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ORIGINAL RESEARCH PAPER

MicroRNA-15b silencing inhibits IL-1β-induced extracellular matrix degradation by targeting SMAD3 in human nucleus pulposus cells

Liang Kang · Cao Yang · Huipeng Yin · Kangcheng Zhao · Wei Liu · Wenbin Hua · Kun Wang · Yu Song · Ji Tu · Shuai Li · Rongjin Luo · Yukun Zhang

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Abstract
Objectives To determine the role of microRNA-15b (miR-15b) in interleukin-1 beta (IL-1β)-induced extracellular matrix (ECM) degradation in the nucleus pulposus (NP).
Results MiR-15b was up-regulated in degenerative NP tissues and in IL-1β-stimulated NP cells, as compared to the levels in normal controls (normal tissue specimens from patients with idiopathic scoliosis). Bioinformatics and luciferase activity analyses showed that mothers against decapentaplegic homolog 3 (SMAD3), a key mediator of the transforming growth factor-β signaling pathway, was directly targeted by miR-15b. Functional analysis demonstrated that miR-15b overexpression aggravated IL-1β-induced ECM degradation in NP cells, while miR-15b inhibition had the opposite effects. Prevention of IL-1β-induced NP ECM degeneration by the miR-15b inhibitor was attenuated by small-interfering-RNA-mediated knockdown of SMAD3. In addition, activation of MAP kinase and nuclear factor-κB up-regulated miR-15b expression and down-regulated SMAD3 expression in IL-1β-stimulated NP cells.
Conclusions MiR-15b contributes to ECM degradation in intervertebral disc degeneration (IDD) via targeting of SMAD3, thus providing a novel therapeutic target for IDD treatment.

Keywords Intervertebral disc degeneration · MicroRNA-15b · SMAD3 · Nucleus pulposus · IL-1β · Extracellular matrix degradation

Liang Kang, Cao Yang and Huipeng Yin have contributed equally to this work.

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L. Kang · C. Yang · H. Yin · K. Zhao · W. Hua · K. Wang · Y. Song · J. Tu · S. Li · R. Luo · Y. Zhang (✉)
Department of Orthopaedics, Union Hospital, Tongji Medical College, Huazhong University of Science and Technology, Jiefang Road, No. 1277, Wuhan 430022, China
e-mail: zhangyukuncom@126.com

W. Liu
Department of Orthopaedics, First Hospital of Wuhan, Wuhan 430022, China

Introduction

Intervertebral disc (IVD) degeneration (IDD) is a primary cause of low back pain, resulting in enormous socioeconomic burden and extreme distress to the affected individual (Andersson 1999). Multiple factors such as age and genetic, biomechanical, and environmental factors have been are related to IDD; however, the cellular and molecular mechanisms underlying IDD have not yet been completely elucidated (Hangai et al. 2008; Power et al. 2011). The

breakdown of balance between extracellular matrix (ECM) synthesis and degradation in the nucleus pulposus (NP) of discs is an important feature of IDD (Risbud and Shapiro 2014). Among the multiple molecular events implicated in IDD, the transforming growth factor-β (TGF-β) signaling pathway acts as an inducer of ECM synthesis and weakens the catabolic effects (van Beuningen et al. 1994). Mothers against decapentaplegic homolog 3 (SMAD3), an essential mediator in the TGF-β signaling pathway, is expressed in normal and degenerative IVDs, particularly in NP cells (Uchiyama et al. 2008; Wan et al. 2014). Downregulation of SMAD3 expression leads to inhibition of type II collagen synthesis but increases MMP13 production, thereby leading to ECM degradation (Chen et al. 2012).

MicroRNAs (miRNAs), a class of short (19–24 nucleotides) noncoding RNAs, are important post-transcriptional regulators of cell proliferation, differentiation, and apoptosis (Bartel 2004). Aberrant miRNA expression is associated with IDD development (Gu et al. 2015; Li et al. 2016). Our previous study also showed that miR-34a is considerably up-regulated in degenerative NP tissues and that inhibition of miR-34a expression prevents ECM degradation in human NP cells via increase in growth differentiation factor 5 (GDF5) levels (Liu et al. 2016). The inflammatory cytokine interleukin-1 beta (IL-1β), the most important member of the IL-1 family, is an important catabolic inducer, which increases in IDD and contributes to its progression (Yang et al. 2015). Several miRNAs, such as miR-27b (Akhtar et al. 2010), miR-146a (Gu et al. 2015) and miR-145 (Yang et al. 2014), are involved in IL-1β-induced ECM degradation. MiR-15b inhibits the TGF-β pathway in neonatal rat cardiomyocytes, suggesting that it is involved in the pathway (Tijsen et al. 2014). The balance between TGF-β and the IL-1β pathway is critical for homeostasis in IVDs. For instance, IL-1β inhibits TGF-β-induced expression of type II collagen and aggrecan (Roman-Blas et al. 2007), whereas TGF-β suppresses the expression and catabolic effect of IL-1β in NP cells (Li et al. 2014). MiR-15b is involved in acute liver failure, cardiac dysfunction, and breast cancer via regulation of cell proliferation, differentiation, and apoptosis (Hand et al. 2009; Kedmi et al. 2015; Roy et al. 2013). However, to our knowledge, no studies have reported the functions of miR-15b in IL-1β-induced NP ECM degradation and IDD pathogenesis.

In this study, we examined the miR-15b expression levels in human degenerative NP tissues and IL-1β-stimulated NP cells and investigated the effects of miR-15b in IL-1β-induced NP ECM degradation. The results indicated that (a) SMAD3 is a direct target of miR-15b and that (b) inhibition of miR-15b expression significantly inhibited IL-1β-induced ECM degradation in NP cells through increase in SMAD3 expression. Thus, miR-15b is a promising target for IDD prevention and treatment.

Materials and methods

Patients and samples

Ten human lumbar NP specimens used as normal controls were collected from 10 patients (n = 4 women and 6 men; mean age, 24.3 years; range, 20–36 years) who had idiopathic scoliosis and were undergoing deformity correction surgery. The degenerative human lumbar NP specimens were collected from 15 patients (n = 6 women and 9 men; mean age, 36.2 years; range, 29–62 years) who had IDD and were undergoing disc excision and spinal fusion surgery. The degree of IDD was assessed according to the modified Pfirrmann grading system (Pfirrmann et al. 2001) by using preoperative MRI scans. The lumbar discs of all patients with IDD were classified as Grades III–V and the discs of those with idiopathic scoliosis were classified as Grade II. The study protocol was approved by the Clinical Research Ethics Committee of Union Hospital, Tongji Medical College, Huazhong University of Science and Technology. Written informed consent was obtained from all participants.

Isolation and culture of human NP cells

The normal tissue specimens from the patients with idiopathic scoliosis were washed twice with phosphate-buffered saline (PBS); then, the NP tissue was carefully separated from the annulus fibrosus under a stereotaxic microscope and cut into small fragments (2–3 mm^3). Subsequently, NP cells were isolated by incubation with 0.25 mg/ml type II collagenase (Invitrogen) for 12 h at 37 °C in Dulbecco's modified Eagle medium (DMEM). Isolated NP cells were

resuspended in DMEM containing 10% (v/v) fetal bovine serum (FBS), 100 μg streptomycin/ml, 100 U penicillin/ml and 1% L-glutamine, and then incubated at 37 °C in a humidified 5% CO_2 atmosphere. Confluent cells were detached by trypsinization, seeded in 35-mm tissue culture dishes in complete culture medium (DMEM supplemented with 10% FBS, 100 μg/ml streptomycin, and 100 U/ml penicillin), and incubated at 37 °C and 5% CO_2. The medium was changed every 3 days. The second-passage cells were used for subsequent experiments.

Transfection

The miR-15b mimic (5′-UAGCAGCACAUCAUG GUUUACA-3′), miR-15b inhibitor (5′-UGUAAAC CAUGAUGUGCUGCUA-3′), mimic control (5′-UUGUACUACACAAAAGUACUG-3′), and inhibitor control (5′-CAGUACUUUUGUGUAGUACAA -3′) were designed and synthesized by RiboBio (Guangzhou, China) and transfected using Lipofectamine 2000 (Invitrogen) into NP cells grown to 80% confluence, according to the manufacturer's instructions. After 24 h of transfection, cells were cultured in serum-free medium for 12 h and then treated with IL-1β (10 ng/ml) for 24 h. They were then harvested for subsequent experiments. For SMAD3 knockdown, short interfering (si) RNA against SMAD3 (siSMAD3) and scrambled siRNA (siScr) (RiboBio) were cotransfected with the miR-15b inhibitor or its negative control into NP cells by using Lipofectamine 2000.

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

Total RNA was extracted from NP tissues and cells using TRIzol. SMAD3, type II collagen, aggrecan, MMP3, MMP13, ADAMTS4, ADAMTS5, and miR-15b expression was quantified by qRT-PCR on a 7500 Realtime PCR System (Applied Biosystems), using the cycling conditions recommended by the manufacturer. The sequences of the specific primers used for qRT-PCR are shown in Supplementary Table 1. All experiments were performed in triplicate. The expression levels of β-actin and U6, as endogenous controls, were used for normalization of expression of each mRNA and miRNA, respectively. Relative expression levels were calculated with the $2^{-\Delta\Delta Ct}$ method.

miR-15b target prediction and luciferase reporter assay

TargetScan (www.targetscan.org), miRanda (www.microrna.org), and Pictar (pictar.mdc-berlin.de/) were used to predict miR-15b target genes. The 3′-UTR of SMAD3 was found to possess a putative miR-15b-binding site. The WT or MUT 3′-UTR segment containing the putative miR-15b-binding site was amplified and inserted into the pGL3 control vector (RiboBio). For luciferase activity analysis, HEK 293 cells were cotransfected with a combination of 200 ng pGL3 vector harboring WT or MUT 3′-UTR and 90 nM miR-15b mimic or its negative control by using Lipofectamine 2000. After 48 h, the cells were harvested and luciferase activity was examined using the Dual Luciferase Reporter Assay System (Promega, Madison, WI, USA). All experiments were carried out three times.

Western blotting

The culture supernatants were collected and the cells were lysed for 20 min in cold radioimmunoprecipitation (RIPA) lysis buffer (Beyotime, Beijing, China). Protein concentrations were measured with an enhanced BCA Protein Assay Kit (Beyotime, Shanghai, China). The extracted proteins were separated using 12% SDS-PAGE and transferred to a PVDF membrane that was blocked with 10% (v/v) non-fat milk and then incubated overnight at 4 °C with antibodies against SMAD3 (1:1000), type II collagen (1:1000), aggrecan (1:1000), MMP3 (1:1000), MMP13 (1:3000), ADAMTS4 (1:1000), ADAMTS5 (1:1000), and β-actin (1:2000) (all from Abcam, Cambridge, MA, USA). β-Actin served as the internal control. After the membrane was washed, it was incubated with horseradish peroxidase (HRP)–conjugated secondary antibody (1:2000; Abcam) for 2 h at 37 °C. The protein bands were visualized using an ECL Chemiluminescence kit (Thermo, Waltham, MA, USA). The experiment was performed in triplicate.

Statistical analysis

The results are expressed as mean ± standard deviation (SD). Statistical analysis was performed using the SPSS 20.0 software (SPSS, Chicago, IL, USA). Differences in mean values between groups were

analyzed using the Student's *t* test or analysis of variance (ANOVA). *P* values of less than 0.05 were considered as statistically significant.

Results

miR-15b expression was upregulated in degenerative NP tissues and in IL-1β-stimulated NP cells

To determine the role of miR-15b in IDD pathogenesis, miR-15b expression in 15 degenerative NP tissues samples and 10 idiopathic scoliosis NP tissues samples was examined by qRT-PCR. miR-15b expression was considerably higher in degenerative NP tissues than in the controls (Fig. 1a). miR-15b expression was detected in IL-1β-stimulated NP cells also by qRT-PCR. miR-15b expression was significantly higher in IL-1β-stimulated NP cells than in non-stimulated NP cells (Fig. 1b).

miR-15b mediated IL-1β-induced ECM degradation in NP cells

To investigate whether miR-15b affects IL-1β-induced ECM degradation, human NP cells were transfected with an miR-15b mimic or miR-15b inhibitor; the cells were then treated with IL-1β for 24 h. We examined the mRNA and protein expression of type II collagen and aggrecan by using qRT-PCR and western blot, respectively. Type II collagen and aggrecan expression in NP cells significantly decreased after IL-1β treatment (Fig. 2a, b, g). miR-15b overexpression increased IL-1β-induced inhibition of type II collagen and aggrecan expression (Fig. 2a, b, g). However, miR-15b suppression significantly reversed the IL-1β-induced downregulation of type II collagen and aggrecan expression (Fig. 2a, b, g). High activities of ECM catabolic proteinases such as MMPs and ADAMTSs are found in degenerative IVD tissue and cells; these enzymes have been linked to ECM degradation and IDD progression (Vo et al. 2013). We therefore used qRT-PCR and western blotting to evaluate MMP3, MMP13, ADAMTS4, and ADAMTS5 expression in NP cells that were transfected with an miR-15b mimic or miR-15b inhibitor and then stimulated with IL-1β. We found that, on stimulation with IL-1β, miR-15b overexpression led to a significant increase in MMP3, MMP13, ADAMTS4, and ADAMTS5 expression at both mRNA and protein levels (Fig. 2c–f, h). In contrast, inhibition of miR-15b had the opposite effect (Fig. 2c–f, h).

miR-15b directly targeted SMAD3

To elucidate the molecular mechanism by which miR-15b affects IL-1β-induced ECM degradation of human NP cells, we identified miR-15b target genes by using TargetScan, miRanda, and Pictar. The 3′-UTR of SMAD3 contains sequences that are complementary to the miR-15b seed sequence (Fig. 3a). A luciferase assay was performed to confirm whether miR-15b

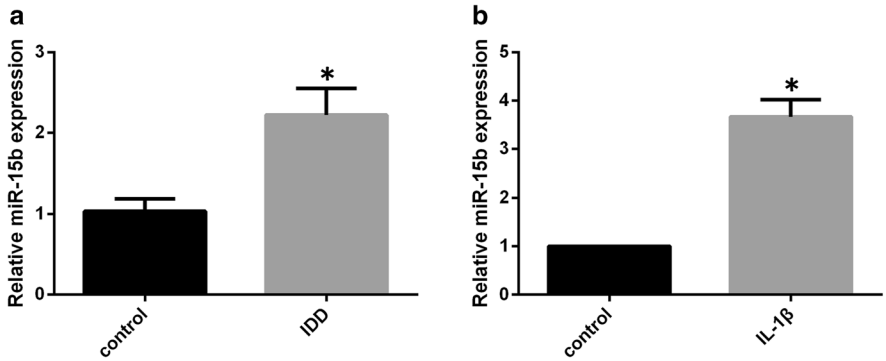

Fig. 1 miR-15b expression was upregulated in degenerative NP tissues and in IL-1β-stimulated NP cells. **a** miR-15b expression in human NP tissues from 15 patients with IDD and 10 patients with idiopathic scoliosis was analyzed by qRT-PCR, with NP tissues from patients with idiopathic scoliosis (n = 10) serving as the control. **b** miR-15b expression in human NP cells that were treated or not treated with IL-1β for 24 h was assessed by qRT-PCR, with untreated cells serving as the control. U6 was used as the internal control. Data are presented as mean ± SD. $^*P < 0.05$

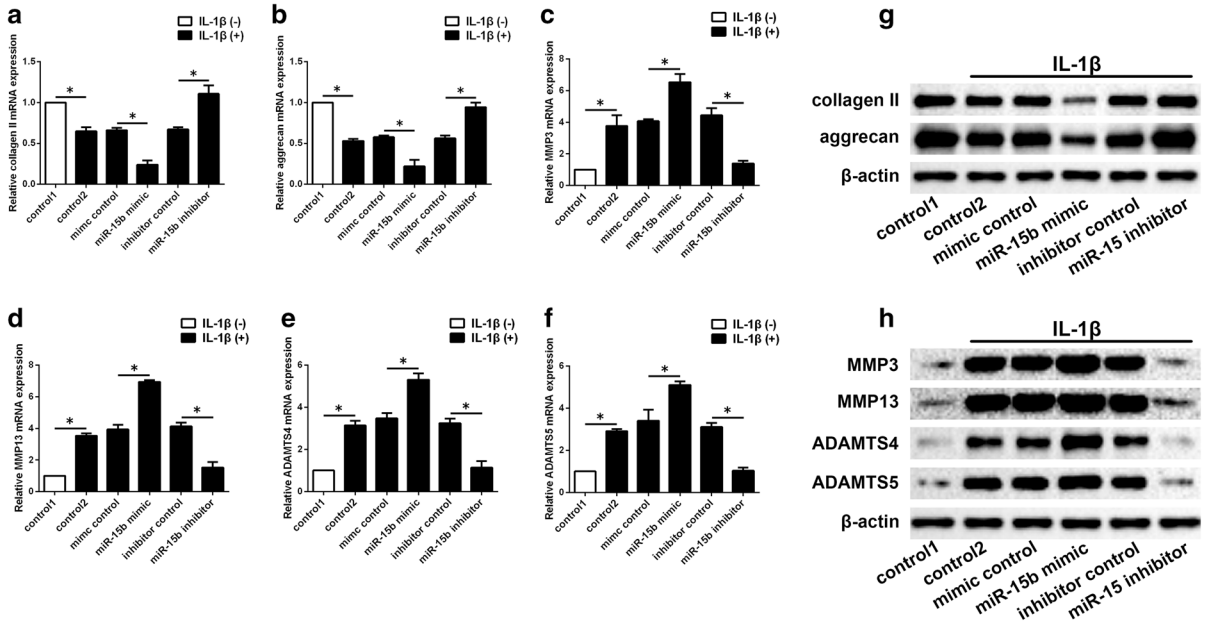

Fig. 2 Modulation of miR-15b affected IL-1β-induced extracellular matrix degradation in NP cells. NP cells were transfected with an miR-15b mimic (100 nM), miR-15b inhibitor (100 nM), or the corresponding negative control for 24 h. Subsequently, they were treated with or not treated with IL-1β (10 ng/ml) for 24 h; untransfected cells that were not treated with IL-1β served as control 1 and untransfected cells treated with IL-1β served as control 2. Type II collagen, aggrecan, MMP3, MMP13, ADAMTS4, and ADAMTS5 mRNA (**a**–**f**) and protein (**g**, **h**) expression levels were analyzed by qRT-PCR and western blotting, respectively. β-Actin was used as an internal control. Data are presented as mean ± SD. $^*P < 0.05$

binds directly to the 3′-UTR of SMAD3. We generated luciferase reporter vectors containing the wild-type (WT) or mutant (MUT) 3′-UTR of SMAD3. Luciferase activity was significantly inhibited in cells cotransfected with the miR-15b mimic and WT 3′-UTR of SMAD3, while cotransfection of the MUT 3′-UTR of SMAD3 evidently abrogated repression of the luciferase activity, which was attributed to miR-15b overexpression (Fig. 3b). Furthermore, to verify this inhibitory effect in NP cells, we transfected the miR-15b mimic and miR-15b inhibitor into the NP cells and then evaluated SMAD3 expression by using qRT-PCR and western blot. SMAD3 mRNA and protein expression levels decreased because of miR-15b overexpression, whereas they increased on miR-15b inhibition (Fig. 3c, d).

miR-15b regulated SMAD3 expression in IL-1β-stimulated NP cells

On treating human NP cells with IL-1β, an inverse relationship between miR-15b and SMAD3 expression was noted. IL-1β stimulation markedly upregulated miR-15b expression at 24, 48, and 72 h and downregulated SMAD3 expression at both mRNA and protein levels in a time-dependent manner in human NP cells (Fig. 4a–c). Next, qRT-PCR and western blot were used to detect SMAD3 expression at mRNA and protein levels, respectively, in human NP cells that were transfected with the miR-15b mimic or miR-15b inhibitor and then treated with IL-1β. SMAD3 mRNA and protein expression was found to be inhibited by miR-15b overexpression but increased on miR-15b silencing in IL-1β-stimulated NP cells (Fig. 4d, e).

miR-15b silencing attenuated IL-1β-induced ECM degradation in NP cells by targeting SMAD3

To confirm whether the effect of miR-15b on IL-1β-induced ECM degradation was due to its direct targeting of SMAD3, we co-transfected human NP cells with the miR-15b inhibitor or inhibitor control along with siSMAD3 or siScr and then treated the cells

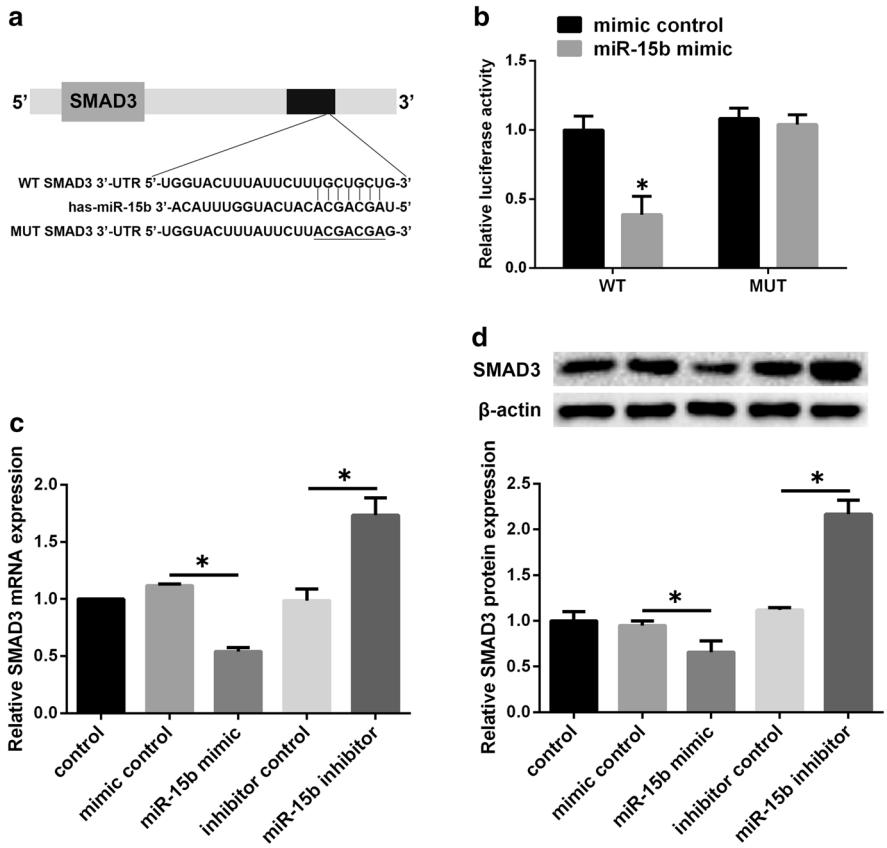

Fig. 3 miR-15b directly targeted SMAD3. **a** Putative miR-15b target site in the SMAD3 3′-UTR, predicted by bioinformatics analysis. **b** Luciferase activity in HEK 293 cells cotransfected with the miR-15b mimic or mimic control and WT or MUT SMAD3 3′-UTR constructs. **c**, **d** The miR-15b mimic, miR-15b inhibitor, or the corresponding negative control was transfected into NP cells for 24 h, with untransfected cells serving as a control. SMAD3 mRNA (**c**) and protein (**d**) expression was assessed using qRT-PCR and western blotting, respectively. β-Actin was used as an internal control. Data are presented as mean ± SD. $^*P < 0.05$

with IL-1β. SMAD3 mRNA and protein expression was significantly suppressed by siSMAD3, and SMAD3 upregulation after transfection with the miR-15b inhibitor was decreased by siSMAD3 cotransfection (Fig. 5a, h). Furthermore, inhibiting miR-15b prevented IL-1β-induced downregulation of type II collagen and aggrecan and upregulation of MMP-3, MMP13, ADAMTS4, and ADAMTS5 mRNA and protein expression, but the effect was attenuated on adding siSMAD3 (Fig. 5b–g, i).

The roles of the NF-κB and MAPK pathways in the regulation of miR-15b expression in human NP cells

The MAPK and NF-κB pathways have been identified within IVD cells as signal transduction pathways following IL-1β stimulation; inhibition of these pathways partially prevents the catabolic effect induced by IL-1β (Studer et al. 2007; Wuertz et al. 2012). Moreover expression of certain miRNAs is regulated by NF-κB and MAPK signaling pathways (Akhtar et al. 2010). Therefore, we investigated whether NF-κB and MAPK signaling is required for IL-1β-induced miR-15b expression in human NP cells. Human NP cells were pretreated with the NF-κB inhibitor SN50 (5 μM) or with various MAPK inhibitors for 2 h: for example, the MEK-1/2 inhibitor PD98059 (10 μM), the p38 MAPK inhibitor SB203580 (1 μM),or the JNK inhibitor SP600125 (10 μM). Subsequently, they were stimulated with IL-1β for 24 h. qRT-PCR analysis showed that IL-1β stimulation markedly upregulated miR-15b expression and that pretreatment with inhibitors resulted in significant suppression in IL-1β-induced miR-15b expression (Fig. 6a). In addition, the downregulation of SMAD3 mRNA expression induced by IL-1β was attenuated by treatment with NF-κB and MAPK inhibitors (Fig. 6b).

Discussion

Accumulating evidence has demonstrated that miRNAs play essential roles in diverse biological and

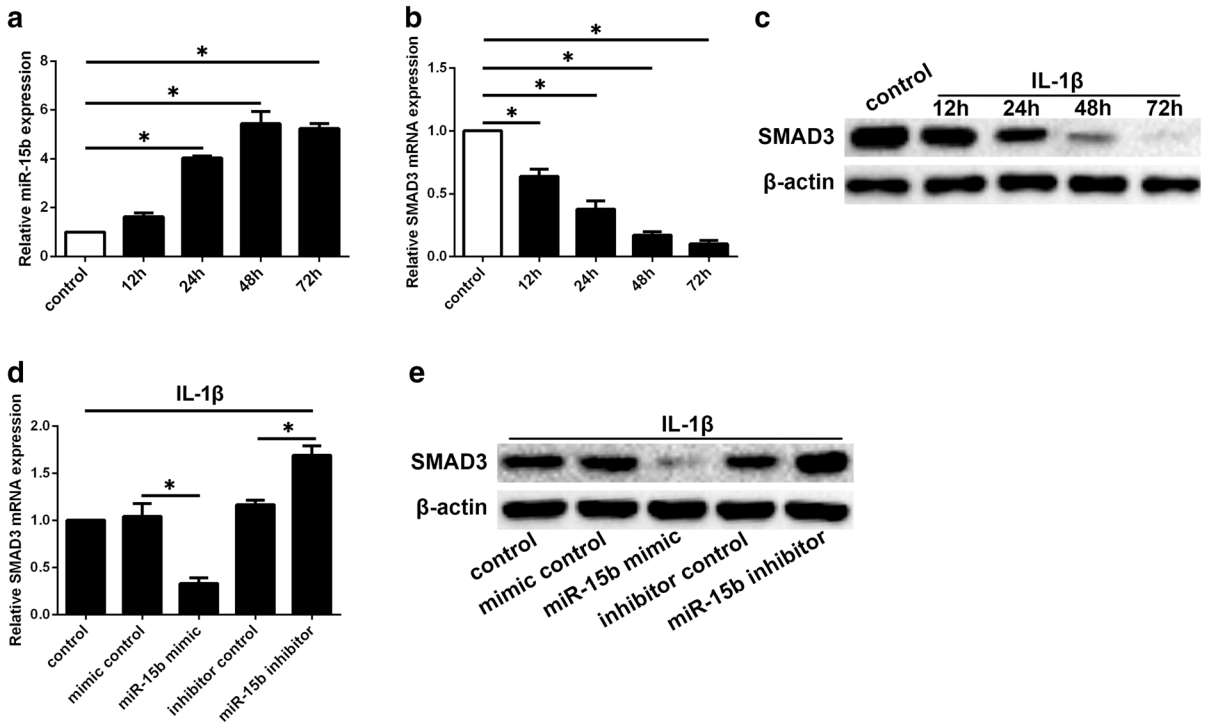

Fig. 4 Regulation of SMAD3 expression by miR-15b in NP cells treated with IL-1β. **a**–**c** NP cells were treated with IL-1β for 12, 24, 48, and 72 h, with untreated cells serving as a control. **a** miR-15b expression in NP cells was determined by qRT-PCR. **b**, **c** SMAD3 mRNA b and protein c expression in NP cells was analyzed by qRT-PCR and western blotting, respectively. U6 and β-actin were used as internal controls for miR-15b and SMAD3 detection, respectively. **d**, **e** SMAD3 mRNA (**d**) and protein (**e**) expression levels in NP cells that were transfected with the miR-15b mimic, miR-15b inhibitor, or the corresponding negative control for 24 h and then treated with IL-1β for 24 h were determined by qRT-PCR and western blotting, respectively. The untransfected cells were considered as the control. β-Actin was used as an internal control. Data are presented as mean ± SD. $^*P < 0.05$

pathological processes by affecting cell proliferation, differentiation, and apoptosis (Bartel 2004). The role of miRNAs in degenerative disc disease in humans has also been investigated. Many miRNAs are involved in IDD pathogenesis (Gu et al. 2015; Li et al. 2016). For example, miR-27b downregulation is involved in loss of type II collagen via direct targeting of MMP13 in human IDD (Li et al. 2016). In the current study, we found that miR-15b expression significantly increased in human degenerative NP tissues and also in IL-1β-stimulated NP cells. MiR-15b overexpression aggravated IL-1β-induced ECM degradation, while miR-15b suppression could reverse these changes via IL-1β stimulation in human NP cells. Moreover, we found that miR-15b regulated SMAD3 expression by directly binding to the 3′-UTR of SMAD3.

IL-1β is a potent inflammatory cytokine that plays a pivotal role in IDD pathogenesis (Yang et al. 2015). Previous studies demonstrated that IL-1β can induce reduction of ECM gene expression in IVD and that IL-1β inhibition could provide an effective therapeutic target for preventing and reversing disc degeneration (Yang et al. 2015). Several miRNAs have been confirmed to be associated with the process of ECM degradation triggered by IL-1β (Gu et al. 2015; Liu et al. 2016). For example, miR-146a reduces IL-1β-dependent inflammatory responses in the IVD (Gu et al. 2015). MiR-15b plays an important role in the progression of breast cancer and development of hemophilic arthropathy (Kedmi et al. 2015; Sen and Jayandharan 2016); however, miR-15b expression levels in degenerative NP tissues and the function of miR-15b in IDD pathogenesis were not known. Here, our results showed that miR-15b significantly increased in degenerative human NP tissues and that IL-1β stimulation upregulated miR-15b expression. These results suggest that miR-15b plays an important role in IL-1β-induced NP ECM degradation in humans.

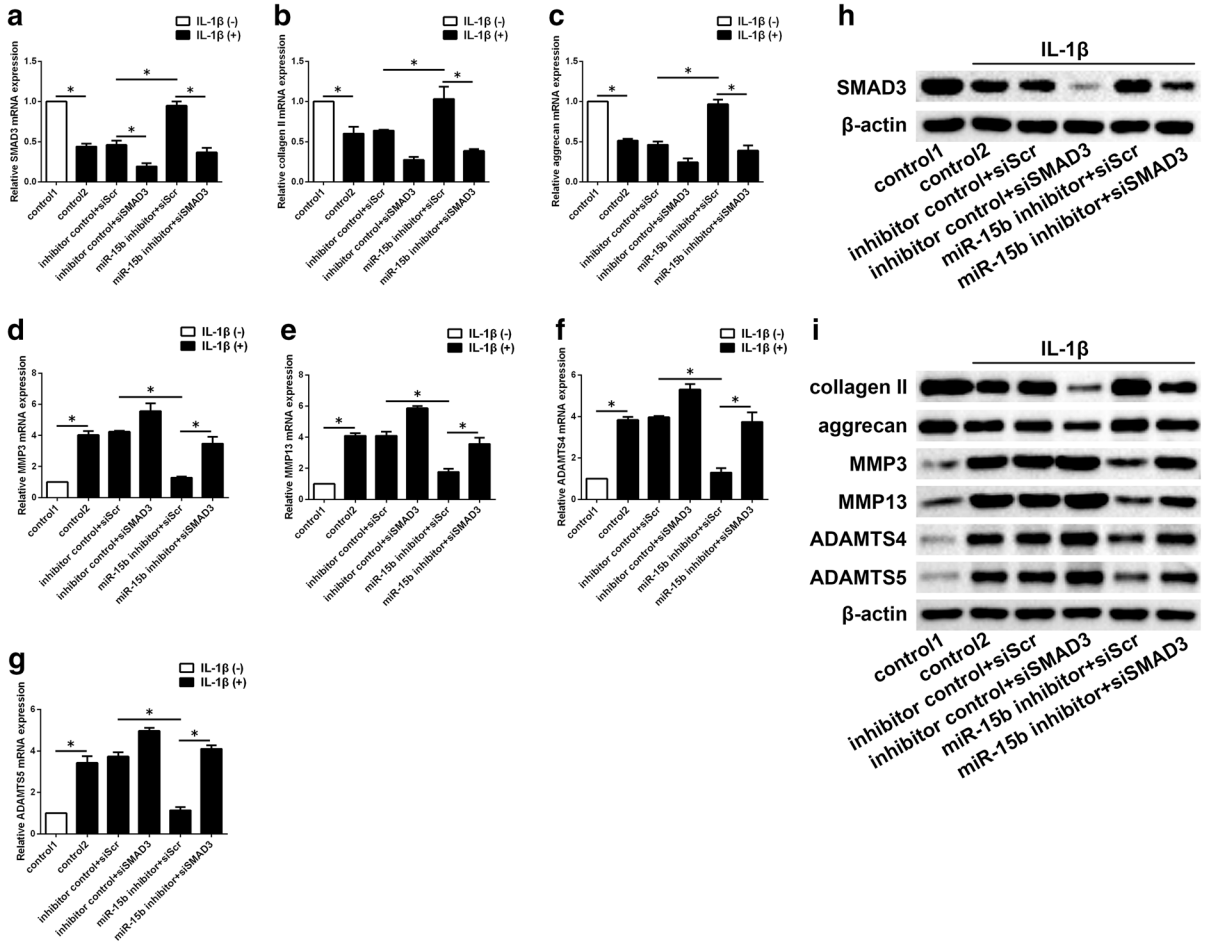

Fig. 5 miR-15b silencing inhibited IL-1β-induced extracellular matrix degradation in NP cells by targeting SMAD3. The NP cells were cotransfected with the miR-15b inhibitor or its negative control and siSMAD3 or siScr for 24 h and then treated with IL-1β for 24 h. The untransfected cells that were not treated with IL-1β were set as control 1 and untransfected cells treated with IL-1β were set as control 2. **a**–**g** SMAD3 (**a**), type II collagen (**b**), aggrecan (**c**), MMP-3 (**d**), MMP13 (**e**), ADAMTS4 (**f**), and ADAMTS5 (**g**) mRNA expression was analyzed by qRT-PCR. **h** SMAD3 protein expression was assessed by western blotting. **i** Type II collagen, aggrecan, MMP3, MMP13, ADAMTS4, and ADAMTS5 protein expression levels were determined using western blotting. β-Actin was used as an internal control. Data are presented as mean ± SD. $^*P < 0.05$

Our results confirmed that miR-15b has a positive effect on IL-1β-induced imbalance between ECM anabolism and catabolism, thereby contributing to NP ECM degradation in humans. miR-15b overexpression increased IL-1β-induced upregulation of MMP3, MMP13, ADAMTS4, and ADAMTS5 expression and downregulation of type II collagen and aggrecan expression, whereas inhibition of miR-15b reversed these changes attributed to IL-1β stimulation. Inhibition of type II collagen and aggrecan expression and elevated expression of MMPs and ADAMTS are intimately involved in ECM breakdown and IDD progression (Vo et al. 2013). Moreover, inhibition of MMP13 and ADAMTS4 function can promote ECM restoration (Le Maitre et al. 2007). These results suggest that miR-15b inhibition protects NP cells from IL-1β-induced ECM degradation.

MiRNAs exert their functions via direct binding to target transcripts to suppress gene expression. We therefore used a bioinformatics approach to predict miR-15b targets; the analysis helped identify a putative miR-15b-binding site in the 3′-UTR of SMAD3 and confirmed that miR-15b directly binds to this site. Furthermore, miR-15b overexpression significantly decreased SMAD3 mRNA and protein expression, whereas miR-15b inhibition increased their levels in

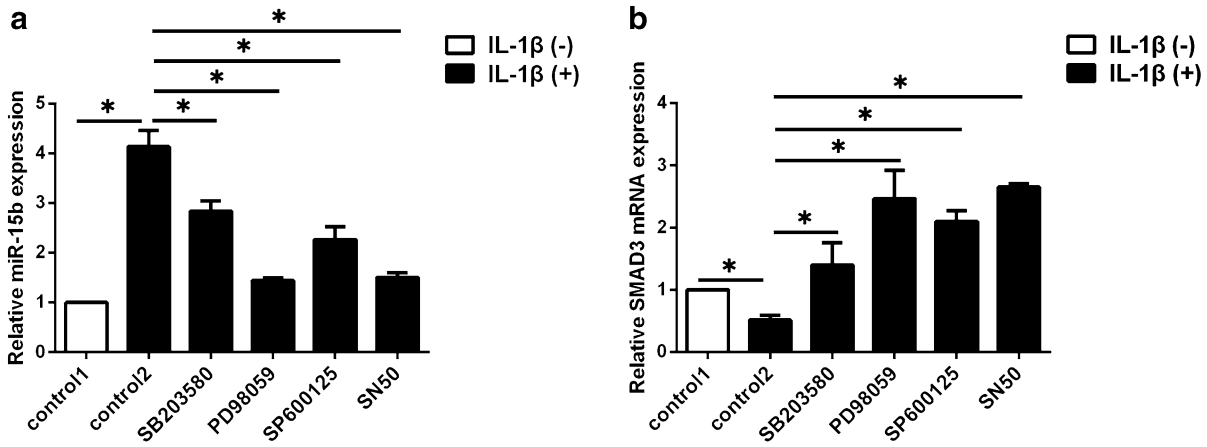

Fig. 6 Roles of the NF-κB and MAPK pathways in the regulation of miR-15b expression in human NP cells. The NP cells were pretreated for 2 h with the NF-κB inhibitor SN50 (5 μM) and the MAPK inhibitors PD98059 (10 μM), SB203580 (1 μM), or SP600125 (10 μM), and then stimulated with IL-1β for 24 h. The cells that were not treated with pathway-specific inhibitors and IL-1β were set as control 1, and untreated cells with pathway-specific inhibitors that were treated with IL-1β were set as control 2. **a** miR-15b expression in NP cells was determined by qRT-PCR. **b** SMAD3 mRNA expression in NP cells was analyzed by qRT-PCR. U6 and β-actin were used as internal controls for miR-15b and SMAD3 detection, respectively. Data are presented as mean ± SD. $*P < 0.05$

human NP cells treated with IL-1β. These results suggest that miR-15b mediates the effect of IL-1β responses in NP cells, probably by repressing SMAD3 expression.

TGF-β signaling plays a critical role in the growth and maintenance of IVD tissue (Jin et al. 2011). SMAD3 is an essential intracellular molecule that is linked to extracellular TGF-β signals. The impairment of TGF-β signals attributed to SMAD3 disruption leads to phenotypes of chondrocytes resembling those in human osteoarthritis (Yang et al. 2001). Moreover, SMAD3 deficiency causes decrease in type II collagen expression, resulting in the morphologic degenerative changes noted in IVD (Li et al. 2009). In the current study, siSMAD3 addition attenuated the prevention of IL-1β-induced downregulation of aggrecan and type II collagen expression and upregulation of MMP3, MMP13, ADAMTS4, and ADAMTS5 expression effected by miR-15b silencing. This finding suggests that miR-15b regulates IL-1β-induced NP ECM degradation by directly targeting SMAD3.

Because both the MAPK and NF-κB pathways have been implicated in the catabolic effects of IL-1β in IDD (Studer et al. 2007; Wuertz et al. 2012), we investigated the possible role of these pathways in the regulation of miR-15b expression in IL-1β-stimulated human NP cells. MAPK and NF-κB signaling is reported to be involved in regulation of miRNA expression in chondrocytes in osteoarthritis (Akhtar et al. 2010). In the current study, we observed that treatment with NF-κB- or MAPK-pathway-specific inhibitors suppressed IL-1β-induced upregulation of miR-15b expression and downregulation of SMAD3 expression in human NP cells. This finding indicates that activation of the NF-κB and MAPK pathways may be necessary for the promotion of miR-15b expression by IL-1β in human NP cells.

In conclusion, miR-15b levels increased in human degenerative NP tissues. miR-15b silencing inhibited IL-1β-induced ECM degradation in human NP cells via increase in SMAD3 expression. Therefore, strategies to downregulate the expression or to prevent the upregulation of miR-15b may have the potential to become a possible therapeutic and/or preventive approach for human IDD.

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Compliance with ethical standards

Conflict of interest The authors declare no conflict of interest.

Supporting information Supplementary Table 1—Sequences of primers used for qRT-PCR.

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