MicroRNA-23a-3p promotes the development of osteoarthritis by directly targeting SMAD3 in chondrocytes

Liang Kang 1, Cao Yang 1, Yu Song, Wei Liu, Kun Wang, Shuai Li, Yukun Zhang*

Department of Orthopaedics, Union Hospital, Tongji Medical College, Huazhong University of Science and Technology, Wuhan, 430022, China

ABSTRACT

Osteoarthritis (OA) is a common chronic degenerative joint disease. Progressive destruction of the integrity of articular cartilage is an important pathological feature, but treatment options that reverse this damage have not been developed. According to recent studies, microRNAs have important regulatory roles in the initiation and progression of OA. In the current study, the biological effects of miR-23a-3p and its expression in OA tissues were examined. We found that miR-23a-3p expression was obviously higher and SMAD3 expression was significantly lower in OA cartilage than in normal tissues. The hypomethylation status of CpG islands in the promoter region of miR-23a-3p was confirmed by methylation-specific polymerase chain reaction in OA cartilage tissues. Furthermore, a bioinformatics analysis and luciferase reporter assay identified SMAD3 as a target gene of miR-23a-3p and SMAD3 expression at both the protein and mRNA levels was inhibited by miR-23a-3p. A functional analysis demonstrated that miR-23a-3p overexpression suppresses type II collagen and aggrecan expression, while miR-23a-3p inhibition had the opposite effects. Small interfering RNA-mediated knockdown of SMAD3 reversed the effects of the miR-23a-3p inhibitor on the expression of type II collagen and aggrecan. Our results suggested that miR-23a-3p contributes to OA progression by directly targeting SMAD3, providing a potential therapeutic target for OA treatment.

© 2016 Elsevier Inc. All rights reserved.

1. Introduction

Osteoarthritis (OA), the most common type of joint disease, is a major cause of pain and disability, and represents a heavy economic burden [1]. Multiple factors are related to the occurrence of OA, including aging, abnormal mechanical loading, trauma, and genetic predisposition [2,3]. OA is characterized by the continuous degradation of articular cartilage, which is mainly attributed to an imbalance between articular chondrocyte extracellular matrix (ECM) synthesis and degradation [4,5]. The degradation of type II collagen and aggrecan, the main components of the ECM, is an important manifestation of OA [6,7]. Current treatments for OA are limited to pain management without reversing the loss of articular cartilage and joint replacement surgery in end-stage OA patients [8]. Therefore, additional studies of the pathogenesis of OA are necessary to develop effective therapeutic strategies.

MicroRNAs (miRNAs) are short (17–25 nucleotides) non-coding RNA molecules. They can silence gene expression by binding to 3′-untranslated region (3′-UTR) sequences in their target messenger RNAs (mRNAs), resulting in the inhibition of translation or mRNA degradation. They play essential roles in diverse cellular processes, such as proliferation, apoptosis, and differentiation [9–11], and have important regulatory roles in OA progression. Differential expression of various miRNAs has been observed between OA and healthy cartilage [12]. For example, overexpression of miR-634 controls the survival and matrix synthesis of human chondrocytes [13], and miR-21 is upregulated in OA chondrocytes and promotes the pathogenesis of OA by targeting GDF-5, the key regulator of chondrogenesis [14].

The miRNA, miR-23a-3p, is broadly involved in cellular processes, including invasion, proliferation, senescence, and ECM synthesis [15–17]. Aberrant miR-23a-3p expression has been detected in many pathological conditions [18,19]. However, miR-23a-3p expression in OA cartilage and its role in disease progression are unclear. SMAD3 mediates transforming growth factor-β (TGF-β) signaling, which is critical in the development,
homeostasis, and repair of cartilage [20,21]. We have shown that SMAD3 expression is remarkably lower in OA cartilage than in normal cartilage [22]. In addition, SMAD3 polymorphisms are associated with the risk of both hip and knee OA [23]. Considering the important roles of miRNAs and SMAD3 in the pathogenesis of OA and the putative SMAD3 binding site for miR-23a-3p, we examined whether miR-23a-3p plays a role in OA progression via SMAD3. Additionally, we examined whether methylation, which is involved in various diseases [24] and affects many miRNA promoters [25–27], is involved in the association between miR-23a-3p and OA.

We discovered that miR-23a-3p expression is significantly upregulated in OA cartilage compared to normal cartilage. Additionally, we found that aberrant miR-23a-3p expression is partially controlled by the hypomethylation of its promoter region in OA cartilage. Moreover, we validated that miR-23a-3p overexpression inhibits chondrocyte ECM synthesis by directly targeting SMAD3. Based on these results, miR-23a-3p is a promising target for OA prevention and treatment.

2. Materials and methods

2.1. Patient samples and cell lines

Human cartilage samples were collected from 10 OA patients (six females and four males; age range 68–76 years) undergoing total knee replacement surgery and from 10 traumatic amputees without rheumatoid arthritis or OA (three females and seven males; age range 43–70 years). OA patients were diagnosed according to the American College of Rheumatology criteria. All samples were collected from the Department of Orthopaedics, Union Hospital. Informed consent was obtained from all patients and the study was approved by the Ethics Committee of Tongji Medical College, Huazhong University of Science and Technology. Chondrocytes were isolated from articular cartilage tissues as described previously [28] and cultured in Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum. Human chondrosarcoma cells (SW1353) have a similar phenotype to chondrocytes [22,29] and thus were used as substitute for cartilage cells to detect the regulatory effects of miR-23a-3p. SW1353 cells were obtained from the Chinese Academy of Sciences (Shanghai, China). SW1353 cells were cultured in Dulbecco’s modified Eagle’s medium with 10% fetal bovine serum, 100 IU/ml penicillin, and 100 µg/ml streptomycin (Invitrogen, Carlsbad, CA, USA) at 37 °C in a humidified 5% CO2 atmosphere. The medium was changed every 2 days.

2.2. RNA extraction and quantitative real time-PCR (qRT-PCR)

Total RNA from cells and cartilage tissues was isolated with TRIzol reagent (Invitrogen) according to the manufacturer’s instructions. A qRT-PCR assay was conducted to quantify SMAD3, type II collagen, aggrecan, and miR-23a-3p using the 7500 Real-time PCR System (Applied Biosystems, Foster City, CA, USA) with the cycling conditions recommended by the manufacturer. The stem-loop primers for miR-23a-3p were purchased from Ribobio (Guangzhou, China) and the other primers used for qRT-PCR are listed in Table 1. All experiments were performed in triplicate. The expression of SMAD3, type II collagen, and aggrecan were normalized to β-actin, and miR-23a-3p levels were normalized to U6. Relative expression levels were determined using the 2−ΔΔCt method.

2.3. Methylation-specific PCR

A methylation-specific PCR (MSP) analysis was performed to detect the methylation status of the miR-23a-3p promoter in OA and normal cartilage. Genomic DNA was extracted using the DNA Extraction Kit (Promega, Madison, WI, USA) and modified with sodium bisulfite according to the manufacturer’s instructions. The primer sequences used for MSP were as follows: methylated, forward 5ʹ-AGTGGTATATGGTAATTTG-3ʹ and reverse 5ʹ-GGCACAACTAACTAATAACGTA-3ʹ; unmethylated, forward 5ʹ-TTTGTTAATGTTGAATTTG-3ʹ and reverse 5ʹ-CACCCACC-TAAATACATAACA-3ʹ. The PCR conditions were as follows: 5 min at 95 °C, followed by 30 cycles of 95 °C for 35 s, 50 °C for 40 s, and 72 °C for 35 s, and a final extension at 72 °C for 10 min. The MSP products were electrophoresed on a 3% agarose gel containing ethidium bromide and visualized using ultraviolet illumination.

2.4. MiR-23a-3p target prediction and luciferase reporter assay

TargetScan (www.targetscan.org), PicTar (pictar.mdc-berlin.de/), and miRanda (www.microrna.org) were used to predict the target genes of miR-23a-3p. SMAD3 possessed a putative miR-23a-3p binding site. A luciferase reporter assay was performed to validate this prediction. The wild-type (WT) or mutant (MUT) 3ʹ-UTR segment of SMAD3 containing the putative miR-23a-3p binding site was amplified and inserted into the pGL3 control vector (RiboBio). SW1353 cells were co-transfected with 200 ng of pGL3 vectors possessing the WT 3ʹ-UTR or MUT 3ʹ-UTR of SMAD3 and 90 nM of miR-23a-3p mimics or miR-Scr (25 scrambled nucleotides) using Lipofectamine 2000 (Invitrogen). After 48 h, SW1353 cells were harvested and luciferase activity was assayed using the Dual Luciferase Reporter Assay System (Promega). All experiments were carried out three times.

2.5. Cell transfection

MiR-23a-3p mimics, the miR-23a-3p inhibitor, and miR-Scr were designed and synthesized by Ribobio. MiR-23a-3p mimics and the miR-23a-3p inhibitor were used to promote and inhibit the expression of miR-23a-3p, respectively. For the knockdown of SMAD3 expression, small interfering RNA (siRNA) against SMAD3 (siSMAD3) and the scrambled siRNA (siScr) were purchased from Ribobio. The transfections of miR-23a-3p mimics, miR-23a-3p inhibitor, miR-Scr, siSMAD3, and siScr in SW1353 cells were performed using Lipofectamine 2000 (Invitrogen) according to the manufacturer’s instructions. After transfection for 48 h, the cells were harvested and used for further analyses.

2.6. Western blot

To analyze protein expression, cells were lysed using RIPA Lysis Buffer and protein concentrations were determined using the BCA Assay (Beyotime, Shanghai, China). Protein samples were separated by 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to a polyvinylidene fluoride membrane. The membrane was blocked with 5% non-fat milk and then incubated with primary antibodies, i.e., anti-SMAD3 (1:1000), anti-Type II collagen (1:1500), anti-aggrecan (1:1500), and anti-β-actin (1:2000) (Abcam, San Francisco, CA, USA), at 4 °C overnight. After washing, the membrane was incubated with the corresponding secondary antibody conjugated to horseradish peroxidase (HRP) (1:1000; Abcam) for 1.5 h at 37 °C. Finally, the protein bands were visualized and detected using the enhanced chemiluminescence (ECL) system. β-actin was used as an internal control. The experiment was repeated three times.
were incubated with primary antibody against type II collagen and blocked with 3% bovine serum albumin for 30 min. The cells miR-23a-3p and found that the 3′-UTR of SMAD3 is a potential binding target (Fig. 2A). To confirm whether miR-23a-3p binds directly to the 3′-UTR of SMAD3 in SW1353 cells, we constructed luciferase reporter vectors containing the WT or MUT 3′-UTR SMAD3. As shown in Fig. 2B, miR-23a-3p effectively reduced luciferase activity in SW1353 cells transfected with the WT 3′-UTR of SMAD3 compared to the miR-Scr groups, while the MUT SMAD3 3′-UTR abolished the suppression by miR-23a-3p. We transfected SW1353 cells with miR-23a-3p mimics, an miR-23a-3p inhibitor, or miR-Scr to further confirm this inhibitory effect. The transfection efficiency was confirmed by qRT-PCR (Fig. 2C). The overexpression of miR-23a-3p significantly inhibited the mRNA and protein expression of SMAD3 in SW1353 cells, while the miR-23a-3p inhibitor increased SMAD3 mRNA and protein levels (Fig. 2D and E). These results indicate that SMAD3 is a target of miR-23a-3p and miR-23a-3p may function in OA via SMAD3 regulation.

2.8. Statistical analysis

Data are expressed as means ± standard deviation (SD). Statistical analyses were performed using SPSS 18.0. Differences in mean values between groups were analyzed by Student’s t-tests or ANOVA. The Pearson correlation coefficient was used to assess the correlation between miR-23a-3p and SMAD3 expression. P-values of less than 0.05 were considered statistically significant.

3. Results

3.1. miR-23a-3p expression was upregulated and SMAD3 was downregulated in OA cartilage

As shown in Fig. 1A, miR-23a-3p expression was significantly higher in OA cartilage than healthy cartilages. The expression of SMAD3 at both the mRNA and protein levels was lower in OA cartilage than normal cartilage (Fig. 1B and C). Additionally, miR-23a-3p expression was inversely correlated with SMAD3 expression (Fig. 1D).

To determine whether the upregulation of miR-23a-3p is associated with DNA hypomethylation status in OA cartilage, the methylation status of the miR-23a-3p promoter was evaluated in normal and OA cartilage. Based on an MSP analysis, methylation was detected in healthy cartilage, but the signals were much higher than those observed in OA cartilage (Fig. 1E), consistent with the high miR-23a-3p expression observed in OA cartilage. These data suggest that hypomethylation of the miR-23a-3p promoter at least partially explains the increased expression of miR-23a-3p in OA cartilage.

3.2. MiR-23a-3p downregulated SMAD3 expression by binding to its 3′-UTR

We used a bioinformatics approach to predict the target genes of miR-23a-3p and found that the 3′-UTR of SMAD3 possesses sequences complementary to the miR-23a-3p seed sequence and accordingly is a potential binding target (Fig. 2A). To confirm whether miR-23a-3p binds directly to the 3′-UTR of SMAD3 in SW1353 cells, we constructed luciferase reporter vectors containing the WT or MUT 3′-UTR SMAD3. As shown in Fig. 2B, miR-23a-3p effectively reduced luciferase activity in SW1353 cells transfected with the WT 3′-UTR of SMAD3 compared to the miR-Scr groups, while the MUT SMAD3 3′-UTR abolished the suppression by miR-23a-3p.

3.3. Overexpression of miR-23a-3p inhibits type II collagen and aggrecan expression in SW1353 cells

To clarify the function of miR-23a-3p in chondrocytes, we inhibited or overexpressed miR-23a-3p in SW1353 cells and observed the chondrocyte ECM components type II collagen and aggrecan. We found that miR-23a-3p mimics significantly downregulated the expression levels of type II collagen and aggrecan compared with the miR-Scr groups, whereas the expression levels of type II collagen and aggrecan were higher in SW1353 cells treated with the miR-23a-3p inhibitor than in the miR-Scr groups (Fig. 3A and B). Moreover, as shown in Fig. 3C, the overexpression of miR-23a-3p decreased the protein expression levels of type II collagen, while the inhibition of miR-23a-3p upregulated type II collagen expression. These results suggested that miR-23a-3p overexpression suppresses chondrocyte ECM synthesis.

3.4. Overexpression of miR-23a-3p inhibits ECM production via SMAD3 in SW1353 cells

To further confirm whether the inhibitory effect of miR-23a-3p on chondrocyte ECM synthesis was due to the direct targeting of SMAD3, we suppressed miR-23a-3p and SMAD3 expression. The expression of SMAD3 was significantly inhibited by siSMAD3. The miR-23a-3p inhibitor upregulated SMAD3 expression, and this effect was reversed by cotransfection with siSMAD3 (Fig. 4A and B). Furthermore, the upregulation of aggrecan and type II collagen expression via transfection with the miR-23a-3p inhibitor was significantly reduced by cotransfection with siSMAD3 (Fig. 4C and D). Immunofluorescence staining confirmed these results (Fig. 4E). Similar differences in type II collagen protein expression between SW1353 transfected with the miR-23a-3p inhibitor alone and cotransfected with siSMAD3 were observed in the immunofluorescence and western blot analyses. These results suggested that SMAD3 is essential in the miR-23a-3p-induced downregulation of aggrecan and type II collagen.

Table 1
Sequences of primers used for RT-PCR.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Oligonucleotide sequence</th>
<th>Product size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Forward (5′-3′)</td>
<td>Reverse (5′-3′)</td>
<td></td>
</tr>
<tr>
<td>SMAD3</td>
<td>CGGCTCTACTACATCGGAGG</td>
<td>GTAGAGAGGCTCAAGGCCCT</td>
</tr>
<tr>
<td>Type II collagen</td>
<td>AGAACCTGGTAGGAGCTAGA</td>
<td>AGCCGGCCTAGAGGTCTATC</td>
</tr>
<tr>
<td>Aggreccan</td>
<td>TGGCCGGGACCACTTGCAGC</td>
<td>TGAATCCAGGCGGTCCCTAGG</td>
</tr>
<tr>
<td>U6</td>
<td>CGCTTCCGGACACATATAC</td>
<td>AAATATGGAAGGCTTACCA</td>
</tr>
<tr>
<td>β-actin</td>
<td>AGGCCAGCATCCCCCAAGGTTCTT</td>
<td>GGGCAGAAAGCTATCTT</td>
</tr>
</tbody>
</table>

L. Kang et al. / Biochemical and Biophysical Research Communications 478 (2016) 467–473
Fig. 1. MiR-23a-3p is upregulated and SMAD3 is downregulated in OA cartilage. (A) The expression of miR-23a-3p in cartilage tissues from 10 osteoarthritis patients and 10 traumatic amputees was analyzed by qRT-PCR. (B–C) The expression of SMAD3 mRNA (B) and protein (C) in cartilage tissues from OA patients and traumatic amputees was analyzed by qRT-PCR and western blotting, respectively. (D) The correlation between Smad3 mRNA expression and miR-23a-3p expression in cartilage samples was analyzed. (E) The DNA methylation status of the miR-23a-3p promoter in OA cartilage tissues and normal cartilage tissues was analyzed by MSP. M, methylated primers; U, unmethylated primers. *P < 0.05, **P < 0.01, compared to normal.

Fig. 2. SMAD3 is a direct target of miR-23a-3p. (A) A putative miR-23a-3p target site in the 3′-UTR of human Smad3 mRNA was predicted in a bioinformatics analysis. (B) Luciferase activity levels of SW1353 cells co-transfected with miR-23a-3p mimics or miR-Scr and wild-type or mutant SMAD3 3′-UTR constructs were measured by a luciferase reporter assay. *P < 0.05, compared to miR-Scr groups. (C–E) SW1353 cells were transfected with miR-23a-3p mimics, miR-23a-3p inhibitors, or miR-Scr for 48 h, and untreated cells were used as a control. (C) The levels of miR-23a-3p were measured by qRT-PCR. (D) SMAD3 mRNA levels were measured by qRT-PCR. (E) The protein expression of SMAD3 was measured by western blotting. *P < 0.05, compared to control groups.
4. Discussion

Alterations in miRNA expression levels are associated with various diseases, including OA [12,30]. The upregulation of miR-30 b, miR-377, miR-22, miR-223, miR-483, and miR-509 as well as the downregulation of miR-337, miR-25, miR-373, miR-26a, miR-210, and miR-299 have been detected in OA cartilage compared to normal cartilage [31]. Additionally, several miRNAs have known roles in the regulation of OA progression. For example, miR-30a promotes articular cartilage degradation via SOX9 inhibition [32]. MiR-16-5p reduces type II collagen and aggrecan expression, while inducing the expression of matrix metalloproteinases and ADAMTS, which is associated with cartilage degradation in OA [22]. Therefore, it is important to characterize the gene networks regulated by these miRNAs in OA. In this study, we found that miR-23a-3p is significantly increased in OA cartilage compared to normal cartilage.

In particular, we found that the 3’-UTR of SMAD3 contained a miR-23a-3p binding site and SMAD3 was significantly downregulated in OA cartilage compared to normal cartilage. SMAD3 also plays a critical role in mediating TGF-β signal transduction [20]. Several studies have demonstrated the essential role of TGF-β/SMAD3 signals in maintaining articular cartilage and preventing OA [21,33–35]. Aberrant TGF-β signals are common in OA, and the impairment of TGF-β signals attributed to SMAD3 disruption leads to phenotypes resembling human OA in experimental models of OA [21,35]. Furthermore, SMAD3 mutations result in cartilage degeneration and OA. SMAD3 regulates the balance between the synthesis and degradation of articular chondrocyte ECM by increasing type II collagen expression and decreasing MMP-13 expression [34]. SMAD3 knockout mice exhibit spontaneous articular cartilage degeneration characterized by chondrocyte hypertrophy, progressive loss of cartilage tissue, and osteophyte formation [35]. Notably, genetic variation in SMAD3 is associated with susceptibility to both hip and knee OA in European populations [23]. Consequently, we speculate that upregulated miR-23a-3p regulates the progression of OA via SMAD3. Our results suggested that miR-23a-3p directly binds to the 3’-UTR of SMAD3. Additionally, the overexpression of miR-23a-3p markedly downregulated the mRNA and protein expression of SMAD3. However, the inhibition of miR-23a-3p apparently increased the mRNA and protein expression of SMAD3. These results demonstrated that miR-23a-3p may play an important role in controlling the development of OA by directly targeting SMAD3.

Articular cartilage ECM regulates many biological processes important for the repair and homeostasis of cartilage, including chondrocyte growth, differentiation, attachment, and survival [36]. The continuous loss of cartilage ECM impairs joint function and is involved in the progression of OA [5–7]. We performed a functional analysis of miR-23a-3p to determine whether it regulates the expression of the ECM components type II collagen and aggrecan. Our results showed that miR-23a-3p mimics significantly suppress the expression of type II collagen and aggrecan, while miR-23a-3p inhibitors induce their expression. These findings suggest that the upregulation of miR-23a-3p promotes the progression of OA by inhibiting chondrocyte ECM synthesis. The upregulation of aggrecan and type II collagen expression, which was induced by the inhibition of miR-23a-3p, was significantly reduced by the introduction of siSMAD3. Our findings suggest that the upregulation of miR-23a-3p decreased SMAD3, thereby suppressing chondrocyte ECM synthesis and contributing to the progression of OA.

DNA methylation is important for gene expression regulation; methylation in promoter CpG islands results in transcriptional silencing [37]. Some studies have reported that the methylation
status of miRNA promoter regions is the main explanation for aberrant expression in a variety of pathological conditions [25–27]. We found that miR-23a-3p is upregulated in OA cartilage. Normal cartilage had higher levels of methylation than OA cartilage. These findings indicate that the upregulation of miR-23a-3p expression in OA is associated with the hypomethylation status of the miR-23a-3p promoter.

In conclusion, we demonstrated that miR-23a-3p levels were increased in OA cartilage and the hypomethylation status of the miR-23a-3p promoter regulates miR-23a-3p expression. MiR-23a-3p inhibits chondrocyte ECM synthesis by targeting SMAD3 and thereby promotes the progression of OA. These results suggest a new therapeutic strategy for OA by targeting miR-23a-3p.

Conflicts of interest

The authors confirm that there are no conflicts of interest with respect to the content of this article.

Acknowledgements

This study was supported by the National Natural Science Foundation of China (No.81272025).

Transparency document

Transparency document related to this article can be found online at http://dx.doi.org/10.1016/j.bbrc.2016.06.071.

References


