

Original Research Article

Evaluation of MiR-181a as a potential therapeutic target in osteoarthritis

Yuqiang Qian^{1,2}, Jiannong Jiang², Jun Peng², Qiang Wang² and Yixin Shen^{1*}

¹Department of Orthopedics, The Second Affiliated Hospital of Soochow University, Suzhou 215004, ²Department of Orthopedics, The Affiliated Yixing Hospital of Jiangsu University, Yixing 214200, China

*For correspondence: **Email:** shenyixin356@hotmail.com; **Tel:** 0086-0510-87921191; **Fax:** 0086-0510-87921191

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Abstract

Purpose: To investigate microRNA-181 (miR-181) as a potential therapeutic target in osteoarthritis (OA).

Methods: MiR-181 expression was evaluated in articular cartilage samples obtained from OA patients undergoing knee arthroplasty and non-OA (control) patients undergoing other orthopedic procedures. Following the isolation of total RNA, miRNA and mRNA expression was determined by real time-polymerase chain reaction (RT-PCR). Luciferase reporter assay and miRNA mimic or inhibitor were then used to establish the molecular target of miR-181 in chondrocytes.

Results: miR-181 family members, namely, miR-181a, miR-181c and miR-181d were significantly up-regulated in articular cartilage obtained from OA patients compared to non-OA control subjects. However, no significant difference in up-regulation of miR-181b expression. B-cell lymphoma 2 (BCL2), a putative target of the miR-181 family, was significantly down-regulated in OA patients compared to control subjects. Furthermore, luciferase reporter assay confirmed direct interaction between miR-181a and three prime untranslated region The 3'UTR of BCL2 in chondrocytes. Transfection of miR-181 mimic resulted in BCL2 suppression in chondrocytes. On the other hand, transfection of miR-181 inhibitor led to increased BCL2 expression and decreased interleukin 1-beta (IL1-β) induced apoptosis.

Conclusion: miR-181 is differentially expressed in articular cartilage of OA patients and leads to down-regulation of BCL2, a regulator of apoptosis. Therefore, miR-181 may be a potential therapeutic target in the treatment of OA.

Keywords: MicroRNA, Osteoarthritis, Apoptosis, B-cell lymphoma 2, Transfection, Chondrocytes

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INTRODUCTION

Osteoarthritis is an age-related chronic health condition [1], which affects over 20 % of the world population. It is associated with loss of mobility as well as impairment in major daily activities [2]. OA results in gradual degeneration of articular cartilage at joints thereby leading to chronic joint inflammation, followed by cartilage damage and pain. It manifests as joint stiffness and slowly develops over several years and is therefore difficult to detect in its early stages [3].

As a consequence, late-stage OA ultimately requires joint replacement to restore mobility. Therapeutic options for treatment of OA are primarily limited to management of symptoms, rather than targeting the underlying disease processes [4]. Therefore, there is an urgent need to identify therapeutic targets that are involved in the pathogenesis of OA that in turn would lead to the development of new therapeutics responsible for alteration of key biological processes underlying OA [5].

A class of non-coding RNAs, microRNAs (miRNAs), are 19-22 nucleotides in length and regulate gene expression at the post-transcriptional level [6]. MiRNAs are found in many cell types including chondrocytes. They are usually transcribed within the nucleus and then processed into mature miRNAs in the cytoplasm [7]. MiRNAs regulate various biological processes via interaction with the 3'UTR of target genes resulting in degradation of mRNA or suppression of translation [8]. MiRNA profiling studies have identified numerous candidates as therapeutic targets which are differentially expressed in OA cartilage [9–12]. However, there have been discrepancies between studies thereby prompting further experimental validation [12].

As an example, miR-181 family comprises of four members namely, miR-181a, miR-181b, miR-181c and miR-181d. They are encoded by three transcripts located at separate genomic loci [13]. However, the aforementioned members share target seed sequence similarity within the main 5-p precursor arm, which binds to target mRNAs. miR-181 family members are reported to be differentially expressed in various cancers namely breast [14], ovarian [15] and hepatic [16]. miR-181 has also been shown to target the anti-apoptotic regulator, BCL2, in hepatocytes [16]. Additionally, a recent study reported that the ratio of BCL2 to BCL2-associated X protein (BAX) expression was found to be lower in OA cartilage compared to normal articular cartilage, which suggests increased apoptosis in late stage OA [17]. However, the role of miR-181 in apoptosis during OA still remains to be elucidated. Therefore, this study aims to establish the expression pattern of miR-181 in OA cartilage as well as its role in apoptosis during OA.

EXPERIMENTAL

Human articular cartilage sample collection

Articular cartilage samples were obtained from OA patients undergoing knee arthroplasty (n = 20, age: 64 ± 5 years). Non-OA cartilage was obtained from patients (n = 10, age: 60 ± 6 years) undergoing other orthopedic knee procedures. Following collection, the samples were snap frozen in liquid nitrogen (-196 °C) and stored prior to further analysis. Written informed consent was taken from all subjects involved in this study. The study was approved by the institutional human ethics committee (approval ref no. NTC-03021-CS) and the knee procedure was conducted in compliance with the Declaration of Helsinki 1964 [18].

RNA isolation and RT-qPCR

Total RNA was isolated using TRIzol (Invitrogen) following the manufacturer's instructions and complementary DNA (cDNA) was synthesized from 500 ng of total RNA using high-capacity RNA-to-cDNA kit (Applied Biosystems). BCL 2 expression was determined using real-time PCR (CFX96, Biorad) with GAPDH as the housekeeping gene. Following are the sequence of primers used: BCL2: Forward Primer: 5'-GATTGTGGCCTTCTTTGAG-3', Reverse Primer: 5'-CAAAGTGGAGCAGAGTCTTC-3' and GAPDH, Forward Primer: 5'-AATCCCATCACC ATCTTCCA-3' and Reverse Primer: 5'-TGGACTCCACGACGTA CTCA-3'. Likewise, miRNA reverse transcription kit (Applied Biosystems) was used to synthesize cDNA from 500 ng total RNA using a specific stem-loop primer. Following this, real-time PCR was performed using stem-loop specific TaqMan assays (Applied Biosystems) for the detection of the following members of the miR-181 family:

- miR-181a
(ACAUCUACAACGCUGUCGGUGAGU)
- miR-181b
(ACAUCUACUUGCUGUCGGUGGG)
- miR-181c
(ACAUCUACAACCUGUCGGUGAGU)
- miR-181d
(ACAUCUACUUGUUGCUGGGUGUU).

Small nuclear RNA U6 was used as an internal reference/housekeeping gene. $2^{-\Delta\Delta CT}$ method was used to determine the fold change in miRNA and BCL2 gene expression between OA and non-OA samples.

Luciferase reporter assay

The putative miR-181 family binding site at position 2896-2903 in the 3'UTR of BCL2 (TargetScan 7.1, www.targetscan.org) was cloned and inserted into the pMIR-report vector system (Ambion, USA) using the following primers, 5'-GCTGTCCTTCAGGGTCTTCC-3' and 5'-CGATGGCCATAGACCCTGTC-3'. Site-directed mutagenesis was then used to create a mutation in the putative miR-181 binding site and the amplified PCR products were finally confirmed by DNA sequencing. Human CHON-001 cells (ATCC, CRL-2846) were then transfected with pMIR-report-BCL2 3'UTR vector (wild or mutant-type). These cells were maintained in DMEM supplemented with 10 % heat-inactivated fetal bovine serum (FBS) and

0.1 mg/mL G418. Chondrocytes transfected with Renilla luciferase reporter acted as an internal control. Following the incubation period (24 h), dual luciferase reporter assay system (Promega, USA) was used to measure the luciferase activity in cell lysates. The assay was conducted in triplicates and results were determined as relative luciferase activity (firefly Luc/Renilla Luc).

MTT assay

Apoptosis was assessed using the 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay. Briefly, CHON-001 cells were plated at a density of 50,000 cells per well. Cells were then treated with 10 ng/mL IL1- β for 24 h to induce apoptosis followed by treatment with miR-181a inhibitor or Phosphate buffered saline (PBS). PBS only was used as a treatment control. After 24 h, cells were washed with PBS followed by incubation with 10 μ L MTT dye for 2 h. This was followed by dye removal and addition of 100 μ L detergent reagent to the well plates. Absorbance was recorded at 570 nm using a microplate reader.

Statistical analysis

In the present study, all data are presented as mean \pm standard error. Statistical analysis was determined by t-test using GraphPad Prism 4.0. Cell culture experiments were based on $n = 4$ and $p < 0.05$ was considered to be statistically significant.

RESULTS

Differential expression of miR-181 and BCL2 in OA cartilage

Figure 1A-C demonstrates a significant up regulation of miR-181a and miR-181c in OA compared to non-OA cartilage. More specifically, miR-181a and miR-181c showed a 2-fold ($p < 0.001$) and 1.6-fold ($p < 0.001$) increase in OA compared to non-OA cartilage respectively. Further, expression of miR-181d increased by 1.5-fold ($p < 0.05$) in OA cartilage compared to control samples (Figure 1D). Although there was an increased expression of miR-181b in OA samples, the increase was not significant (Figure 1B).

Additionally, BCL2 expression in OA and non-OA cartilage was studied since it is a putative target of miR-181 family as identified by the TargetScan algorithm. Results indicated a significant down regulation of BCL2 in OA cartilage compared to non-OA cartilage ($p < 0.05$; Figure 2A).

MiR-181a binds to 3'UTR of BCL2

In order to determine whether miR-181a can bind to and regulate BCL2 expression, luciferase reporter assays were performed in a human chondrocyte cell line, CHON-001. Relative luciferase activity (firefly Luc/Renilla Luc) of the wild type pMIR-3'UTR-BCL2 reporter vector decreased in chondrocytes that were transfected with miR-181a mimic, when compared to chondrocytes transfected with mutant pMIR-3'UTR-BCL2 report vector or an empty pMIR-report vector, containing a mutated miR-181a binding site (Figure 2B) thereby indicating potential binding of miR-181a mimic to the 3'UTR of BCL2 in chondrocytes.

MiR-181a modulates BCL2 expression in chondrocytes

In order to establish the role of miR-181a in regulation of BCL2 expression at the post-transcriptional level, chondrocytes were transfected with miR-181a mimic or miR-181a inhibitor. It was observed that transfection with miR-181a mimic led to down regulation of BCL2 expression (Figure 3). In contrast, transfection of miR-181a inhibitor resulted in significant upregulation of BCL2 expression compared to transfection of miR-181a mimic ($p < 0.001$) or scrambled miRNA control ($p < 0.001$, Figure 3).

MiR-181a inhibition suppresses IL1-B induced apoptosis in chondrocytes

To determine the role of miR-181a in apoptosis, chondrocytes were incubated with IL1- β and miR-181a inhibitor or PBS. The results indicated that IL1- β treatment led to significant apoptosis in chondrocytes after 24 h and 48 h compared to PBS treated controls ($p < 0.01$, Figure 4). However, concomitant treatment with miR-181a inhibitor caused only a modest reduction in IL1- β induced apoptosis after 24 h compared to PBS treated controls, but a significant reduction in apoptosis after 48 h ($p < 0.01$, Figure 4).

DISCUSSION

In the present study, it was found that miR-181 family members are differentially expressed in OA cartilage. MiR-181 family members are predicted to target several genes. Here, it was demonstrated that miR-181a regulates BCL2 at the post-transcriptional level following miR-181a binding to the 3'UTR of BCL2 in chondrocytes. Furthermore, it was shown that miR-181a mimic augmented apoptosis via down regulation of BCL2;

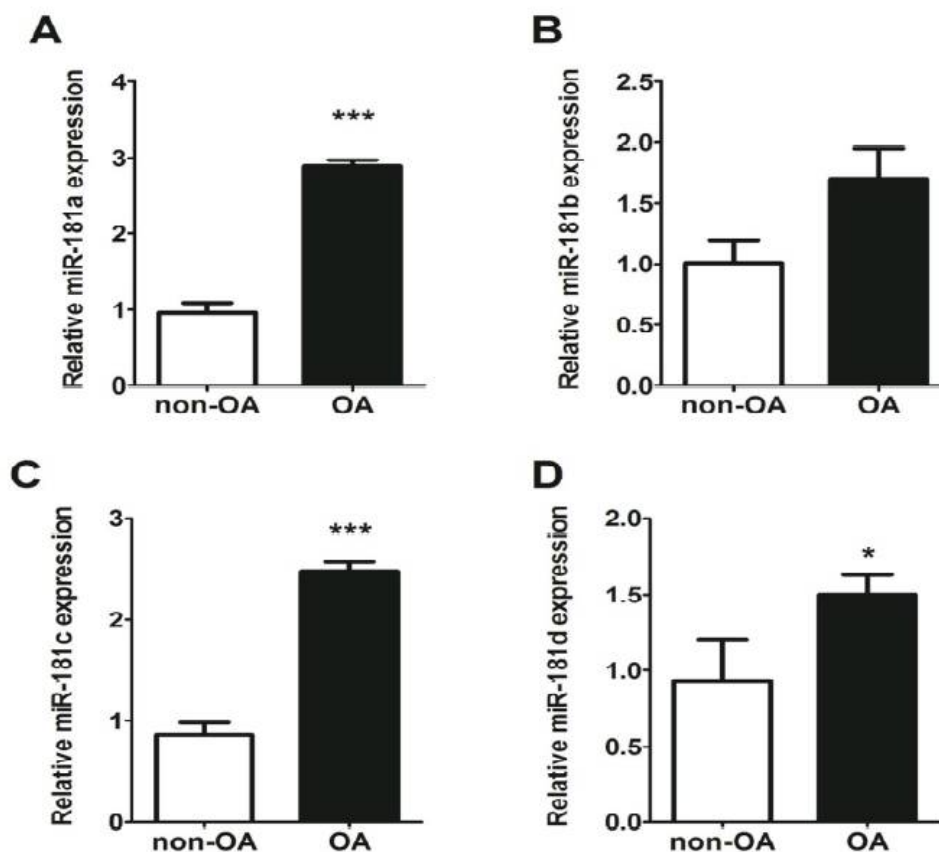


Figure 1: Differential expression of (A) miR-181a, (B) miR-181b, (C) miR-181c, (D) miR-181d in OA and non-OA articular cartilage measured by Real time polymerase chain reaction (RT-PCR). Data shown as mean \pm SE; *** p <0.001 indicates significance in miR-181a and miR-181c expression levels between OA and non-OA articular cartilage; * p < 0.05 indicates significance in miR-181d expression levels between OA and non-OA articular cartilage

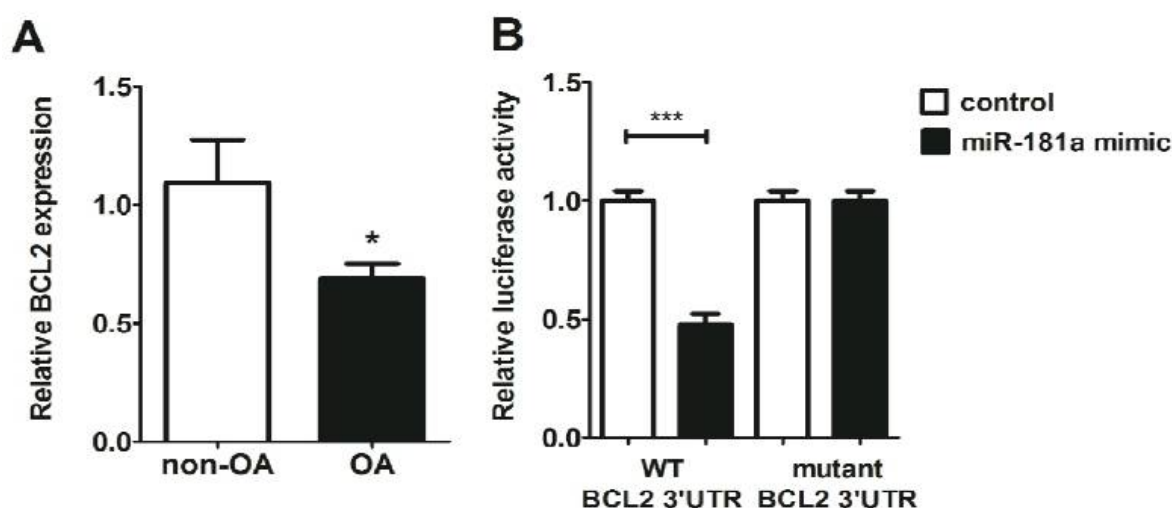


Figure 2: (A) Differential BCL2 expression in OA and non-OA cartilage measured by RT-PCR. (B) miR-181a binds to the 3'UTR of BCL2. Human CHON-001 cells transfected with a wild-type pMIR-report vector containing the putative miR-181 family-binding site at position 2896-2903 in the 3'UTR of BCL2 or a mutant site, and a miR-181a mimic or scrambled control. Data are expressed as relative luciferase activity (Firefly Luc/Renilla Luc). Data are represented as mean \pm SE; * p <0.05 indicates significance in relative BCL2 expression levels between OA and non-OA cartilage; *** p < 0.001 indicates significance in relative luciferase activity between wild-type pMIR-report vector laden chondrocytes transfected with miR-181a mimic or scrambled control

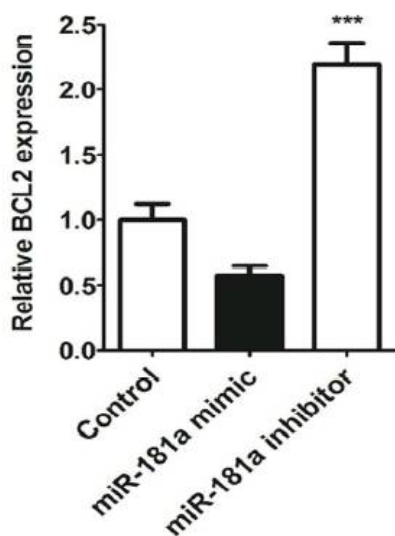


Figure 3: Transfection of miR-181a mimic or miR-181 inhibitor differentially modulates BCL2 expression in human CHON-001 cells. BCL2 expression was determined by RT-PCR and normalized to the housekeeping gene, glyceraldehyde 3-phosphate dehydrogenase (GAPDH). Data are shown as mean \pm SE; *** p < 0.001 indicates significance between chondrocytes treated with miR-181a inhibitor and miR-181a mimic or untreated control.

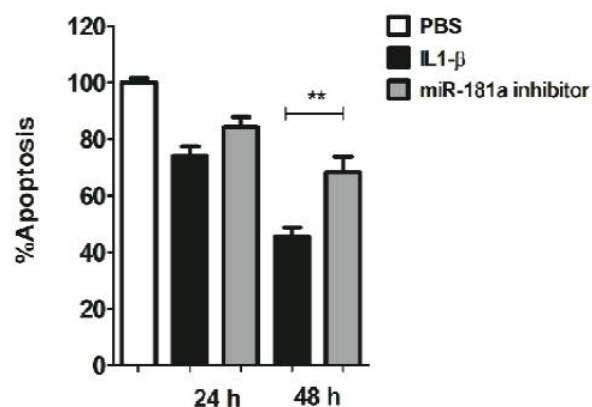


Figure 4: miR-181a inhibition suppresses interleukin 1-beta (IL1- β) induced apoptosis in human CHON-001 cells as determined by MTT assay. Cells were incubated in 10 ng/mL IL1- β for 24 h and treated with miR-181a inhibitor or PBS (negative control). Data are shown as mean \pm SE; ** p < 0.01 indicates significance in % apoptosis between IL1- β + PBS treated cells and IL1- β + miR-181a inhibitor treated cells.

however, miR-181a inhibitor attenuated IL1- β induced apoptosis in chondrocytes. Therefore, miR-181a demonstrates potential as a therapeutic target for the treatment of OA.

In the first set of experiments, differential expression of miR-181 family members in OA cartilage was determined. Of the various members, a prominent increase in miR-181a in

OA subjects was observed. While there are several hundred-gene targets that harbor putative miR-181a binding sites within their 3'UTR region, only few have been experimentally validated. We identified BCL2 as the target gene of interest since it is a negative regulator of apoptosis [17], a key process involved in the degeneration of cartilage during the pathogenesis of OA [19]. It was found that BCL2 expression was lower in OA cartilage as compared to normal cartilage, which is consistent with post-transcriptional suppression by miR-181a. Luciferase reporter assays further confirmed the potential binding of miR-181a to the 3'UTR of BCL2 in chondrocytes. This is in agreement with previous studies wherein miR-181a binding to the 3'UTR of BCL2 was demonstrated in hepatocytes [14].

MiR-181 family members are transcribed as precursor miRNAs from three different transcripts on three different chromosomes before being processed into four functional mature miRNA sequences [13].

Previous studies have suggested that miR-181 is transcriptionally regulated by transforming growth factor beta (TGF- β) signaling pathway [20] and that TGF- β signaling plays a role in the pathogenesis of OA [21]. A member of transforming growth factor β cytokine superfamily, TGF- β regulates the expression and secretion of various extracellular matrix components, including procollagen genes and cross-linking enzymes such as PLOD2, LOX, COL1A1, COL5A1, and TIMP1 [22]. Additionally, TGF- β primarily acts via activation of Smad, a signal transducer protein which translocates to the nucleus and binds to Smad responsive promoter regions, thereby resulting in transcriptional modulation of target miRNAs and mRNAs [25]. Importantly, TGF- β has also been shown to cause up regulation of miR-181a in hepatocytes [16] and breast cancer cells [14]. Furthermore, TGF- β levels are reported to be elevated in the synovial fluid of OA patients [23]. In addition, animal OA models have demonstrated that inhibition of TGF- β signaling attenuates the development of OA [24]. Hence, it will be important to establish how miR-181 transcription is regulated in cartilage in order to aid therapeutic targeting strategies in OA. However, further studies are necessary to confirm that differences in miR-181 levels observed in OA cartilage are driven partly by changes in TGF- β as demonstrated in earlier studies. Interestingly, recent studies have shown intra-articular injection of miRNAs as a promising route to deliver therapeutic miRNA mimic or inhibitor to the synovial cavity.

Several past studies have used miRNA arrays to identify differentially expressed miRNAs between OA and normal cartilage. Multiple candidate miRNAs have been identified in miRNA profiling studies, however, there is poor agreement between various studies. Discrepancies between studies may be attributed to multiple factors including differences in patient characteristics, stage of OA, concomitant medications, tissue sample preparations or miRNA profiling platform. Of the various miRNAs, seven were found to be differentially regulated in chondrocytes from OA or normal cartilage taken during autopsy. They include miR-483-5p (up regulated in OA chondrocytes) and miR-149*, miR-582-3p, miR-1227, miR-634, miR-576-5p and miR-641 (up regulated in healthy chondrocytes) [26]. To the best of our knowledge, this is the first study to demonstrate the role of miR-181a as a therapeutic target for modulation of apoptosis in OA. Past reviews have also highlighted miR-145 and miR-146a as candidate therapeutic targets for OA [12]. However, given the importance of miRNAs in multiple biological and disease processes, more putative candidate OA-associated miRNAs need to be identified in future studies.

CONCLUSION

MiR-181 represents a potential therapeutic target for the prevention or management of OA via modulation of BCL2 and apoptosis. In future studies, it will be important to establish whether therapeutic targeting of miR-181a can indeed decrease the rate of OA progression *in vivo*. Hence, further experimental validation of miRNA targets is essential to establish the role of miRNAs in OA development or progression.

DECLARATIONS

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Conflict of Interest

No conflict of interest associated with this work.

Contribution of Authors

The authors declare that this work was done by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by them.

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