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MicroRNA-132 upregulation promotes matrix degradation in intervertebral disc degeneration

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ABSTRACT

MicroRNAs (miRNAs) have been shown to be involved in the pathogenesis of intervertebral disc degeneration (IDD). This experiment was designed to study the expression and role of the miRNA, miR-132, in IDD. MiR-132 expression in human nucleus pulposus (NP) tissue was assessed by quantitative real-time PCR. The methylation status of the miR-132 was assessed with methylation-specific PCR and bisulfite sequencing PCR. The regulation of growth differentiation factor5 (GDF5) expression by miR-132 was evaluated by luciferase reporter assay. Moreover, we investigated the function of miR-132 on IDD *in vivo* using a classic needle-punctured rat tail model. These results showed that miR-132 expression was upregulated during IDD and this upregulation was associated with hypomethylation of its promoter. MiR-132 overexpression led to increased expression of ECM catabolic factors, including MMP13 and ADAMTS4, in NP cells while levels of anabolic proteins, such as type II collagen and aggrecan, were diminished. *GDF5* was identified as a direct target of negative regulation by miR-132. MAPK/ERK signaling was also found to be associated with miR-132-induced ECM degradation. In addition, we showed that miR-132 inhibition effectively attenuated NP ECM degradation in IDD *in vivo*. Our findings demonstrated that miR-132 promotes ECM degradation by human NP cells by direct targeting of *GDF5*. Hence, miR-132 represents a potential therapeutic target in the treatment of IDD.

1. Introduction

Low back pain (LBP) is the most common type of musculoskeletal disorder; it severely affects the health of individuals and constitutes a heavy economic burden for societies [1,2]. IDD is generally considered to be involved in LBP [3]. Intervertebral disc (IVD) are located between adjacent vertebrae in the vertebral column. Each IVD is composed of a nucleus pulposus (NP), annulus fibrosus, and cartilage end plates. IDD begins at the NP and is characterized by a imbalance between extracellular matrix (ECM) synthesis and degradation [4]. Where ECM catabolic activity prevails over anabolic activity in the NP, type II collagen and aggrecan degrade, leading to NP tissue resorption and dehydration, and reductions in disc height and ability to resist mechanical load, which are the main pathological features of IDD and the main triggers of LBP [5]. However, the molecular mechanisms underlying these pathological processes remain poorly understood.

Current treatments for LBP caused by IDD, including conservative approaches and surgical procedures such as spine fusion and discectomy, are limited to symptom relief. These operations could not preserve the function of IVD, and increase the mechanical load on adjacent IVD [6,7]. Thus, the need for early therapeutic inhibition of the IDD process is increasing.

Micro (mi)RNAs are small endogenous noncoding RNAs that negatively regulate mRNA stability and/or repress mRNA translation by binding to the 3′-untranslated region (3′-UTR) of target mRNAs [8]. They have essential function in cell proliferation, apoptosis, ECM metabolism, and inflammatory diseases [9,10]. Differential expression of some miRNAs has been found between normal and degenerative NP tissues, and is associated with the development and progression of IDD [11,12]. For example, miR-155 is downregulated in IDD, and contributes to the upregulation of MMP16 *in vivo*. MMP16 further degrades aggrecan and type II collagen, leading to the degeneration of IVDs [13]. Downregulation of miR-27b causes loss of type II collagen by directly targeting MMP13 in human IDD [12]. Recently, we showed that miR-34a is considerably upregulated in degenerative NP and that inhibition of miR-34a expression prevents ECM degrada-

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tion in human NP cells [14], emphasizing the impact of miRNAs in the progression of IDD.

miR-132 is one of the most studied miRNA molecules and is associated with multiple cellular processes, including apoptosis, proliferation, and ECM metabolism [15–17]. As a multi-functional miRNA, miR-132 is expressed in diverse tissues [15,18]. The abnormal miR-132 expression is involved in a variety of diseases, including osteoarthritis [19]; however, the expression and function of miR-132 in IDD remain unknown. Bioinformatic target prediction indicated that a putative binding site for miR-132 may be located in the 3′-UTR of human growth differentiation factor 5 (*GDF5*), an important factor involved in chondrogenesis and chondrocyte differentiation [20]. GDF5 is expressed in both nondegenerate and degenerate disc, particularly in the NP; however, a slight reduction in the proportion of cells in the NP expressing GDF5 is detected during degeneration [21]. In addition, treatment of human NP cells with GDF5 induced a dramatic increase in type II collagen and aggrecan expression and increased accumulation of proteoglycans [21]. The *GDF5* single-nucleotide polymorphism rs143383 is associated with the risk of lumbar disc degeneration [22]. Considering the important roles of miRNAs and GDF5 in the pathogenesis of IDD, and the results of our bioinformatic target prediction, we hypothesized that miR-132 may be involved in the pathogenesis of IDD *via* direct targeting of *GDF5*. Accordingly, we used both *in vitro* and *in vivo* assays to study the roles of miR-132 in IDD and further elucidate the molecular mechanisms underlying IDD.

2. Materials and methods

2.1. Patient tissue samples

Experimental protocols were 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. Control lumbar NP tissue samples were collected from 14 patients (n = 5 women and 9 men; mean age, 20.5 years; range, 17–34 years) with idiopathic scoliosis undergoing deformity correction surgery. Degenerative NP lumbar samples were collected from 27 patients (n = 11 women and 16 men; mean age, 44 years; range, 29–70 years) with IDD, undergoing disc excision and spinal fusion surgery. The degree of degeneration of IVDs was assessed from pre-operative magnetic resonance imaging scans according to the modified Pfirrmann grading system [23]. The lumbar discs of all patients with IDD were classified as Grades III–V and those of the 14 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 14 patients with idiopathic scoliosis as described previously [14]. After isolation, 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. 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. Establishment of rat IVD model

Three-month-old Sprague-Dawley (SD) rats were used. Animals were purchased from the Experimental Animal Center of Tongji Medical College (Wuhan, China). All animal studies were approved by the Institutional Animal Research Committee of Tongji Medical College. The animal experiments were carried out in accordance with protocols approved by the Institutional Animal Care and Use Committee. Rats (n = 60) were randomly divided into six groups (n = 10 per group): control, punctured, agomir-132 control-treated punctured group, agomir-132-treated punctured group, antagomir-132 control-treated punctured group, and antagomir-132-treated punctured group. A rat model of IDD was generated using the annulus fibrosus needle puncture method. In brief, general anesthesia (10 mg/kg xylazine and 90 mg/kg ketamine hydrochloride) was administered. Subsequently, coccygeal discs C6–C7 were punctured using a syringe needle. The syringe needle was inserted into the C6–C7 disc in a vertical direction, and then rotated in the axial direction by 180° and held for 10 s. The puncture was made parallel to the endplates through the AF into the NP using a 31-gauge needle, which was inserted 1.5 mm into the disc to depressurize the nucleus. The other segments were left undisturbed as controls. agomir-132, antagomir-132, and their corresponding negative controls (agomir-132 control and antagomir-132 control) were designed and synthesized by RiboBio (Guangzhou, China). agomir-132 or antagomir-132 were injected into disc C6–C7 of the rats in the agomir-132-treated punctured group and antagomir-132-treated punctured group, respectively. The C6–C7 level discs of rats were exposed, but not punctured, to serve as controls (sham group), and rats with punctured C6–C7 level discs without treatment with miR-132 agomir, antagomir or their negative control equivalents, served as puncture control groups. All rats were sacrificed by lethal anesthetic overdose on the 28th day after the operation. Discs were collected and used for further analysis.

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

Total RNA was isolated from NP tissue and cells using TRIzol reagent (Invitrogen) according to the manufacturer's instructions. *GDF5*, type II collagen, aggrecan, *MMP13*, *ADAMTS4*, and miR-132 expression were quantified by qRT-PCR on a 7500 Real-time PCR System using the cycling conditions recommended by the manufacturer. Primers used for qRT-PCR are listed in Table 1. Reactions were

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

Gene	Oligonucleotide sequence		Product size (bp)
	Forward (5–3′)	Reverse (5–3′)	
has-GDF5	CGATAAGACCGTGTATGAGT	CTCGCAGTGGAAAGCCTCGT	196
has-Type II collagen	TCCAGATGACCTTCCTACGC	GGTATGTTTCGTGCAGCCAT	209
has-Aggrecan	TGAGCGGCAGCACTTTGAC	TGAGTACAGGAGGCTTGAG	287
has-MMP13	GCAGTCTTTCTTCGGCTTAG	CATTGTATTCACCCACATCAG	104
has-ADAMTS4	CACCCAAGCATCCGCAATCC	CATACCCAGCGTGTCGCAAGT	234
rat-GDF5	CTGTTCCTGGTGTTTGGGCGTAC	AGCCTTGAGGTTCTTGCTGGGTC	174
rat-Type II collagen	GAAGAGCGGAGACTACTGGATTG	TGGACGTTAGCGGTGTTGGGAG	243
rat-Aggrecan	TCGCAAGTCCCTTCCACATC	TCAAGGCGTCCTGAAGTGTC	149
rat-MMP13	GTGACTCTTGCGGGAATCCT	CAGGCACTCCACATCTTGGT	151
rat-ADAMTS4	CATATGCAACGTCAAGGCTCC	GTTGACAGGGTTTCGGATGC	214
U6	CGCTTCGGCAGCACATATAC	AAATATGGAACGCTTCACGA	100
β-actin	AGCGAGCATCCCCCAAAGTT	GGGCACGAAGGCTCATCATT	285