

Original Paper

The Role of MicroRNA-381 in Chondrogenesis and Interleukin-1- β Induced Chondrocyte Responses

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Key Words

Osteoarthritis • Chondrogenesis • Cartilage degeneration • MiR-381 • Metalloproteinase-13

Abstract

Aim: The molecular pathways regulating cartilage degradation are unclear. miR-381 was identified as a putative regulator of chondrogenesis related genes. Here, we examined its role in chondrogenesis and osteoarthritic cartilage degeneration. **Methods:** miR-381 expression was assessed *in vitro* in response to IL-1 β stimulation in primary human (PHC) and mouse (PMC) chondrocytes, and ATDC5 derived chondrocytes; and *in vivo* in mouse embryos and human osteoarthritic cartilage. The effects of miR-381 on chondrogenesis and NF- κ B signaling were assessed using a synthetic RNA mimic or inhibitor and luciferase assay, respectively. Upstream regulators of miR381 were probed using siRNA or overexpression plasmids for Sox9 and Runx2. **Results:** miR-381 expression was elevated in chondrogenic and hypertrophic ATDC5 cells. miR-381 was induced *in vitro* by IL-1 β in ATDC5 cells, PMCs, and PHCs, and was expressed in areas of cartilage degradation or absorption *in vivo*. Overexpression of Runx2 or Sox9 increased miR-381 expression in ATDC5 cells. miR-381 suppressed expression of collagen, type II, alpha 1, and enhanced expression of metalloproteinase-13 (MMP-13), but did not regulate NFKBIA and NKRF activity. **Conclusion:** miR-381 was highly expressed during chondrogenesis and in arthritic cartilage. It may contribute to absorption of the cartilage matrix by repressing type II collagen and inducing MMP-13.

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Introduction

Osteoarthritis (OA) is characterized by degeneration of joint cartilage. The prevalence of radiographic OA, diagnosed based on the inter-bone distance in the affected joint, ranges C. Hou and F. Meng contributed equally to the present study.

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from 19-28% in the knee and 7-27% in the hip [1]. However, the frequency of symptomatic OA, defined by pain, aching, or stiffness is much less frequent. In general, symptomatic OA affects less than 10% of the population, although rates as high as 17% have been reported [1]. The risk factors for developing OA include increased age, female gender, race, and genetic predispositions [1]. The current treatments for OA can relieve symptoms like pain and tenderness, but cannot cure the disease or stop its progression. An incomplete understanding of the molecular mechanisms that regulate cartilage formation and degeneration has slowed the development of novel OA therapies.

The pathogenesis of OA is thought to have a major inflammatory component that drives cartilage degradation and inhibits cartilage formation. The proinflammatory cytokine, interleukin-1 β (IL-1 β), leads to the upregulation of matrix metalloproteinase (MMP) enzymes, which degrade the extracellular cartilage matrix (ECM) [2]. The ECM is secreted by chondrocytes and contributes to the mechanical function of cartilage. MMP-13 in particular has the capacity to degrade a wide range of matrix molecules, and is thought to be one of the major effectors induced by IL-1 β in OA [3, 4]. Type II collagen is a major component of the ECM, and an MMP-13 target, that is degraded in osteoarthritic cartilage [5-7]. The transcription factor SRY-related high mobility group-box-9 (Sox9) initiates type II collagen expression, and inhibits the expression and activity of Runx2, a key regulator of collagen degradation that upregulates MMP-13 [8, 9].

While chondrogenic differentiation is regulated by many different factors, including retinoic acid receptor signaling, microRNAs (miRNAs), small non-coding RNAs that post-transcriptionally regulate gene expression, have been implicated in regulating all stages of ossification [10-13]. For example, miR-140 regulates cartilage and bone formation [14, 15], and miR-365 increases chondrocyte proliferation and differentiation [16]. miRNAs have also been implicated in the development of arthritic cartilage [13, 17]. For example, IL-1 β significantly represses miR-558, which relieves its inhibition of Cyclooxygenase-2 (Cox2) and increases cartilage catabolism [18]. Another example is miR-194, which inhibits expression of Sox5 and thereby inhibits chondrogenic differentiation in adipose stem cells [19]. We were the first to describe an approximately 3-fold upregulation in miR-381 when human mesenchymal stem cells differentiate to chondrocytes [20]. We also identified chondrogenesis related genes that are putative targets of miR-381 including NFKBIA; CEBP β ; Runx2; Sox-2, 4, 5, 6, and 9; SMAD-3, 4, and 5; MAPK-1 and 9; BMPR2, and MMP7 [20]. To date, there is very little known about miR-381. It has been most thoroughly characterized in cancer models where it interacts with cell cycle genes. miR-381 has been reported to act as an "oncomir" in glioma progression by enhancing proliferation of glioma cells, and to suppress lung adenocarcinoma by inhibiting cell migration and invasion [21-23]. Given the role of miRNAs in regulating cartilage homeostasis, and the putative chondrogenesis targets of miR-381, we hypothesized that miR-381 might have a role in OA pathogenesis. Here we investigated the regulation and molecular mechanism of action for miR-381 in chondrogenesis and cartilage degeneration.

Materials and Methods

Primary chondrocyte isolation

This study adhered to the standards of the Ethics Committee on Human Experimentation at the First Affiliated Hospital of Sun Yat-Sen University, China and the Declaration of Helsinki (2000). After obtaining informed consent, primary human chondrocytes (PHCs) were isolated from three hip or knee joints during amputation surgery to treat osteosarcoma at the First Affiliated Hospital of Sun Yat-Sen University. Patients with osteoarthritis and local or systemic immunological disorders were excluded based on the clinical diagnosis. In a second patient set, degraded joint cartilage was collected from OA hips (n = 3) during total hip arthroplasty surgery. Cartilage was cut into pieces 1 mm in diameter, and digested with DMEM/F12 (Hyclone, Logan, UT, USA) supplemented with 2% penicillin and streptomycin (Gibco, Grand Island, NY,

USA), 5% FBS (Gibco, 12657), and 0.4% Pronase (Roche, Indianapolis, IN, USA) for 90 min. The tissue pieces were then transferred to a second digestion solution consisting of DMEM/12 (Hyclone) supplemented with 2% penicillin and streptomycin (Gibco), 5% FBS (Gibco), and 0.025% Collagenase P (Roche) for 7 h on a 37°C stir-plate. The chondrocytes were seeded in flasks with DMEM/F12 plus 5% FBS, 2% penicillin, and streptomycin.

Mice were cared for in accordance with guidelines of Animal Center of the First Affiliated Hospital of Sun Yat-Sen University. Primary mouse chondrocytes (PMCs) were isolated as described previously [24]. Briefly, newborn mice (5 days old) were washed with 70% ethanol and sacrificed under general anesthesia. The cartilage was obtained from the femoral head, femoral condyle, and tibial plateau and digested with 3 mg/mL collagenase D in complete culture medium (M199 (Gibco 11150-059) supplemented with 10% FBS and 1% penicillin and streptomycin) for 90 minutes at 37°C with agitation. Then the cartilage was digested again in collagenase D overnight at 37°C. The cells were dislodged from the cartilage with agitation, filtered through a 48 µm nylon mesh, and then seeded in a culture flask at a density of 8,000 cells/cm². Early-passage PMCs, defined as no more than four passages, were used in these experiments. For a second set of experiments, pregnant C57BL/6J mice were purchased from the Animal Center of the First Affiliated Hospital of Sun Yat-Sen University. Embryos were collected on E10.5 and E14.5 and used to detect miR-381 expression in areas undergoing active cartilage absorption in the early limb buds.

Cell culture

ATDC5 mouse cells (Riken Cell Bank; Ibaraki, Japan) were cultured in DMEM/F12 supplemented with 5% FBS, and 1% penicillin and streptomycin at 37°C in a humidified 5% CO₂ atmosphere. The culture medium was changed every 2 days. The cells were split when they reached confluence. All of the experiments were completed using cells passaged fewer than 20 times.

Inducing chondrogenesis in ATDC5, mouse, and human cells

Chondrogenesis was induced in ATDC5 cells for 14 days. The chondrogenic culture medium was changed every day. PMCs were grown in M199 medium (Gibco, 11150-059) supplemented with 10% FBS, 1% penicillin and streptomycin, bFGF (Peprotech, Rocky Hill, NJ, USA), EGF (Peprotech), and insulin (Sigma, St. Louis, MO, USA) at 37°C in a humidified 5% CO₂ atmosphere. PHCs were cultured in DMEM/F12 supplemented with 5% FBS, 1% penicillin and streptomycin, and ITS (insulin, transferrin, and selenous acid) + Premix (BD Biosciences, San Jose, CA, USA catalog: 354352) as previously described [25-29] at 37°C in a humidified 5% CO₂ atmosphere. Chondrogenic ATDC5 cells, PHCs, and PMCs were treated with recombinant IL-1β (Peprotech, 200-01B) using various doses and times [30-32]. Where indicated, the cells were transfected with an mmu-miR-381 mimic, a synthetic miRNA with the same sequence as mature miR-381 (micrON™ mmu-miR-381-3p mimic, miR10000746-1-5), an miR-381 inhibitor, synthetic miRNA that interferes with miR-381 (micrOFF™ mmu-miR-381-3p inhibitor, miR20000746-1-5), or negative control miRNA, synthetic miRNA with a nonsensical sequence (micrON™ miRNA mimic control, miR01201-1-5; micrOFF™ miRNA inhibitor control, miR02201-1-5; Ribobio, Guangzhou, China).

Alcian blue staining

Cultured cells were fixed in formalin for 4 hours at room temperature and stained with alcian blue 8GX (Cyagen) for 20 min at room temperature.

In situ hybridization

Tissue from mouse embryos at days E10.5 and E14.5 were harvested and processed with human joint cartilage obtained from OA hip patients. The tissues were fixed in DEPC treated with 4% paraformaldehyde at 4°C overnight and embedded in paraffin. 5 µm sections were incubated with 40 µg/mL Proteinase K (Promega, Madison, WI, USA catalog: V3021) at room temperature overnight. The following day, the sections were incubated with 5'-labeled RNA probe (Exiqon, 38188) at 56°C overnight. Endogenous alkaline phosphatase was blocked at room temperature for 1 h, followed by incubation with anti-Digoxigenin-AP (1:500, Roche, 11093274) at 4°C overnight. The nitroblue tetrazolium (NBT)/BCIP (Sigma, N1911) staining reaction was then performed, the sections were counterstained with safrinin O and dehydrated with ethanol. Negative control staining was performed using PBS.

RNA extraction, reverse transcription, and quantitative real-time reverse transcription-polymerase chain reaction (qRT-PCR)

Total RNA was extracted with miRNeasy Mini Kit (QIAGEN, USA) following the manufacture's instruction. The concentration and purity of the extracted RNA was analyzed using the Epoch Multi-Volume Spectrophotometer System (BIOTEK, USA). cDNA was generated using the PrimeScript® miRNA cDNA Synthesis Kit (Takara, Japan) following the manufacture's instruction. qRT-PCR was performed using SYBR® Premix Ex Taq™ II (Takara, Japan) and the BioRad IQ5 system according to the manufacturer's instruction. The specific primers are listed in Table 1. The reverse primer for miRNA-381 was the Uni-miR qPCR Primer (Takara, Japan, Cat: D352). The melting curve was used to monitor the quality of the pRT-PCR reactions. The fold change in mRNA expression was calculated using the $\Delta\Delta C_t$ method. All samples were measured in triplicate.

Transfection

ATDC5 cells were cultured in DMEM/F12 supplemented with 5% FBS until they reached 60% confluence. Lipofectamine® 2000 Transfection Reagent (Invitrogen, 11668) was used to transfect cells according to the manufacturer's instruction. After 6 hours, the transfected ATDC5 cells were differentiated to chondrocytes using medium containing ITS+ Premix.

Construction of eukaryotic expression plasmids

Eukaryotic expression GV230 plasmids were purchased from Genechem (Shanghai, China). The plasmids were constructed using standard DNA techniques. Runx2 was cloned using the following primers: 5' -TAC CGG ACT CAG ATC TCG AGA TGG CGT CAA ACA GCC TCT TC-3', 5' - GAT CCC GGG CCC GCG GTA CCG TAT ATG GCC GCC AAA CAG ACT C-3'. Sox9 was cloned using the primers: 5'-TCC GCT CGA GAT GAA TCT CCT GGA CCC CTT C-3', 5'-ATG GGG TAC CGT GGG TCT GGT GAG CTG TGT GTAG-3'. The amplified DNA was inserted into the *XhoI/KpnI* restriction sites of GV230. The final plasmids were verified by sequencing.

Target prediction, construction of luciferase reporter vector, and luciferase reporter assay.

The potential binding sites of miR-381 were predicted using miRanda, miRDB, and TargetScan software. The DNA sequences containing predicted seed sequences of NFKBIA and NKRF 3' -UTR were amplified by PCR using the primers: m-NFKBIA-3UTR-F: CCG ACT CGA GGT GGA AAG TGG CAA AAA G; m-NFKBIA-3UTR-R: ATT GCG GCC GCC TGT CTG TAA AAA TCT GTT; m-NKRF-F: CCG CCT CGA GGA TTT TAC TAA GTT GTC; and m-NKRF-R: ATT GCG GCC GCTT TTG ATC AAC TTT GG. The seed sequences were mutated using standard PCR techniques with the following primers: m-NFKBIA-mut-F:ATG TGG AGA ACA TAT TTT GTA CAA ATA GAG TT; m-NFKBIA-mut-R:GTA CAA AAT ATG TTC TCC ACA TTC TTT TTG C; m-NKRF-mut-F:CTG TCC AGA ACA TAA CTT AGC TTA ATC CAG CAG; and m-NKRF-mut-R: AAG CTA AGT TAT GTT CTG GAC AGA ATT CTC TAG.

Table 1. Primer sequences for qPCR

Gene	Primer sequences
mmu-U6	Forward CTCGCTTCGGCAGCACA
	Reverse AACGCTTCACGAATTTGCGT
mmu-GAPDH	Forward TGTGTCCGTCGTGGATCTGA
	Reverse TTGCTGTTGAAGTCGCAGGAG
mmu/hsa-mir-381	TATACAAGGGCAAGCTCTCTGT
mmu-col2a1	Forward CCCGCCTTCCCATTTATTGAC
	Reverse GGGAGGACGGTTGGGTATCA
mmu-Sox9	Forward GGGGGTGAGCTTTGATTAATTC
	Reverse GGGATTTAAGGCTCAAGGTGTTT
mmu-Col10a1	Forward TTCTGCTGCTAATGTTCTTGACC
	Reverse GGGATGAAGTATTGTGTCTTGGG
mmu-Runx2	Forward ATGCTTCATTGCGCTCACAAA
	Reverse GCACTCACTGACTCGGTTGG
mmu-mmp13	Forward ATGCATTCAGCTATCCTGGCCA
	Reverse AAGATTGCATTTCTCGGAGCCTG
mmu-TNF-alpha	Forward GACGTGGAAGTGGCAGAAGAG
	Reverse TTGGTGTTTGTGAGTGTGAG
hsa-mmp13	Forward TCCTGATGTGGGTGAATACAATG
	Reverse GCCATCGTGAAGTCTGGTAAAAAT

The amplified DNA sequences were inserted into pmiR-RB-REPORT™ Vector to generate wild type (WT) or mutated (MUT) NFKBIA/NKRF 3' -UTR luciferase vectors. To generate mutant NFKBIA and NKRF 3' -UTR vectors, we switched the predicted seed sequences CTTGTATA and CTTGTATT to GAACATAT and GAACATAA, respectively. All vectors were verified by sequencing.

For the reporter assay, 1.2×10^4 cells were cultured in a 96-well plates with 100 μ L of culture medium for 24 h. The mmu-miR-381 mimic (5 pmol) and vector (100 ng) were cotransfected into each well. The transfected cells were cultured in fresh culture medium for an additional 48 h. The luciferase reporter assay was carried out following the manufacturer's instructions (Dual-Glo® Luciferase Assay System, Promega). Luminescence was measured using the Veritas 9100-002 luminometer.

Statistical analysis

All of the experiments were performed at least three times. The results are expressed as the mean and standard deviation (mean \pm SD). Both parametric and non-parametric inferential statistics were utilized in this study depending on whether the data was normally distributed. The independent t-test and Mann-Whitney U test were used to compare between two groups as appropriate. For multiple group comparisons, the one-way ANOVA and Kruskal-Wallis tests were utilized. A *p* value less than 0.05 was considered statistically significant. All analyses were performed using IBM SPSS Version 20 (SPSS Statistics V20, IBM Corporation, Somers, New York).

Results

miR-381 was highly expressed in vitro during late stage chondrogenesis in ATDC5 cells and IL-1 β induced chondrocytes

We have previously shown that stem cells derived from human adipose tissue express miR-381 when they are induced to differentiate to chondrocytes *in vitro* [20]. Here, ATDC5 cells were induced to differentiate to chondrocytes *in vitro* using ITS + Premix. miR-381 was significantly upregulated in chondrogenic ATDC5 cells compared to control cells at 14 (3.07 ± 0.34 vs. 14.58 ± 1.7 ; $p < 0.001$), 21 (2.23 ± 0.23 vs. 8.50 ± 1.38 ; $p = 0.002$), and 28 (2.86 ± 0.26 vs. 240.60 ± 11.41 ; $p = 0.001$) days (Fig. 1A). Expression of miR-381 generally increased over time in the ATDC5 cells, although a slight decrease was observed at day 20 (Fig. 1A). Chondrocyte differentiation was verified through significantly increased expression of the chondrogenic markers collagen, type II, alpha 1 (Col2a1), Sox9, Collagen, type X, alpha 1 (Col10a1), and Runx2 (Fig. 1A). Sox9 and Col2a1 expression were noticeably increased at day 14 while Runx2 and Col10a1 peaked late at day 28 (Fig. 1A). Representative images of alcian blue staining in ITS + premix treated ATDC5 cells from 0-35 days are shown in Fig. 1B. Cartilage staining became stronger with prolonged chondrogenesis, indicating the accumulation of matrix, and peaked after 14 days.

To establish whether miR-381 expression was regulated by inflammation, we assessed the expression of miR-381 in IL-1 β treated chondrogenic ATDC5 cells and primary chondrocytes. In the PHCs, IL-1 β significantly increased the expression of miR-381 1.6 \pm 0.2-fold ($p < 0.001$; Fig. 2A). MMP-13 mRNA was undetectable in PHCs in the absence of IL-1 β after 40 cycles, but became detectable (0.00060 \pm 0.0001-fold; $p < 0.001$) after 24 h of stimulation with IL-1 β (data not shown). Similarly, in PMCs (Fig. 2B) and ATDC5 cells (Fig. 2C) miR-381 was significantly upregulated in response to IL-1 β after 24 h in culture by 1.44 \pm 0.42-fold ($p = 0.031$) and 14.28 \pm 0.93-fold ($p < 0.001$), respectively. Similar to the PHCs, in PMCs and ATDC5 cells MMP-13 was significantly upregulated by 7.76 \pm 2.20-fold ($p < 0.001$) and 16.74 \pm 3.55-fold ($p < 0.001$), respectively after 24 h. In the ATDC5 cells, MMP-13 expression remained elevated (19.06 \pm 2.23-fold; $p < 0.001$) up to 7 d in culture. IL-1 β treatment also increased the expression of TNF- α (31.5 \pm 5.62-fold; $p < 0.001$), another inflammatory cytokine, in PMCs at 24 h. In ATDC5 cells, Col2a1 expression was significantly reduced after 24 h with IL-1 β (0.81 \pm 0.08-fold; $p = 0.002$) and was further reduced at 7 d (0.30 \pm 0.08-fold; $p < 0.001$). Interestingly, across the *in vitro* models, expression of miR-381, MMP-13, and Col2a1 expression were altered in response to IL-1 β in a time dependent

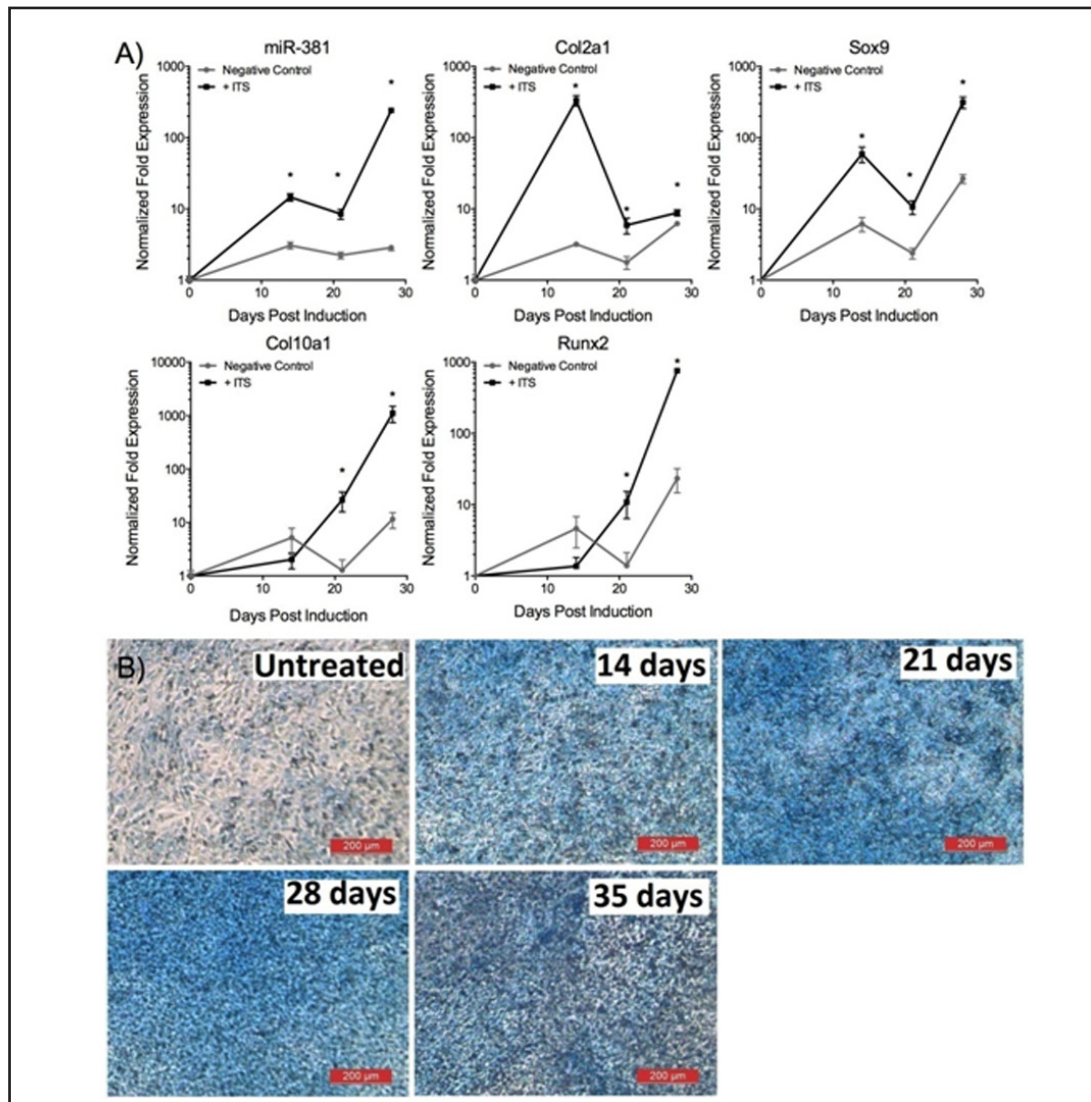


Fig. 1. Elevated expression of miR-381 during chondrogenesis. ATDC5 cells were induced to chondrogenesis by incubating with media containing insulin, transferrin, and selenous acid (ITS) + Premix for the indicated amount of time. (A) Gene expression and collagen deposition in treated cells was compared to untreated cells by qPCR. The expression levels of miR-381, chondrogenic markers (Col2a1 and Sox9) and hypertrophic markers (Col10a1 and Runx2) were measured. Untreated ATDC5 cells (F) or ATDC5 cells treated for 14 (G), 21 (H), 28 (I), or 35 days (J) were fixed with formalin and stained with alcian blue to detect collagen deposition. * $p < 0.05$. The data are representative of an average of 6 independent assays, each with 3 samples per group.

manner. Taken together, these results suggested that inflammation might contribute to regulating miR-381 mediated chondrogenesis.

miR-381 is expressed in in vivo models of late stage of chondrogenesis and arthritis

To confirm our *in vitro* findings in a more physiologically relevant *in vivo* model, we stained mouse embryos and arthritic cartilage from OA patients by *in situ* hybridization to determine whether miR-381 was present. The negative control and Safrinin O counterstain for each representative image is shown. miR-381 was observed in the early cartilage tissues of the embryonic mouse limb bud, especially in the areas of the toe webs that were being absorbed (Fig. 3A). miR-381 was also evident in areas of endochondral ossification in the toe

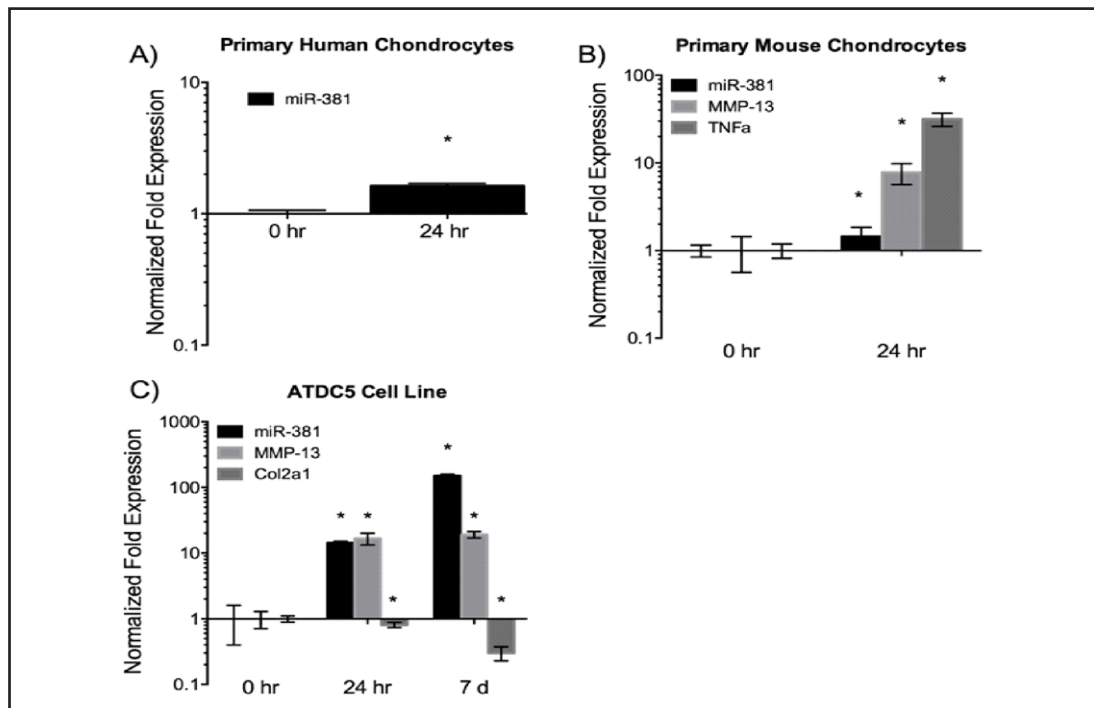
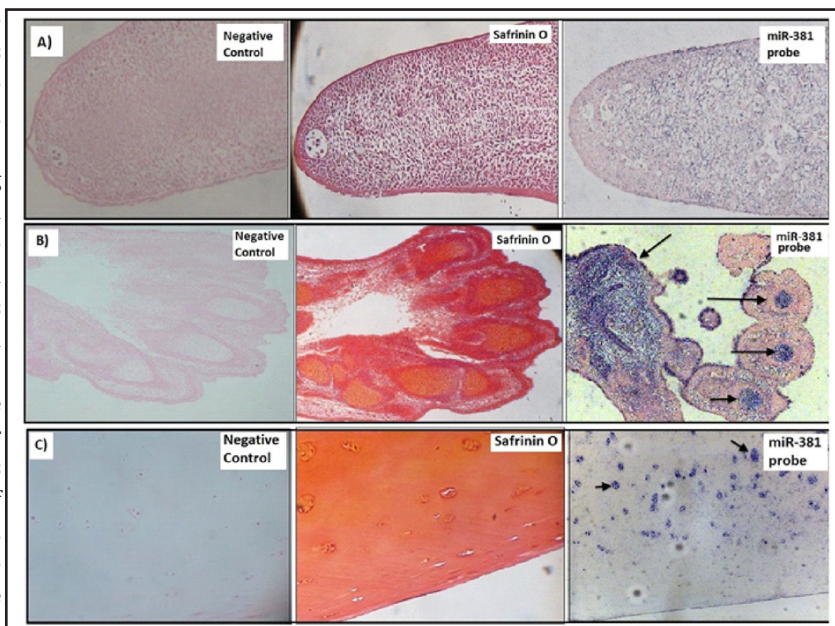


Fig. 2. miR-381 expression is elevated in chondrocytes following treatment with IL-1 β . Primary human chondrocytes (PHCs; A) and primary mouse chondrocytes (PMCs; B) were treated with 1 ng/mL of IL-1 β for 4 h. ATDC5 cells were induced to become chondrocytes insulin, transferrin, and selenous acid (ITS) + Premix for 14 days prior to treatment with 1 ng/mL IL-1 β for the indicated amount of time (C). The expression levels of miR-381, TNF- α , MMP-13, and Col2a1 expression were measured by qPCR and shown in fold induction relative to unstimulated cells. * $p < 0.05$. The data are representative of an average of 3 independent assays, each with 5 samples per group.

Fig. 3. miR-381 is evident in mouse embryos and cartilage from osteoarthritic patients. miR-381 expression *in vivo* was assessed using *in situ* hybridization with nitroblue tetrazolium (NBT)/BCIP-labeled probes (blue). Tissues were counterstained with nuclear Safrinin O (red). Representative staining is shown for miR-381 in limb buds (A) and claws (B) of E10.5 mouse embryos. Endochondral ossification is highlighted in the toe webs and metacarpal bones (black arrows) in (B). (C) miR-381 was present in chondrocytes isolated from osteoarthritic patients. The cartilage was cut and spread to provide a full depth view, and the bottom of the photo was the cartilage surface. Black arrows designate strongly stained chondrocytes in deeper layers of the cartilage.



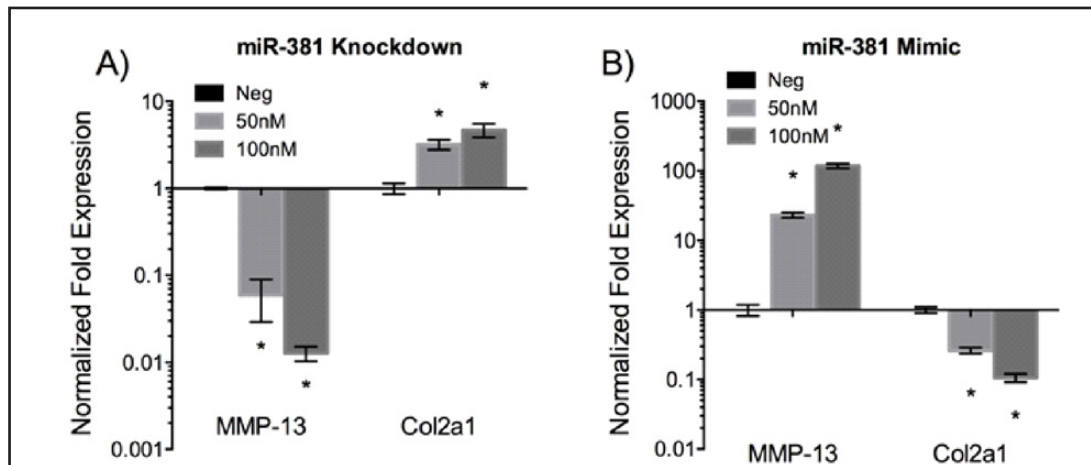


Fig. 4. Effect of miR-381 on Col2a1 and MMP-13. ATDC5 cells transfected with a synthetic RNA miR-381 inhibitor (A) or synthetic RNA mimic(B) were treated with insulin, transferrin, and selenous acid (ITS) + Premix to induce chondrogenesis. ATDC5 cells were treated with a non-sense synthetic RNA as the negative control. The expression levels of MMP-13 and Col2a1 were determined with qPCR. * $p < 0.05$. The data are representative of an average of 3 independent assays, each with 6 samples per group.

and metacarpal bones (Fig. 3B). In the OA patients, miR-381 was expressed in chondrocytes from joint cartilage (Fig. 3C). The anti-miR-381 probe stained chondrocytes in the deep layer of the articular cartilage, which are primarily hypertrophic chondrocytes [33-35]. These data suggested that miR-381 is involved in late stage chondrogenesis and endochondral ossification, and is present in osteoarthritic joints *in vivo*.

miR-381 repressed type II collagen expression and enhanced MMP-13 expression

To establish a mechanistic link between miR-381 and chondrogenesis and cartilage degradation, we either inhibited miR-381 or over expressed an miR-381 mimic in ATDC5 cells. The ATDC5 cells were induced to differentiate into chondrocytes and transfected with either the inhibitor for 7 days (Fig. 4A) or the mimic for 3 days (Fig. 4B). The expression of MMP-13 and Col2a1 was assessed by qRT-PCR and compared to negative control ATDC5 cells. The miR-381 mimic induced a dose dependent decrease in the expression of Col2a1 (50nM: 0.26 ± 0.06 -fold, $p = 0.004$; 100nM: 0.11 ± 0.04 -fold, $p = 0.003$), and a dose dependent increase in the expression of MMP-13 (50nM: 23.04 ± 4.37 -fold, $p = 0.019$; 100nM: 117.82 ± 19.43 -fold, $p < 0.001$). In contrast, the miR-381 inhibitor induced a dose dependent increase in Col2a1 expression (50nM: 3.19 ± 0.32 -fold, $p = 0.008$; 100nM: 4.69 ± 2.05 -fold, $p = 0.02$) and dose dependent decrease in MMP-13 expression (50nM: 0.06 ± 0.07 -fold, $p < 0.001$; 100nM: 0.01 ± 0.01 -fold, $p < 0.001$). Neither the miR-381 mimic nor the inhibitor affected expression of Sox9, Runx2, or Col10a1 (data not shown).

Runx2 and Sox9 upregulated expression of miR-381

Given the roles of Sox9 and Runx2 in regulating Col2a1 and MMP-13 expression, we next investigated whether Runx2 and Sox9 also regulated miR-381 expression during ATDC5 chondrogenesis. The expression levels of Runx2 and Sox9 were increased or decreased using targeted siRNA or plasmids that overexpressed Runx2 (Fig. 5A) or Sox9 (Fig. 5B). Targeted siRNA significantly reduced the expression of Runx2 (0.77 ± 0.13 -fold, $p < 0.05$) and significantly reduced the expression of miR-381 (0.17 ± 0.03 -fold, $p < 0.05$; Fig. 5A). However, transfecting a plasmid containing the Runx2 gene significantly increased expression of both Runx2 (1.95 ± 0.64 -fold, $p < 0.01$) and miR-381 (2.79 ± 0.75 -fold, $p < 0.05$). Similar results were observed for Sox9, although the reduction in miR-381 expression when Sox9 was knocked down was not significant (Fig. 5B). Thus, it is likely that both Runx2 and Sox9 contributed to regulating the expression of miR-381 *in vitro*.

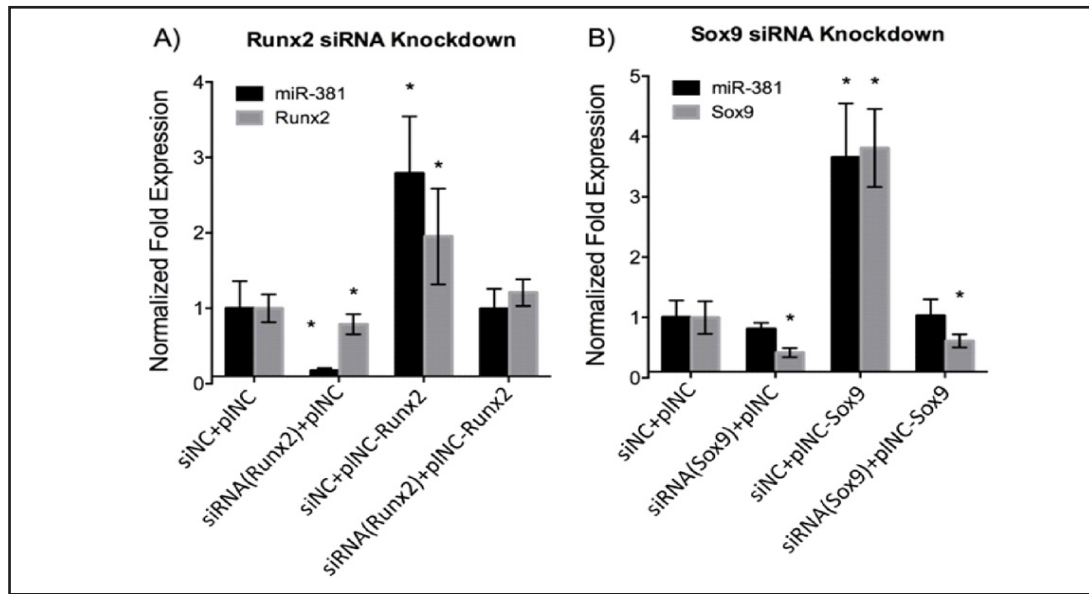


Fig. 5. Regulation of miR-381 by Runx2 and Sox9. ATDC5 cells were cotransfected with negative control siRNA (siNC), Runx2 (A) or Sox9 (B) siRNA and empty vector (GV230; plNC) or a GV230 plasmid containing Runx2 (A) or Sox9 (B). Chondrogenesis of ATDC5 was induced using insulin, transferrin, and selenous acid (ITS) + Premix and the expression levels of miR-381, Sox9, and Runx2 were measured with qPCR. * $p < 0.05$. The data are representative of an average of 4 independent assays, each with 6 samples per group.

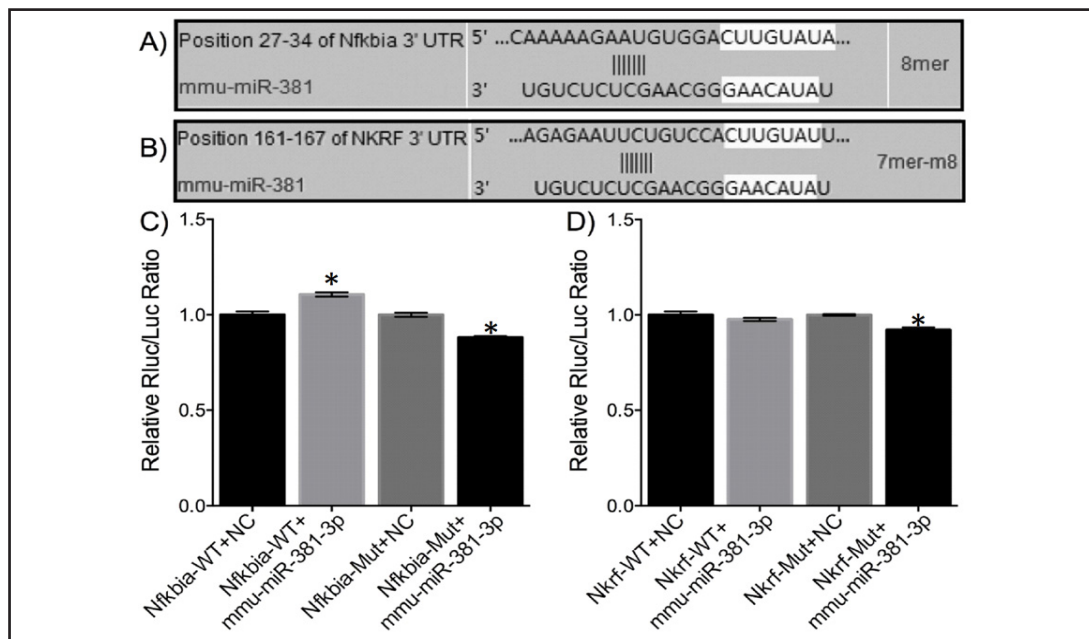


Fig. 6. NFKBIA and NKRF regulation by miR-381. (A) Wild type 3'-UTR and mutant of NFKBIA (Nfkbia-WT; Nfkbia-Mut) or (B) Wild-type and mutant 3'-UTR of NKRF (NKRF-WT; NKRF-Mut) were inserted into luciferase reporter vectors. The constructed vectors and miR-381 mimic (mmu-miR-381-3p) or non-sense negative control (NC) were cotransfected into cells. The fluorescence intensity was measured. * $p < 0.05$. The data are representative of an average of 4 independent assays, each with 3 samples per group.

miR-381 does not target NFKBIA or NKRF

NF- κ B has been reported to enhance MMP-13 expression and repress type II collagen expression [36, 37]. Based on our previous work [20], we hypothesized that miR-381 might

affect the expression of nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha (NFKBIA) and NFKB repressing factor (NKRF), which are NF- κ B inhibitors [38]. The putative miR-381 binding sites for NFKBIA (Fig. 6A) and NKRF (Fig. 6B) are shown. Luciferase reporter assays with the wildtype or mutant 3' UTR of NFKBIA and NKRF were performed in the presence or absence of the overexpressed miR-381 mimic. The mutant 3' UTR sequence prevented the binding of the miR-381 mimic. Transfection of the miR-381 mimic slightly increased transcription from the wildtype 3' UTR of NFKBIA (1.1 ± 0.02 -fold, $p = 0.006$), suggesting that NFKBIA is not a target of miR-381 mediated repression (Fig. 6C). Surprisingly, when the NFKBIA seed sequence was mutated, the miR-381 mimic slightly reduced luciferase expression from the mutant 3' UTR (0.88 ± 0.01 -fold, $p = 0.0008$), however this was not considered to be biologically relevant as the mutant seed sequence is not found *in vivo*. Similar results were observed from NKRF (Fig. 6D), indicating that neither NF- κ B inhibitors were likely to be targets for miR-381 mediated repression.

Discussion

We hypothesized that miR-381 might play a role in cartilage degeneration and chondrogenesis. To test this hypothesis, we used *in vitro* models (PHCs, PMCs, and ATDC5 cells) and examined miR-381 expression in two different *in vivo* models of cartilage degeneration/adsorption, cartilage from arthritic joints and from mouse limb buds during embryogenesis. We demonstrated that miR-381 was expressed in the *in vitro* systems during differentiation to chondrocytes and *in vivo* in areas of active cartilage degeneration. Furthermore, miR-381 expression is induced by IL-1 β , which is consistent with the inflammatory milieu commonly seen in OA [39], and regulated by Runx2 and Sox9. Finally, miR-381 increased the expression of MMP-13 and decreased Col2a1 expression. Together these data suggest that miR-381 plays an active role in regulating cartilage degeneration.

Kawai et al. proposed that chondrogenesis in ATDC5 cells progresses in stages based on the kinetics of TGF- β 2 and fibronectin expression [40]. He proposed that TGF- β 2 and fibronectin were active during the early (7-12 days) stages of chondrogenesis. In contrast, we observed that miR-381 expression was elevated particularly during late-stage (after day 28 in ATDC5 cells) of endochondral ossification. Sox9 and Runx2 play central roles in the early- and late-stages of endochondral ossification. Runx2 has also been reported to participate in cartilage degradation and ossification under inflammatory conditions [41]. Regarding the slight decrease in miR-381, Col2a1, and Sox9 expression levels at day 21, the decrease and subsequent rebound are consistent with the expression patterns of proteoglycan in previous studies [42, 43], and likely reflects an intrinsic characteristic of ATDC5 cells as they transition from the early to late stage of chondrogenesis. Our data suggested that the expression levels of Sox9 and Runx2 are positively correlated with transcription of miR-381, indicating they may regulate expression of miR-381. The mechanism of regulation remains to be fully elucidated and warrants further study.

MMP-13 is a key regulator of OA and collagen degradation that has been shown to participate in late-stage endochondral ossification [8, 44]. It is induced by CEBP β and Runx2, both putative targets of miR-381 [9], and constitutive activation of Runx2 enhances MMP-13 expression [45-48]. miR-381 increased the expression of MMP-13 in ATDC5 chondrocytes and was associated with reduced collagen deposition and Col2a1 expression. MMP-13 has been shown to be regulated by other miRNAs including miR-27a, miR-27b, miR-146, and miR-9 [49-52]. In the case of miR-27b, the regulation is direct through the interaction of the miRNA and the MMP-13 3' UTR [49]. However, miRNA regulation can also be indirect as in the case of miR-146, which regulated MMP-13 through Irak1 and Traf6 [50]. Our data suggest that miR-381 may be a signaling intermediate between Runx2 and MMP-13 during cartilage degradation. In the context of inflammation, as would be expected in OA patients, IL-1 β stimulation increased the expression of MMP-13 in PHCs, PMCs, and ATDC5 cells, and

concurrently upregulated miR-381 in all three systems. These data strongly suggest a role for miR-381 in OA pathogenesis via MMP-13 regulation.

The NF- κ B pathway has also been reported to enhance MMP-13 expression and repress type II collagen expression [36, 53]. Two key inhibitory components of the NF- κ B signaling pathway, NFKBIA and NKRF, were predicted to be targets of miR-381. However, neither NFKBIA nor NKRF were targeted by miR-381 in chondrogenesis. A comprehensive literature search did not identify any published studies that have used agomir/antagomir, or similar RNA-based, strategies in clinical trials or pre-clinical animal models to treat OA. *In vitro* work has suggested that other promising candidates for RNA-based OA therapy include miR-140, miR-27a/b, and miR-22 [30, 49, 51, 54]. However, MMP-13 inhibitors have already been developed to treat OA [55] and targeting miR-381 may be another viable therapeutic strategy to treat OA.

In this study, we are the first to characterize the role of miR-381 in chondrogenesis and cartilage degeneration. The mechanistic insights we were able to offer into miR-381 function in chondrocytes were limited, as the luciferase assay did not conclusively show that miR-381 bound to the 3' UTR of NFKBIA and NKRF. This leaves open the possibility that miR-381 acts on chondrocytes through other mechanisms. Overall, our data suggest that miR-381 is a viable therapeutic target for preventing cartilage degradation. Therapeutic strategies incorporating microRNA have been applied in several disease states, including cancer, diabetes, and cardiovascular disease [56-58]. miR-155 has also been targeted recently as a therapeutic in inflammatory diseases including rheumatoid arthritis [59]. The strategies fall broadly into two categories: (1) inhibitory anti-sense RNA and (2) exogenous miRNA like molecules [56]. In the case of miR-381, an inhibitory anti-sense RNA therapeutic could be a candidate for OA treatment.

Disclosure Statement

None of the authors have a potential conflict of interest or the appearance of a conflict of interest with regard to the work.

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