Decreased miR-214–3p activates NF-κB pathway and aggravates osteoarthritis progression

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**ABSTRACT**

**Background:** Osteoarthritis (OA), a disease with whole-joint damage and dysfunction, is the leading cause of disability worldwide. The progressive loss of hyaline cartilage extracellular matrix (ECM) is considered as its hallmark, but its exact pathogenesis needs to be further clarified. MicroRNA(miRNA) contributes to OA pathology and may help to identify novel biomarkers and therapies against OA. Here we identified miR-214–3p as an important regulator of OA.

**Methods:** qRT-PCR and in situ hybridization were used to detect the expression level of miR-214–3p. The function of miR-214–3p in OA, as well as the interaction between miR-214–3p and its downstream mRNA target (IKKβ), was evaluated by western blotting, immunofluorescence, qRT-PCR and luciferase assay. Mice models were introduced to examine the function and mechanism of miR-214–3p in OA in vivo.

**Findings:** In our study, we found that miR-214–3p, while being down-regulated in inflamed chondrocytes and OA cartilage, regulated ECM metabolism and cell apoptosis in the cartilage. Mechanically, the protective effect of miR-214–3p downregulated the IKK-β expression and led to the dysfunction of NF-κB signaling pathway. Furthermore, intra-articular injection of miR-214–3p antagonist in mice joints triggered spontaneous cartilage loss while miRNA-214–3p agomiRNA alleviated OA in the experimental mouse models.

**Interpretation:** Decreased miR-214–3p activates the NF-κB signaling pathway and aggravates OA development through targeting IKKβ, suggesting miR-214–3p may be a novel therapeutic target for OA.

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1. Introduction

Osteoarthritis (OA) is a highly prevalent joint disorder with whole-joint damage and dysfunction, mainly presented in aged population, leading to chronic pain, joint stiffness, and severe physical disability [1,2]. It is characterized by the loss of articular cartilage in addition to other joint disruption, including subchondral bone remodeling, osteophyte formation, synovium inflammation and meniscal damage [3,4]. Present OA management is widely divided into nonpharmacological, pharmacological, and surgical treatments [5,6]. Nonpharmacological treatments, such as exercise, weight loss, and physical therapy, are recommended for early-stage OA patients. Pharmacological treatments are mainly focused on pain management with analgesics and anti-inflammatory medication. Surgical treatment is most widely used for patients in the late phase of the disease process [7,8]. However, the specific mechanisms leading to OA have not been fully elucidated, current OA treatments are limited and insufficient to prevent the initiation and progression of the disease. Thus, further study on pathogenesis of the disease and exploring new therapeutic strategies is of great clinical significance.

MicroRNAs (miRNAs), approximately 22 nucleotides (nt) long, are a class of non-coding, single-stranded RNAs, which repress mRNA translation or promote its degradation by interacting with the 3’ untranslated regions (UTRs) of the target genes [9,10]. Recent studies have shown that miRNAs are involved in the development and progression of OA by regulating chondrocyte apoptosis and proliferation.
Research in context

Evidence before this study

An increasing number of studies have revealed that miRNA is tightly associated with the initiation and progression of OA, but its effect and underlying mechanism remain unclear. Recent studies on other diseases have demonstrated that miR-214–3p has the ability to regulate numerous targets in pathogenesis of disease. As cartilage deterioration is a key pathogenic event in the development of OA, we investigated the mechanical role of miR-214–3p expression on articular cartilage degradation and cell apoptosis.

Added values of this study

Our in vitro and in vivo studies demonstrated that miR-214–3p could effectively protect the cartilage from damage through inhibition of NF-kB signaling pathway, and the miR-214–3p/IKKβ/NF-kB axis may represent a promising potential therapeutic target.

Implications of all the available evidence

This is the first report of a role for miR-214–3p in the regulation of cartilage metabolism in OA, with specific relevance to a common arthritis disease impacting on human health. Its protective effect was mediated by targeting IKKβ, leading to the blockage of NF-kB signaling pathway. These data suggest that the miR-214–3p/IKKβ/NF-kB axis may stand for a novel opportunity for intervention of OA and other inflammatory arthritis diseases.

extracellular matrix (ECM) metabolism and inflammatory response [11–13]. Thus, revealing the roles of miRNAs and their potential target regulators is critical for understanding the molecular mechanisms of OA and identifying new biomarkers or therapeutic targets for OA.

The nuclear factor-kappa B (NF-kB) signaling is essential in a wide range of biological processes, such as ECM degradation, cell cycle progression, apoptosis and inflammation [14,15]. Under the stimulation of inflammatory factors, the inhibitor of nuclear factor kappa B kinase (IKK) complex leads to leb phosphorylated in the cytoplasm and degraded subsequently. The NF-kB dimers are then released into the nucleus, resulting in the transcription of downstream target genes [16,17]. As a key catalytic subunit of IKK complex, IKKβ functions crucially in NF-kB activation and in many diseases [18,19]. Researchers have found that several miRNAs target IKKβ, such as miR-199b and miR-200b, in some disorders [20,21]. Previous studies have confirmed that NF-kB signaling is implicated in OA pathophysiology through various effects and thus representing a potential therapeutic target for OA treatment [22–24]. However, the molecular mechanism in regulating IKKβ/NF-kB pathway in OA remains poorly understood.

In the present study, we identified a miRNA, miR-214–3p, which was significantly downregulated in IL-1β-treated chondrocytes and cartilage tissues from OA patients and the experimental mouse models. Further, we investigated that downregulation of miR-214–3p activated the NF-kB signaling pathway and aggravated OA development through targeting IKKβ. We believe our findings provide new insights into the role of miRNAs and the IKKβ/NF-kB pathway in OA and are valuable for developing novel therapeutic strategies.

2. Materials and methods

2.1. Ethics

This study was approved by the Ethics Committees of Zhujiang Hospital, Southern Medical University, and all aspects of the study comply with the criteria established by the Declaration of Helsinki. The OA patients provided informed consent to participate in this study. The human knee cartilage study was approved by the Ethics Committee of Zhujiang Hospital, Southern Medical University (Guangzhou, China) (2019-KY-022–03). Animal handling and experimental procedures were performed with the approval from Zhujiang Hospital of Southern Medical University Ethics Committee (LAEC-2019–004).

2.2. Human cartilage tissue collection

OA cartilage samples were aseptically collected from patients undergoing total knee replacement surgery for end-stage knee OA (n = 30). The human knee cartilage study was approved by the Ethics Committee of Zhujiang Hospital, Southern Medical University (Guangzhou, China) (2019-KY-022–03). Written informed consent was obtained from all patients. The inclusion criteria included: 1) age 45–79, gender unlimited; 2) knee pain and other manifestations of arthritis. Secondary OA and other inflammatory joint diseases were ruled out based on clinical data. Clinical characteristics of the patients were collected as Supplementary Table S1. Human knee cartilage tissues of undamaged and damaged areas were fixed in 4% paraformaldehyde (PFA) for 36 h, and then changed to decalcification solution, SAF/004 Fast Green or RNA in situ hybridization were performed on these human articular cartilage tissue sections.

2.3. Human primary chondrocyte isolation and cell culture

Human chondrocytes (HCs) were isolated and cultured according to previous protocols [25]. Human cartilages of undamaged areas were eliminated under sterile conditions. Subsequent to the elimination of connective tissue, as well as perichondrium, rest of the cartilages were minced and washed thrice with PBS. Tissues were digested by trypsin (Gibco Life Technology, NY) for 20 min. Subsequent to supernatant elimination, PBS was utilized to wash the cartilages. Using a three to five times volume of a type II collagenase solution (ThermoFisher, NY), a twelve-hour digestion was carried out at 37 °C. The cell suspension was centrifuged at 40 g for 5 min, the supernatant was taken to another centrifuge tube, remaining cells continued to be digested, and the cells were harvested again after 24 h. Centrifuged the supernatant at 300 g for 5 min, poured out the excess liquid, resuspended the cell pellet with 5 mL DMEM/F12 (Gibco Life Technology, NY) containing 10% fetal bovine serum (Gibco Life Technology, NY), and cultured in 25 cm² culture flasks in a 37 °C humidified atmosphere with 5% CO₂. The medium was changed every 3 days.

2.4. Primary culture of mice chondrocytes

Femoral heads and femoral condyles of C57BL/6 newborn mice were used to isolate primary chondrocytes as described previously [26]. The basic procedure is similar to that described above for the extraction of human chondrocytes. After isolation, cells were cultured in 25 cm² culture flasks with DMEM/F12 (Gibco Life Technology, NY) containing 10% fetal bovine serum (Gibco Life Technology, NY). Non-adherent cells were removed, and adherent chondrocytes were cultured and expanded for further experiments. Primary chondrocyte cells were used in the experiments prior to the second passage. Primary chondrocyte from human and mice were validated by immunofluorescence of type II collagen and Toluidine blue staining.

2.5. RNA extraction and quantitative real-time polymerase chain reaction (qRT-PCR)

Total RNA was isolated from human and mice chondrocytes using the TRIzol kit (TaKaRa, Japan) according to the manufacturer's
instructions. Reverse transcription was performed using 1000 ng total RNA and a PrimeScript RT Reagent Kit (TAKARA) or PrimeScript RT Master Mix (TAKARA), which were used to investigate the expression of miRNA and mRNA, respectively. For miRNA assay, the reverse reactions were incubated at 42 °C for 15 min followed by inactivation at 85 °C for 5 s. qRT-PCR amplification was assessed in a CFX Connection Real-Time System (Bio-Rad) by using the SYBR Premix Ex Taq II kit (Takara). The following cycling conditions were used: 95 °C for 30 s, followed by 40 cycles of 95 °C for 5 s and 60 °C for 30 s. All reactions were performed in duplicate and normalized to the internal reference U6 for miRNA and GAPDH for mRNAs. The 2$^{-\Delta\Delta Ct}$ method was used to evaluate the relative miRNA/mRNA expression levels. Primers are listed in Supplementary Table S2A.

2.6. In situ hybridization (ISH)

Both human and mouse knee tissues were fixed with 4% PFA followed by decalcification with ethylenediaminetetraacetic acid (EDTA). ISH was used to evaluate miR-214-3p expression levels based on the manufacturer’s protocol (Boster Biological Technology Co., Ltd, China) as described previously [27]. Briefly, the endogenous peroxidase activity was blocked with 3% hydrogen peroxide for 10 min, and then the slides were digested with pepsin diluted with 3% citric acid for 30 min at 37 °C. The sections were fixed with 4% PFA containing 1/1000 diethylpyrocarbonate (DEPC) at room temperature for 10 min, and then prehybridized solution was added for incubation at 42 °C for 2 h. After hybridizing with miR-214-3p probe at 42 °C overnight, the sections were washed with SSC, and then the blocking solution, biotinylated mouse anti digoxin, strept avidin-biotin complex and biotinylated peroxidase were added successively. Finally, the slides were stained with DAB. The sections were dehydrated and observed using a microscope.

2.7. MiRNAs transfection and cell treatments

HCs were seeded in 6-well plate at density of 1.5 × 10^5/well to reach about 70% confluence after 48 h. Then cells were transfected with miR-214–3p mimic, scramble, miR-214–3p inhibitor and negative control (GenPharma Co. Shanghai, China) at a concentration of 30 nM by using Lipofectamine 2000 reagents (Life Technologies Corporation, Carlsbad, CA, USA) according to the manufacturer’s instructions. Cells were cultured with IL-1β (Sigma-Aldrich, St. Louis, Mo, USA) at the concentration of 10 ng/mL for 24 h before harvested. After 48 h transfection, the cells were collected and then were used for further experiments. All miRNA sequences were listed in Supplementary Table S2B.

2.8. Western blotting

Proteins were extracted from HCs by radio-immunoprecipitation assay buffer (RIPA, Beyotime, China), and protein concentrations were quantified by bicinchoninic acid (BCA) protein assay kit (Beyotime, China). Protein components were separated by 10% or 12% SDS-PAGE gels and transferred onto polyvinylidene fluoride (PVDF) membranes (Immobilon P, Millipore). Membranes were blocked in 10 mM Tris-buffered saline (TBS) containing 5% nonfat skimmed milk and probed for 2 h. After the incubation with a high affinity anti-COL2A1 antibody (1:1000, Abcam Cat# ab34712, RRID: AB_731,688), anti-SOX9 (1:1000, Proteintech Cat# 67,439–1-lg, RRID: AB_2,882,675), anti-MMP13 antibody (1:1000, Abcam Cat# ab84594, RRID: AB_10,562,126), anti-MMP3 antibody (1:1000, Abcam Cat# ab52915, RRID: AB_881,243), anti-cleaved-Caspase 3 (1:1000, Proteintech Cat# 19,677–1-AP, RRID: AB_10,733,244), anti-Bcl-2 (1:1000, Proteintech Cat# 12,789–1-AP, RRID: AB_2,227,948), anti-IKKβ (1:1000, Abcam Cat# ab124957, RRID: AB_10,975,710), anti-p-iKBx (1:1000, Cell Signaling Technology Cat# 2859, RRID: AB_561,111), anti-p-p65 (1:1000, Cell Signaling Technology Cat# 3033, RRID: AB_331,284), anti-p65 (1:1000, Cell Signaling Technology Cat# 8242, RRID: AB_10,859,369), and anti-GAPDH antibody (1:5000, Proteintech Cat# 60,004–1-lg, RRID:AB_2,107,436) in 5% BSA dilution at 4 °C overnight, washed with TBST, and then incubated with a secondary antibody (1:5000, Abclonal Cat# AS014, RRID: AB_2,769,854; Abclonal Cat# AS003, RRID: AB_2,769,851) for 1 h at room temperature. After washes, enhanced chemiluminescent imaging of the blots were detected using ECL (merck millipore) and a chemiluminescence system (Bio-Rad, USA) and processed using Image Lab Software.

2.9. Immunofluorescence staining

HCs were seeded on 15 mm cell slides (Nest Biotechnology) in 24-well plates. 4% PFA was first used to fix cells for 15 min, and 0.5% Triton X-100 was performed to permeate cells for 20 min. After blocked by 5% BSA for 30 min, chondrocytes were incubated with a primary antibody at 4 °C overnight and then with fluorescent Alexa Fluor® 555-conjugated secondary antibody in dark at 37 °C for 1 h. The antibodies used were anti-IKKβ (1:200, Abcam Cat# ab124957, RRID: AB_10,975,710), anti-p65(1:400, Cell Signaling Technology Cat# 8242, RRID: AB_10,859,369), and fluorescent Alexa Fluor® 555-conjugated secondary goat anti-rabbit antibodies (1:500, Cell Signaling Technology Cat# 4413, RRID: AB_10,694,110). Fluorescence images were obtained using Nikon Ti2-E.

2.10. Apoptosis detection

Apoptosis was detected using the Caspase-3/7 cell apoptosis detection kit (RiboAPO, China) according to the manufacturer’s instructions. Briefly, we removed the common medium, added mixed staining medium into each well, and incubated at 37 °C for 45 min. During dyeing and incubation, we diluted reagent B with fresh medium at the ratio of 100:1 to prepare an appropriate amount of 1 × PI staining medium. After incubation, removed the mixed staining medium, added 1 × PI staining medium into each well, and incubated at room temperature for 5 min. Washed twice with PBS carefully, and replaced it with fresh medium. Specimens were detected by fluorescence microscopy Nikon Ti2-E. Caspase-3/7 green fluorescence, PI red fluorescence and hoechst33342 blue fluorescence could distinguish apoptotic cells, dead cells and living cells, respectively.

2.11. Luciferase assay

HEK-293T cells were seeded in 6-well plates 24 h before transfection. 500 ng plasmids of IKKB1 3’UTR-wt and IKKB1 3’UTR -mut, 20 nmol miR-214–3p and NC were cotransfected with Lipofectamine 3000 (Invitrogen; Carlsbad, CA, USA) according to the manufacturer’s instructions. After 48 h incubation, firefly and Renilla luciferase activities were calculated by the Promega Dual-Luciferase system according to the manufacturer’s instructions. Luciferase Assay Reagent II (LAR II) (Luciferase Assay Reagent, Promega) was used to measure firefly luciferase activities while Stop & Glo® Reagent (Luciferase Assay Reagent, Promega) was used to measure renilla luciferase activities. Firefly/Renilla luciferase were measured to evaluate relative luciferase activity.
2.14. Histological, immunofluorescence and tunel analysis

The knee joints from human and mice were fixed in 4% PFA (bio-sharp) and then decalcified with EDTA. For Safranin O-Fast Green staining, each paraffin-embedded sample was sectioned at 4 μm, and was stained with 0.2% Safranin O solution for 15 min and 0.2% Fast Green solution for 5 min (Sigma-Aldrich, USA). For immunofluorescence, after conventional dewaxing, the sections were repaired with EDTA repair solution in a boiling water bath for 30 min. Then, 0.5% Triton (Regan) was used to permeate the membrane for 20 min intraperitoneally, the right knee joint of mice was exposed to stereomicroscope through a medial capsular incision. Then the medial meniscocellular ligament was resected, and the medial meniscus was displaced medially. Finally, the incision was stitched, and the skin was closed. Mice in the sham and OA groups underwent articular injection. The right knee articulation was harvested to conduct a further section for 1 h at 37 °C in dark. Evaluated the section under a fluorescence and tunel analysis. The decreased expression of miR-214 3p might contribute to its pathogenesis. To test it, we transfected miR-214 3p inhibitor or mimics into chondrocytes and assessed the effect of miR-214 3p on IL-1β-induced ECM degradation and cell apoptosis. As expected, transfection of chondrocytes with miR-214 3p inhibitor destabilized miR-214 3p and caused a 74% decrease in miR-214 3p level while miR-214 3p was elevated by 375.7-fold in the cells transfected with miR-214 3p mimics compared to the cells transfected with a scramble control (Fig. 2a). To determine whether miR-214 3p in chondrocytes contributes to the ECM degradation and cell apoptosis, HCs were treated with or without IL-1β in the presence of miR-214 3p mimic or inhibitor. MiR-214 3p mimic or inhibitor significantly inhibited COL2A1 and SOX9 mRNA levels while increased MMP3 and MMP13 expression (Fig. 2b-e). In contrast, transfection of the cells with miR-214 3p mimic caused elevated expression of COL2A1 and SOX9 but decreased expression of MMP3 and MMP13 (Fig. 2f-i). Consistent with the mRNA levels, western blotting further confirmed the function of miR-214 3p in ECM metabolism (Fig. 2j and k; Supplementary Fig. 2a-h). To explore the effects of miR-214 3p overexpression or inhibition on IL-1β-induced HCs apoptosis, we detected apoptosis-related proteins including cleaved-caspase 3 and Bcl2. The results showed that miR-214 3p inhibition increased the expression of cleaved-caspase 3 and decreased Bcl2 expression, while miR-214 3p overexpression decreased cleaved-caspase 3 and increased Bcl2 expression (Fig. 2l and m; Supplementary Fig. 2i-l). To further demonstrate if miR-214 3p influences human chondrocytes apoptosis, immunofluorescence assay of caspase-3/7 and PI was performed. Caspase 3/7/PI+ cells were indicative of early apoptotic cells, while caspase 3/7/PI− labeled late apoptotic or dead cells. Consistently, IL-1β-induced HCs apoptosis and cell death was also significantly increased after miR-214 3p inhibition while markedly decreased with miR-214 3p overexpression.

2.16. Role of funding source

The funders had no role in study design, data collection, interpretation and analysis, decision to publish or preparation of the manuscript.

3. Results

3.1. MiR-214 3p is decreased in IL-1β-stimulated chondrocytes and OA cartilage tissues

To identify the expression level of miR-214 3p in chondrocytes, we incubated human and mice chondrocytes with IL-1β or vehicle. We found that IL-1β treatment of human and mice chondrocytes decreased miR-214 3p expression in a time- and dose-dependent manner (Fig. 1a and b). We then examined the expression of miR-214 3p by ISH and compared its expression level between damaged and undamaged regions of articular cartilage from human OA patients. The OA cartilage damaged area exhibited obvious proteoglycan loss as shown by reduced Safranin O staining and significantly reduced miR-214 3p expression in damaged regions compared with undamaged regions of arthritic cartilage. In the undamaged cartilage regions, miR-214 3p was located in the cytoplasm of chondrocytes (Fig. 1c). To determine if downregulated expression of miR-214 3p and increased cartilage degeneration were conserved in an experimental mouse model of OA induced by DMM surgery, ISH analysis showed an apparent decrease in the number of miR-214 3p positive chondrocytes in OA cartilage compared with sham group (Fig. 1d). These results indicated that miR-214 3p expression was downregulated in inflamed chondrocytes and knee OA cartilage.

3.2. MiR-214 3p partially inhibits IL-1β-induced ECM degradation and apoptosis in HCs

The decreased expression of miR-214 3p in OA indicated that miR-214 3p might contribute to its pathogenesis. To test it, we transfected miR-214 3p inhibitor or mimics into chondrocytes and assessed the effect of miR-214 3p on IL-1β-induced ECM degradation and cell apoptosis. As expected, transfection of chondrocytes with miR-214 3p inhibitor destabilized miR-214 3p and caused a 74% decrease in miR-214 3p level while miR-214 3p was elevated by 375.7-fold in the cells transfected with miR-214 3p mimics compared to the cells transfected with a scramble control (Fig. 2a). To determine whether miR-214 3p in chondrocytes contributes to the ECM degradation and cell apoptosis, HCs were treated with or without IL-1β in the presence of miR-214 3p mimic or inhibitor. MiR-214 3p inhibitor significantly inhibited COL2A1 and SOX9 mRNA levels while increased MMP3 and MMP13 expression (Fig. 2b-e). In contrast, transfection of the cells with miR-214 3p mimic caused elevated expression of COL2A1 and SOX9 but decreased expression of MMP3 and MMP13 (Fig. 2f-i). Consistent with the mRNA levels, western blotting further confirmed the function of miR-214 3p in ECM metabolism (Fig. 2j and k; Supplementary Fig. 2a-h). To explore the effects of miR-214 3p overexpression or inhibition on IL-1β-induced HCs apoptosis, we detected apoptosis-related proteins including cleaved-caspase 3 and Bcl2. The results showed that miR-214 3p inhibition increased the expression of cleaved-caspase 3 and decreased Bcl2 expression, while miR-214 3p overexpression decreased cleaved-caspase 3 and increased Bcl2 expression (Fig. 2l and m; Supplementary Fig. 2i-l). To further demonstrate if miR-214 3p influences human chondrocytes apoptosis, immunofluorescence assay of caspase-3/7 and PI was performed. Caspase 3/7/PI+ cells were indicative of early apoptotic cells, while caspase 3/7/PI− labeled late apoptotic or dead cells. Consistently, IL-1β-induced HCs apoptosis and cell death was also significantly increased after miR-214 3p inhibition while markedly decreased with miR-214 3p overexpression.

2.12. Bioinformatics analysis of mRNA–miRNA interactions

Online databases including miRDB (http://mirdb.org/), Targetscan (http://www.targetscan.org/) and starbase (http://starbase.sysu.edu.cn/) were used to predict the targets of miR-214 3p. These data were intersected with NF-kB core genes [28]. A Venn diagram was constructed to show the overlapping interactions by a web-based tool (http://bioinformatics.psb.ugent.be/webtools/Venn/).
Collectively, these results suggested that overexpression of miR-214/C03p partially reversed IL-1β-induced cartilage matrix degradation and cell apoptosis in HCs.

3.3. MiR-214/C03p directly targets IKBKB

Numerous studies showed that miRNAs in the cytoplasm could bind to 3′-UTR of target mRNAs and subsequently induce their degradation [10]. MiR-214/C03p was reported to directly target β-catenin and inhibit WNT/β-catenin pathway [29, 30]. However, in our study, we found that miR-214/C03p had no significant effect on β-catenin (Supplementary Fig. 3a and b). To explore potential miR-214/C03p targeting genes associated with NF-κB pathway, we performed bioinformatics analysis with human public database, including miRDB, TargetScan and starBase and merged them with 17 NF-κB core genes [28]. The result showed that IKBKB could be the potential targeted gene of miR-214/C03p (Fig. 3a). Next, immunofluorescence analysis detected a significance increase of IKKB expression in the OA-damaged cartilage as compared to the undamaged cartilage (Fig. 3b). Similar results were also found in the mice OA models (Supplementary Fig. 3c). qRT-PCR and western blotting analysis results demonstrated that the IKBKB mRNA and protein (IKKB) were negatively linked to miR-214/C03p in IL-1β-treated HCs (Fig. 3c-f; Supplementary Fig. 3d and e). Immunofluorescence assays consistently confirmed that miR-214/C03p significantly inhibited the expression of IKKB (Fig. 3g). Target prediction algorithms showed that human and mouse IKKB 3′-UTR regions were identified as the target of miR-214/C03p, which contained putative seed sequences (Fig. 3h). To further verify whether miR-214/C03p directly binds with the 3′-UTR of IKBKB mRNA, we conducted luciferase activity assay. MiR-214/C03p mimics markedly repressed the luciferase activity of the reporter gene in the cells transfected with wild type (WT) 3′-UTR of IKBKB, but not in those transfected with mutated (Mu) 3′-UTR of IKBKB. Taken together, our data showed that miR-214/C03p directly targeted IKBKB in chondrocytes.

3.4. MiR-214–3p inhibits the activation of NF-κB signaling pathway

IKKβ is a pivotal protein phosphorylating IκBα, leading to its degradation and activates the NF-κB signaling pathway. To detect the activation of NF-κB pathway, we next determined the phosphorylation of IκBα and p65 in HCs after transfection with miR-214–3p mimics or inhibitor. The results suggested that IL-1β markedly
activated the NF-κB pathway by increasing p- IkBα and p-p65, whereas miR-214–3p inhibition significantly increased the IkBα and p65 phosphorylation without alteration of total p65 in IL-1β-stimulated chondrocytes (Fig. 4a). However, miR-214–3p overexpression dramatically decreased p-IκBα and p-p65 (Fig. 4b). As IkBα is an inhibitor of the nuclear translocation NF-κB dimers (p50–p65) isoform, we investigated whether miR-214–3p delayed NF-κB activation through suppressing p65 translocation to the nucleus. Immunofluorescence assays showed that nuclear translocation of p65 was increased after transfection with miR-214–3p inhibitor, while miR-214–3p overexpression exerted opposite effects (Fig. 4c and d). Collectively, these results suggested that miR-214–3p significantly suppressed the activation of NF-κB pathway in inflammatory cytokine stimulated chondrocytes.
3.5. MiR-214–3p exerts biological functions in chondrocytes via targeting IKKβ

Next, we conducted rescue experiments to examine whether the effects of miR-214–3p expression on OA phenotypes were achieved through IKKβ. Chondrocytes were transfected with two different siRNAs (siIKKβ1 and siIKKβ2) to successfully inhibit IKKβ expression (Fig. 5a). Co-transfection of miR-214–3p inhibitor and siIKKβ into chondrocytes reversed the effect of miR-214–3p inhibition on down-regulating COL2A1 and SOX9 expression and increasing the expression of MMP3 and MMP13 by qRT-PCR (Fig. 5b–e). Changes in the protein levels of these ECM anabolic and catabolic markers also supported this reversed effects by western blotting analysis (Fig. 5f; Supplementary Fig. 4a–d). Furthermore, increased cleaved-caspase 3 and decreased Bcl2 were observed in the IL-1β and miR-214–3p inhibitor group compared with the control group, while cleaved-caspase 3 was significantly downregulated and Bcl2 was elevated in the group with siIKKβ co-transfection (Fig. 5g; Supplementary Fig. 3e and f).

Moreover, immunofluorescence assays suggested that the induction of cell apoptosis observed after miR-214–3p silence was also suppressed by inhibiting IKKβ (Fig. 5h). Therefore, our data demonstrated that miR-214–3p inhibited the ECM catabolism and chondrocyte apoptosis by targeting IKKβ.

3.6. Intra-articular (IA) delivery of miR-214–3p alleviates OA in mouse models

To determine the in vivo role of miR-214–3p during OA progression, we conducted IA injection in non-surgical mice with miR-214–3p antagomir (or its NC) and OA mice with miR-214–3p agomir (or NC) for 10 weeks (Fig. 6a and b). Safranin O and Fast Green staining (SOFG) showed miR-214–3p inhibition with antagomir spontaneously induced cartilage destruction while miR-214–3p overexpression attenuated OA progression in DMM-induced OA mice with the injection of miR-214–3p agomir (Fig. 6c and d). The staining also showed that inhibition of miR-214–3p might increase the...
4. Discussion

This study elucidated the biological role of miR-214–3p in the initiation and progression of OA. We presented a pathway in which inflammatory cytokines induced the downregulation of miR-214–3p in chondrocytes. Decreased miR-214–3p activated the NF-κB signaling pathway and promoted OA development through targeting IKKβ (Fig. 6k). Thus, our findings demonstrated that miR-214–3p might be a novel potential therapeutic target for OA prevention and treatment.

MiRNAs, a type of conserved endogenous non-coding RNAs, regulate target miRNA expression via translational suppression or mRNA degradation [31,32]. Evidences have demonstrated that certain miRNAs could directly target multiple mRNAs related to the development of OA, and play crucial roles in mediating the biological functions of chondrocytes [33,34]. For instance, miR-218–5p was upregulated in OA patients and could target the seed region of the PIK3C2A mRNA 3’UTR, leading to articular cartilage degradation [35]. Moreover, miR-127–5p was reported to directly target cartilage matrix protein MMP13 and regulate Col2a1 expression to stimulate ECM destruction and cell apoptosis [36]. IA injection of LNA-miR-181a-5p ASO could reduce loss of chondrocyte components in preclinical models of lumbar facet joint and knee OA [37]. Although miR-214–3p were mainly focused on its down-expression in tumor tissues and its inhibition of apoptosis and promotion of the bone formation, its role in OA cartilage degradation remained unclear [38,39]. Herein, we found that miR-214–3p had a protective effect against chondrocyte degradation. Overexpression of miR-214–3p reduced the expression of the matrix metalloproteinases, such as MMP3 and MMP13, and inhibited cell apoptosis but increased the expression of COL2A1 and SOX9. Therefore, to our knowledge, this is the first report to highlight the biological significance of miR-214–3p in protection of OA.

IKKβ belongs to IKK complex that represents an indispensable member of NF-κB family. Recent studies have indicated that IKKβ, in addition to its role as a initiator of NF-κB pathway activation, could be involved in maintaining the dynamic homeostasis of ECM microenvironment [40,41]. For example, icariin could reduce the production of inflammatory factors and inhibit chondrocyte apoptosis through affecting its hub gene IKKβ [42]. Furthermore, pomegranate fruit extract suppressed the activation of NF-κB pathway by inhibiting the expression of IKKβ, thereby exerting chondroprotective effect [43]. Several studies have demonstrated that miRNAs affected cartilage metabolism by inhibiting or activating NF-κB pathway, thus alleviating or aggravating the progression of OA [44–46]. In our research, we analyzed the downstream genes of miR-214–3p and found that miR-214–3p may target multiple genes. However, our results suggest that miR-214–3p is related to inflammatory factors, and could directly target IKKβ, which could activate NF-κB pathway to disrupt the equilibrium of ECM metabolism. Moreover, we revealed that IKKβ was upregulated in the joint damaged zone, and was regulated by miR-214–3p. In OA damaged cartilage, decreased miR-214–3p maintained IKKβ expression and promoted the activation of NF-κB pathway. Knockdown of IKKβ significantly reversed the pro-apoptosis and cartilage destruction of a miR-214–3p antagonist, confirming that IKKβ was the direct target of miR-214–3p to suppress ECM degeneration and chondrocyte apoptosis. Hence,
unraveling the role of miR-214–3p on IKKβ/NF-κB pathway will shed light on the prevention and treatment of OA.

IA miRNA treatments have come to light in recent years. Clinicians may have more options to provide patients with effective and reliable therapies that have fewer side effects [47]. In previous studies, IA gene delivery systems mainly focused on LNA-miR-ASO, lentiviruses, adenovirus vectors and atelocollagen [37,48,49]. These biologic delivery systems mainly focused on LNA-miR-ASO, lentiviruses, adenovirus vectors and atelocollagen [37,48,49]. These biologic delivery systems have exhibited good safety, but they may have biotoxic effects or off-target effects. Currently, a plenty of studies have demonstrated that IA injection of synthetic antago-miR-483 significantly delayed the onset of OA [51]. In this study, miRNA-214–3p and IKKβ inhibition. The data were normalized to GAPDH. (f) Western blotting analysis of COL2A1, SOX9, MMP3, MMP13 protein levels in chondrocytes after miR-214–3p inhibition or a combination of miR-214–3p and IKKβ inhibition. The data were normalized to GAPDH. (g) Western blotting analysis of cleaved-caspase 3 and Bcl2 expression in chondrocytes after miR-214–3p stimulation. (h) Representative immunofluorescence images of caspase-3/7 (green) and PI (red) after miR-214–3p knockdown or/with IKKβ inhibition (left) and the quantification (right). PI, propidium iodide. Scale bars, 100 μm. ns: no significant difference, *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001. All data are shown as means ± SEM of three independent experiments in (b), (c), (d), (e) and (h). Student’s t-test and one-way ANOVA were used for comparison between two groups and multiple groups, respectively.

This study has some limitations. First, according to the data from miRBase, miR-214–3p is homologous to human and mouse. We investigated the involvement of miR-214–3p in regulating human chondrocyte ECM metabolism and cell apoptosis and explored its possible underlying molecular mechanisms in OA pathogenesis both in vitro and in vivo. Our results suggested that miR-214–3p was decreased after IL-1β stimulation in both human and mice primary chondrocytes. Thus, it is reasonable to suppose that miR-214–3p can exert the protective effects in mice primary chondrocytes. Of note, the mechanism of the decrease in miR-214–3p levels during the deteriorative process remains undetectable. Previous studies revealed that m6A mark acted as a key post-transcriptional modification that promoted the initiation of miRNA biogenesis [52]. Moreover, H3K4me3 demethylase, bound to the miRNA promoter, which led to inhibition of its transcription and expression [53]. SnorRNAs, involving in ribosome biogenesis and RNA modification, acted as endogenous sponges that regulate miRNA expression [54]. These studies suggested that several mechanisms were involved in miRNA biogenesis. In this study, we found that decreased miR-214–3p activated NF-κB pathway and promoted osteoarthritis. However, the detailed mechanism that inflammatory cytokines induce the decrease of miR-214–3p remains further investigation.
In conclusion, our study revealed the biological role of miR-214–3p in OA development. Decreased miR-214–3p promotes ECM degradation and chondrocyte apoptosis via activation of NF-κB pathway. Intra-articular delivery of miR-214–3p agomir may be a novel promising approach in OA therapy.

**Contributions**

ST and CD designed the experiments and directed the study. YC, ST, XN, ZZ, GR, WH and ZZ conducted experiments, data analysis and interpretation. YC and ST wrote the manuscript. All authors...
have read and verified the underlying data, and approved the final version of the manuscript.

Declaration of Competing Interest

The authors declare no conflict of interest.

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Data Sharing Statement

All reagents used in this work are available upon request and a brief statement describing the purpose for their use.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/ebiomed.2021.103283.

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