilage remodeling.^{11–13} However, the role of miR-21 in the progression of osteoarthritis and its underlying mechanism has remained unclear. GDF-5 acts as a signal for chondrogenesis and promotes chondrocyte differentiation.¹⁴ Mutations in GDF-5 cause epiphyseal dysplasia, characterized by early-onset osteoarthritis affecting the hip and hand joints.^{15,16} Moreover, a functional polymorphism in the 5'-UTR of GDF-5 is directly associated with susceptibility to osteoarthritis.^{17,18} Given the important roles of miRNAs and GDF-5 in osteoarthritis development,^{16,19} it is important to identify the potential effects of the miR-21-GDF-5 pathway in

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the development of chondrogenesis disorder-related osteoarthritis development so that therapeutic approaches can be further developed.

In the present study, we predicted GDF-5 as a putative target of miR-21 via bioinformatics analysis. We further explored the biological effects and the potential mechanisms of miR-21 in osteoarthritis by detecting the expression of miR-21 in osteoarthritis patients and we found that miR-21 was elevated in osteoarthritis. Overexpression of miR-21 could attenuate the process of chondrogenesis, which is characterized by chondrocyte proliferation and the expression of type-II-collagen, type-X-collagen, glycosaminoglycan and aggrecan genes in chondrocytes. We also analyzed the expression of genes encoding catabolic factors, including matrix metalloproteinases (MMP1, MMP2, MMP3, MMP9 and MMP13) as well as inflammatory cytokines (CCL2, CSF1 and CCL5) in chondrocytes. We found that miR-21 significantly increased the levels of MMP1, MMP2, MMP3 and MMP9. A subsequent luciferase activity assay and western blot analysis confirmed that miR-21 repressed the expression of GDF-5 by inducing GDF-5 mRNA decay. These results reveal a model that furthers our understanding of the molecular regulation of miRNAs in osteoarthritis and the potential therapeutic approaches that may be applied.

METHODS

Cell lines and tissue samples

Human articular chondrocytes (CH8 cell lines) were obtained from the Chinese Academy of Sciences (Shanghai, China). The cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum at 37°C with 5% CO₂. Ten paired osteoarthritic tissues and matched normal tissues from traumatic amputees were collected from our department. Informed consent from each patient was obtained, and this work was approved by the Ethics Committee of Union Hospital, Tongji Medical College.

RNA extraction and mRNA quantification

In the present study, we first discarded the perichondrium and cut the collected cartilage tissue into small pieces. We then placed these fragments into a homogenizer, in which they were ground thoroughly for RNA extraction. Total RNA was extracted using Trizol reagent (Invitrogen, Carlsbad, CA, USA). cDNA expression was quantified by real-time PCR using an Applied Biosystems model 7900HT Fast Real-Time PCR System. Briefly, the RNA was denatured for 10 min at 72°C and immediately placed on ice for 5 min. Denatured RNA was added to a mixture of MMLV-RT, MMLV-RT buffer and dNTPs and incubated for 1 h at 42°C. Then, the reagent was inactivated by heating at 95°C for 2 min. Relative gene expression was calculated using the $2^{-\Delta\Delta Ct} = 2^{-[(Ct \text{ target gene} - Ct \text{ GAPDH}) - (Ct \text{ target gene} - Ct \text{ GAPDH})]}$ method. MiR-21 was normalized to U6. Primers for miR-21 were designed and purchased from GeneCopoeia, Rockville, MD, USA. All experiments were carried out at least in triplicate.

Plasmids and transfection

MiR-21 precursor (Catalog# HmiR0202-MR01) and inhibitor (Catalog# HmiR-AN0312-AM01) constructs were purchased from GeneCopoeia. The pEGFP-N1-GDF-5 plasmid was generated using the following primers: sense 5'-CCGCTCGAGCAGCACTGGCC

CTCTGTCTTC-3', antisense 5'-GAATGCGGCCGCAGGCACAGTTT TGCTTTTATCTCATCAATATAC-3'. The amplified sequences were inserted into pEGFP-N1 within the XhoI/HindIII sites. Transfection was performed using Lipofectamine 2000 reagent (Invitrogen). Transfected cells were harvested after 48 h for RNA isolation and western blot.

Western blot

Chondrocytes were extracted with protein lysis buffer (50 mM Tris-HCl [pH 8.0], 150 mM NaCl and 1% NP-40) supplemented with protease inhibitor cocktail (2 µg ml⁻¹ each of PMSE, leupeptin, aprotinin and pepstatin A). The chondrocytes were lysed on ice for 30 min and centrifuged at 12 000 for 10 min prior to collection for the bicinchoninic acid assay. The extracts were separated by 8% SDS-polyacrylamide gel electrophoresis, transferred to a polyvinylidene difluoride membrane (Millipore, Darmstadt, Germany), and incubated with anti-GDF-5 (Sigma, St Louis, MO, USA; 1:1000 dilution) and anti-β-actin (Abcam, Cambridge, MA, USA; 1:2000 dilution) antibodies for 24 h at 4°C. The blots were developed using a chemiluminescent substrate kit (Pierce, Rockford, IL, USA) and exposed to CL-XPosure film. Finally, the blots were scanned and analyzed quantitatively using Kodak Digital Science 1D software (Eastman Kodak, Rochester, NY, USA).

In vitro cell proliferation assay

At 48 h after transfection, the cells were seeded into 96-well plates at 6 × 10³ cells per well. Cell viability was measured with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT). Optical densities were measured at 490 nm.

Colony formation assay

CH8 cells transfected with miR-21 precursor/inhibitor or the corresponding controls were seeded in a 10-cm dish and maintained in complete culture medium. After 21 days, CH8 cells were fixed with methanol and subsequently stained with 0.1% crystal violet. The colonies were counted manually.

Flow cytometric analysis of apoptosis

A total of 5 × 10⁵ cells were recovered by centrifugation for apoptosis evaluation via double staining with annexin V-fluorescein isothiocyanate (annexin V-FITC) and propidium iodide²⁰ (BioVision, St Lake Worth, FL, USA) according to the manufacturer's instructions, followed by flow cytometric analysis. Cell Quest software (Becton, Franklin Lakes, NJ, USA) was used to determine the percentage of apoptotic cells.

Luciferase reporter assay

Chondrocytes were transfected with 0.25 µg of p-MIR-report plasmid (Ambion, Austin, TX, USA) containing the 3'-UTR of mouse GDF-5 RNA and 50 nM of miR-21 precursor using Lipofectamine 2000 (Invitrogen). Luciferase activity was measured 48 h later in cell lysates using a luciferase assay kit (Promega, Madison, WI, USA).

Statistical analysis

All results are expressed as the means ± s.e.m. Statistical analysis was carried out using SPSS 13.0 (SPSS, Chicago, IL, USA). For statistical comparisons, we used Student's *t*-test or unpaired, one-way analysis of variance (ANOVA) followed by least significant difference (LSD) and Dunnett's T3 tests. Differences were considered significant when *P* < 0.05.

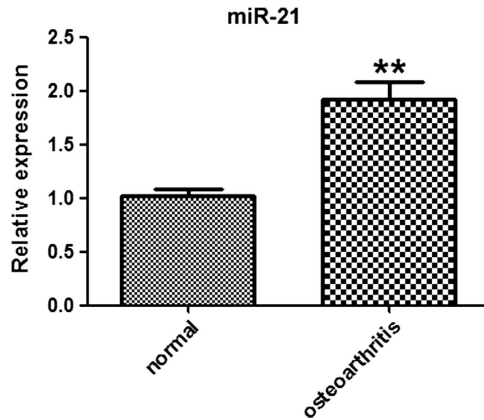


Figure 1 MiR-21 was increased in osteoarthritis patients. Quantitative real-time PCR was performed to examine the expression of miR-21 using cartilage tissues from osteoarthritis patients and cartilage tissues from traumatic amputees. $n=10$. * $P<0.05$, ** $P<0.01$ compared with control.

RESULTS

MiR-21 expression is increased in osteoarthritis patients

We initially assessed miR-21 expression levels in osteoarthritis patients. As shown by RT-PCR analysis, the expression levels of miR-21 were significantly ($P<0.05$) increased in tissues from osteoarthritis patients compared with tissues from traumatic amputees (Figure 1)

MiR-21 inhibits chondrocyte proliferation

Because miR-21 was markedly increased in osteoarthritis, it might function as a promoter of osteoarthritis. Therefore, we tested whether overexpression or downregulation of miR-21 in CH8 cells would affect chondrocyte growth. In MTT assay, cells transfected with the miR-21 precursor grew more slowly than the control group, while the miR-21 inhibitor caused an increase in growth (Figure 2a). To further test the effect of miR-21 on the growth of CH8 cells, a colony formation assay was performed. CH8 cells transfected with miR-21 showed

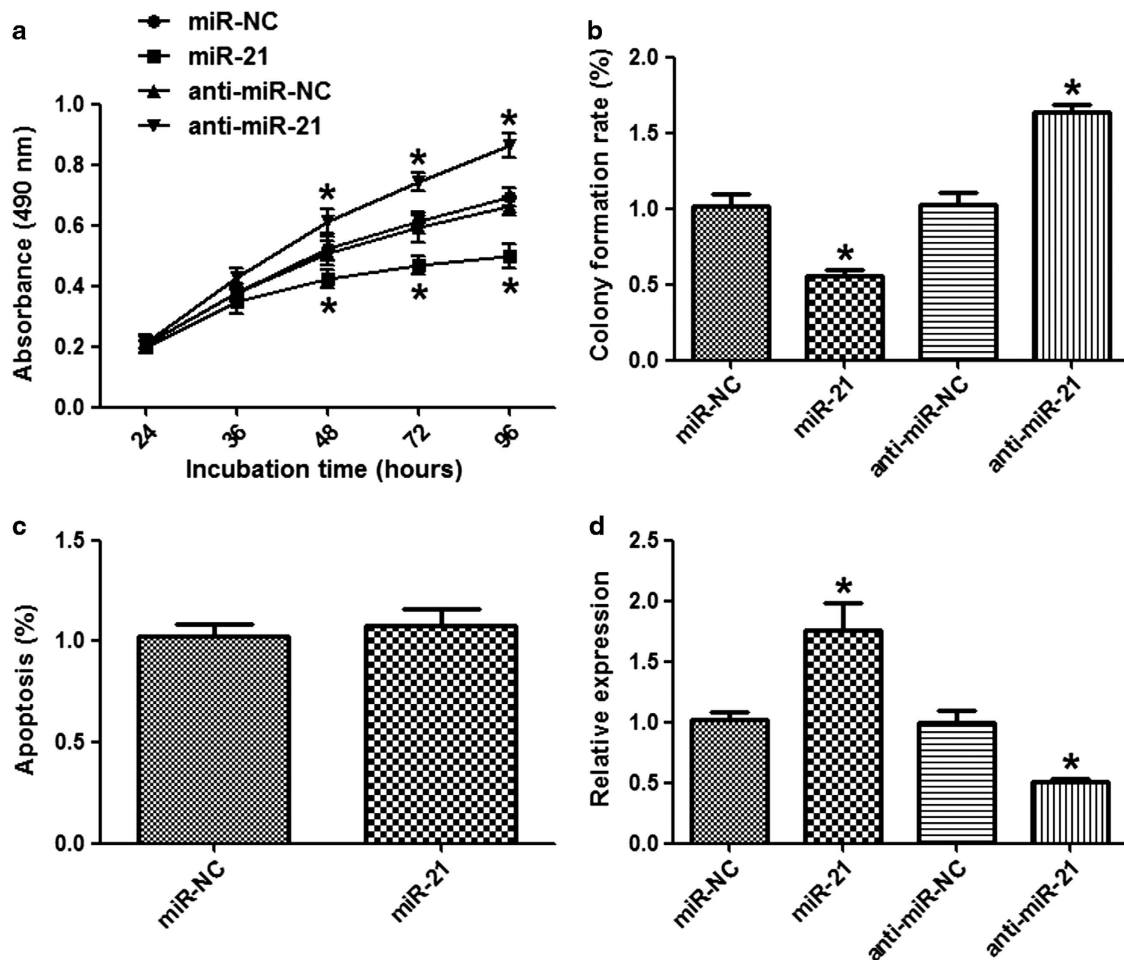


Figure 2 MiR-21 inhibited the proliferation of chondrocytes. CH8 cells were transfected with an miR-21 precursor, an miR-21 inhibitor or the corresponding controls. (a) At 24, 48, 36, 72 or 96 h after transfection, an MTT assay was performed to examine CH8 proliferation. (b) Representative results of a colony formation assay in CH8 cells transfected with an miR-21 precursor or inhibitor or the corresponding controls. (c) Apoptosis of CH8 cells was unchanged after miR-21 overexpression. (d) Quantitative real-time PCR was performed to examine miR-21 expression in CH8 cells transfected with an miR-21 precursor or inhibitor or the corresponding controls. $n=6$. * $P<0.05$ compared with control. MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide.

decreased colony formation compared with cells transfected with the control vector. However, CH8 cells transfected with miR-21 inhibitor showed more colony formation compared with cells transfected with the control inhibitor (Figure 2b). We further used flow cytometric analysis to determine the effect of miR-21 on the apoptosis of CH8 cells; however, no significant difference was detected between the miR-21 precursor and the control (Figure 2c). The data from the colony formation assay were consistent with those obtained with the MTT assay, indicating that overexpression of miR-21 could promote CH8 cell proliferation *in vitro*. The transfection efficiency was analyzed by quantitative real-time PCR (Figure 2d).

MiR-21 inhibits the maturation of CH8 cells and the process of chondrogenesis

To understand whether miR-21 has a negative effect on the chondrogenesis process, an miR-21 precursor and inhibitor were transfected into CH8 cells and the markers of

chondrogenesis, including type-II-collagen, type-X-collagen, glycosaminoglycan and aggrecan were investigated. Infection with the miR-21 precursor (Figure 3a) caused a decrease in the gene expression pattern of type-II-collagen, type-X-collagen, glycosaminoglycan and aggrecan. However, the miR-21 inhibitor caused significantly increased expression of hallmark genes of chondrogenesis (Figure 3b). Collectively, these data suggest that miR-21 suppresses the maturation of CH8 cells and the chondrogenesis process.

We further investigated the expression of genes encoding catabolic factors, including MMP and inflammatory cytokines, in chondrocytes because matrix-degrading enzymes and pro-inflammatory cytokines have a key role in the pathogenesis of osteoarthritis.^{21,22} We found that miR-21 overexpression significantly increased the levels of MMP1, MMP2, MMP3 and MMP9 (Figure 3c). The miR-21 inhibitor caused significant decreases in the expression patterns of MMP1, MMP2, MMP3 and MMP9 (Figure 3d). However, the levels of pro-inflammatory cytokines were not altered significantly.

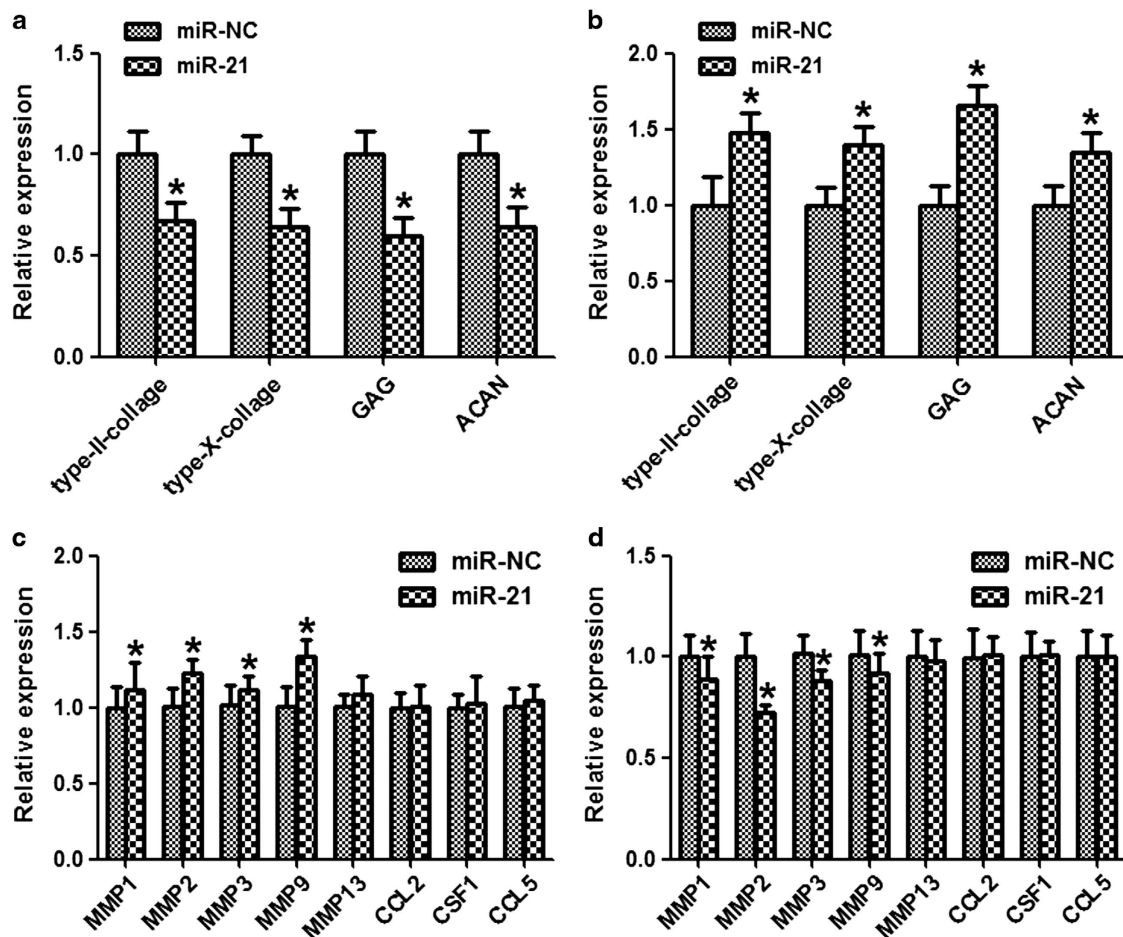


Figure 3 MiR-21 inhibited the maturation of CH8 cells and the chondrogenesis process. (a) CH8 cells transfected with an miR-21 precursor showed decreased expression of chondrogenesis markers compared with those transfected with the control. (b) An miR-21 inhibitor increased the expression of chondrogenesis markers in CH8 cells. (c) Levels of matrix metalloproteinases and inflammatory cytokines in CH8 cells transfected with an miR-21 precursor. (d) Levels of matrix metalloproteinases and inflammatory cytokines in CH8 cells transfected with an miR-21 inhibitor. $n=6$. * $P<0.05$ compared with control.

MiR-21 expression is inversely correlated with GDF-5 expression

Quantitative RT-PCR was utilized to detect the expression of GDF-5 in 10 osteoarthritic tissue samples and 10 matched tissue samples from traumatic amputees. The results showed that GDF-5 mRNA was significantly decreased in osteoarthritis (Figure 4a). Furthermore, the protein level of GDF-5 was also downregulated in osteoarthritic tissues compared with the normal tissue samples (Figures 4b and c). Moreover, the GDF-5 mRNA level was inversely correlated with miR-21 expression in the same osteoarthritic tissues (Figure 4d).

GDF-5 is a direct target of miR-21

TargetScan 6.2 was used to investigate the downstream target of miR-21. GDF-5 was predicted to be a target of miR-21. To confirm direct targeting of GDF-5 by miR-21, we amplified the wild-type (WT) GDF-5 3'-UTR containing the target sequences, or a mutant variant thereof, into a luciferase reporter vector and detected the effects of miR-21 on the luciferase activity in CH8 cells. As shown in Figure 5a, miR-21 suppressed the luciferase activity of the WT GDF-5 3'-UTR,

while mutation of the miR-21 binding sites (Mut) blocked this suppressive effect. Western blot analysis demonstrated that transfection of miR-21 in CH8 cells inhibited GDF-5 expression, while the miR-21 inhibitor elevated endogenous expression of GDF-5 protein (Figure 5b). Quantitative RT-PCR analysis showed similar levels of GDF-5 expression (data not shown), indicating that miR-21 suppressed GDF-5 expression post-transcriptionally.

DISCUSSION

MiRNAs have essential roles in modulating and maintaining normal physiological conditions. However, their levels are changed in response to pathological disorders, thereby resulting in the pathogenesis of degenerative diseases, such as osteoarthritis. Thus, it is important to determine how miRNA expression is regulated in osteoarthritis. Our present studies indicated a key role for miR-21 in the pathogenesis of osteoarthritis. The expression of miR-21 was significantly increased in human osteoarthritis tissue. Using TargetScan 6.2 to screen the targets of miR-21, GDF-5 was predicted to be a target of miR-21. Our results further showed that the GDF-5

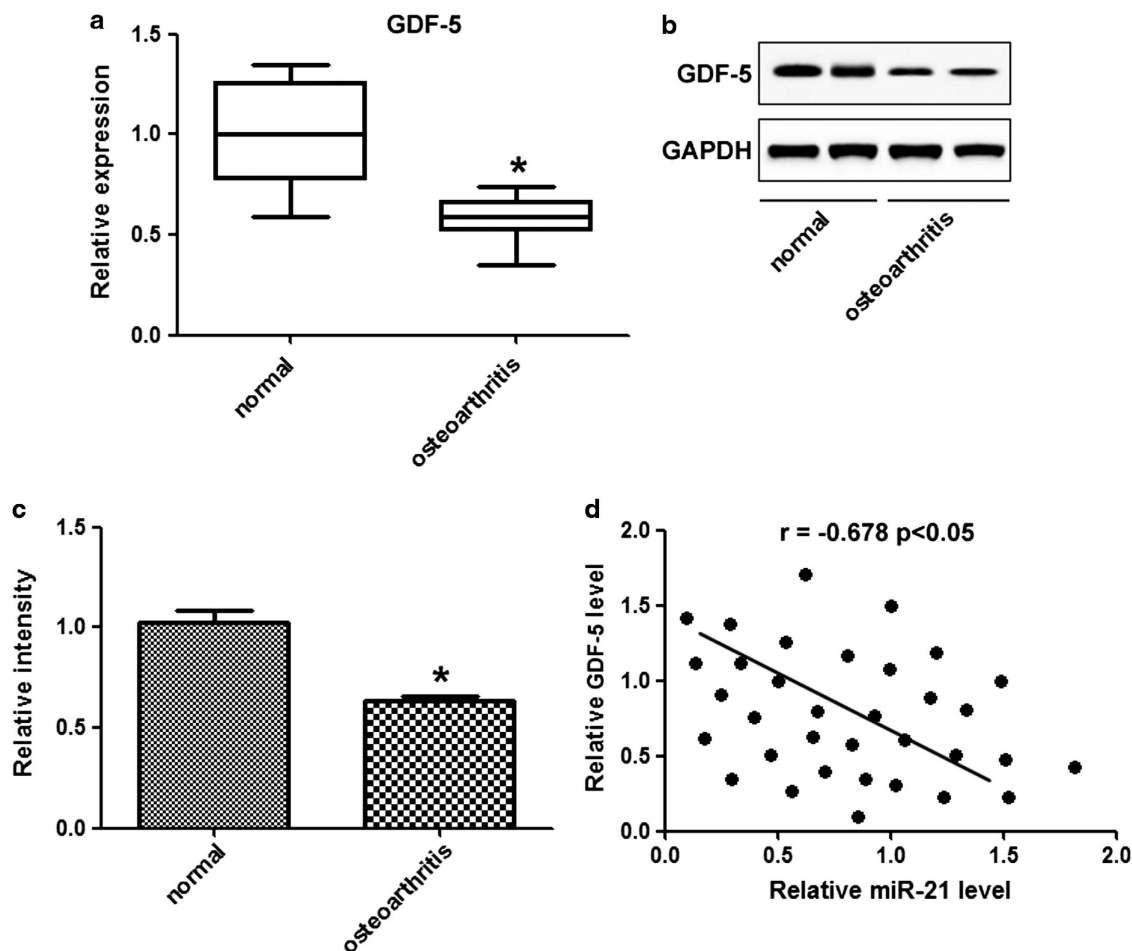


Figure 4 MiR-21 expression was inversely correlated with GDF-5 expression. (a) The level of GDF-5 mRNA in tissues from osteoarthritis patients was analyzed by qPCR. (b) The level of GDF-5 protein in tissues from osteoarthritis patients was analyzed by western blot. (c) Relative intensity of the western blot results. (d) The correlation between miR-21 expression and the GDF-5 protein level was analyzed. $n = 6$. * $P < 0.05$, ** $P < 0.01$ compared with control.

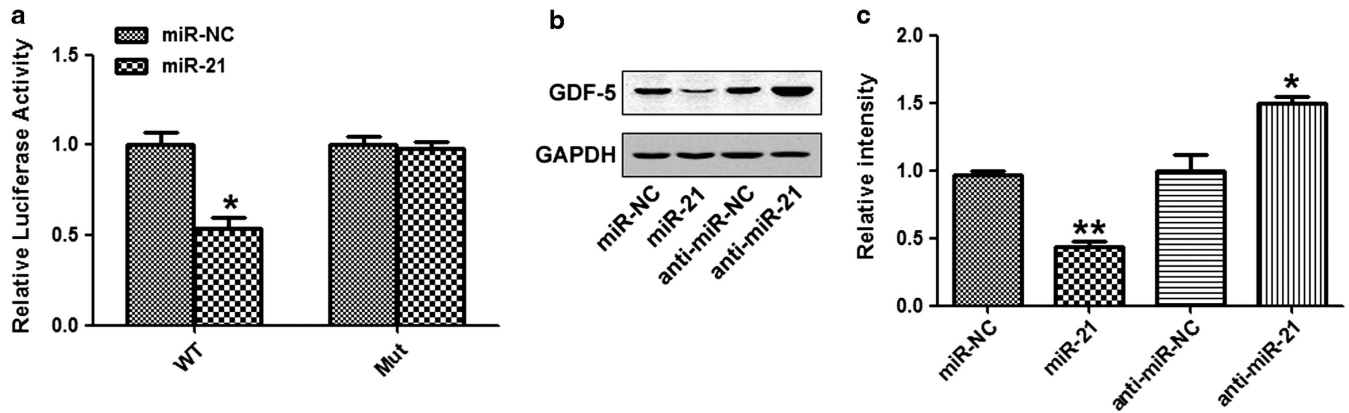


Figure 5 GDF-5 was a direct target of miR-21. (a) CH8 cells were co-transfected with an miR-21 precursor or negative control (miR-NC) plus a GDF-5 3'-UTR fragment containing either the miR-21 target sequence (WT) or a mutated sequence (Mut). (b) Expression of GDF-5 protein was detected by western blot in cells transfected with an miR-21 precursor or inhibitor or the corresponding controls. GAPDH was used as an internal control. (c) Relative intensity measurements provide a quantitative means of examining protein bands on the western blot. $n=6$, * $P<0.05$, ** $P<0.01$ compared with control.

expression level was inversely correlated with miR-21 in human osteoarthritis tissue. In addition, GDF-5, as a key regulator of the chondrogenesis process, has been reported to have an important role in cartilage formation. We next proposed a potential role for the miR-21-GDF-5 regulatory pathway in controlling the development of osteoarthritis.

In accordance with this hypothesis, we first validated the role of miR-21 in the pathogenesis of osteoarthritis. Transfection of a synthetic miR-21 precursor *in vitro* led to significant suppression of CH8 proliferation. The results of MTT and colony formation assays suggested that forced miR-21 overexpression inhibited the proliferation of CH8 cells, whereas downregulation of miR-21 expression increased CH8 proliferation. Similar effects were observed in the levels of chondrogenesis markers, including type-II-collagen, type-X-collagen, glycosaminoglycan and aggrecan. Because matrix-degrading enzymes and pro-inflammatory cytokines have a key role in the pathogenesis of osteoarthritis,^{21,22} we further evaluated the expression levels of genes encoding catabolic factors, including MMPs and pro-inflammatory cytokines. We found that the levels of MMP1, MMP2, MMP3 and MMP9 were significantly increased when cells were transfected with synthetic miR-21 precursor and correspondingly decreased in the miR-21 inhibitor group. Previous studies also showed that miR-21 could manipulate the levels of MMPs in other tissues, especially MMP2 and MMP9.^{23,24} However, the levels of pro-inflammatory cytokines were not changed, indicating that miR-21 does not exert its biological effects on osteoarthritis through pro-inflammatory cytokines. Our study suggests that miR-21 overexpression might promote osteoarthritis.

We further determined whether miR-21 acts as a promoter of osteoarthritis by targeting GDF-5, as predicted by bioinformatics analysis. As an important mediator in chondrogenesis, GDF-5 was downregulated in osteoarthritis patients, as mentioned above. A luciferase activity assay further suggested that GDF-5 is directly targeted by miR-21, which induces GDF-5

mRNA decay. GDF-5 expression was also inversely correlated with miR-21 in osteoarthritis patients. GDF-5 is closely related to the bone morphogenetic protein family and is a member of the TGF-beta superfamily. Overexpression of GDF-5 increases chondrocyte cell growth and differentiation in chondrogenesis, while downregulation of GDF-5 attenuates this process.²⁵⁻²⁷

To our knowledge, this study provides the first evidence of a role for miR-21 in osteoarthritis by directly targeting GDF-5. Further studies examining the functional and clinical implications of miR-21 and its target GDF-5 during osteoarthritis treatment are needed. Our results may provide important information for future studies exploring the beneficial effects of targeting miR-21 or GDF-5 as a biological means for the treatment of osteoarthritis.

CONCLUSION

MiR-21 promotes the pathogenesis of osteoarthritis by targeting GDF-5. MiR-21 can suppress GDF-5 expression, and its overexpression can attenuate the process of osteoarthritis. Our findings provide novel evidence that may be useful for future studies exploring therapeutic approaches for osteoarthritis by targeting miR-21.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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