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Original Article

Knockdown of microRNA-96-5p resists oxidative stress-induced apoptosis in nucleus pulposus cells

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Abstract: Background: Intervertebral disc degeneration (IVDD) often leads to low back pain, which severely affects people's quality of life. Oxidative stress (OS) can accelerate nucleus pulposus cell (NPCs) senescence and apoptosis. Exploring the mechanism underlying OS-induced apoptosis is of utmost importance to aid in the development of IVDD treatment. Methods: In the current study, we tested the function of microRNA-96-5p in H_2O_2-treated NPCs. Apoptosis and mitophagy-related proteins were examined by western blot. Reactive oxygen species (ROS) generation, mitochondrial membrane potential, and apoptosis of NPCs were evaluated by flow cytometry. A luciferase reporter assay was conducted to confirm the interaction between microRNA-96-5p and Forkhead Box Protein O1 (FOXO1). Results: H_2O_2 treatment enhanced apoptosis in NPCs and upregulated the microRNA-96-5p expression. It was shown that knockdown of microRNA-96-5p attenuated H_2O_2-induced OS and apoptosis. FOXO1 is a direct target of microRNA-96-5p, and knockdown of microRNA-96-5p enhanced PINK1/Parkin-mediated mitophagy by up-regulating FOXO1. Conclusions: Collectively, knockdown of microRNA-96-5p enhanced PINK1/Parkin-mediated mitophagy by up-regulating FOXO1. Our results facilitate the understanding of the role of microRNA-96-5p in IVDD and the mechanism of H_2O_2-induced oxidative damage.

Keywords: MicroRNA-96-5p, nucleus pulposus cells, intervertebral disc degeneration, FOXO1, mitophagy

Introduction

Intervertebral disc degeneration (IVDD) can cause low back pain (LBP) [1]. However, current treatments face challenges in repairing or regenerating the structure and function of intervertebral discs. Current studies have pointed out that the pathogenesis of disc degeneration involves oxidative stress (OS) [2]. The nucleus pulposus, which constitutes the core of the intervertebral disc (IVD), plays a vital role in maintaining the extracellular matrix (ECM), such as collagen 2 and aggrecan [3]. IVDD leads to the rupture of the annulus fibrosus and the degeneration of cartilage endplates, and promotes reactive oxygen species (ROS) production through the inflammatory response, which aggravates the OS reaction in nucleus pulposus cells (NPCs) [3]. OS induces mitochondrial apoptosis in nasopharyngeal carcinoma and participates in the evolution of IVDD [4]. Nevertheless, the precise mechanisms of OS-induced NPCs apoptosis still need more research to verify.

MicroRNAs (miRNAs) are a kind of non-coding RNAs ranging from 17 to 23 nucleotides [5]. Mounting evidence suggests that miRNA has extensive and crucial effects on a range of fundamental biological processes, including cell proliferation, apoptosis, tumorigenesis, and cancer progression [6]. Dysregulation of miRNAs is also associated with IVDD. For example, miR-640 aggravates IVDD through regulating nuclear factor κB (NF-κB) and Wingless-Int (WNT) pathway [7]. Furthermore, knockdown of miR-143 inhibited apoptosis through modulating Bcl-2 expression in IVDD [8]. The oncogenic microRNA-96-5p is relevant to the progression of hepatocellular carcinoma, nasopharyngeal carcinoma, and gastric adenocarcinoma [9, 10]. Nevertheless, the effect and mechanism of microRNA-96-5p in OS-induced oxidative damage and cell apoptosis of NPCs remain

unknown and require further investigation. In the current study, we examined the biological function of microRNA-96-5p in H_2O_2-induced OS. Additionally, the functional relationship between microRNA-96-5p and FOXO1 was evaluated. The results revealed that knockdown of microRNA-96-5p alleviated H_2O_2-induced OS through regulating mitochondrial autophagy.

Materials and methods

Cell culture and transfection

NPCs were bought from Applied Biological Materials (Richmond, BC, Canada) and cultured routinely in a complete culture medium [DMEM/F-12 containing 10% FBS (Gibco, USA)] and 1% penicillin/streptomycin. The NPCs were maintained at 37°C in an atmosphere containing 5% CO_2. Transfection experiments were conducted using Lipo 3000 transfection reagent (Thermo Fisher, USA). The microRNA-96-5p inhibitor (5'-UAAAGUGCUCUGGCUAGUGCC-3'), mimic (5'-UAGCAGCACGUAAAUAUGCUUG-3'), and negative control oligonucleotides (F: 5'-UUCUCCGAACGUGUCACGUTT-3') and (R: 5'-ACGUGACACGUUCGGAGAATT-3') were bought from Sangon Biotech (Shanghai, China). For FOXO1 overexpression, the coding sequence of FOXO1 (NP_002006.2) was integrated into pCMV-HA vector to create pCMV-FOXO1-HA.

Flow cytometric analysis

Apoptotic cells were examined by flow cytometric analysis (FCA) using an Annexin V-FITC Apoptosis Detection kit (Beyotime, China) according to the instructions. Apoptotic cells were evaluated using a COULTER ® EPICS ® XL™ Flow Cytometer (USA).

Western blot analysis

NPCs were fully lysed using radioimmunoassay precipitation (RIPA) buffer (Beyotime, China). In order to obtain high-purity proteins, lysates were placed into a cryogenic centrifuge with parameters set at 10000 g and 4°C for 15 min. The content of protein was detected by bicinchoninic acid (BCA) protein assay kit (Beyotime, China). Protein samples were isolated with 10% SDS-PAGE and moved to PVDF membranes, followed by incubation with primary antibodies: anti-Collagen-2 (1:2000, ab34712), anti-Aggrecan (1:3000; ab3778), anti-Bax (1:1500; ab32503), anti-Bcl-2 (1:2500; 15071), anti-cleaved caspase3 (1:2000; 9661), anti-PINK1 (1:2000; 6946), anti-Parkin (1:4000; 4211), anti-LC3 (1:2000; ab192890), and anti-FOXO1 (1:2000; ab179450), and then by incubation with the secondary antibodies: HRP-linked anti-mouse IgG (1:8000; 7076P2), HRP-linked anti-rabbit IgG (1:5000; 7074P2). These reagents were purchased from Abcam and Cell Signaling Technology. ImageJ 1.51p software was used for Western blot quantification.

JC-1 fluorescent probe staining

A mitochondrial membrane potential (MMP) assay kit with JC-1 (Beyotime, China) was used to detect MMP. Briefly, NPCs were incubated with 5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolylcarbocyanine iodide (JC-1) solution at 37°C for 15 min and cleaned twice with JC-1 dye buffer. After JC-1 staining, the fluorescence strength of JC-1 was analyzed using flow cytometer or a fluorescence microscope. The JC-1 Red: JC-1 Green ratio was calculated to assess the loss of MMP after the flow cytometry assay.

Cell viability assay

The Cell Counting Kit CCK-8/WST-8 assay (Beyotime, China) was applied to calculate cell viability following the specification. NPCs were transferred to 96-well plate at 0, 12, 24, and 48 h after transfection, and MTT solution was added into the 96-well plate, which was then cultivated for 2 h at 37°C. The relative viability of cells was calculated by measuring the absorbance at 490 nm.

H_2O_2 treatment

For hydrogen peroxide (H_2O_2) treatment, NPCs were transferred to 24-well plates at a density of 2×10^5 per well. When cell confluence reached 90%, cells were intervened with 150 μM H_2O_2 (Sigma, UK) for 6 h. Cells were cleaned with PBS and collected for subsequent experiments.

ROS and intracellular reductive substances assays

Flow cytometry analysis of ROS was performed using a ROS Assay Kit (Solarbio, China). After

treatment, cells were cultivated with 5 μM 2',7'-Dichlorodihydrofluorescein diacetate (DCFH-DA) at 37°C for 30 min, and subsequently analyzed by flow cytometer. For reduced glutathione (r-GSH) assay, cells were cultivated in 100 μl mixed GSH-Glo™ reagent (Promega Corporation, USA) for 0.5 h at 25°C and subsequently in 100 μl reconstituted Luciferin Detection Reagent (Promega Corporation, USA) for 20 min at 25°C. The fluorescence signal was visualized using a Fluoroskan luminescence scanner (Thermo Fisher Scientific). A SOD Assay Kit (Dojindo, Japan) was used to measure superoxide dismutase (SOD) activity. After incubation at 37°C for 0.5 h, OD values were observed at 450 nm.

Luciferase reporter assay

Dual luciferase reporter assay was used to detect the association between microRNA-96-5p and FOXO1. The pmirGLO vector was used to carry mutant (MUT) or wild type (WT) FOXO1 bound to microRNA-96-5p. MUT-FOXO1 or WT-FOXO1 was co-transfected with microRNA-96-5p into NPCs cells. At 48 h after transfection, luciferase activity was measured using a dual luciferase assay system (Promega, USA). WT-FOXO1: 5'-AAUCAUGACAGCAAAGUGCCAAA-3', MUT-FOXO1: 5'-AAUCAUGACAGCAAACACGGUUU-3'.

Statistical analysis

Data analysis was performed using GraphPad Prism (GraphPad Prism 9.4.0, USA). The measurement data were expressed as mean ± standard deviation (SD). Statistical analysis was conducted using ANOVA followed by Tukey's post hoc test. $P<0.05$ was regarded as statistically significant.

Results

Knockdown of microRNA-96-5p alleviated the impairment of extracellular matrix (ECM) synthesis and cell viability caused by H_2O_2

To demonstrate the function of microRNA-96-5p in NPCs, we first examined microRNA-96-5p expression in H_2O_2-treated NPCs. In contrast to the control group, microRNA-96-5p expression was increased in H_2O_2-treated NPCs (**Figure 1A**).

To investigate the action of microRNA-96-5p in NPCs, gain-of-function or loss-of-function tests were performed by transfecting NPCs with microRNA-96-5p overexpression or microRNA-96-5p inhibitor. RT-qPCR results revealed that microRNA-96-5p overexpression and microRNA-96-5p inhibitor could upregulate and decrease the expression of microRNA-96-5p, respectively (**Figure 1B**). CCK8 assay revealed that NPCs transfected with microRNA-96-5p inhibitor showed higher cell viability than inhibitor negative control group, whereas transfection of microRNA-96-5p mimic showed the opposite trend (**Figure 1C**). Western blot assay demonstrated that H_2O_2 treatment caused a reduction in the expression of ECM-associated protein (Collagen-2 and Aggrecan), which was restored by microRNA-96-5p knockdown (**Figure 1D**). Furthermore, NPCs presented with a long spindle shape and grew in a flower formation before H_2O_2 treatment, whereas H_2O_2-treated cells showed more vacuole formation, decreased refractivity, and cell shrank (**Figure 1E**). However, the cell growth level of the microRNA-96-5p knockdown group was better and the survival rate was higher, indicating that microRNA-96-5p knockdown attenuated the cytotoxicity to the cells caused by H_2O_2.

Knockdown of microRNA-96-5p suppressed H_2O_2-induced cell apoptosis

Next, we tested whether microRNA-96-5p was associated with H_2O_2-induced NPCs apoptosis. Flow cytometry results revealed that the knockdown of microRNA-96-5p significantly reduced H_2O_2-induced NPCs apoptosis (**Figure 2A**). In addition, the knockdown of microRNA-96-5p decreased Bax and cleaved-caspase3 expression and increased Bcl-2 expression in H_2O_2-treated NPCs. The expression of the Bax protein is related to the promotion of cell apoptosis, while the expression of the Bcl-2 protein is more favourable to inhibiting cell apoptosis (**Figure 2B**). Therefore, these results indicate that the knockdown of microRNA-96-5p can reduce the sensitivity of oxidative-stress-induced apoptosis.

Knockdown of microRNA-96-5p alleviated H_2O_2-induced oxidative damage

The DCFH-DA staining results revealed that H_2O_2 treatment increased ROS value in NPCs,

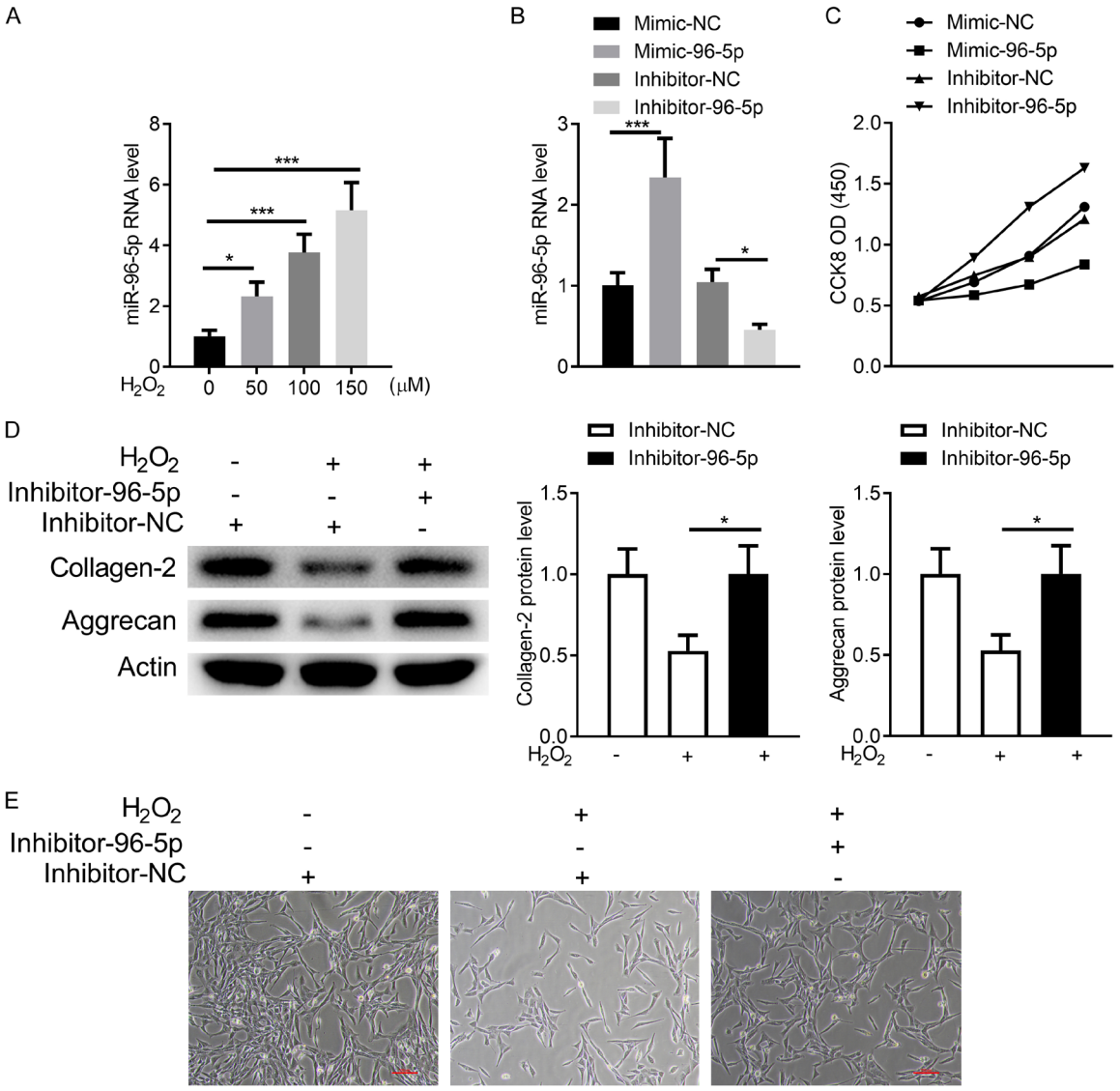

Figure 1. MicroRNA-96-5p knockdown reduced ECM synthesis in H_2O_2-treated NPCs. A. NPCs were treated with H_2O_2 (150 μM), and then expression of microRNA-96-5p was examined by qPCR. B. NPCs were transfected with mimic-NC (negative control), mimic-96-5p, inhibitor-NC, or inhibitor-96-5p, and then expression of microRNA-96-5p was examined by qPCR. C. NPCs were transfected with mimic-NC (negative control), mimic-96-5p, inhibitor-NC, or inhibitor-96-5p, and then cell viability was examined by CCK-8/WST-8 assay. D. NPCs were transfected with inhibitor-NC or inhibitor-96-5p; cells were then treated with H_2O_2 (150 μM). Protein levels of Collagen-2 and Aggrecan were examined by western blot. E. NPCs were transfected with inhibitor-NC or inhibitor-96-5p; cells were then treated with H_2O_2 (150 μM). Cell morphology was detected by a microscope. *P<0.05, ***P<0.001.

which was prevented by microRNA-96-5p inhibitor transfection (**Figure 3A**). It showed that the knockdown of microRNA-96-5p inhibited the oxidizing reaction in NPCs. The red fluorescence of JC-1 decreased, and the green fluorescence of JC-1 increased, indicating the loss of MMP. Our findings showed that the knockdown of microRNA-96-5p prevented H_2O_2-induced MMP loss (**Figure 3B**). Furthermore, we found that the knockdown of microRNA-96-5p enhanced the expression of PINK1 and Parkin proteins, which are known to be associated with mitophagy. At the same time, the knockdown of microRNA-96-5p also enhanced the LC3-II/LC3-I ratio (**Figure 3C**). Together, these results suggested that the knockdown of microRNA-96-5p alleviated H_2O_2-induced oxidative damage.

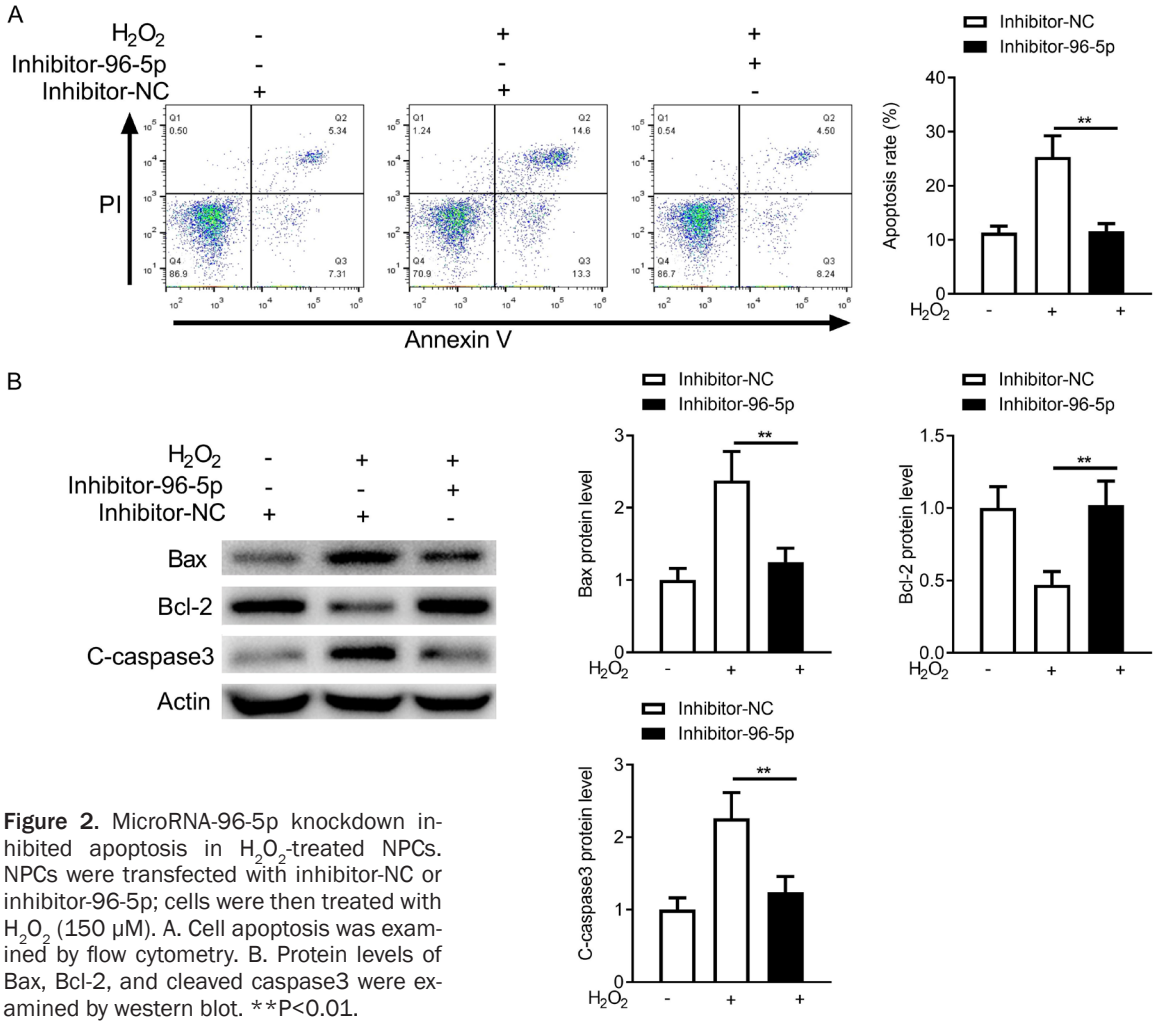

Figure 2. MicroRNA-96-5p knockdown inhibited apoptosis in H_2O_2-treated NPCs. NPCs were transfected with inhibitor-NC or inhibitor-96-5p; cells were then treated with H_2O_2 (150 μM). A. Cell apoptosis was examined by flow cytometry. B. Protein levels of Bax, Bcl-2, and cleaved caspase3 were examined by western blot. **P<0.01.

FOXO1 is a direct target of microRNA-96-5p

We used TargetScan (http://genes.mit.edu/targetscan) to predict the potential targets of microRNA-96-5p. Our study revealed conserved binding site(s) of miR-96-5p in 1343 genes. We focused on FOXO1 as a candidate target gene and found that FOXO1 is involved in regulating mitosis through the PINK1/Parkin pathway [11]. Thus, we investigated the correlation between microRNA-96-5p and FOXO1 by predicting the binding sites of microRNA-96-5p in the 3'-UTR of FOXO1 mRNA using the TargetScan database (**Figure 4A**). In order to further verify our hypothesis, we performed a dual-luciferase reporter assay. The luciferase activity was inhibited by 73% in cells transfected with microRNA-96-5p mimic fused to FOXO1, compared to the control group (**Figure 4B**). Furthermore, the protein level of FOXO1 was reduced in the microRNA-96-5p overexpression group, while it was upregulated in the microRNA-96-5p inhibitor group (**Figure 4C**). Taken together, these results indicated the direct binding of microRNA-96-5p to the 3'-UTR of FOXO1.

MicroRNA-96-5p influenced H_2O_2-induced oxidative damage through targeting FOXO1

Subsequently, we performed rescue experiments to investigate whether FOXO1 is required for microRNA-96-5p to exert its function. As anticipated, the overexpression of microRNA-96-5p resulted in reduced expression levels of FOXO1, PINK1, Parkin, and the LC3-II/LC3-I ratio, while knockdown of microRNA-96-5p led to the opposite effects (**Figure 5A**). After H_2O_2 treatment, the expressions of FOXO1, PINK1, Parkin, and the LC3-II/LC3-I ratio were decreased in microRNA-96-5p-knockdown-cells. Nevertheless, overexpression of FOXO1

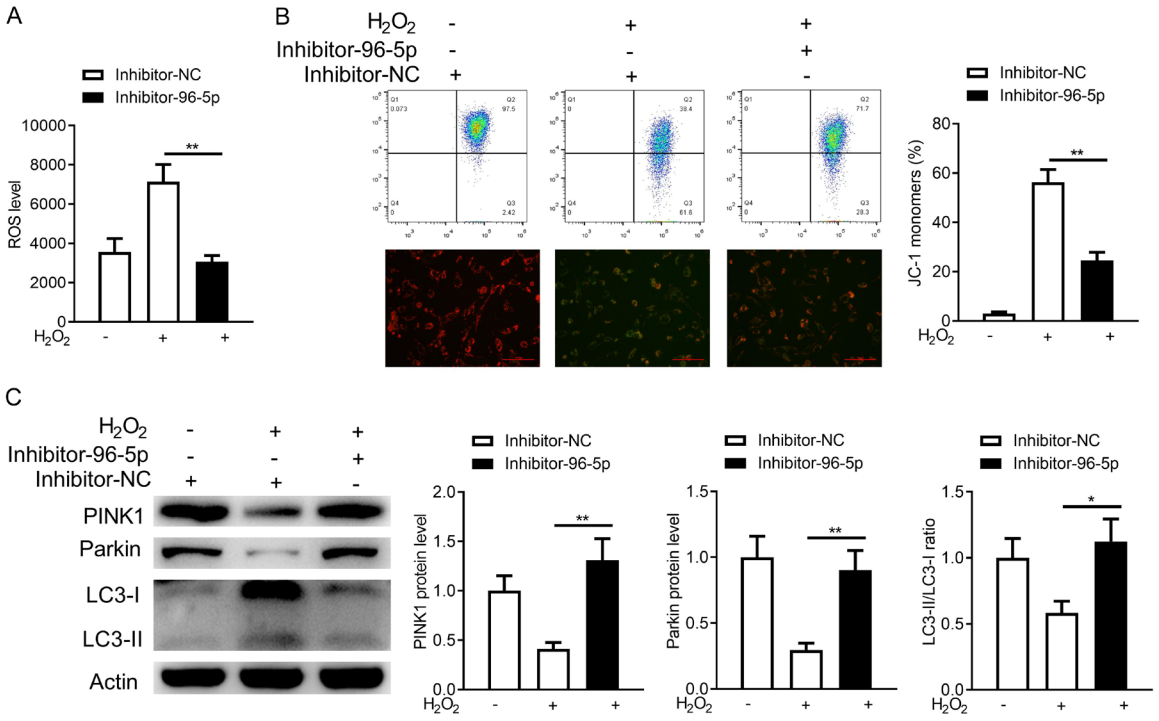

Figure 3. MicroRNA-96-5p knockdown alleviated oxidative damage in H_2O_2-treated NPCs. NPCs were transfected with inhibitor-NC or inhibitor-96-5p; cells were then treated with H_2O_2 (150 μM). A. Intracellular ROS production was measured by DCFH-DA assay. B. Mitochondrial membrane potential was measured by JC-1 assays. Magnification, 20×; scale bar, 50 μm. C. Protein levels of PINK1, Parkin, and LC3 were examined by western blot. *P<0.05, **P<0.01.

partially reversed these declines (**Figure 5B**). Similar results were obtained when testing for apoptosis-associated proteins and flow cytometry analysis of apoptosis (**Figure 5C** and **5D**). Furthermore,overexpression of microRNA-96-5p resulted in further increased ROS levels, while FOXO1 overexpression inhibited this effect (**Figure 5E**). Therefore, these results together revealed that microRNA-96-5p enhanced H_2O_2-induced oxidative damage by suppressing FOXO1 expression.

Discussion

IVDD is considered to be closely associated with the apoptosis of NPCs. Our study is based on understanding the molecular mechanism of NPCs death, which is crucial for the development of new methods for IVDD treatment. IVDD is caused by a lot of factors, among which strong OS can lead to disc cell apoptosis [12]. To explore the mechanism of apoptosis induced by strong OS, we established an *in vitro* model of OS by exposing NPCs cells to H_2O_2.

Emerging evidence has demonstrated that microRNA-96-5p are closely related to the regulation of NPC death. Surprisingly, literature on the function of microRNA-96-5p in IVDD is scarce. In the current study, our results showed that H_2O_2 treatment increased microRNA-96-5p expression (**Figure 1A**). Furthermore, it was observed that the apoptotic rate of NPCs upregulated significantly, and the main effector molecules of apoptosis including Bax and cleaved-caspase3, were also significantly upregulated in H_2O_2-treated NPCs, indicating the occurrence of apoptosis in NPCs. However, knockdown of microRNA-96-5p inhibited H_2O_2-induced apoptosis (**Figure 2**). Different molecular mechanisms for microRNA-96-5p underlying apoptosis have been identified in different cell models. For example, Li et al. [13] found that microRNA-96-5p alleviated malathion-induced apoptosis of human kidney cells through regulating the ER stress marker DNA damage inducible transcript 3 (DDIT3). Conversely, Tian et al. [14] showed that microRNA-96-5p inhibited cell growth and migration and accelerated cell

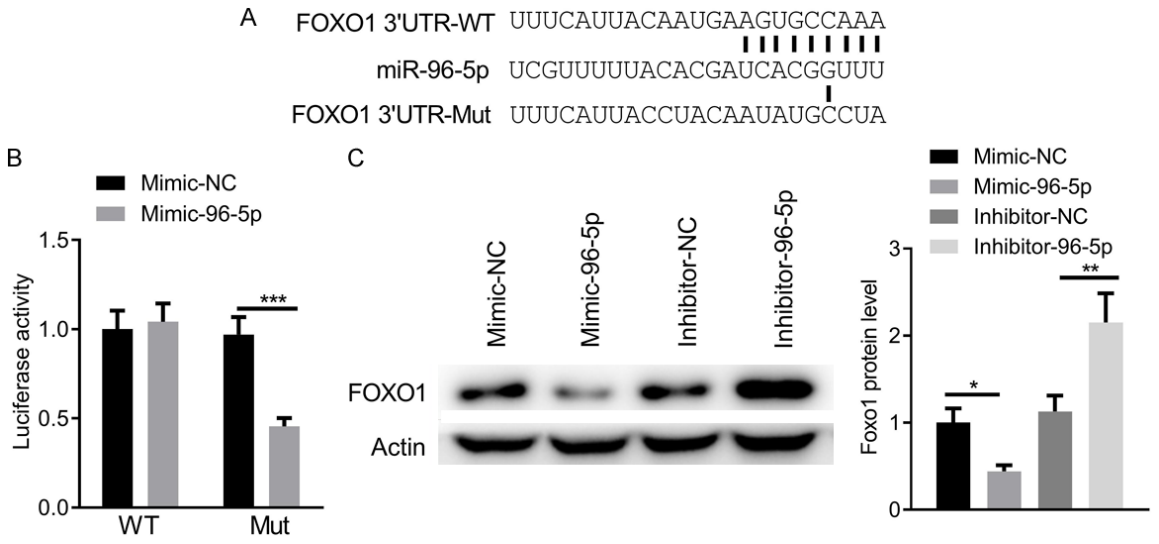

Figure 4. FOXO1 is a target of MicroRNA-96-5p. A. Schematics of the putative binding sites of microRNA-96-5p in 3'UTR of FOXO1. B. Luciferase reporter assay in NPCs co-transfected with microRNA-96-5p mimic, a luciferase reporter containing wild-type (WT) FOXO1 3'UTR or a mutant (Mut) version. C. NPCs were transfected with mimic-NC (negative control), mimic-96-5p, inhibitor-NC, or inhibitor-96-5p. Protein level of FOXO1 was examined by western blot. *P<0.05, **P<0.01, ***P<0.001.

apoptosis of vascular smooth muscle cells (VSMCs). IVDD is typically characterized by the loss of ECM components such as aggrecan and collagen II [15]. ECM is essential for regulating survival, morphology and differentiation of NPCs. Our study also found a reduction in the levels of aggrecan and collagen II in H_2O_2-treated NPCs, whereas knockdown of microRNA-96-5p increased these proteins level, suggesting that microRNA-96-5p promoted degradation of ECM in NPCs (**Figure 1E**).

Scavenging excess ROS production under environmental stress is considered to be a promising method for the treatment of IVDD [16]. Our results revealed that H_2O_2 treatment resulted in higher levels of ROS and lower activity of GSH and SOD, but the changes were mitigated by microRNA-96-5p knockdown (**Figure 3**). The balance of MMP homeostasis is fundamental to cell survival, and loss of MMP may cause a series of responses that lead to apoptosis [17]. It was also revealed that knockdown of microRNA-96-5p significantly reduced H_2O_2-induced MMP loss (**Figure 3**). These data suggested that microRNA-96-5p knockdown attenuated H_2O_2-induced mitochondrial dysfunction. OS-induced autophagy can clear damaged organelles. An appropriate value of autophagy protects cells from damage caused by endogenous stress, whereas overdose autophagy induces apoptosis [18, 19]. Autophagy includes macroautophagy, micro-autophagy, chaperone-mediated autophagy, and mitophagy, in which mitophagy refers to the biological process by which cells selectively clear damaged mitochondria through autophagy [20]. When mitophagy occurs, PINK1 accumulates on the outer membrane of mitochondria, recruits and activates Parkin, which then leads to ubiquitination of mitochondrial surface proteins [21]. Parkin has been shown to be involved in the pathogenesis of IVDD and may be a potential therapeutic target for IVDD [22]. Moreover, Parkin-mediated mitophagy and nuclear factor E2-related factor 2 (Nrf2)-mediated antioxidant system counteracts the apoptosis induced by OS [23]. In this study, the results revealed that knockdown of microRNA-96-5p enhanced the expression PINK1 and Parkin (**Figure 3C**), indicating that microRNA-96-5p is involved in PINK1/Parkin-mediated mitophagy. According to bioinformatics analyses using TargetScan, FOXO1 was suggested to be a potential target of microRNA-96-5p (**Figure 4A**). It was reported that FOXO1 promoted mitophagy in diabetic mice through modulating PINK1/Parkin pathway [11]. The current study provides evidence demonstrating the negative regulation of FOXO1 by microRNA-96-5p (**Figure 4**). Subsequent rescue experiments revealed the oppos-

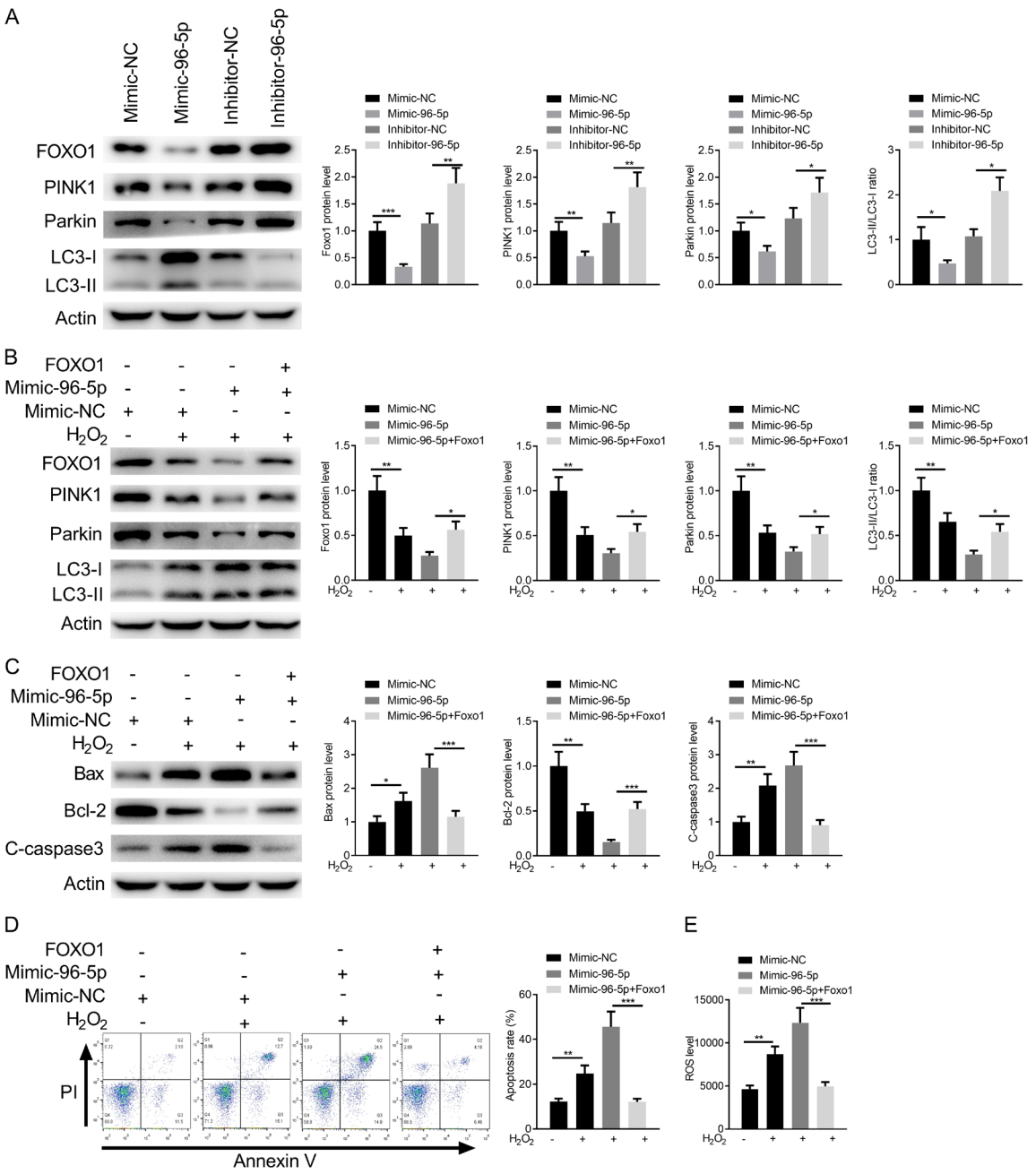

Figure 5. MiR-298 knockdown regulated mitophagy depended on FOXO1 expression. A. NPCs were transfected with mimic-NC (negative control), mimic-96-5p, inhibitor-NC, or inhibitor-96-5p. Protein levels of FOXO1, PINK1, Parkin, and LC3 were examined by western blot. B. NPCs were transfected with mimic-NC (negative control), mimic-96-5p, or pCMV-FOXO1, cells were then treated with H_2O_2 (150 μM). Protein levels of FOXO1, PINK1, Parkin, and LC3 were examined by western blot. C. NPCs were transfected with mimic-NC (negative control), mimic-96-5p, or pCMV-FOXO1, and cells were then treated with H_2O_2 (150 μM). Protein levels of Bax, Bcl-2, and cleaved caspase3 were examined by western blot. D. NPCs were transfected with mimic-NC (negative control), mimic-96-5p, or pCMV-FOXO1, and cells were then treated with H_2O_2 (150 μM). Cell apoptosis was examined by flow cytometry. E. NPCs were transfected with mimic-NC (negative control), mimic-96-5p, or pCMV-FOXO1, cells were then treated with H_2O_2 (150 μM). Intracellular ROS production was measured by DCFH-DA assay. *P<0.05, **P<0.01, ***P<0.001.

ing roles of microRNA-96-5p and Foxo1 in the regulation of mitophagy and apoptosis in NPCs treated with H_2O_2 (**Figure 5**). A study reported that the expressions of FOXO1 and FOXO3, but not Foxo4, were significantly reduced in human degenerated discs [24]. Further, Foxo proteins are critical regulators of intervertebral disk during aging [25]. We deduced that under our

experimental conditions, H_2O_2-induced microRNA-96-5p expression suppressed Foxo1, leading to a reduction in mitophagy of NPCs.

However, our study has some limitations. For example, although we have performed a series of experiments in cell culture, we have not yet validated the function of microRNA-96-5p in animal models or in clinical settings. Future studies should investigate the effects of microRNA-96-5p *in vivo*, which will help to further elucidate the role of this miRNA in the regulation of mitophagy and apoptosis.

In summary, our study demonstrated that knockdown of microRNA-96-5p inhibited H_2O_2-induced oxidative damage through promoting mitophagy. Additionally, microRNA-96-5p regulated mitophagy through targeting Foxo1. The present study revealed the molecular mechanisms of OS-induced apoptosis in NPCs. These results provide valuable reference data into the complicated relationship between microRNA-96-5p and its downstream target, FOXO1, and their respective roles in regulating mitophagy and apoptosis.

Disclosure of conflict of interest

None.

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