EBioMedicine 65 (2021) 103283

Contents lists available at ScienceDirect

EBioMedicine

journal homepage: www.elsevier.com/locate/ebiom

Research paper

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

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ARTICLE INFO

Article History:
Received 25 October 2020
Revised 12 February 2021
Accepted 28 February 2021
Available online 11 March 2021

Keywords:
Osteoarthritis
miR-214–3p
NF-κB
IKKβ

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 (*IKBKB*), 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 antagomir in mice joints triggered spontaneous cartilage loss while miRNA-214–3p agomir 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.

Funding: This study was financially supported by grants from the National Natural Science Foundation of China (81,773,532, 81,974,342).

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,

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https://doi.org/10.1016/j.ebiom.2021.103283

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-κB signaling pathway, and the miR-214−3p/IKKβ/NF-κB 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-κB signaling pathway. These data suggest that the miR-214−3p/IKKβ/NF-κB 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-κB) 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 IκBα phosphorylated in the cytoplasm and degraded subsequently. The NF-κB 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-κB 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-κB 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-κB 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. Furthermore, we investigated that downregulation of miR-214−3p activated the NF-κB 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-κB 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. Safranin O/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 cm^2 culture flasks in a 37 °C humidified atmosphere with 5% CO_2. 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 cm^2 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 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 $2^{-\triangle\triangle Ct}$ method was used to evaluate the relative mRNA/miRNA 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 slices 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-iKBα (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-Ig, 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 *IKBKB* 3′UTR-wt and *IKBKB* 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.

2.12. 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-κB 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/).

2.13. Animal experiments

Animal handling and experimental procedures were performed with the approval from Zhujiang Hospital of Southern Medical University Ethics Committee (LAEC-2019–004). A total of 20 adult (aged 10 weeks) male C57BL/6 mice weighing about 25 g were divided into four groups randomly. Groups included the following: sham with negative control (NC) (miR-214–3p antagomir NC), sham with antagomir (miR-214–3p antagomir), OA with NC (miR-214–3p agomir NC), and OA with agomir (miR-214–3p agomir). A model of OA was induced by surgical destabilization of the medial meniscus (DMM) surgery. Briefly, after anesthetized with amobarbital sodium (25 mg/kg) intraperitoneally, the right knee joint of mice was exposed to stereomicroscope through a medial capsular incision. Then the medial meniscotibial 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 of miR-214–3p antagomir or NC and miR-214–3p agomir or NC into the articulation every two weeks. A total of 10 μL solution (approximately 4 mol/mL) was slowly injected into right knees. All mice were sacrificed at 70 days after initial surgery or the first injection. The right knee articulation was harvested to conduct a further evaluation.

2.14. Histological, immunofluorescence and tunel analysis

The knee joints from human and mice were fixed in 4% PFA (biosharp) 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 and 5% BSA (MCE, China) was used for blocking for 1 h. After that, the slices were incubated with primary antibodies (diluted 1:100 by BSA, MMP3: Abcam Cat# ab52915, RRID: AB_881,243; MMP13:Proteintech Cat# 18,165–1-AP, RRID: AB_2,144,858) at 4 °C overnight, washed thrice with PBST and incubated with the fluorescent Alexa Fluor® 555-conjugated secondary antibody (Cell Signaling Technology Cat# 4413, RRID: AB_10,694,110) at 37 °C for 1 h. Finally, the nuclei were stained with DAPI. Fluorescence images were acquired using Nikon Ti2-E. For TUNEL staining, after incubation with 0.5% Triton (Regan) for 15 min, rinsing slide(s) three times in PBS for 5 min each, adding 25 μL TUNEL reaction mixture (Sigma, Roche) on the section for 1 h at 37 °C in dark. Evaluated the section under a fluorescence microscope in the range of 523–555 nm (green).

2.15. Statistical analysis

Data are reported as the mean ± SEM or SD based on at least triplicate samples. Statistical analysis was performed by unpaired two-tailed Student's *t*-test, one-way analysis of variance (ANOVA) or Mann-Whitney U test for comparisons between groups. $P < 0.05$ (two-sided) was considered statistically significant for all of the statistical calculations. PRISM 6.0 (GraphPad Software, San Diego, CA, USA) was applied to data analysis.

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 (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

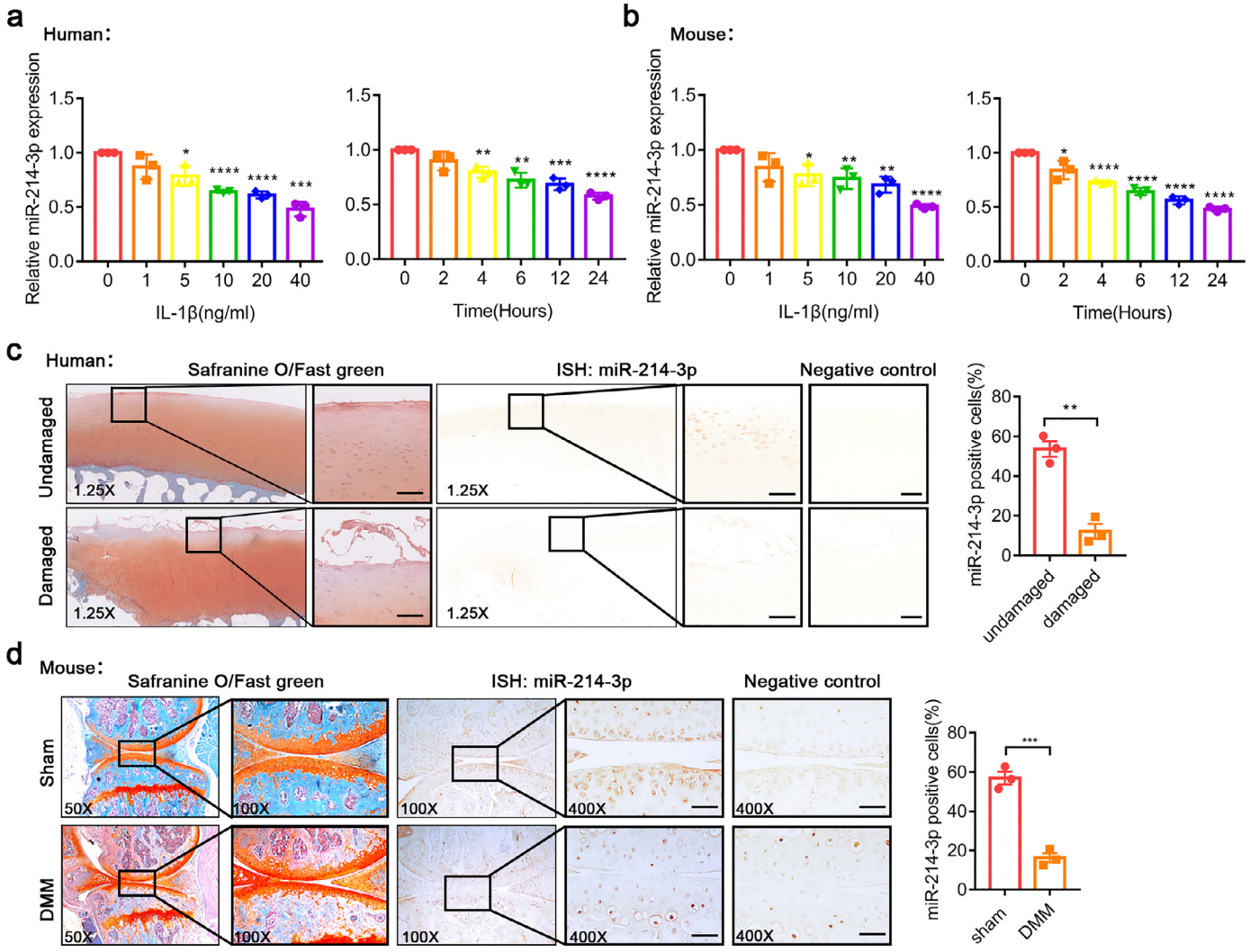

Fig. 1. MiR-214−3p is downregulated in IL-1β stimulated human chondrocytes and OA cartilage tissues. (a) qRT-PCR analysis of miR-214−3p expression in primary human chondrocytes stimulated with 10 ng/mL IL-1β for 0, 2, 4, 6, 12 or 24 h and at 0, 1, 5, 10, 20, or 40 ng/mL for 24 h. (b) Dose and time-dependent downregulation of miR-214−3p expression in murine primary chondrocytes. (c) Safranin O/fast green staining (left panel) and in situ hybridization (ISH) (right panel) of the cartilage from OA patients. Scale bars, 50 μm. (d) Safranin O/fast green staining (left panel) and ISH (right panel) of the cartilage from sham or DMM-induced OA mice knee tissues. Scale bars, 50 μm. $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$, $^{****}P<0.0001$. All data are shown as means ± SEM of three independent experiments in (a) and (b). Student's *t*-test was used for comparison.

(Fig. 2n-p). Collectively, these results suggested that overexpression of miR-214−3p partially reversed IL-1β-induced cartilage matrix degradation and cell apoptosis in HCs.

3.3. MiR-214−3p 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-214−3p was reported to directly target β-catenin and inhibit WNT/β-catenin pathway [29, 30]. However, in our study, we found that miR-214−3p had no significant effect on β-catenin (Supplementary Fig. 3a and b). To explore potential miR-214−3p 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-214−3p (Fig. 3a). Next, immunofluorescence analysis detected a significance increase of IKKβ 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 (IKKβ) were negatively linked to miR-214−3p in IL-1β-treated HCs (Fig. 3c-f; Supplementary Fig. 3d and e). Immunofluorescence assays consistently confirmed that miR-214−3p significantly inhibited the expression of IKKβ (Fig. 3g). Target prediction algorithms showed that human and mouse IKKβ 3′UTR regions were identified as the target of miR-214−3p, which contained putative seed sequences (Fig. 3h). To further verify whether miR-214−3p directly binds with the 3′-UTR of *IKBKB* mRNA, we conducted luciferase activity assay. MiR-214−3p 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*. (Fig. 3i). Taken together, our data showed that miR-214−3p 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

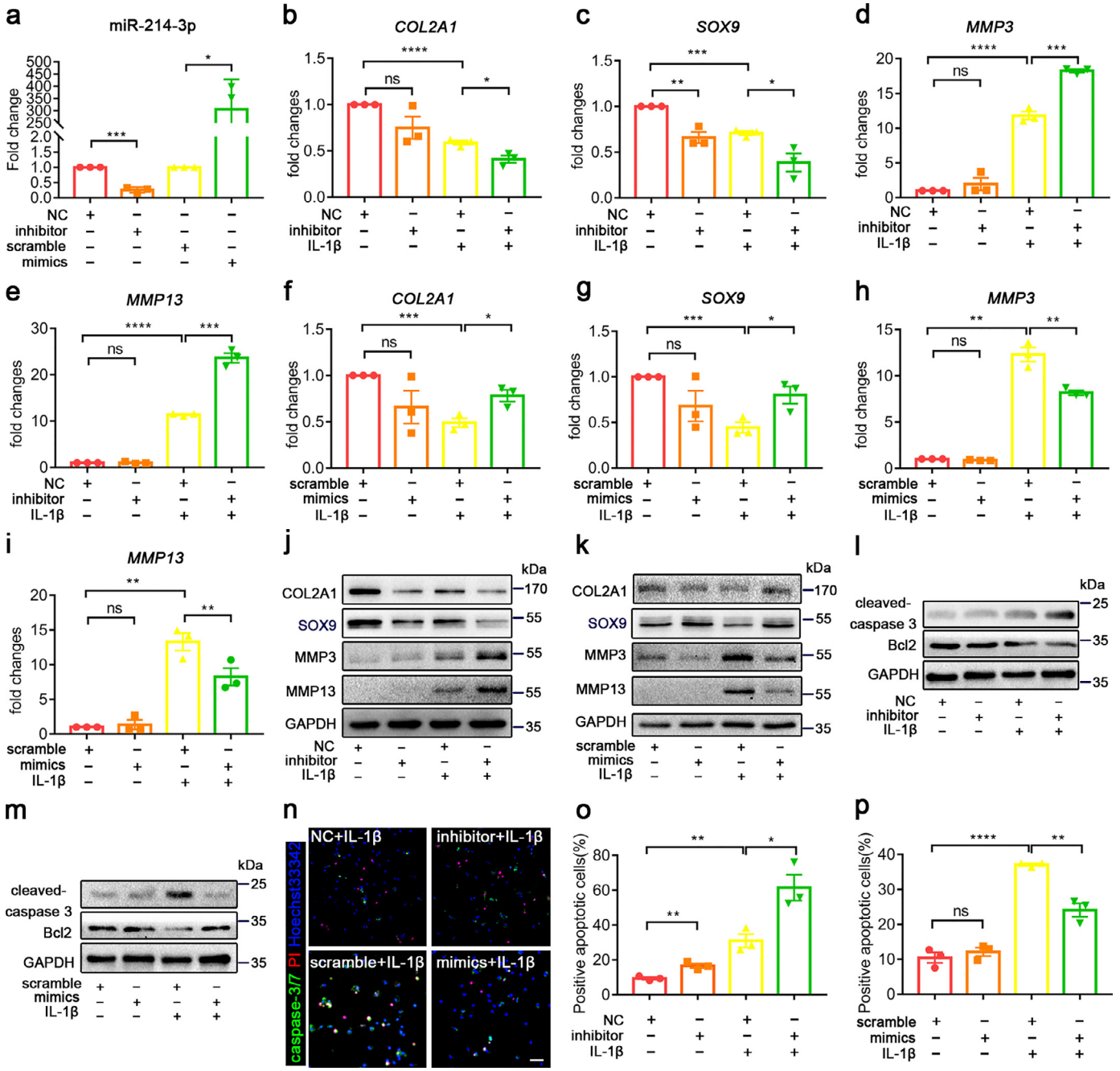

Fig. 2. MiR-214–3p inhibits IL-1β induced extracellular matrix degradation and apoptosis in chondrocytes. (a) Human chondrocytes were transfected with miR-214–3p inhibitor or negative control and miR-214–3p mimic or scramble at a final concentration of 30 nM. After 24 h of transfection, miR-214–3p levels in human chondrocytes were evaluated by qRT-PCR. (b-e) qRT-PCR analysis of *COL2A1* (b), *SOX9* (c), *MMP3* (d) and *MMP13* (e) in human chondrocytes transfected with miR-214–3p inhibitor or negative control with or without IL-1β stimulation. (f-i) qRT-PCR analysis of *COL2A1* (f), *SOX9* (g), *MMP3* (h) and *MMP13* (i) in human chondrocytes transfected with miR-214–3p mimics or scramble with or without IL-1β stimulation. (j, k) Western blotting analysis of COL2A1, SOX9, MMP3 and MMP13 protein levels in chondrocytes after miR-214–3p inhibition (j) and overexpression (k). (l, m) Western blotting analysis of cleaved-caspase 3 and Bcl2 expression in chondrocytes after miR-214–3p inhibition (l) and overexpression (m). (n) Representative immunofluorescence images of caspase-3/7 (green) and PI (red) after miR-214–3p knockdown or overexpression with IL-1β. PI, propidium iodide. Scale bars, 100 μm. (o, p) Quantification of positive apoptotic cells after miR-214–3p inhibition (o) or overexpression (p). 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 (a), (b), (c), (d), (e), (k), (f), (g), (h), (i), (o)and (p). Student's *t*-test and one-way analysis of variance (ANOVA) were used for comparison between two groups and multiple groups, respectively.

activated the NF-κB pathway by increasing p-iκBα and p-p65, whereas miR-214–3p inhibition significantly increased the IκBα 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 IκBα 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 transfected 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.

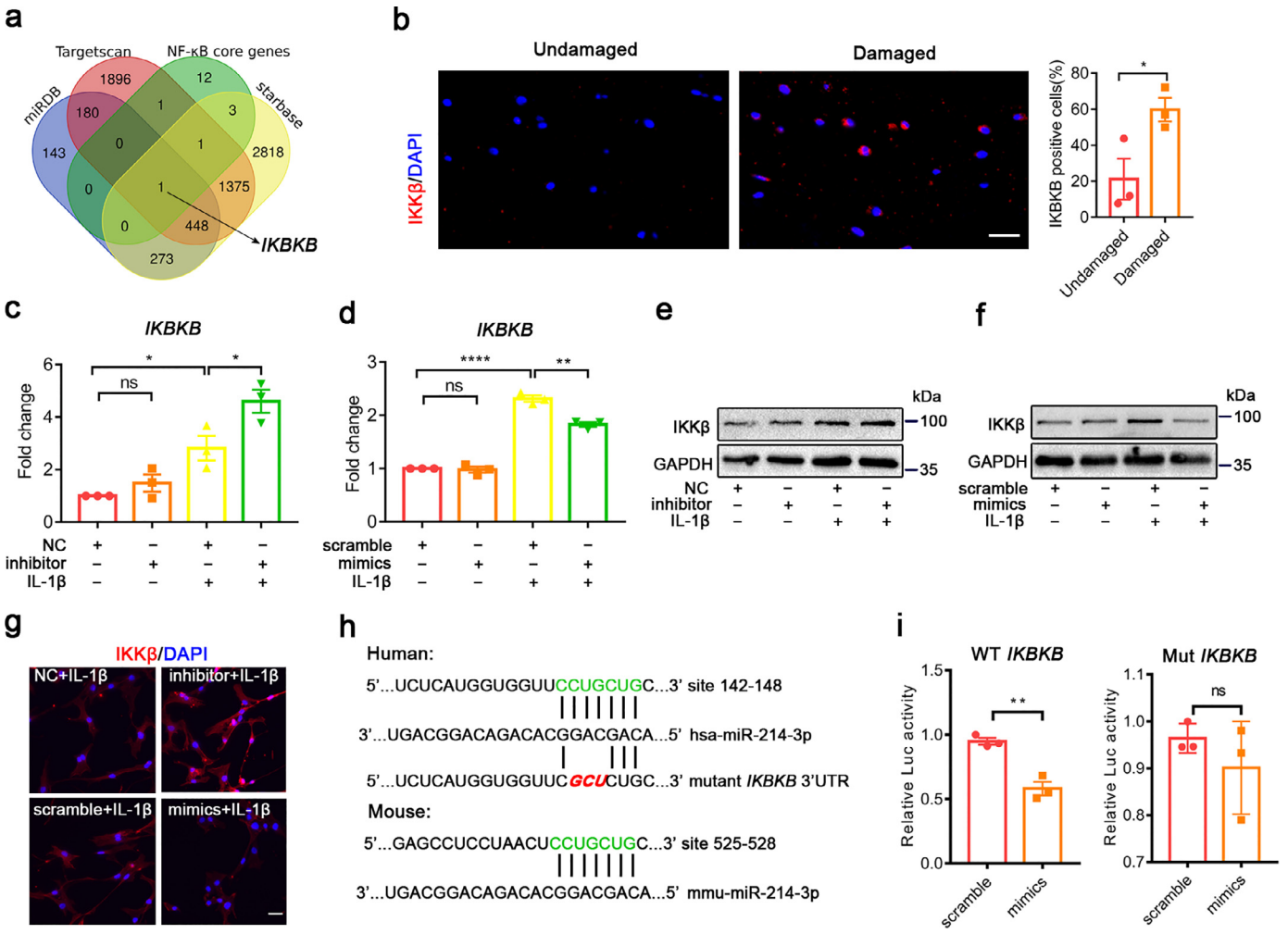

Fig. 3. Identification of *IKBKB* as a direct target of miR-214−3p in chondrocytes. (a) Venn diagram display the overlapping of the human target genes of miR-214−3p, as predicted by Targetscan, miRDB, starbase and NF-κB core genes. (b) Immunofluorescence analysis (left) and quantification data (right) of IKKβ of human cartilage in undamaged and damaged areas in OA patients. (c, d) qRT-PCR analysis of *IKBKB* in human chondrocytes transfected with miR-214−3p inhibitor (c) or miR-214−3p mimics (d) with or without IL-1β stimulation. (e, f) Western blotting analysis of IKKβ protein levels in chondrocytes after miR-214−3p inhibition (e) or overexpression (f). The data were normalized by GAPDH. (g) Representative images of IKKβ assayed by immunofluorescence confocal microscopy in human chondrocytes with miR-214−3p knockdown or overexpression with IL-1β. DAPI, 4′,6-diamidino-2-phenylindole. Scale bar, 25 μm. (h) Sequence alignment of a putative miR-214−3p binding site within the 3′-UTR of *IKBKB* mRNA shows a high level of sequence conservation and complementarity with miR-214−3p. (i) HEK-293T cells were co-transfected with miR-214−3p mimics or scramble and luciferase reporter constructs of the wild-type IKBKB-3′UTR (3′UTR-wt) or the mutated IKBKB-3′UTR (3′UTR mut). Luciferase activity was measured after transfection. Luciferase reporter assay revealed that miR-214−3p exclusively decreased luciferase activity of the wild-type reporter plasmids. 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) and (i). Student's *t*-test and one-way ANOVA were used for comparison between two groups and multiple groups, respectively.

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 (siIKKβ−1 and siIKKβ−2) 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 downregulating *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

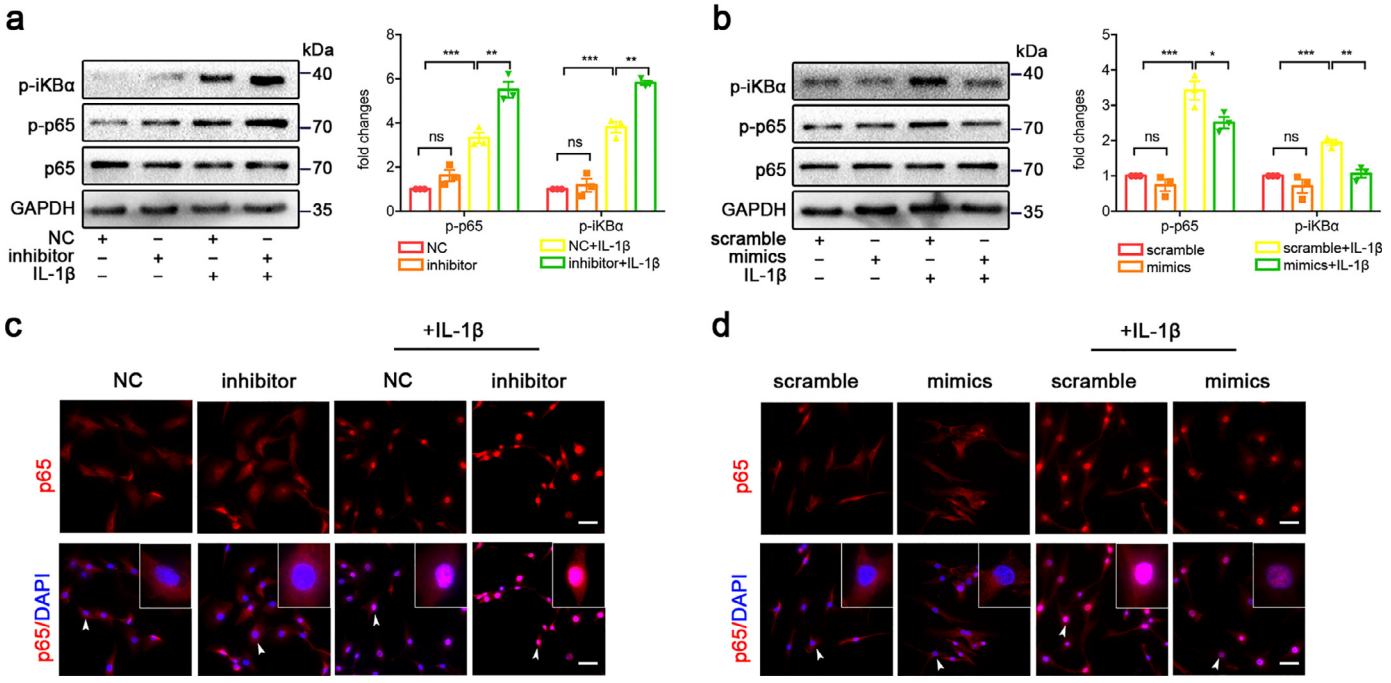

Fig. 4. MiR-214–3p inhibits the activation of NF-κB signaling pathway. (a) Western blotting analysis and quantification data of p-iKBα and p-p65 in chondrocytes after miR-214–3p inhibition. The data were normalized to GAPDH. (b) Western blotting analysis and quantification data of p-iKBα and p-p65 in chondrocytes after miR-214–3p overexpression. The data were normalized to GAPDH. (c, d) Representative images of p65 translocation assayed by immunofluorescence confocal microscopy in human chondrocytes with miR-214–3p knockdown (c) or overexpression (d). DAPI, 4′,6-diamidino-2-phenylindole. Scale bar, 25 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 (a) and (b). Student's *t*-test and one-way ANOVA were used for comparison between two groups and multiple groups, respectively.

synovitis and meniscus degeneration while overexpression miR-214–3p might attenuate the synovitis and meniscus degeneration during OA progression (Supplementary Fig. 5). To analyze the effect of miR-214–3p on the catabolic activities of cartilage, we examined the pathological changes in articular cartilage. The injection of miR-214–3p antagomir aggravated the deteriorative changes in the cartilage matrix while miR-214–3p agomir alleviated these in OA, as showed catabolic response (Fig. 6e-h) and decreased apoptotic cells (Fig. 6i and j) by immunofluorescence. These results revealed that miR-214–3p might impart cartilage protective effects by attenuating articular cartilage apoptosis and inhibiting ECM catabolism in vivo.

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 mRNA 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 *IKBKB* [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,

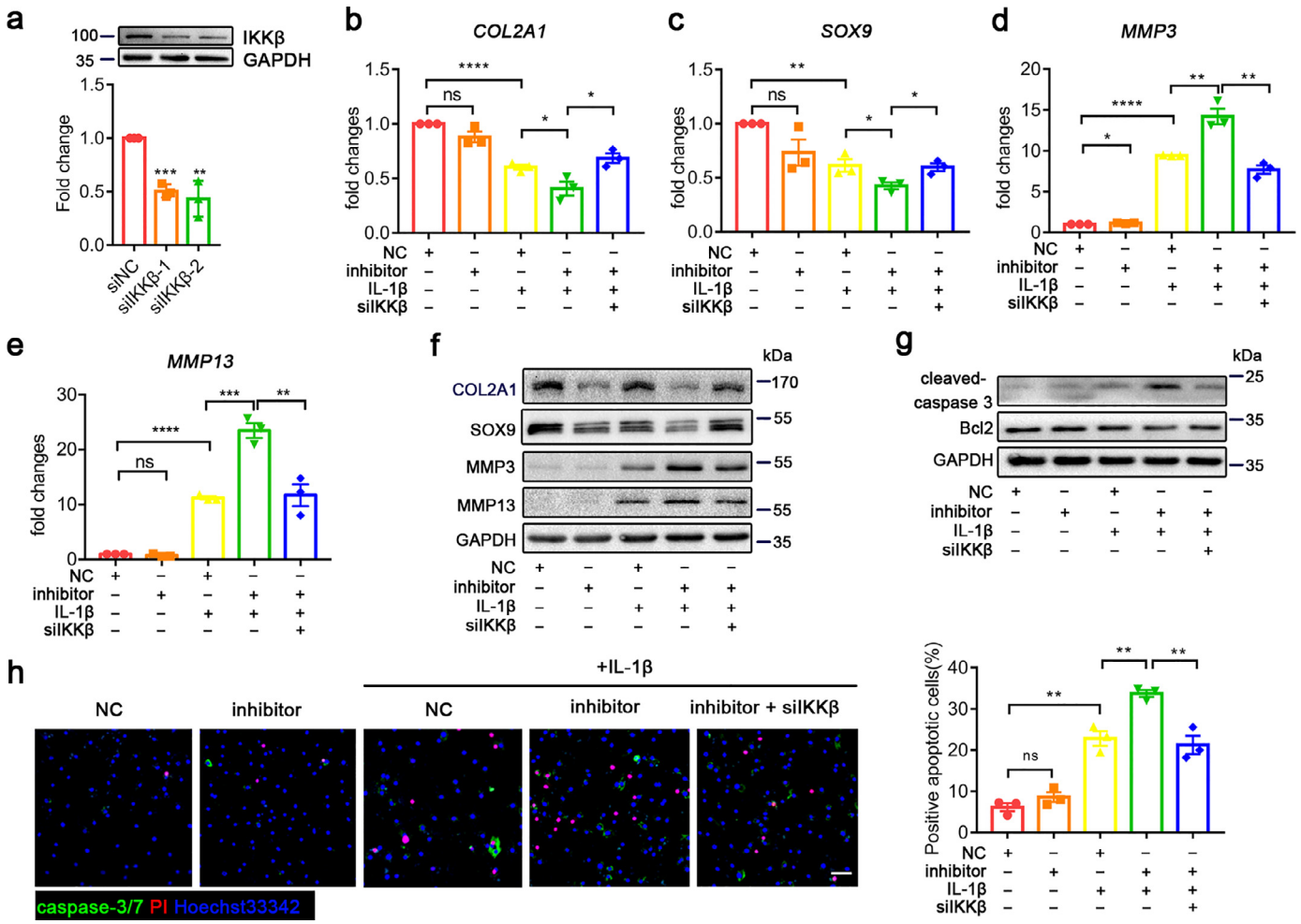

Fig. 5. MiR-214−3p exerts biological functions in HCs through targeting *IKBKB*. (a) Human chondrocytes were transfected with two siRNAs (siIKKβ−1 and siIKKβ−2) at a final concentration of 30 nM. After 48 h of transfection, the knockout efficiency of IKKβ was demonstrated by western blot. GAPDH was used as the internal control. (b-e) qRT-PCR analysis of *COL2A1* (b), *SOX9* (c), *MMP3* (d) and *MMP13* (e) in human chondrocytes with miR-214−3p inhibition or a combination of miR-214−3p and IKKβ inhibition with or without IL-1β stimulation. (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 inhibition or a combination of miR-214−3p and IKKβ inhibition. The data were normalized to GAPDH. (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.

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 have exhibited good safety, but they may have biotoxic effects or off-target effects. Currently, a plenty of studies have reported the application of modified miRNAs in the joint. Si et al. demonstrated that IA injection of miRNA-140 attenuated OA progression by regulating ECM dynamic balance, and may serve as a new therapeutic agent for the treatment of OA [50]. Wang et al. reported that IA injection of synthetic antago-miR-483−5p suppressed chondrocyte hypertrophy, cartilage and ECM destruction, and consequently delayed the onset of OA [51]. In this study, miRNA-214−3p agomir, a specially modified miRNA agonist, was administered intra-articularly in DMM-induced OA mouse model. Our results showed that overexpression miR-214−3p could ameliorate surgically induced OA in mice. Therefore, our study indicates that miR-214−3p delivery may be a promising way in the treatment of OA. Further study will be conducted to confirm its therapeutic potential in humans.

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 chondrocytes 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]. SnoRNAs, involving in ribosome biogenesis and RNA modification, acted as endogenous sponges that regulate miRNA expression [54]. These studies suggested that several mechanism 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.

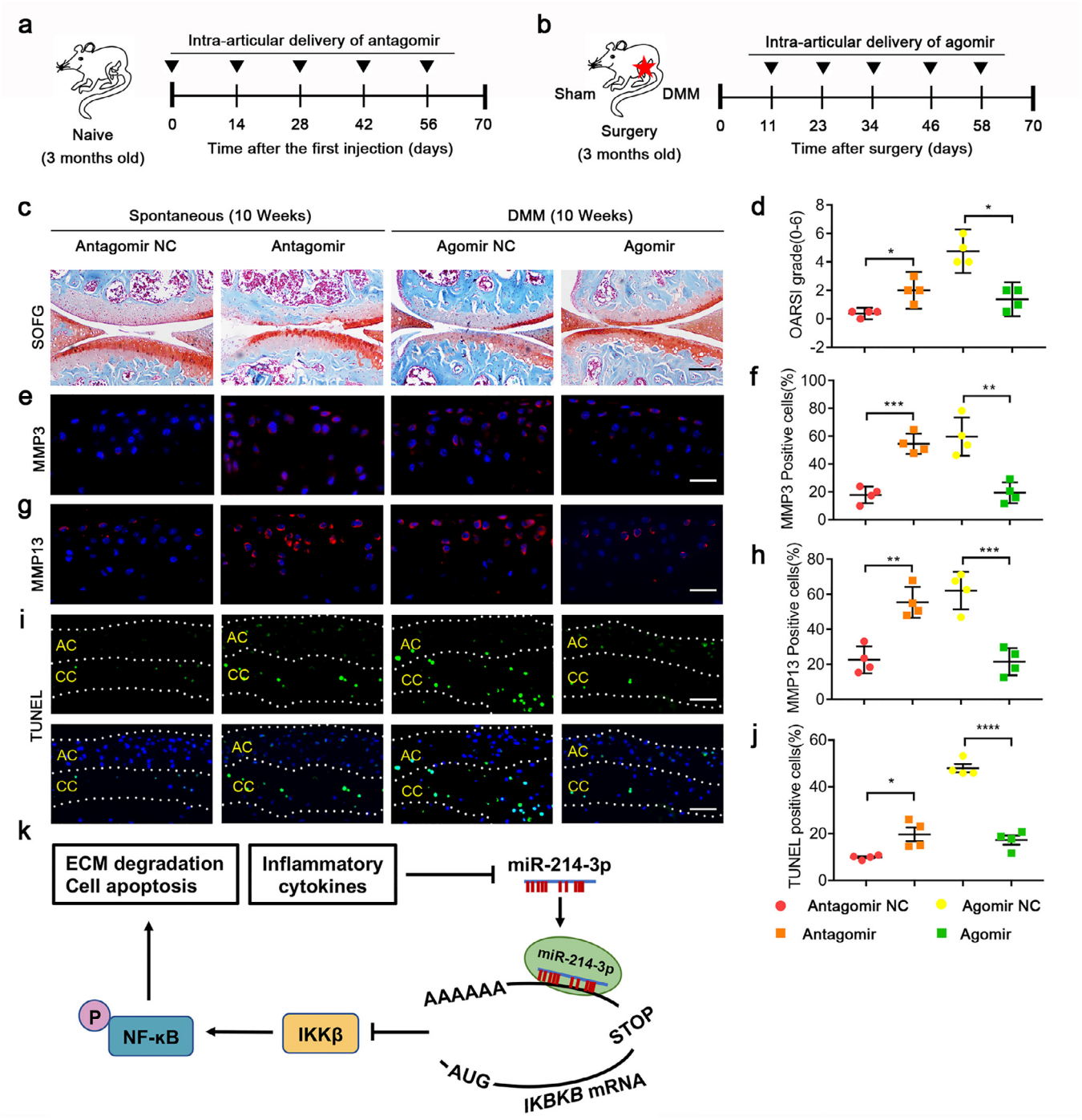

Fig. 6. MiR-214−3p alleviates OA in vivo. (a-b) Schematic diagram showing the design of the OA therapeutic experiment targeting miR-214−3p. (c) Mice in spontaneous group were injected with miR-214−3p antagomir or antagomir NC, while DMM-induced OA mice were injected with miR-214−3p agomir or agomir NC. The articular cartilage was stained with Safranin-O/Fast green (SOFG). Scale bars, 100 μm. (d) OARSI scoring was measured according to SOFG staining results (n = 4 mice per group). (e, g) Expression of MMP3 (e) and MMP13 (g) in articular cartilage were detected by immunofluorescence. Scale bars, 400 μm. (f, h) Quantification of (e) and (g) (n = 4 mice per group). (i) TUNEL staining of the chondrocytes in cartilage (CC) and articular cartilage (AC) in the indicated groups at 10 weeks (n = 4 mice per group). Blue fluorescence (DAPI) indicating total cells; green fluorescence (fluorescein isothiocyanate) indicating TUNEL positive cells. Scale bars, 100 μm. (j) Quantification of E (n = 4 mice per group). ns: no significant difference, $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$, $^{****}P<0.0001$. Means ± 95% confidence interval (CI) with Mann-Whitney U test for OARSI score in (d). Data are shown as means ± SD in (f) (h) and (j). Student's t-test was used for comparison in (f) (h) and (j). OARSI, Osteoarthritis Research Society International. TUNEL, terminal dexynucleotidyl transferase(TdT)-mediated dUTP nick end labeling. (k) Schematic of the working hypothesis. The downregulation of miR-214−3p in the inflammatory cytokines-stimulated human chondrocytes and OA cartilage tissues is directly associated with ECM catabolism and cell apoptosis. MiR-214−3p inhibits NF-κB pathway by directly targeting IKKβ.

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.

Acknowledgements

We want to thank Central Laboratory of Zhujiang Hospital (Southern Medical University, Guangzhou, Guangdong, China) for providing guidance and technical assistance in the study. We would like to acknowledge the funding of the National Natural Science Foundation of China (81773532, 81974342).

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/j.ebiom.2021.103283.

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