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PINK1 protects against oxidative stress induced senescence of human nucleus pulposus cells via regulating mitophagy

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ABSTRACT

Intervertebral disc degeneration (IDD) is closely related with aging, whereas mitochondrial dysfunction is a common feature of aging in which results cell senescence. Phosphatase and tensin homolog (PTEN)-induced putative kinase protein 1 (PINK1) is a mitochondrial-targeted serine/threonine kinase, which plays a protective role against mitochondrial dysfunction with mitochondrial quality control by activating PINK1/Parkin mediated mitophagy. This study aimed to investigate the protective role of PINK1 against mitochondrial dysfunction and human nucleus pulposus cell (NPC) senescence. We found that mitochondrial dysfunction and NPC senescence could be induced under sublethal oxidative stress by 150 μM H_2O_2. Moreover, down-regulation of PINK1 tended to aggravate NPC senescence under oxidative stress. Therefore, mitophagy was evaluated in NPCs to further reveal the underlying mechanism. Results showed that sublethal oxidative stress induced mitochondria dysfunction and mitophagy in NPCs. Furthermore, depletion of PINK1 utilizing short hairpin RNA targeting PINK1 (PINK1-shRNA) impaired mitophagy, and exasperated NPC senescence under oxidative stress. In summary, these results suggested that PINK1 played as a protective role in clearance of damaged mitochondrial and alleviating cell senescence under oxidative stress, whose mechanism is associated with regulating mitophagy. These findings may provide a better understanding in pathomechanism of IDD and potential therapeutic approaches for IDD treatment.

1. Introduction

Intervertebral disc degeneration (IDD), which results in instability, stenosis, and deformity of the spinal motion segment, has been recognized as one of the leading causes of low back pain (LBP) and motor deficiency [1]. IDD is a complicated process that involves both age-related changes [2] and tissue damage caused by multiple stresses [3]. In a degenerative intervertebral disc, cellular senescence accumulates and is linked to reduced proliferation, compromised self-repair, increased inflammatory response, and enhanced catabolic metabolism of extracellular matrix (ECM) [4].

Oxidative stress has been widely demonstrated to play a primary role in the regulation of senescence and degeneration [5]. Recent studies have revealed that the pathomechanism of IDD is closely associated with reactive oxygen species (ROS) and oxidative stress [6]. As a major ROS-generating organelle, mitochondrion is also the target organelle of damage by ROS. Furthermore, mitochondria function has been shown to be a key factor in the development of pro-ageing features of senescence [7,8]. Hence, we hypothesized that interference of mitochondrial homeostasis can impair the resistance to oxidative stress and aggravate senescence in human NPCs.

Phosphatase and tensin homolog (PTEN)-induced putative kinase protein 1 (PINK1) is a mitochondrial-targeted serine/threonine kinase, which plays a protective role against mitochondrial dysfunction and apoptosis with mitochondrial quality control by activating PINK1/Parkin mediated mitophagy, a selective type of autophagy [9,10]. The importance of PINK1 in the mitochondria is affirmed in cell-protective properties for counteracting oxidative stress [11]. The functional role of PINK1 has been largely reported in neurodegenerative and aging-related diseases [11–13]. However, no study has concerned the role of PINK1 in human IDD, and

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whether it can alleviate NPCs senescence via regulating PINK1/Parkin mediated mitophagy. Hence, we conduct the present study to detect the relationship between PINK1 and senescence in human degenerative NPCs and to understand whether PINK1/Parkin mediated mitophagy is involved in the protective effect of PINK1 against oxidative stress induced senescence in degenerative human NPCs.

2. Materials and methods

2.1. NP sample source

All the NP tissues were acquired from lumbar spine surgery patients in our department. The relatively normal human intervertebral disc (IVD) tissues used as controls were obtained from patients with lumbar vertebral fracture (LVF) who did not have a documented medical history of LBP. Human IDD tissues were obtained from patients with lumbar disc herniation (LDH). The degree of IDD was assessed according to the Pfirrmann classification system by utilizing pre-operative MRI scans [14]. Samples from IDD patients were grades III-V, and those samples from LVF patients were grade I. Written informed consent was obtained from all tissue donors before the surgery, and the study protocol was subject to approval by the Ethics Committee of Chongqing Medical University (Chongqing, China).

2.2. NPC isolation and culture

NP tissues from donors were isolated and cultured as described previously [15]. In brief, NP samples were separated from harvest IVD tissues microscopically according to their morphological difference. After washing with PBS solution, NP tissues were isolated and sequentially digested by 0.25% trypsin solution and 0.2% type II collagenase (sigma, USA) at 37 °C for 3–5 h. Tissue debris was removed by a 200-μm filter by enzymatic digestion and were cultured into a monolayer by incubation in DMEM/F-12 medium (HyClone, USA) supplemented with 10% fetal bovine serum (FBS) (Corning, USA), 100μU/mL streptomycin, and 1% penicillin. NPCs were incubated at 37 °C in an atmosphere containing 5% CO_2. Cells at passage II were used in vitro experiments.

2.3. Immunohistochemical staining

NP specimens were fixed with 4% paraformaldehyde for 24 h and then embedded in paraffin and sectioned at 4 mm for Immunohistochemical (IHC) staining. The IHC staining procedure was performed with a streptavidin-peroxidase immunohistochemical kit (Wuhan Boster Biological Technology, Ltd., Wuhan, China) according to the manufacturer's protocol. Briefly, the sections were treated with 3% H_2O_2 for 15 min at room temperature to eliminate endogenous peroxidase activity and were subsequently incubated with 0.125% trypsin for 30 min at 37 °C to retrieve the antigen, before being blocked with normal goat serum for 15 min at room temperature. The sections were incubated with rabbit anti-PINK1 (1:1000; Abcam, USA) primary antibodies overnight at 4 °C. Then, the sections were incubated with secondary antibody goat anti-rabbit IgG-HRP (1:1000; Beyotime, China) and counterstained with haematoxylin. The resulting sections were photographed under a microscope (Leica, Germany). Five fields which distributed in the whole section were counted (200 ×), and the average positive rate was counted.

2.4. Cell proliferation assay

CCK-8 assays were performed to detect the proliferation of NPCs obtained from the IDD patients and those obtained from the LVF patients according to the manufacturer's instructions. Briefly, 1×10^4 cells/well were seeded in 96-well plates and incubated at 37 °C in an atmosphere containing 5% CO_2 for 24 h. Then, 10 ml of CCK-8 solution was added to 90 ml of culture media in each well and continuously incubated for another 2 h in the incubator at 37 °C. Finally, the absorbance of the sample from each well was measured using an automicroplate reader (BD, USA).

2.5. Senescence-associated β-galactosidase staining

NPCs (1×10^6/well/group) were cultured in 6-well plates (Thermo Fisher Scientific, USA) for 24 h, fixed with fixative solution, and subjected to senescence-associated β−galactosidase (SA-β-Gal) staining. The staining process was performed according to the overnight instructions presented in the SA-β-Gal kit (Beyotime, China). The cells were then photographed under a microscope in order to detect any SA-β-Gal activity (Leica, Germany). Five fields which distributed in the whole well were counted (100 ×), and the average positive rate was counted.

2.6. ROS detection

The intracellular production of ROS was evaluated by DCFH-DA (Beyotime, China), which would be oxidized into a fluorescent green dichlorofluorescein (DCF) by reacting with ROS. Briefly, treated NPCs were washed and incubated with DCFH-DA (10 mg/ml) in 1 ml of DMEM/F12 at 37 °C for 20min. Then, NPCs were washed and analyzed by flow cytometry at an excitation wavelength of 488 nm and an emission wavelength of 525 nm. The mean fluorescence intensity (MFI) of 1×10^4 cells was recorded.

2.7. Mitochondrial membrane potential detection

The fluorescent probe JC-1 (Beyotime, China) was used to identify the mitochondrial membrane potential (ΔΨm). Briefly, treated NPCs were washed in PBS buffer and incubated with 5 mg/ml of JC-1 at 37 °C for 15min. The stained cells were observed by confocal microscopy and analyzed by flow cytometry using a FACScan (Becton Dickinson, CA). Depolarized ΔΨm resulted in decreased red and increased green fluorescence. Results were expressed as the ratio of green/red fluorescence intensity values.

2.8. Lentivirus and shRNA transfection

For depletion of PINK1 in NPCs, short hairpin RNA targeting PINK1 (PINK1-shRNA (Santa Cruz Biotechnology, Dallas, TX, U.S.A.)) was transfected into NPCs by using recombinant lentiviral vector (GeneChem, Shanghai, China). Scrambled shRNAs with no known mammalian homology (non-targeting shRNA (Santa Cruz Biotechnology, Dallas, TX, U.S.A.)) were used as negative controls. For transfection, cells were seeded into 6-well plates, incubated for 24 h, and then transfected according to the manufacturer's instructions. After subsequent treatments, cells were harvested for all the experiments.

2.9. Treatment protocol of cell culture

To investigate the resistance to oxidative stress when down-regulating PINK1 in NPCs, human NPCs were seeded at a density of 2.5×10^6/T-25 flasks, or were seeded at a density of 1×10^6/well in 6-well plates. First, PINK1-shRNA was used to infect the cells for 72 h. They were subsequently incubated with 150 μM H_2O_2 for 1 h to induce sublethal oxidative stress according to previous study [16]. Then, the H_2O_2 was removed from the culture, and the NPCs

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