Experimental Cell Research 352 (2017) 322–332

Contents lists available at ScienceDirect

Experimental Cell Research

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

# Sirtuin 6 prevents matrix degradation through inhibition of the NF-κB pathway in intervertebral disc degeneration

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ARTICLE INFO

*Keywords:*
Intervertebral disc degeneration
Sirtuin 6
Matrix metalloproteinase
Extracellular matrix
Nuclear factor-κB

ABSTRACT

Intervertebral disc degeneration (IDD) is marked by imbalanced metabolism of the extracellular matrix (ECM) in the nucleus pulposus (NP) of intervertebral discs. This study aimed to determine whether sirtuin 6 (SIRT6), a member of the sirtuin family of nicotinamide adenine dinucleotide-dependent deacetylases, protects the NP from ECM degradation in IDD. Our study showed that expression of SIRT6 markedly decreased during IDD progression. Overexpression of wild-type SIRT6, but not a catalytically inactive mutant, prevented IL-1β-induced NP ECM degradation. SIRT6 depletion by RNA interference in NP cells caused ECM degradation. Moreover, SIRT6 physically interacted with nuclear factor-κB (NF-κB) catalytic subunit p65, transcriptional activity of which was significantly suppressed by SIRT6 overexpression. These results suggest that SIRT6 prevented NP ECM degradation in vitro via inhibiting NF-κB-dependent transcriptional activity and that this effect depended on its deacetylase activity.

## 1. Introduction

Lower back pain (LBP) is a common musculoskeletal disorder; health surveys indicate a prevalence of 15–45%, and 70–80% of the population has suffered at least one episode of LBP at some point during their lives [1]. This results in a global burden of severe healthcare and socioeconomic costs [2]. Investigators have demonstrated that intervertebral disc (IVD) degeneration (IDD) is the major cause of LBP [3]. Because the pathological mechanisms of IDD remain unclear, current treatments for LBP caused by IDD, including conservative approaches and surgical procedures such as spine fusion and discectomy, are limited to symptom relief. As no effective measures are available to reverse the pathology of IDD, it often results in recurrences of disc disease or loss of disc function [4,5]. Therefore, therapeutic inhibition of the early stages of the IDD process is of great necessity.

The IVD is an avascular organ composed of the nucleus pulposus (NP), annulus fibrosus (AF), and cartilaginous endplates. NP cells play an important role in maintaining extracellular matrix (ECM) homeostasis [6]. The NP ECM is mainly composed of type II collagen and proteoglycans (mainly aggrecan), which enable the disc to maintain water content and to withstand a mechanical load [7]. When ECM catabolic activities prevail over anabolic activities in the NP, IDD usually occurs [8]. The identification of the molecular mechanisms underlying this pathological process can provide insight into the pathogenesis of IDD, as well as a basis for the development of effective therapeutic strategies to inhibit IDD progression.

Matrix metalloproteinases (MMPs) are the most important enzymes that can cleave collagen and aggrecan in NP ECM [9–12]. Previous studies have demonstrated that many members of MMPs are highly expressed in degenerative IVD tissues and cells, which results in ECM degradation and IDD development [9]. Consequently, correcting the anabolism/catabolism imbalance in the disc might prevent or even reverse the progression of IDD.

Sirtuins are a family of evolutionarily conserved nicotinamide adenine dinucleotide ($NAD^+$)-dependent deacetylases that target histone and non-histone proteins [13]. Mammals possess seven sirtuins (SIRT1–SIRT7) with different biological functions and subcellular localizations [14–18]. Recently, sirtuins have attracted considerable research attention because of their important roles in aging-related disease. The physiological and pathological functions of SIRT1 in IDD have been well studied. SIRT1 overexpression significantly inhibited interleukin-1 beta (IL-1β)-induced ECM degradation in human NP

*Abbreviations:* AF, annulus fibrosus; ECM, extracellular matrix; IDD, intervertebral disc degeneration; IL-1β, interleukin-1 beta; IVD, intervertebral disc; LBP, lower back pain; MMP, matrix metalloproteinase; NAD, nicotinamide adenine dinucleotide; SIRT, sirtuin

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http://dx.doi.org/10.1016/j.yexcr.2017.02.023
Received 4 January 2017; Received in revised form 11 February 2017; Accepted 15 February 2017
Available online 16 February 2017
0014-4827/ 

**Table 1**
Sequences of primers used for quantitative real-time PCR.

| Gene | Oligonucleotide sequence Forward (5–3′) | Reverse (5–3′) | Product size (bp) |
|---|---|---|---|
| SIRT6 | GAATGTGCCAAGTGTAAGACG | TCATCGGCGAGTGCCAGGTC | 188 |
| Type II collagen | AGAACTGGTGGAGCAGCAAGA | AGCAGGCGTAGGAAGGTCAT | 142 |
| Aggrecan | TGAGCGGCAGCACTTTGAC | TGAGTACAGGAGGCTTGAGG | 287 |
| MMP3 | TTCCTTGGATTGGAGGTGAC | AGCCTGGAGAATGTGAGTGG | 248 |
| MMP13 | CCCAACCCTAAACATCCAA | AAACAGCTCCGCATCAACC | 147 |
| IL-6 | TTCGGTCCAGTTGCCTTCTC | GCCTCTTTGCTGCTTTCACA | 227 |
| MMP9 | GAGACCGGTGAGCTGGATAG | TACACGCGAGTGAAGGTGAG | 236 |
| RANTES | CAGAGAAGAAATGGGTTCGG | GGGGTAGGATAGTGAGGGGA | 144 |
| β-actin | AGCGAGCATCCCCCAAAGTT | GGGCACGAAGGCTCATCATT | 285 |

cells [19]. Administration of resveratrol, an activator of SIRT1, protects against puncture-induced disc degeneration in mice [20], and MMP3 is markedly downregulated in NP cells treated with resveratrol [21]. In addition, SIRT1 inhibits the apoptosis of degenerative NP cells in humans [22]. SIRT6, a nuclear sirtuin, has been recently implicated in the regulation of multiple pathophysiological processes, including aging, DNA damage repair, inflammation, and metabolic homeostasis [23–25]. Notably, SIRT6 is decreased in osteoarthritic cartilage and has an overall protective effect on osteoarthritis in mice when injected into the knee joint using lentiviral vectors [26]. Moreover, SIRT6 overexpression attenuates the upregulation of MMP13 and the down-regulation of type II collagen induced by IL-1β in chondrocytes [26], and depletion of SIRT6 increases the expression of MMP-1 and MMP-13 in normal human chondrocytes [27]. These findings suggest that SIRT6 plays an important role in aging-associated degenerative diseases. In addition, it has been found that NP cells have the same morphology and avascular supply as chondrocytes, and the incidence of degenerative disc disease increases markedly with age. However, to our knowledge, SIRT6 expression in degenerative NP tissues and its role in the pathogenesis of IDD remain unknown.

Based on previous findings, we hypothesized that SIRT6 plays an important role in IDD by regulating NP ECM metabolism. Hence, the purpose of this study was to determine SIRT6 expression level in human NP tissue, and investigate the role of SIRT6 in anabolic and catabolic processes in human NP cells. Further, we explored the mechanistic pathway of SIRT6 involvement in IDD in vitro, using gain- and loss-of-function approaches.

## 2. Materials and methods

### 2.1. Patient tissue samples

The study protocol was approved by the Ethics Committee of Tongji Medical College, Huazhong University of Science and Technology. Written, informed consent was obtained from all participants in our study. Lumbar NP samples were collected as normal controls from 10 patients (n=5 women and 5 men; mean age, 22.1 years; range, 17–34 years) with idiopathic scoliosis undergoing deformity correction surgery. Degenerative lumbar NP samples were collected from 30 patients (n=13 women and 17 men; mean age, 39.5 years; range, 29–70 years) with IDD, undergoing disc excision and spinal fusion surgery. The degeneration degree of IVD was assessed according to the modified Pfirrmann grading system [28] from pre-operative magnetic resonance imaging scans. The lumbar discs of all patients with IDD were classified as Grades III–V and the discs of the 10 patients with idiopathic scoliosis were classified as Grade II.

### 2.2. Isolation and culture of human NP cells

Human NP cells were isolated from the discs of the 10 patients with idiopathic scoliosis. Briefly, the NP was carefully separated from the AF under a stereotaxic microscope and cut into small fragments (2–3 $mm^3$). NP cells were isolated by enzymatic digestion for 12 h at 37 °C in Dulbecco's Modified Eagle's Medium (DMEM)/F12 (Gibco, Grand Island, NY, USA) with 0.25 mg/mL type II collagenase (Invitrogen, Carlsbad, CA, USA). After isolation, the NP cells were resuspended in DMEM/F12 containing 15% fetal bovine serum (FBS; Gibco) and 1% penicillin-streptomycin and incubated at 37 °C in a humidified 5% $CO_2$ atmosphere. When the NP cells grew to 80% confluence, they were detached by trypsinization and subcultured in culture flasks. During passaging, no significant changes in morphology were observed between primary (passage 0) and later-passage (passage 2) cells. Therefore, we used second-passage cells cultured in a monolayer for experiments.

### 2.3. RNA extraction and quantitative real-time PCR (qRT-PCR)

Total RNA was isolated from NP tissue and cells with TRIzol reagent (Invitrogen) according to the manufacturer's instructions. After determination of the RNA concentration using spectrophotometry, RNA was reverse transcribed with PrimeScript™ RT Master Mix (TakaRa, Dalian, China) according to manufacturer's instructions. qPCR was performed to quantify SIRT6, type II collagen, aggrecan, MMP3, MMP13, IL-6, MMP9, RANTES, and β-actin mRNA expression levels. qPCR was conducted using the One Step SYBR® PrimeScript™ RT-PCR Kit (TakaRa). β-Actin was used for normalization. The primers used for qRT-PCR are listed in Table 1. Relative mRNA expression levels were calculated by the $2^{-\Delta\Delta Ct}$ method. All experiments were carried out in triplicate.

### 2.4. Western blotting

NP cells were harvested and lysed in RIPA lysis buffer. Nuclear and cytosolic proteins were isolated using a Nuclear/Cytosol Fractionation Kit (BioVision, Mountain View, CA, USA) according to the manufacturer's instructions. Protein concentrations were measured with the Enhanced BCA Protein Assay Kit (Beyotime, Shanghai, China). Proteins were separated by 12% sodium dodecyl sulphate polyacrylamide gel electrophoresis and transferred to a polyvinylidene fluoride membrane (Millipore, Billerica, MA, USA). After blocking with Tris-buffered saline with Tween-20 containing 5% non-fat milk, the membranes were incubated overnight at 4 °C with antibodies against the following proteins: SIRT6 (1:2000), type II collagen (1:1000), aggrecan (1:1000), MMP3 (1:1000), MMP13 (1:4000), and β-actin (1:2000) (all from Abcam, Cambridge, MA, USA); and nuclear factor (NF)-κB p65 (1:2000), inhibitor of κB alpha (IκBα) (1:2000), and Lamin B1 (1:1000) (Cell Signaling Technology, Danvers, MA, USA). After washing, the membrane was incubated with horseradish peroxidase (HRP)-conjugated secondary antibody (1:2000; Abcam) for 2 h at 37 °C. Protein bands were visualized and detected using the enhanced chemiluminescence system. β-Actin and Lamin B1 were used as loading controls. The experiment was performed in triplicate.