MicroRNA-200c-3p suppresses intervertebral disc degeneration by targeting RAP2C/ERK signaling

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MicroRNA-200c-3p suppresses intervertebral disc degeneration by targeting RAP2C/ERK signaling by SCH772984 partially reversed the proinflammatory effect of the miR-200c-3p inhibitor on LPS-treated NP cells. Thus, miR-200c-3p inhibits NP cell apoptosis, inflammatory cytokine levels and ECM degradation in IDD by targeting RAP2C/ERK signaling.

Introduction

Intervertebral disc degeneration (IDD) is a prevalent complicated disease of the spine that reduces the quality of life of patients (1). IDD is usually associated with myelopathy, radiculopathy and lower back pain, with an incidence rate of >90% (2). IDD is affected by several factors, such as apoptosis, inflammatory factors, degradative enzymes, mechanical load, cellular senescence and heredity (3). However, the exact mechanisms responsible for IDD remain elusive.

MicroRNAs (miRNAs/miRs) are small non-coding RNAs that modulate their target genes by regulating mRNA stability and translation, as well as controlling gene expression at the post-transcriptional level by pairing with target mRNAs at the 3' untranslated region (3'UTR) (4). Increasing evidence has revealed that miRNAs are involved in the progression of IDD. For instance, miR-140-5p overexpression reduces lipopolysaccharide (LPS)-induced inflammation and degeneration of intervertebral discs by downregulating Toll-like receptor 4 (TLR4) (5). Moreover, miR-132 contributes to extracellular matrix (ECM) degradation by directly targeting growth and differentiation factor 5 (GDF5) in NP cells (6). miR-200c-3p is involved in inflammatory diseases (7), and it has been shown that the expression levels of miR-200c-3p in the synovial fluid of 150 patients with knee osteoarthritis were significantly lower compared with those in 54 healthy controls (8). miR-200c-3p has also been reported to inhibit apoptosis and inflammation in cigarette smoke extract-stimulated 16HBE cells (9). However, the mechanism of action of miR-200c-3p on IDD progression remains unknown.

Ras-related proteins (RAPs) organize various biological processes, including metabolic turnover, cytoskeletal organization, cell cycle, differentiation and cell adhesion (10). RAP contains a broad family of nucleotide-binding proteins, including five members: RAP1A, RAP1B, RAP2A, RAP2B and RAP2C (11). Previous studies have revealed that RAP2C was involved in multiple diseases, such as laryngeal squamous cell carcinoma (12). However, to the best of our knowledge, the
role of RAP2C in the regulation of IDD progression has not been previously reported. It has been shown that RAP2C activates ERK signaling (13), which has been identified as crucial in the modulation of IDD progression (14,15). Pro-inflammatory macrophages promote the degeneration phenotypes of nucleus pulposus (NP) cells partly via JNK and ERK signaling (16). However, the association of ERK signaling with miR-200c-3p and RAP2C in IDD modulation is yet to be fully elucidated.

Thus, the present study aimed to investigate the role and underlying mechanism of miR-200c-3p in the modulation of IDD development.

Materials and methods

**IDD clinical samples.** A total of 22 intervertebral disc tissues from patients with IDD (age, 41-64 years; 10 male and 12 female) and 9 normal samples (age, 40-49 years; 4 male and 5 female) used in this study were obtained from Qingdao No. 6 People's Hospital between May 2018 and December 2019. All patients with chronic lower back pain (lasting for >3 months) underwent MRI examinations (17,18). The 22 patients with IDD underwent fusion surgery and intervertebral disc excision. The 9 normal tissues were obtained from patients who underwent traumatic lumbar fracture. Patients who were treated with chemotherapy or radiotherapy were excluded from this study. The samples were immediately frozen in liquid nitrogen and stored at -80˚C before further analysis. The samples used in this study were obtained with written approval from patients. This study conformed to the experimental guidelines of the World Medical Association and the Ethics Committee of Qingdao No. 6 People's Hospital (approval no. 201907).

**Cell culture and treatment.** Human NP cells were obtained from ScienCell Research Laboratories, Inc. The cells were cultured in DMEM (Gibco; Thermo Fisher Scientific, Inc.) containing 15% FBS (Gibco; Thermo Fisher Scientific, Inc.), 0.1 mg/ml streptomycin (Beijing Solarbio Science & Technology Co., Ltd.) and 100 U/ml penicillin (Beijing Solarbio Science & Technology Co., Ltd.) at 37˚C with 5% CO₂. Lentiviral plasmids (GV248) carrying RAP2C short hairpin RNA (shRNA) and the corresponding negative control (NC) shRNA were synthesized and obtained from Shanghai GenePharma Co., Ltd. The sequence of RAP2C shRNA was 5'-GAA GCA AAGA UCA GUG UUG U-3' and reverse, 5'-GCC TCA CAC AGG TCC CCT TCG TA-3'; the ratio of the lentiviral plasmid, packaging vector (Shanghai GenePharma Co., Ltd.) and envelope vector (Shanghai GenePharma Co., Ltd.) was 4:3:3. A second-generation lentiviral particles were collected using 72,000 x g/min (Invitrogen; Thermo Fisher Scientific, Inc.) at 37˚C for 12 h. The second-generation lentiviral plasmids were used as the interim cell line, and they were transfected with 20 µg lentiviral plasmid using Lipofectamine (Invitrogen; Thermo Fisher Scientific, Inc.) at 37˚C for 12 h. The NP cells were plated into 24‑well plates (2x10⁵/well), which were used as the interim cell line, and they were transfected with 100 pmol pcDNA-3.1 vectors or 50 nM miRNA constructs using Lipofectamine® 3000 (Invitrogen; Thermo Fisher Scientific, Inc.), according to the manufacturer's instructions, at 37˚C for 6 h. The sequences of each construct were as follows: miR-200c-3p mimic sense, 5'-UAAUCAGCGGUUAUGAGGA-3' and antisense, 5'-CAUCAUACCCGGCCAGUAUAU-3'; mimic NC sense, 5'-UUCUCCGAACGUGUCACGUUTT-3' and antisense, 5'-ACGUGACAGCUCCGAGAATT-3'; miR-200c-3p inhibitor, 5'-UCCAUUAUACCCGGCCAGUAUAU-3'; and inhibitor NC, 5'-CAGAUUUUGUUGUUGAUAAC-3'. NP cells were harvested 24 h after transfection for use in subsequent experiments.

To construct the IDD cell model, NP cells were plated into 6-well plates (3x10⁵/well) and treated with LPS (1 µg/ml; Sigma-Aldrich; Merck KGaA) for 24 h at 37˚C after 24 h of transfection. The ERK inhibitor, SCH772984 (Selleck Chemicals), was used at a dosage of 10 µmol/l for 24 h at 37˚C.

**Reverse transcription-quantitative PCR (RT-qPCR).** Total RNAs were extracted using TRIzol® (Invitrogen; Thermo Fisher Scientific, Inc.) and were treated with RQ1 RNase-Free DNase (Promega Corporation) for 30 min at 37˚C. The quality of total RNA was detected at an absorbance 260/280 nm ratio using NanoDrop life spectrophotometer (Thermo Fisher Scientific, Inc.). cDNA was synthesized with the Maxima First-strand cDNA Synthesis kit according to the manufacturer's instructions (Thermo Fisher Scientific, Inc.). qPCR was performed using the SYBR Real-time PCR I kit (Takara Bio, Inc.). The thermocycling conditions used for the qPCR were as follows: Initial denaturation for 3 min at 95˚C; followed by 37 cycles of denaturation at 94˚C for 1 min, annealing at 60˚C for 1 min and extension at 72˚C for 1 min, followed by a final extension step at 72˚C for 7 min. Relative quantification was performed using the 2^(-ΔΔCt) method (19). The standard controls for miRNA and mRNA were U6 and GAPDH, respectively (20). Quantitative determination of the RNA levels was performed in three independent experiments. Data are presented as the mean ± SD. The primer sequences were as follows: miR-200c-3p NCBI Reference Sequences (RefSeq), NC: 000012.12; miR-200c-3p forward, 5'-GGGGTATGGGGAAGTGGTATTTA-3' and reverse, 5'-CACACACACCAATCTTAAAACACT-3'; RAP2C NCBI RefSeq, XM_035008753.1; RAP2C forward, 5'-GGGCGCAATACGCCAGATAAACTCA-3' and reverse, 5'-ACAGGTGTTAACAGGCTGTTGTC-3'; collagen II NCBI RefSeq, XM_00895124.2; collagen II forward, 5'-CTGGTGATGTTGAGGGTA-3' and reverse, 5'-CCCTTGTTGAAAATTGTTTTT-3'; GAPDH NCBI RefSeq, NM_001256799.3; GAPDH forward, 5'-CATGTTGCAAACGGGAAGA-3' and reverse, 5'-GCC...
CAATACGACCAATCAGAG-3'; and U6 NCBI RefSeq, NC_015438.3; U6 forward, 5'-GCT TCG GCA GCA CAT ATA CTA A-3' and reverse, 5'-AAC GCT TCA CGA ATT TGC GT-3'.

ELISA. The levels of TNF-α (cat. no. KH3013), IL-6 (cat. no. KAC1261) and IL-1β (cat. no. KAC1211) were analyzed using ELISA kits (eBioscience; Thermo Fisher Scientific, Inc.) according to the manufacturer's instructions. All standards and samples were tested on a SpectraMax M5 microplate reader device (Molecular Devices, LLC) at an absorbance of 450 nm. A standard curve was prepared, and the concentration of the samples was calculated according to the absorbance value. Data are presented as the mean ± SD of three experiments.

Analysis of cell apoptosis. In total, ~2x10^5 NP cells were plated into 6-well plates. Apoptosis was analyzed using the Annexin V-FITC Apoptosis Detection kit (Cell Signaling Technology, Inc.) according to the manufacturer's instructions. Briefly, ~2x10^5 NP cells were collected and washed with binding buffer, followed by flow cytometry analysis on a flow cytometer (BD Accuri™ C6 Plus; BD Biosciences). The apoptotic rate was calculated as the percentage of early + late apoptotic cells using FlowJo software (v10.7; FlowJo, LLC). Data are presented as the mean ± SD of three experiments.

Luciferase reporter gene assay. TargetScan (http://www.targetscan.org/vert_72) was used to verify the potential interaction between RAP2C and miR-200c-3p. Luciferase reporter gene assays were performed using a dual-luciferase reporter assay system (Promega Corporation). The RAP2C wild type or mutant 3'-untranslated region sequences containing the miR-200c-3p binding site were constructed and subcloned into the psiCHECK2 vector (Promega Corporation). Briefly, the miR-200c-3p or control mimic, and the vector containing...
RAP2C and RAP2C mutant fragments were transfected into the cells (3x10^5/well) using Lipofectamine 3000 for 48 h, followed by the analysis of luciferase activities, in which Renilla was used as a normalized control reporter. Data are presented as the mean ± SD of three experiments.

**Western blot analysis.** Total proteins were extracted from cells using RIPA buffer (Cell Signaling Technology, Inc.). Protein concentrations were measured using the BCA Protein Quantification kit (Abbkine Scientific Co., Ltd.). An equivalent concentration of protein (50 µg/lane) was separated via SDS-PAGE (12% polyacrylamide gels) and transferred onto PVDF membranes (MilliporeSigma). The membranes were blocked with 5% milk at 25˚C for 60 min and incubated over-night at 4˚C with the primary antibodies for RAP2C (1:3,000; cat. no. ab97805; Abcam), ERK (1:1,000; cat. no. ab32537; Abcam), phosphorylated (p)-ERK (1:1,000; cat. no. ab194776; Abcam) and β-actin (1:5,000; cat. no. ab8227; Abcam), in which β-actin served as the control. Then, the goat anti-rabbit IgG H&L (HRP) secondary antibodies (1:5,000; cat. no. ab7090; Abcam) were added to membranes for 1 h at 25˚C, followed by visualization using an Odyssey CLx Infrared Imaging system (LI-COR Biosciences). Image Studio™ Lite (v4.0; LI-COR Biosciences) was used for analysis. Data are presented as the mean ± SD of three experiments.

**Statistical analysis.** Data are presented as the mean ± SD of three experiments and statistical analysis was performed using GraphPad Prism 7 (GraphPad Software, Inc.). An unpaired Student’s t-test was used to compare two groups. One-way ANOVA was used for multiple group comparisons and Tukey’s test was used for post hoc analysis. P<0.05 was considered to indicate a statistically significant difference.

**Results**

**miR-200c-3p is downregulated in IDD and inhibits LPS-induced NP cell apoptosis.** To assess the potential association between miR-200c-3p and IDD, the expression level of miR-200c-3p was analyzed in patients with IDD and normal controls. The expression level of miR-200c-3p was decreased in the intervertebral disc tissues of patients with IDD (n=22) compared with that in normal subjects (n=9) (Fig. 1A), suggesting a potential association between miR-200c-3p and IDD development. Next, the expression of miR-200c-3p was examined in LPS-treated NP cells. LPS treatment reduced the expression of miR-200c-3p in a time-dependent manner (Fig. 1B), indicating that miR-200c-3p may be involved in IDD progression. NP cell incubation with LPS for 24 h was selected for the next experiment.

To evaluate the role of miR-200c-3p in the development of IDD in vitro, LPS-treated NP cells were transfected with miR-200c-3p mimic or miR-200c-3p inhibitor and the efficiency was validated (Fig. 1C). As expected, LPS treatment increased the apoptosis of NP cells, in which the miR-200c-3p inhibitor enhanced, while the miR-200c-3p mimic suppressed...
miR-200c-3p targets RAP2C in nucleus pulposus cells. (A) Interaction of miR-200c-3p and RAP2C 3’UTR was identified via bioinformatics analysis using TargetScan. (B) NP cells were transfected with control mimic or miR-200c-3p mimic. (C) Luciferase activities of RAP2C WT and RAP2C with the miR-200c-3p-binding site mutant (RAP2C MUT) were determined using luciferase reporter gene assays. (D) mRNA expression level of RAP2C was assessed using reverse transcription-quantitative PCR in the cells. (E) Protein expression level of RAP2C was measured via western blotting. Data are presented as the mean ± SD. *P<0.05, **P<0.01 vs. control. ns, no significance; miR, microRNA; WT, wild-type; MUT, mutant; RAP2C, Ras-related protein 2C; UTR, untranslated region.

Figure 3. miR-200c-3p targets RAP2C in nucleus pulposus cells. (A) Interaction of miR-200c-3p and RAP2C 3’UTR was identified via bioinformatics analysis using TargetScan. (B) NP cells were transfected with control mimic or miR-200c-3p mimic. (C) Luciferase activities of RAP2C WT and RAP2C with the miR-200c-3p-binding site mutant (RAP2C MUT) were determined using luciferase reporter gene assays. (D) mRNA expression level of RAP2C was assessed using reverse transcription-quantitative PCR in the cells. (E) Protein expression level of RAP2C was measured via western blotting. Data are presented as the mean ± SD. *P<0.05, **P<0.01 vs. control. ns, no significance; miR, microRNA; WT, wild-type; MUT, mutant; RAP2C, Ras-related protein 2C; UTR, untranslated region.

this effect (Fig. 1D), suggesting that miR-200c-3p inhibits LPS-induced NP cell apoptosis.

miR-200c-3p attenuates inflammatory cytokine levels and ECM degradation in LPS-treated NP cells. Since inflammatory factors have been shown to be involved in the pathological processes of IDD (21). The effect of miR-200c-3p on the levels of inflammatory factors was determined in LPS-treated NP cells. The levels of inflammatory cytokines in the culture medium of NP cells, including TNF-α, IL-6 and IL-1β, were increased by LPS stimulation, in which the miR-200c-3p inhibitor enhanced, but the miR-200c-3p mimic reduced this phenotype in the cells (Fig. 2A-C). This suggests that miR-200c-3p inhibits inflammatory cytokine levels in LPS-treated NP cells. Moreover, the expression levels of collagen II and aggrecan were significantly decreased by the miR-200c-3p inhibitor but were upregulated by the miR-200c-3p mimic in the LPS-treated NP cells (Fig. 2D and E), indicating that miR-200c-3p attenuates ECM degradation in LPS-treated NP cells.

miR-200c-3p targets RAP2C in NP cells. miR-200c-3p has a recognized target site in RAP2C 3’UTR, as determined using TargetScan (Fig. 3A). To examine the effect of miR-200c-3p on RAP2C, the NP cells were transfected with miR-200c-3p mimic (Fig. 3B). Notably, the miR-200c-3p mimic inhibited luciferase activity of wild-type RAP2C but failed to affect the RAP2C with the miR-200c-3p-binding site mutant in the NP cells (Fig. 3C). Furthermore, the mRNA and protein expression levels of RAP2C were significantly down-regulated by miR-200c-3p mimic transfection in NP cells (Fig. 3D and E), suggesting that miR-200c-3p can target RAP2C in NP cells.

RAP2C promotes apoptosis, inflammatory cytokine levels and ECM degradation in LPS-treated NP cells. LPS-treated NP
cells were infected with lentiviral plasmids carrying RAP2C shRNA or corresponding control shRNA or transfected with the vector carrying the complete RAP2C coding sequence for overexpression. The efficiency of RAP2C shRNA and RAP2C overexpression was validated in the cells (Fig. 4A). It was demonstrated that the LPS-induced apoptosis of NP cells was significantly enhanced by RAP2C overexpression and reduced by RAP2C knockdown (Fig. 4B), suggesting that RAP2C promotes NP cell apoptosis. Moreover, the upregulation of TNF-α, IL-6 and IL-1β by LPS treatment was increased by the overexpression of RAP2C but was suppressed by RAP2C knockdown in the NP cells (Fig. 4C-E), indicating that RAP2C activates the inflammatory cytokine levels. Furthermore, RAP2C overexpression inhibited and RAP2C knockdown increased the expression levels of collagen II and aggrecan in NP cells (Fig. 4F and G), suggesting that RAP2C contributes to ECM degradation in LPS-treated NP cells.

**RAP2C enhances apoptosis, inflammatory cytokine levels and ECM degradation by activating ERK signaling.** It has been reported that the activation of ERK signaling contributes to the development of IDD (14,22). The phosphorylation of ERK is required for activation of ERK signaling (23,24). The expression level of RAP2C was significantly upregulated by the overexpression of RAP2C, whereas the ERK inhibitor SCH772984 partially reduced this upregulation in NP cells. Moreover, the knockdown of RAP2C significantly decreased RAP2C expression (Fig. 5A). The knockdown of RAP2C increased the ratio of p-/total ERK protein expression (Fig. 5A). Moreover, RAP2C induced LPS-treated NP cell apoptosis, and SCH772984 partially blocked this phenotype (Fig. 5B). In addition, SCH772984 partially blocked the elevated levels of TNF-α, IL-6 and IL-1β caused by the overexpression of RAP2C in LPS-treated NP cells (Fig. 5C-E). In addition, RAP2C overexpression-inhibited expression of collagen II and aggrecan was rescued by SCH772984 treatment in LPS-treated NP cells (Fig. 5F and G). Taken together, these data suggest that RAP2C enhances apoptosis, inflammatory cytokine levels and ECM degradation by activating ERK signaling.

**miR-200c-3p suppresses IDD by targeting RAP2C/ERK signaling.** Knockdown of miR-200c-3p with a miR-200c-3p inhibitor promoted the expression of RAP2C. However,
SCH772984 treatment blocked miR-200c-3p inhibitor-induced RAP2C expression in NP cells. The miR-200c-3p mimic inhibited the expression of RAP2C (Fig. 6A). The miR-200c-3p mimic also increased the ratio of p-/total ERK protein expression (Fig. 6A). Moreover, the miR-200c-3p inhibitor-induced apoptosis of LPS-treated NP cells was partially inhibited by RAP2C shRNA or SCH772984 (Fig. 6B). In addition, the levels of TNF-α, IL-6 and IL-1β enhanced by the miR-200c-3p inhibitor were partially decreased by RAP2C knockdown or SCH772984 treatment in NP cells (Fig. 6C-E). RAP2C knockdown and SCH772984 treatment also partially restored the expression levels of collagen II and aggrecan reduced by miR-200c-3p inhibitor (Fig. 6F and G). Collectively, these results suggest that miR-200c-3p suppresses IDD by targeting RAP2C/ERK signaling.

Discussion

IDD is a complex disorder characterized by genotypic and phenotypic alterations, such as ECM degradation, apoptosis and inflammation (25). miRNAs are essential regulators of multiple physiological and pathological processes, and are involved in the modulation of IDD progression. It has been reported that TLR4/NF-κB signaling increases the expression of miR-625-5p, thereby contributing to the pathological process of IDD by modulating collagen type I α 1 (26).
miR-200c-3p contributes to the proliferation and matrix degradation of NP cells by regulating the expression of GDF5 in IDD (27). Moreover, miR-24-3p upregulation is able to increase IDD by targeting insulin-like growth factor-binding protein 5 and ERK signaling (28), while miR-640 induces IDD by modulating Wnt and NF-κB signaling (29). Contrary to the aforementioned results, miR-200c-3p expression was significantly decreased in the intervertebral disc tissues of patients with IDD and it inhibited apoptosis, inflammatory cytokine levels and ECM degradation in IDD by targeting RAP2C/ERK signaling.

RAP2C is an evolutionarily conserved Ras-like GTPase that serves as a molecular switch that pairs receptor signaling to remodel the actin cytoskeleton, cell adhesion and cell polarity (30,31). Previous studies have identified a correlation between miRNAs and RAP2C in multiple pathological processes (10,32,33). Spinal cord ischemia/reperfusion injury promotes neurocyte apoptosis by reducing RAP2C via sponging miR-204 (34). miR-188-5p increases apoptosis and represses proliferation in breast cancer cells by regulating RAP2C/MAPK signaling (35). In addition, it has been shown that RAP2C can activate ERK signaling (12). Similarly, in the present study, RAP2C was targeted by miR-200c-3p in NP cells, whereby RAP2C promoted apoptosis, inflammatory cytokine levels and ECM degradation in LPS-treated NP cells by activating ERK signaling.

ECM degradation plays a critical role in the development of IDD, in which collagen II and aggrecan serve as markers of ECM degradation (36). In the present study, the expression levels of collagen II and aggrecan were decreased...
by miR-200c-3p inhibitors but upregulated by miR-200c-3p mimics in LPS-treated NP cells. This finding was in agreement with a previous study showing that long non-coding RNA nuclear paraspeckle assembly transcript 1 downregulated aggrecan, collagen II, thrombospondin-type motifs 5 and MMP13 by modifying ERK/MAPK signaling in NP cells (37). Consistently, TGF-β1 inhibits the expression of C-C motif chemokine ligand 3/4 via ERK signaling and restricts IDD and inflammation-induced pain (38). Moreover, autophagy alleviates compression-related NP cell apoptosis in IDD via MEK/ERK/nuclear respiratory factor 1/autophagy related 7 signaling (39). The present study demonstrated that ERK signaling was involved in the miR-200c-3p/RAP2C-mediated apoptosis, inflammatory cytokine levels and ECM degradation in NP cells.

The current study has some limitations. The expression levels of aggrecan and collagen II were determined only via RT-qPCR, while levels of inflammatory factors were assessed using ELISA alone. In future studies, the results of the present study should be verified using in vivo models and the regulation of IDD by miR-200c-3p requires further investigation.

In conclusion, the present study demonstrated that miR-200c-3p inhibited NP cell apoptosis, inflammatory cytokine levels and ECM degradation in IDD by targeting RAP2C/ERK signaling. Moreover, miR-200c-3p and RAP2C may serve as potential targets for IDD therapy.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

JC and KX designed the study. MJ performed the experiments. HR analyzed data. JC and KX confirm the authenticity of all the raw data. KX wrote the paper. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The protocol of this research has been approved by the Ethics Committee of Qingdao No.6 People's Hospital. All patients have signed written informed consent.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have competing interests.

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