MiR-184 Regulates Proliferation in Nucleus Pulposus Cells by Targeting *GAS1*

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■ OBJECTIVE: **The precise mechanism of nucleus pulposus proliferation in the degeneration of the intervertebral disk pathogenesis remains to be implicated. MicroRNAs (MiRNAs) are a class of 18−22 nucleotides, which are small, noncoding RNAs that inhibit protein translation by binding to the 3′-UTR of target gene. Recent studies have shown that miRNAs play a crucial role in various cell biologies such as cell proliferation, invasion, migration, and cell cycle. However, the role of miR-184 in nucleus pulposus proliferation is still unknown.**

■ METHOD: **qRT-PCR was performed to measure the expression of miR-184. CCK-8 assay, qRT-PCR, and Western blot were used to measure the functional role of miR-184 in nucleus pulposus (NP) cells. Western blot and Luciferase assays were done to find the miR-184 target gene.**

■ RESULT: **We demonstrated that expression of miR-184 was upregulated in degenerative NP tissues compared with that in the control NP tissues, and the expression of miR-184 was positively correlated with disk degeneration grade. We identified Growth Arrest Specific Gene 1 (*GAS1*) as a direct target gene of miR-184 in NP cells, and ectopic expression of miR-184 promoted NP cells proliferation. In addition, we found that *GAS1* expression was downregulated in degenerative NP tissues compared with that in the control NP tissues and the *GAS1* expression was inversely correlated with the grade of disk degeneration. Moreover, we demonstrated that miR-184 overexpression could induce AKT phosphorylation and ectopic expression of *GAS1* decreased the miR-184 overexpressing NP cells proliferation.**

■ CONCLUSION: **These results demonstrated that miR-184 and the *GAS1*/Akt pathway may be a potential therapeutic target for intervertebral disc degeneration.**

INTRODUCTION

Low back pain (LBP) is a major health problem that causes a serious burden on economies and the health care system.[1,2] Degeneration of the intervertebral disk is a principal cause of LBP.[3-5] Intervertebral disk degeneration is thought to be associated with genetics, toxins, systemic factors, and age. However, the precise causes of intervertebral disk degeneration are still unclear.[6-8]

Micro ribonucleic acids (miRNAs) are a category of small (about 22 nucleotides), highly conserved, endogenous, noncoding RNAs, which control gene expression through silencing mRNAs (messenger RNAs).[9-11] miRNAs play crucial roles in many cell pathologic processes including cell proliferation, development, stress response, apoptosis, differentiation, and invasion.[11-15] Increasing data have proven that miRNAs play important roles in the initiation and development of tumors and act as a tumor suppressor gene or oncogene.[16-18] Importantly, studies also found that miRNAs also act as critical regulators in intervertebral disk degeneration.[7,19,20] However, the role of miRNA-184 in intervertebral disk degeneration has not been well explained.[21]

In our study, we showed that the expression of miR-184 was upregulated in degenerative nucleus pulposus (NP) tissues compared with that in the control NP tissues and the expression of miR-184 was positively correlated with disk degeneration grade. We identified Growth Arrest Specific Gene 1 (GAS1) as a direct target gene of miR-184 in NP cells and discovered that ectopic

Key words
- *GAS1*
- Intervertebral disk degeneration
- MicroRNAs
- miR-184
- Nucleus pulposus

Abbreviations and Acronyms
GAS1: Growth Arrest Specific Gene 1
LBP: Low back pain
miRNAs: microRNAs
mRNAs: Messenger RNAs
NP: Nucleus pulposus

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MATERIALS AND METHODS

Tissues and Cell Cultures

Human lumbar nucleus pulposus tissues were obtained from idiopathic scoliosis patients ($n = 4$; range age 11–14 years) and intervertebral disk degeneration patients during surgery (Table S1). Magnetic resonance imaging (MRI) of the lumbar spine of these patients was performed during the reoperation time; disk degeneration degree was classified using the modified Pfirrmann method. NP cells were isolated from idiopathic scoliosis patients' lumbar NP tissues and cultured as previously shown. NP cells were kept in DMEM (Dulbecco's modified Eagle medium, Gibco, Thermo Fisher Scientific, Waltham, Massachusetts, USA) supplied with FBS (Gibco), streptomycin, and penicillin in a 5% CO_2 (v/v), 37°C environment.

Ribonucleic Acid Extraction and qRT-PCR

RNA was isolated from samples and cells using Trizol (Invitrogen, Thermo Fisher Scientific, Carlsbad, California, USA). Complementary DNAs were synthetized using M-MLV reverse kit (Invitrogen). The expression of miRNA and mRNA was measured by qRT-PCR on an IQ5 PCR system. U6 was used for an internal control to miRNA, and GAPDH was employed as a control for *GAS1*. The following sequences were used: miR-184, 5′-TGGACGGAGAACTGATAAGGGT-3′; U6, 5′-CTCGCTTCGGCAGCACATATA-3′; Hes1, forward primer 5′-TGAAGGATTCCAAAAATAAAATTCTCTGGG-3′ and reverse primer 5′-CGCCTCTTCTCCATGATAGGCTTTGATGAC-3′; GAPDH, forward primer 5′-ATTCCATGGCACCGTCAAGGCTGA-3′, reverse primer 5′-TTCTCCATGGTGGTGAAGACGCCA-3′.

Dual Luciferase Assays

A wild-type *GAS1* 3′UTR and mutant *GAS1* 3′UTR from the human cDNA library were amplified by polymerase chain reaction (PCR), confirmed by sequencing, and then cloned into the miRNA target expression vector (Genechem Incorporation, Shanghai, China). MiR-184 mimic and scramble and the reporter vector were cotransfected using Lipofectamine 2000 (Invitrogen) according to the manufacturer's information. The luciferase activity was measured by the Dual-Luciferase Assay System (Promega, Madison, Wisconsin, USA).

Oligonucleotides, Constructs, and Transfections

miR-184 mimics, scramble mimic and pCDNA-GAS, were obtained from Dharmacon (Austin, Texas, USA) and transfected in NP cells using Lipofectamine 2000 (Invitrogen) at a concentration of 30 nM according to the manufacturer's instructions.

Western Blotting

Protein was extracted from treated cells using protein extraction reagent (Pierce, Thermo Fisher Scientific). Protein was separated by 12% SDS and then transferred to PVDF membranes (Merck Millipore, Billerica, Massachusetts, USA). The primary antibodies *GAS1* and GAPDH (Santa Cruz) were incubated overnight at 4°C. The bands were measured by the enhanced chemiluminescence system (Merck Millipore).

CELL PROLIFERATION AND CELL CYCLE

NP cells were cultured in 96 well plates and measured with CCK-8 (Cell Counting Kit-8, Dojindo, Rockville, Maryland, USA) at different time points per the manufacturer's information. For the cell cycle, we fixed cells with ethanol overnight at −20°C and followed the steps provided by the manufacturer (Molecular Probes Inc., Eugene, Oregon, USA). Cells were then washed twice in PBS, resuspended in FACS buffer, and analyzed by FACS flow cytometry (Becton Dickinson, Franklin Lakes, New Jersey, USA).

STATISTICAL ANALYSIS

Data were analyzed using SPSS 17.0. and shown as mean ± standard deviation. Analysis of variance (ANOVA) or Student's t-test was performed to measure the statistical significance between differences, and $P < 0.05$ was mean statistically significant.

RESULTS

MiR-184 was upregulated, and *GAS1* was downregulated in degenerative NP tissues. The expression of miR-184 was increased in degenerative NP tissues compared with the control NP tissues (Figure 1A). The expression of *GAS1* was decreased in degenerative NP tissues compared with the control NP tissues (Figure 1B).

The expression of miR-184 was positively related with degeneration grade. We further measured the expression of miR-184 in 40 degenerative NP tissues (Figure 2A and B). The expression of miR-184 was positively related with Pfirrmann classification disk degeneration grade (Figure 2C; $r = 0.56$, $P < 0.01$).

The expression of *GAS1* was correlated with degeneration grade. We further measured the expression of *GAS1* in 40 degenerative NP tissues (Figure 3A and B). The expression of *GAS1* was inversely correlated with Pfirrmann classification disk degeneration grade (Figure 3C; $r = 0.51$, $P < 0.01$).

GAS1 was a direct target gene of miR-184. A putative miR-184 seed sequence was expected in the 3′ UTR region of *GAS1* (Figure 4A). As shown in Figure 4B, the luciferase activity was significantly decreased in NP cells with miR-184 transfection compared with the scramble treated; however, mutation of the predicted seed sequence of miR-184 on the *GAS1* 3′ UTR rescued this effect (Figure 4B). Overexpression of miR-184 inhibited the *GAS1* protein expression in NP cells (Figure 4C and D).

MiR-184 promoted NP cell proliferation. The miR-184 mimics transfection efficiency was confirmed in NP cells by qRT-PCR (Figure 5A). Ectopic expression of miR-184 promoted the NP cell proliferation (Figure 5B). Moreover, this effect was further confirmed by cell cycle (Figure 5C).

GAS1 was involved in the effect of miR-184-induced NP cell proliferation. The expression of *GAS1* was upregulated in NP cells after being transfected by *GAS1* vector (Figure 6A). Overexpression of miR-184 induced Akt phosphorylation in NP cells (Figure 6B). Moreover, overexpression of *GAS1* inhibited Akt phosphorylation