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MicroRNA-145 overexpression attenuates apoptosis and increases matrix synthesis in nucleus pulposus cells

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ABSTRACT

Aims: Lower back pain is often associated with intervertebral disc degeneration (IDD), which results from a decrease in nucleus pulposus (NP) cells and an imbalance between the degradation and synthesis of extracellular matrix (ECM) components. Multiple microRNAs play crucial roles in the modulation of NP cell apoptosis and matrix degradation. miR-145 is an important microRNA related to degenerative diseases such as osteoarthritis. Here, the effect of miR-145 in IDD was elucidated. The aim of this study was to explore the role and mechanism of miR-145 in the apoptosis of NP cells and in matrix metabolism in NP cells.

Materials and methods: Real-time PCR, western blotting and flow cytometry analysis were used to observe the effect of miR-145 on NP cell apoptosis in the absence or presence of oxidative stress. Cell transfection, loss-of-function experiments using an ADAM17 inhibitor or lentiviral shADAM17, immunofluorescence, real-time PCR and western blotting were performed to demonstrate the role and mechanism of miR-145 in NP cell matrix metabolism.

Key findings: miR-145 attenuated NP cell apoptosis in the absence and presence of oxidative stress. Moreover, miR-145 overexpression increased and miR-145 suppression decreased matrix synthesis. ADAM17, which is expressed in degenerative discs, is the target of miR-145. ADAM17 gene suppression with lentiviral shRNA or an inhibitor enhanced matrix synthesis in NP cells. In addition, siADAM17 reversed the matrix degradation induced by miR-145 inhibition.

Significance: miR-145 suppresses apoptosis and promotes ECM synthesis in NP cells. miR-145 is thus a potential therapeutic microRNA for IDD.

1. Introduction

Low back pain (LBP) is often associated with intervertebral disc degeneration (IDD), which is an extremely common musculoskeletal disorder [1,2]. Although much existing evidence suggests that IDD is triggered or influenced by multiple factors, such as aging, genetics and lifestyle, the etiology and pathogenesis of IDD remain unclear [3–5]. Intervertebral discs (IVDs) are composed of an inner gel-like nucleus pulposus (NP) and an outer fibrocartilaginous annulus fibrosus (AF). NP cells maintain the homeostasis of the extracellular matrix (ECM), which comprises proteoglycans (mainly aggrecan) and collagens (mainly collagen II) [6]. Decreases in NP cells and declines in ECM deposition are hallmarks of IDD [6–8]. Evidence has revealed that multiple factors, including serum deprivation, oxidative stress and inflammation, are related to the decreases in NP cells and ECM metabolism. However, the underlying etiology is still not fully understood [9–11].

MicroRNAs (miRNAs), which are widespread in eukaryotic cells, are a class of small endogenous noncoding RNAs. Through preferential

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Table 1
Primer sequences for real-time PCR.

Species	Gene	Sense (5′ to 3′)	Antisense (5′ to 3′)
Rat	Col2a1	AGCAAGAGCAAGGAGAAGAAGCA	TGGACAGTAGACGAGGAAAGTCA
	Aggrecan	ACACGGCTCCACTTGATTCTT	CTTGGTCTTTGTGACTCTGCG
	ADAM17	CAAAGCCATCATCATCCACAT	TTCGCATTATCAAGCCGTTCC
	GAPDH	TCTCTGCTCCTCCCTGTTC	ACACCGACCTTCACCATCT
Human	Col2a1	GGCAATAGCAGGTTCACGTACA	CGATAACAGTCTTGCCCCACTT
	Aggrecan	CAGTTGTCTCCTCTTCTACGG	GAACGGTCTACCTCTACCCTAA
	ADAM17	ATGAATGGCAAATGTGAGAAACGAG	CAATGGACAAGAATGCTGAAAGGAA
	GAPDH	AGAAAAACCTGCCAAATATGATGAC	TGGGTGTCGCTGTTGAAGTC
	HPRT1	CGAGATGTGATGAAGGAGATGG	TTGATGTAATCCAGCAGGTCAG

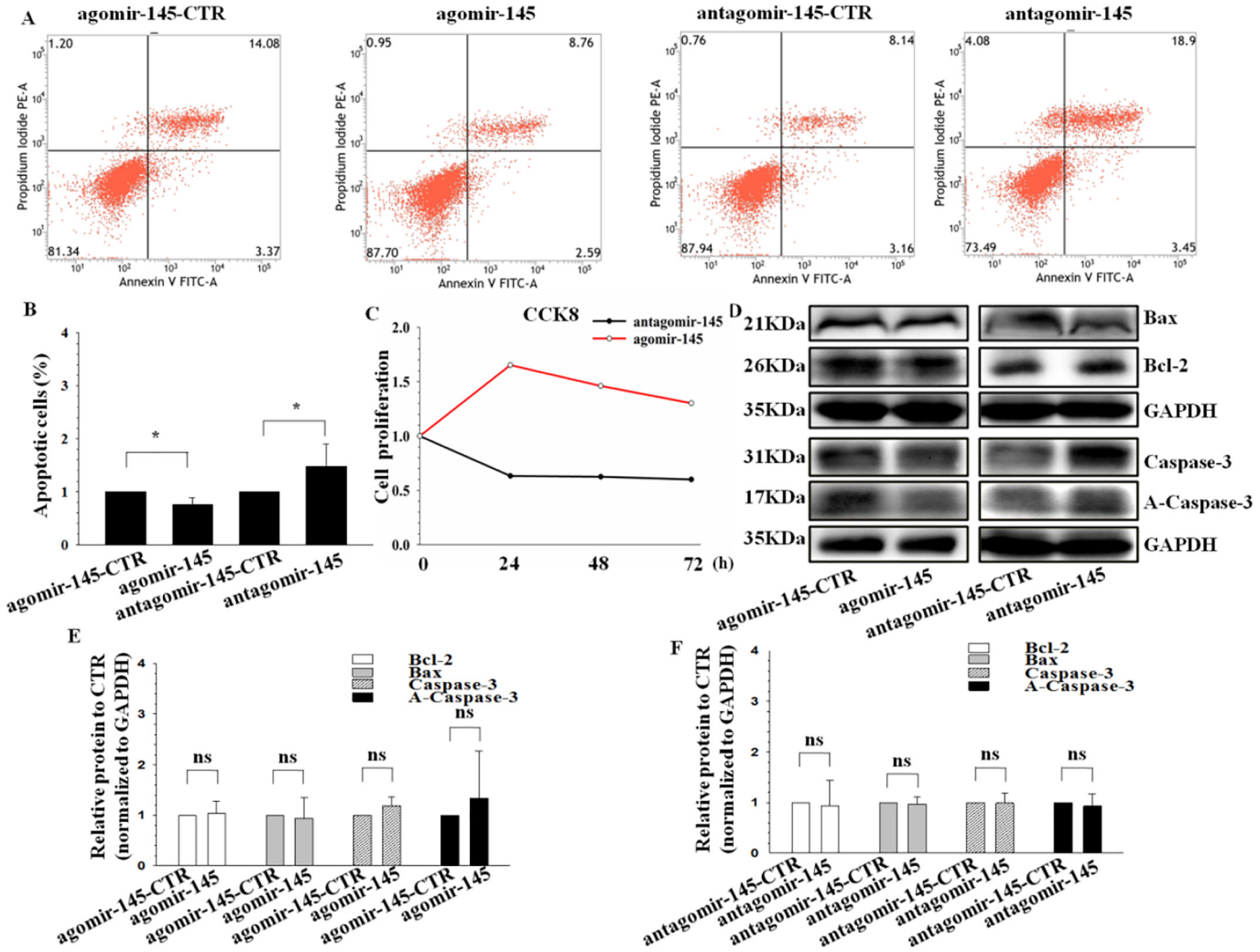

Fig. 1. miR-145 suppressed apoptosis in rat NP cells.
(A, B) Flow cytometry was used to determine the rate of apoptosis in rat NP cells transfected with agomir-145-CTR, agomir-145, antagomir-145 or antagomir-145-CTR for 48 h. (C) The proliferation of rat NP cells transfected with agomir-145 or antagomir-145 was demonstrated by CCK-8 assay. (D-F) Western blotting (D) and subsequent densitometric analysis (E, F) showed the protein levels of Bcl-2, Bax, caspase-3 and activated caspase-3 (A-caspase-3). GAPDH was used as a loading control. The data are presented as the mean ± SD. $^*P < 0.05$ vs. the control group.

binding to the 3′-untranslated regions (3′-UTRs) of specific messenger RNAs (mRNAs), miRNAs negatively regulate gene expression and in turn control a wide variety of biological processes, such as cell differentiation, proliferation, autophagy and apoptosis [12,13]. Previous studies have demonstrated that microRNAs play a vital role in IDD [14–18]. For example, microRNA-155 (miR-155) regulates apoptosis by targeting Fas-associated protein with death domain (FADD) and caspase-3 and controls ECM degradation via CCAAT/enhancer-binding protein β (C/EBP β) [14,15], while miR-138-5p promotes TNF-α-induced apoptosis by targeting SIRT1 through PTEN/PI3K/Akt signaling [16]. In addition, miR-494 promotes TNF-α-induced apoptosis by targeting JunD, and miR-494 overexpression promotes ECM degradation through Sox9 [17,18].

As a protective microRNA in osteoarthritis, a common degenerative disease, miR-145 attenuates TNF-α and IL-1β-driven cartilage matrix degradation [19,20]. However, the role of miR-145 is dependent on cell type [21,22]. For example, miR-145 overexpression suppresses apoptosis in rat infarcted myocardial cells and human non-small-cell lung

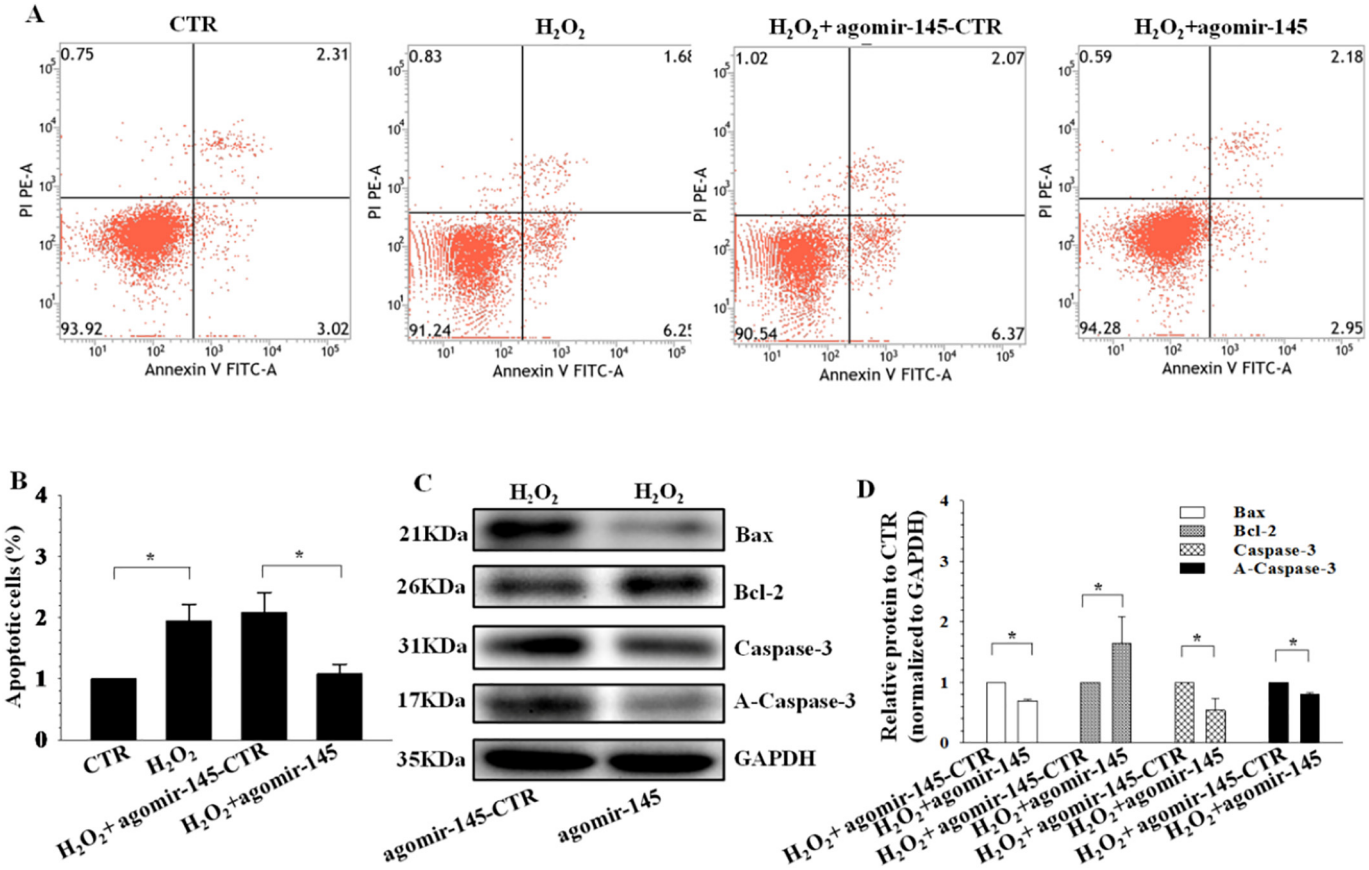

Fig. 2. miR-145 repressed the apoptosis induced by oxidative stress in rat NP cells.
(A, B) Flow cytometry determined the rate of apoptosis in rat NP cells transfected with agomir-145-CTR or agomir-145 in the presence of H_2O_2 for 48 h, with untransfected cells serving as the controls. (C, D) Western blotting (C) and subsequent densitometric analysis (D) showed the protein expression of Bcl-2, Bax, caspase-3 and activated caspase-3 (A-caspase 3); GAPDH was used as a loading control. The data are presented as the mean ± SD. *$P < 0.05$ vs. the control group.

cancer A549 cells but promotes apoptosis in osteosarcoma cells and activated hepatic satellite cells [21–24]. Whether and how miR-145 controls apoptosis and matrix metabolism in NP cells remain to be elucidated.

The present study investigated the role and mechanism of miR-145 in the apoptosis and matrix synthesis of NP cells. The findings herein provide insight into the role of miR-145 in IDD pathogenesis.

2. Materials and methods

2.1. Plasmids and reagents

Rat miR-145 mimics (agomir-145), mimic controls (agomir-145-CTR), miR-145 inhibitors (antagomir-145), and inhibitor controls (antagomir-145-CTR) were purchased from RiboBio Co., Ltd. (Guangzhou, China). A segment of the wild-type 3′-UTR of rat ADAM17 mRNA containing the putative miR-145 binding sequence was amplified and inserted into a pmiR-RB-Report™ vector to create a 3′-UTR/ADAM17 plasmid. Both wild-type and mutant pmiR-3′-UTR/ADAM17-Report™ constructs were developed by Genechem, Shanghai, China. The wild-type sequence was 5′-GAGGATACAGTTCCCACAGA-3′, and the mutant sequence was 5′-GAGGATAACTGGAACACAGA-3′. The lentiviral human ADAM17 shRNA (LV-shADAM17) target sequence was 5′-TATGTCGA TGCTGAACAAA-3′, and media containing LV-shADAM17 and scrambled negative control shRNA (LV-shControl) were also purchased from Genechem. The target sequence of rat siADAM17 is 5′-CACCTGCGAC TTGAGAAGCTT-3′ and the target sequence of mock construct is 5′-TTCTCCGAACGTGTCACGT-3′, both of them were developed by Gene Pharma, Shanghai.

TNF-α was purchased from Peprotech, Rocky Hill, NJ, USA, and H_2O_2 was purchased from Sigma/Merck Millipore, Darmstadt, Germany. The ADAM17 inhibitor TAPI-1 was obtained from ApexBio, Houston, TX, USA. An anti-collagen IIa antibody was purchased from Abzoom, Brussels, Belgium. Anti-ADAM17, anti-B, anti-Bax, Bcl-2, anti-caspase 3, anti-activated caspase 3 and anti-aggrecan antibodies were obtained from Abcam, Cambridge, UK, and an anti-GAPDH antibody was obtained from Proteintech. Horseradish peroxidase-conjugated rabbit or mouse IgG was purchased from ABclonal, MA, USA.

2.2. Isolation and culture of NP cells

In accordance with the Institutional Review Board guidelines of Sun Yat-sen University, the experimental animal center of Sun Yat-sen University provided Sprague-Dawley rats, and all experimental procedures were approved by the animal care and use committee of Sun Yat-sen University. Normal human NP tissue for human NP cell separation was obtained from patients suffering from thoracolumbar fractures, and each patient consented to sample collection.

Rat and human NP cells were isolated as described previously [25]. Briefly, NP tissues were digested with 0.2% pronase for 1 h, washed three times with phosphate-buffered saline (PBS) and then resuspended in Dulbecco's modified Eagle's medium (DMEM; Gibco, Grand Island, NY, USA) containing 10% fetal bovine serum (FBS, Gibco), 100 U/mL penicillin and 100 U/mL streptomycin. The obtained NP cells were incubated at 37 °C with 5% CO_2, and the medium was changed every other day. To investigate the effects of cytokines, oxidative stress or the ADAM17 inhibitor TAPI-1, NP cells were treated with TNF-α, H_2O_2 or TAPI-1, respectively, for 24 h after overnight culture in serum-free

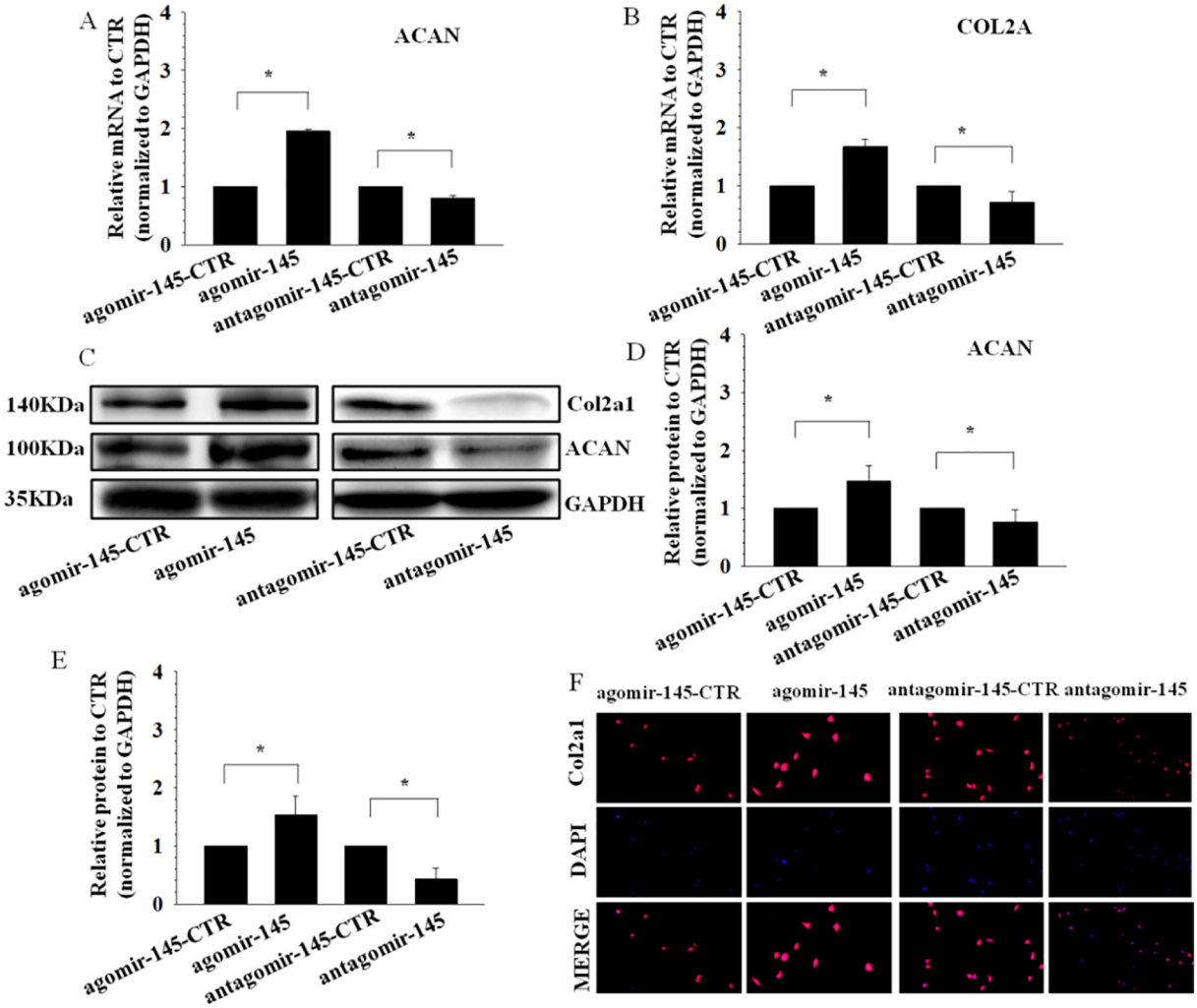

Fig. 3. miR-145 increased matrix metabolism in rat NP cells.
(A, B) Real-time PCR analysis was performed to evaluate the mRNA expression of aggrecan (A) and collagen IIa (B) after a single transfection with agomir-145 or miR-155 inhibitors. (C, D, E) Western blotting (C) and subsequent densitometric analysis (D) demonstrated that aggrecan (C, E) and collagen IIa (D, E) protein were significantly upregulated in rat NP cells after transfection with agomir-145. (F) Immunofluorescence staining of type II collagen in miR-145 mimic- or miR-145 inhibitor-transfected NP cells. Col2a1: collagen IIa; ACAN: aggrecan. The data are presented as the mean ± SD of three independent experiments. *$P < 0.05$.

medium.

2.3. Human NP tissue collection and grading

Human IVD tissue samples were obtained from patients undergoing anterior lumbar interbody fusion (ALIF) for degenerative disc disease under a protocol approved by the Institutional Review Board of Thomas Jefferson University (Protocol #08D.525). In the laboratory, the tissue was immediately separated sharply into NP and AF based on gross examination. Only the innermost portion of NP was used. The degree of IVD degradation was evaluated according to the Pfirrmann system [26]. The characteristics of the human tissue used are listed in Supplemental Table 1, and the grades of the IVDs were as follows: grade 3: $n = 8$, grade 4: $n = 10$, grade 5: $n = 6$.

2.4. Cell apoptosis detection using flow cytometry

After transfection with agomir-145, agomir-145-CTR, antagomir-145 or antagomir-145-CTR for 48 h, NP cells were collected and re-suspended for Annexin V and propidium iodide (PI) staining using a FITC Annexin V Apoptosis Detection Kit I (BD Pharmingen, San Diego, CA, USA) according to the manufacturer's protocol. Then, flow cytometry (FACSCalibur, BD Biosciences, San Diego, CA, USA) was used to analyze the apoptotic cells.

2.5. Cell counting kit-8

After transfection ot NP cells with plasmids of agomir-145 and antagomir-145 in 96-well plates, Cell counting kit-8 (CCK-8, Dojindo, Kumamoto, Japan) was used to determine relative cell proliferation according to the manufacturer's instructions. The absorbance at 450 nm was measured by using a microplate reader (Thermo Multiskan Mk3, USA). Data shown are representative of three independent experiments.

2.6. Cell transfection and dual luciferase reporter assays in rat NP cells

According to the manufacturer's instructions, 20 μM rat agomir-145 or agomir-145-CTR were transiently transfected into rat NP cells using Lipofectamine 2000 reagent (Invitrogen) at a final oligonucleotide concentration of 50 nM. antagomir-145 or antagomir-145-CTR were similarly transfected at a final oligonucleotide concentration of 100 nM. After 48 h of transfection, the NP cells were harvested for RNA and protein extraction.

To detect the activity of the pmiR-3′-UTR/ADAM17-Report™

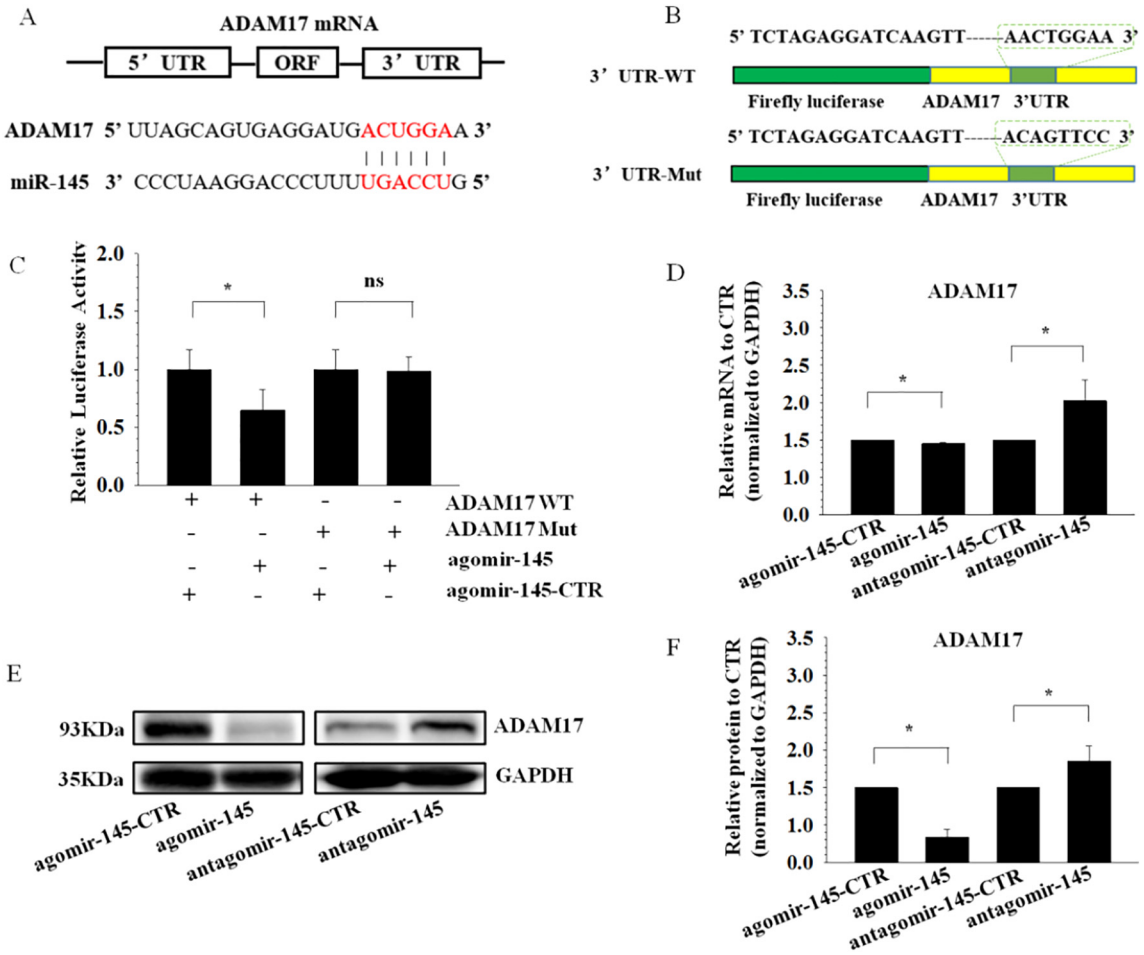

Fig. 4. ADAM17 is a direct target of miR-145.
(A) Alignment of the rat miR-145 target site with the ADAM17 3′-UTR. (B) Introduction of the wild-type and mutant sequences into the luciferase reporter construct. (C) Dual luciferase reporter assay results for the wild-type (WT) and mutant (Mut) ADAM17 3′-UTR reporters following agomir-145 transfection. (D) Real-time PCR analysis was used to evaluate ADAM17 mRNA levels after a single transfection with agomir-145 or antagomir-145. (E, F) Western blotting (E) and subsequent densitometric analyses (F) revealed that ADAM17 protein expression was altered by transfection with agomir-145 and antagomir-145. WT: ADAM17 3′-UTR wild-type construct; Mut: ADAM17 3′-UTR mutation construct. The data are presented as the mean ± SD of three independent experiments. *$P < 0.05$.

construct, the NP cells were cotransfected with 0.4 μg of wild-type or mutant pmiR-3′-UTR/ADAM17-Report™ construct and 0.2 μg of a pGL-3 control vector with either miR-145 or a negative control. Two days later, cells were harvested and assayed using a dual luciferase reporter assay (Promega, WI, USA). Firefly luciferase values were normalized to the Renilla signals. All luciferase assays were performed in triplicate.

2.7. Immunofluorescence analysis of rat NP cells

After 48 h of transfection, NP cells were fixed with 4% paraformaldehyde for 15 min at 37 °C, washed three times with PBS and permeabilized with 0.1% Triton X-100. After rinsing with PBS, the cells were blocked with 10% goat serum for 1 h, incubated with an anti-collagen IIa antibody (1:50) for 16 h at 4 °C and incubated for 2 h at room temperature with a Cy3-conjugated goat anti-rabbit IgG (1:100). Finally, the cells were incubated with 0.1 mg/mL DAPI (Cell Signaling Technology Inc., MA, USA) for 15 min. PBS was used to replace the primary antibodies as a negative control. Images were collected with a fluorescence microscope (Nikon, Eclipse 80i, Japan).

2.8. Knockdown of ADAM17

Human NP cells were cultured at a density of 0.5×10^6 cells per 10-cm plate. After 3 days, the medium was replaced with medium containing LV-shADAM17 or LV-shControl particles together with 6 mg/mL polybrene (EMD Millipore, Billerica, MA, USA) in accordance with the manufacturer's transfection protocol. Then, 24 h later, the medium containing the viral particles was removed and replaced with normal culture medium (DMEM containing 10% FBS and antibiotics). The NP cells were harvested 5 days later for protein extraction.

2.9. Quantitative real-time polymerase chain reaction (PCR) analysis

Total RNA from human NP tissue was extracted using an RNeasy Mini Kit (Qiagen) according to the manufacturer's instructions. Before elution from the column, the RNA was treated with RNase-free DNase I (Qiagen).The purified, DNA-free RNA was converted to complementary DNA (cDNA) using RNA to cDNA EcoDry Premix (Oligo dT; Clontech Laboratories, Inc.). Total RNA was extracted from the NP cells using TRIzol reagent according to the manufacturer's instructions (TaKaRa, Dalian, China). Total DNA-free RNA was also used to synthesize cDNA using an EcoDry double-primed cDNA synthesis kit (Clontech Laboratories, Mountain View, CA). Quantitative real-time PCR was performed using SYBR Green PCR Master Mix (Applied Biosystems) on a StepOnePlus Real-Time PCR System. The amount of PCR product from human NP tissue was estimated using the relative standard curve quantification method, and the amount of PCR product from NP cells was calculated using the $2^{-\Delta\Delta Ct}$ method.

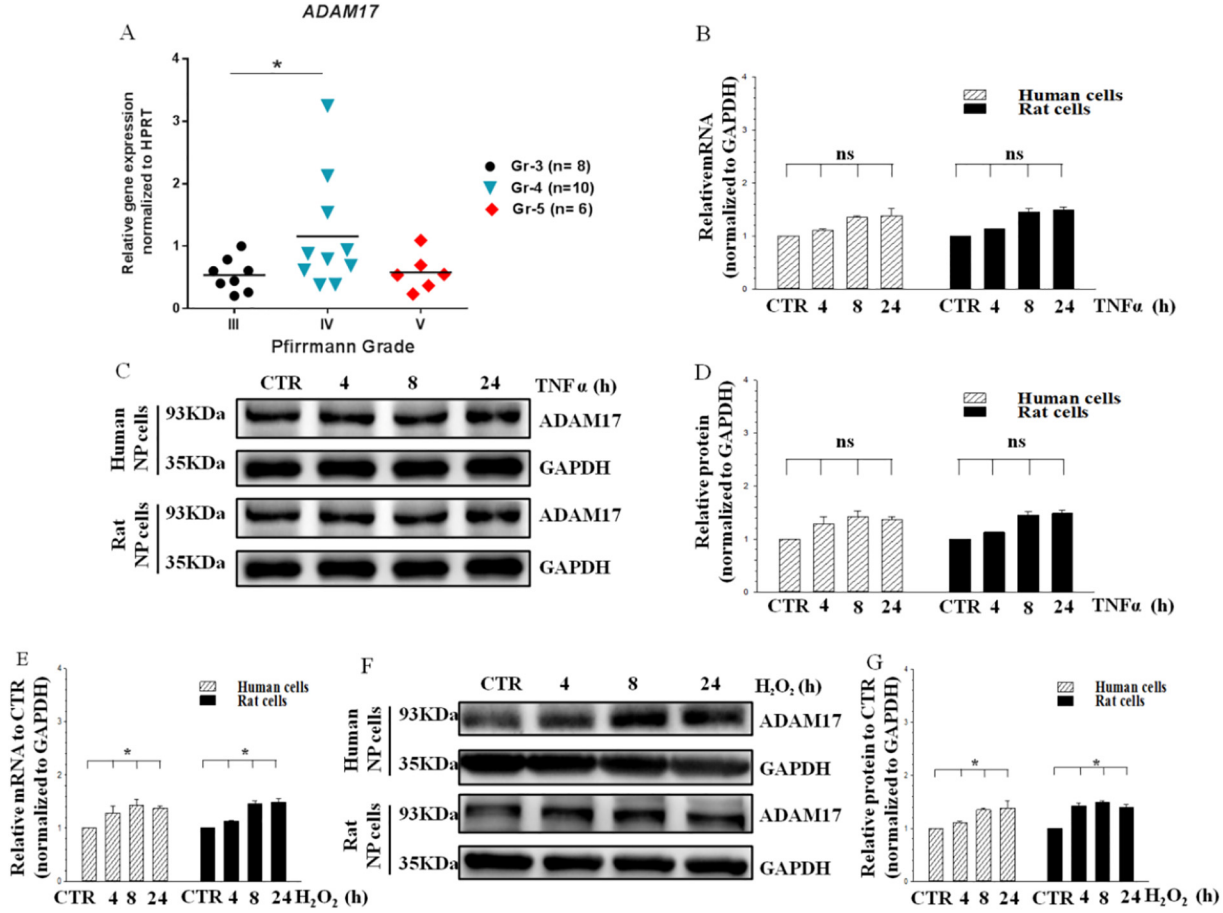

Fig. 5. Expression of ADAM17 in degenerative human NP tissue and regulation by TNF-α and H_2O_2 in NP cells.
(A) Disc tissue from patients was collected and classified according to the Pfirrmann grading system. ADAM17 expression in human NP tissue ($n = 24$) was determined by real-time PCR. (B) Effect of TNF-α on the expression of ADAM17 mRNA in human and rat NP cells. (C, D) Western blotting (C) and subsequent densitometric analysis (D) revealed the expression of ADAM17 protein after TNF-α stimulation in human and rat NP cells. (E) Real-time PCR revealed the effect of H_2O_2 on the expression of ADAM17 mRNA in human and rat NP cells. (F, G) Western blotting (F) and subsequent densitometric analysis (G) demonstrated the expression of ADAM17 protein after H_2O_2 stimulation in human and rat NP cells. HPRT or GAPDH were used as an internal control. CTR: control; Gr-: grade. The data are presented as the mean ± SD. *$P < 0.05$; ns: not significant.

The gene-specific primers are described in Table 1. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used to normalize the mRNA expression in human and rat NP cell samples, and hypoxanthine phosphoribosyltransferase (HPRT) was used to normalize the expression of mRNA in human NP tissue. Each sample was analyzed in duplicate, and the expression scores were calculated using the $2^{-\Delta\Delta Ct}$ method.

2.10. Western blotting analysis

After harvesting, the NP cells were lysed with lysis buffer containing 1 × protease inhibitor cocktail (Roche Diagnostics, Basel, Switzerland), NaCl (5 mM), NaF (200 μM), Na_3VO_4 (200 μM) and dithiothreitol (0.1 mM) for 30 min. Following quantification with the BCA reagent, the total cell proteins were heat-denatured, electrophoresed on sodium dodecyl sulfate (SDS)- polyacrylamide gels and transferred to polyvinylidene difluoride membranes (Millipore, MA). Subsequently, TBST (10 mM Tris, pH 8.0, 150 mM NaCl and 0.1% Tween 20) with 5% BSA (Bovine Serum Albumin V) was used to block the membranes for 1 h; the membranes were then incubated overnight at 4 °C with primary antibodies (1:1000 anti-ADAM17, 1:1000 anti-Bax, 1:1000 anti-Bcl-2, 1:1000 anti-caspase 3, 1:1000 anti-activated caspase 3, 1:1000 anti-aggrecan, 1:1000 collagen IIa and 1:3000 anti-GAPDH). The secondary antibodies (rabbit or mouse IgG conjugated to horseradish peroxidase) were then incubated with the membranes at a dilution of 1:2000 or 1:5000 (only for GAPDH) at room temperature for 1 h, and the immunoreactive bands were detected using an enhanced chemiluminescence reagent system (Affinity Biosciences, OH, USA).The band intensity was determined by densitometric analysis using Image J software (National Institutes of Health, USA). The expression of GAPDH, the internal control, was used to normalize the expression of the other proteins.

2.11. Statistical analysis

All data are presented as the mean ± SD of at least three experiments. SPSS 13.0 software was used for analysis, and differences between two groups were analyzed using independent sample *t*-tests. One-way ANOVA was used to analyze differences among three or four groups. Differences were considered statistically significant at $P < 0.05$.

3. Results

3.1. miR-145 suppressed apoptosis in rat NP cells

To determine whether miR-145 modulates the apoptosis of NP cells, flow cytometry analysis and western blotting were used. Flow cytometry analysis revealed that miR-145 overexpression caused by agomir-145 suppressed the rate of NP cell apoptosis compared to that in the

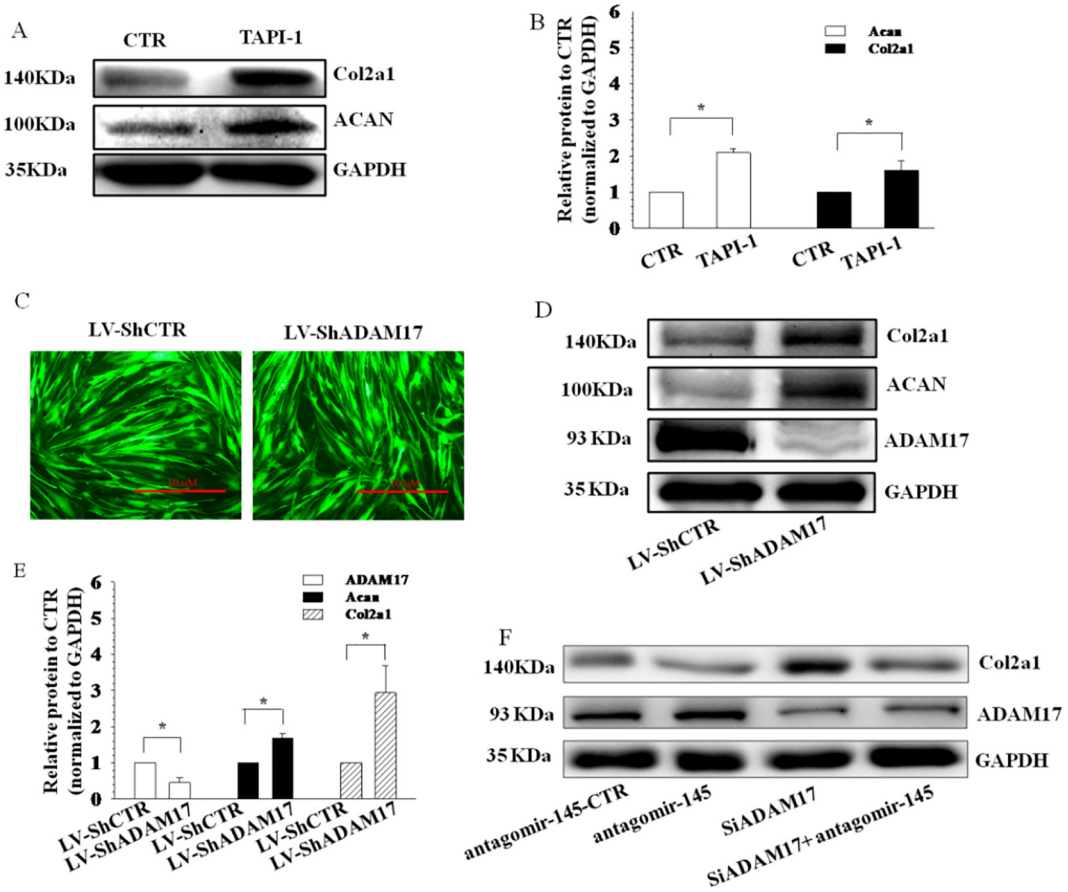

Fig. 6. ADAM17 suppression enhanced matrix metabolism in NP cells.
(A,B) Western blotting (A) and subsequent densitometric analysis (B) revealed that collagen IIa and aggrecan protein expression in rat NP cells was increased by the ADAM17 inhibitor. GAPDH was used as the control. (C) Immunofluorescence imaging of human NP cells treated with LV-shControl and LV-shADAM17. (D, E) Western blotting (D) and subsequent densitometric analysis (E) demonstrated that ADAM17 suppression by LV-shADAM17 markedly increased collagen IIa and aggrecan protein expression in human NP cells. (F, G) Western blotting (F) and subsequent densitometric analysis (G) demonstrated the effect of siADAM17 on matrix degradation induced by antagomir-145. Col2a1: collagen IIa; ACAN: aggrecan; CTR: control. The data are presented as the mean ± SD of three independent experiments. *$P < 0.05$.

control group; in contrast, antagomir-145 increased the rate of apoptosis (Fig. 1A and B). Moreover, CCK-8 assay was used to demonstrate the proliferation and the results showed that 50 nM agomir-145 increased, but 100 nM antagomir-145 suppressed the proliferation at 24, 48 and 72 h (Fig. 1C). However, western blotting and subsequent densitometric analysis revealed that the protein expression of Bcl-2, Bax, caspase-3 and activated caspase-3 was insensitive to transfection with agomir-145 and inhibitors (Fig. 1D–G).

3.2. miR-145 attenuated the apoptosis induced by oxidative stress in rat NP cells

Oxidative stress is an important factor for the induction of programmed cell death [27]. Apoptosis, a form of programmed cell death, is induced by oxidative stress in NP cells [28,29]. To further verify the effect of miR-145 on apoptosis, oxidative stress was used to induce apoptosis. Flow cytometry analysis revealed that oxidative stress indeed increased the rate of apoptosis in NP cells, whereas miR-145 attenuated this effect (Fig. 2A and B). Moreover, western blotting and subsequent densitometric analysis revealed that in the presence of oxidative stress, miR-145 overexpression with 50 nM agomir-145 markedly increased the protein expression of Bcl-2 to 170% of control levels and significantly suppressed the protein expression of Bax, caspase-3 and activated caspase-3by 25%, 40% and 20%, respectively (Fig. 2C and D).

3.3. miR-145 increased matrix metabolism in rat NP cells

To clarify whether miR-145 controls the expression of the ECM, real-time PCR and western blotting were performed in NP cells. Real-time PCR revealed that miR-145 overexpression with agomir-145 significantly enhanced the mRNA expression of aggrecan (Fig. 3A) and collagen IIa (Fig. 3B) to 200% and 170% of control levels, respectively. However, miR-145 suppression with antagomir-145 significantly decreased the mRNA expression of aggrecan (Fig. 3A) and collagen IIa (Fig. 3B) by 20% and 40%, respectively. In parallel, western blotting and subsequent densitometric analyses showed that miR-145 overexpression significantly enhanced and miR-145 suppression markedly suppressed the aggrecan protein expression to 130% and 80% of control levels, respectively (Fig. 3C and D). Moreover, the expression of collagen IIa protein was significantly increased by miR-145 overexpression to 135% of control levels and was suppressed by miR-145 inhibition to 60% of control levels (Fig. 3C and E). In addition, immunofluorescence staining of type II collagen in transfected NP cells also demonstrated that miR-145 overexpression increased and miR-145 inhibition suppressed the protein expression of collagen IIa (Fig. 3F).

3.4. ADAM17 is a direct target of miR-145

To clarify the mechanism by which miR-145 enhances ECM synthesis in NP cells, a bioinformatics approach was used, and as reported

previously [20], the 3′-UTR of ADAM17 contains sequences that are complementary to the miR-145 seed sequence (Fig. 4A and B). To determine whether ADAM17 is a direct target of miR-145, luciferase reporter vectors containing wild-type or mutant ADAM17 3′-UTRs were generated. Compared to that in the control group, luciferase activity was markedly suppressed in the group cotransfected with agomir-145 and the wild-type ADAM17 3′-UTR. However, this effect was abolished in the group cotransfected with the agomir-145 and mutant ADAM17 3′-UTR (Fig. 4C).

In agreement with the reporter assays, real-time PCR revealed that 50 nM agomir-145 markedly suppressed ADAM17 mRNA expression. Moreover, 100 nM antagomir-145 significantly enhanced ADAM17 mRNA expression (Fig. 4D). Consistent with these findings, western blotting and subsequent densitometric analysis revealed that the protein expression of ADAM17 was markedly decreased by 50 nM agomir-145 to 34% and increased by 100 nM miR-155 inhibitors to 136% of control levels (Fig. 4E and F).

3.5. The expression and regulation of ADAM17 in NP tissue and cells

ADAM17 is expressed in adult IVDs and is slightly reduced in the IVDs of older individuals [30]. Through real-time PCR, we observed that ADAM17 mRNA was expressed in the degenerative discs of 24 cases. Moreover, the expression of ADAM17 was significantly higher in discs of Pfirrmann grade 4 than in discs of grade 3 (Fig. 5A). Interestingly, although high expression of the inflammatory cytokine TNF-α is a hallmark of IDD [31], the expression of ADAM17 mRNA was insensitive to TNF-α stimulation (Fig. 5B), and 50 ng/mL TNF-α had no effect on ADAM17 protein expression (Fig. 5C and D). However, 250 μM H_2O_2 increased the expression of ADAM17 mRNA (Fig. 5E). Moreover, western blotting and subsequent densitometric analysis showed the expression of ADAM17 protein was unregulated by 250 μM H_2O_2 (Fig. 5F and G).

3.6. ADAM17 suppression enhanced matrix metabolism in human NP cells

Although TNF-α had no effect on ADAM17 protein expression, ADAM17 expression was higher in degenerative discs than in normal NP tissue. It has remained unclear whether ADAM17 controls the expression of matrix genes in NP cells. To address this uncertainty, the ADAM17 inhibitor TAPI-1 was used. Western blotting and subsequent densitometric analysis demonstrated that ADAM17 inhibition significantly enhanced the protein expression of aggrecan and collagen IIa in rat NP cells to approximately 300% and 150% of control levels, respectively (Fig. 6A and B).

Moreover, loss-of-function experiments were performed with LV-shADAM17. Herein, excellent transfection efficiency of human NP cells was obtained with LV-shADAM17 (Fig. 6C). Moreover, western blotting and subsequent densitometric analysis revealed that ADAM17 protein expression was markedly suppressed by LV-shADAM17to approximately 40% of control levels. Correspondingly, compared to LV-shControl, LV-shADAM17 markedly enhanced the protein expression of aggrecan and collagen IIa in human NP cells by approximately 70% and 220%, respectively (Fig. 6D and E).

In addition, to observe whether siADAM17 could reverse the effect of antagomir-145, a cotransfection experiment was performed with miRNA-145 inhibitors and siADAM17. Western blotting revealed that siADAM17 reversed the suppression of collagen IIa protein expression induced by antagomir-145 (Fig. 6F).

4. Discussion

The present investigation demonstrates, for the first time, that miR-145 overexpression suppresses NP cell apoptosis in the presence and absence of oxidative stress. A second major observation was that ADAM17, a TNF-α-activating enzyme, is expressed at higher levels in degenerative discs than in normal discs and is insensitive to the inflammatory cytokine TNF-α. Another novel observation was that miR-145 overexpression enhanced and miR-145 inhibition suppressed matrix synthesis by targeting ADAM17, and siADAM17 could reverse the effect of antagomir-145 on matrix synthesis in NP cells.

In IVD, NP cells are responsible for the synthesis and regulation of the matrix [31]. Disc degeneration is the pathological basis of degenerative disc disease and results from decreases in NP cells and an imbalance between the degradation and synthesis of ECM components [6,8,32]. Apoptosis is a pattern of programmed cell death, and previous studies have shown that apoptosis of NP cells is present in degenerative IVDs and is altered by compression and hypoxia [33–37]. In addition, miRNAs negatively regulate gene expression and control apoptosis [13]. Accumulating evidence suggests that miRNAs, including miR-494 and miR-143, play a vital role in the apoptosis of NP cells [14,38].

miR-145 is one of the microRNAs that controls cell apoptosis, and the function of miR-145 on cell apoptosis is dependent on cell type [21,22]. Herein, the effect of miR-145 on the apoptosis of NP cells was examined using flow cytometry and western blotting. Flow cytometry analysis demonstrated that miR-145 overexpression significantly decreased and miR-145 suppression markedly enhanced the apoptosis rate of NP cells, although the expression of apoptosis-related genes was insensitive to the overexpression or suppression of miR-145. Previous studies have revealed that oxidative stress may induce apoptosis [28,39]. To further clarify the role of miR-145 in NP cell apoptosis, NP cells were first treated with H_2O_2, and the results demonstrated that miR-145 markedly suppressed the NP cell apoptosis rate induced by oxidative stress and controlled the expression of apoptosis-related proteins. Taken together, the results revealed that miR-145 attenuates the apoptosis of NP cells in the presence and absence of oxidative stress. In addition, as to the effect of miR-145 on NP cells proliferation, our results revealed that miR-145 increased NP cell proliferation, which is contrary to the reported that miR-145 increases cell apoptosis and in the meantime, inhibits cell proliferation in many kinds of cells [22,23].

Previous studies have reported that microRNAs modulate ECM degradation in NP cells [15,16,38]. For example, miR-34a controls the IL-1β-induced decrease in type II collagen and aggrecan expression in NP cells [24]. miR-132 inhibition has also been reported to effectively attenuate ECM degradation in NP cells [40]. In chondrocytes, miR-145 also controls the catabolic degradation induced by inflammatory cytokines [19,20]. In the current study, we revealed that miR-145 significantly controlled collagen IIa and aggrecan expression in NP cells. These results revealed that miR-145 is also a protective factor in NP cells.

Members of the a disintegrin and metalloproteinase with thrombospondin motifs (ADAMTS) and a disintegrin and metalloproteinase (ADAM) families play crucial roles in degenerative cartilage disease [41]. In the process of IDD, multiple ADAMTSs, such as ADAMTS4 and ADAMTS5, are very important for matrix degradation [6,11,15]. However, among the ADAMs, only ADAM8 and ADAM17 have been reported to be involved in the process of IDD [29,42]. The current study revealed that ADAM17 mRNA is expressed in degenerative human NP tissue and demonstrated, for the first time, that the expression of ADAM17 is higher in discs with a Pfirrmann grade of 4 than in those with a grade of 3. In addition, the present study demonstrated that ADAM17 is insensitive to TNF-α, the hallmark of IDD, perhaps because ADAM17 causes the shedding of TNF-α receptors, as reported in other cells [43]. Oxidative stress is confirmed to be involved in intervertebral disc degeneration [29]. Consistent with previous reports by Scott and Brill A [44,45], our experiments revealed oxidative stress, induced by H_2O_2, activated the expression of ADAM17 in rat and human NP cells. Additionally, TGF-β is a very important growth factor for matrix metabolism in intervertebral disc [46]. Although miR-145 is refractory to TGF-β in NP cells, ADAM17 is activated by TGF-β in plentiful cells [47,48], the effect of other factors, including TGF-β, is worthy to be studied.

In addition, some studies have demonstrated that ADAM17 is regulated by miR-145 in cancer cells [49–51]. Does a similar process occur in NP cells? Our results revealed that miR-145 markedly suppressed ADAM17 expression and that ADAM17 suppression enhanced matrix synthesis in NP cells. Moreover, a loss-of-function experiment with siADAM17 reversed the suppression of type II collagen and aggrecan expression induced by antagomir-145, which further verified that miR-145 modulates matrix metabolism through ADAM17 in NP cells. Mesenchymal stem cells (MSCs) increase the matrix synthesis. However, it is interesting that direct co-culturing of NP cells and MSCs strongly downregulates the levels of miR-145 and TGF-b type I receptor inhibitor aborts the suppression [48]. Because TGF-β has no effect on the expression of miR-145 in NP cells [48], perhaps the decrease of miR-145 is the reaction of MSCs on NP cells, which is the process of tissue repair after intervertebral disc injury as reported by Nazari et al., mast cells, which is involved in wound healing after myocardial infarction, suppress the expression of miR-145 in MSCs [52].

Taken together, these findings provide novel insights and the first evidence that miR-145 controls matrix metabolism through ADAM17 in NP cells. miR-145 is a protective factor in IVDs and a potential therapeutic microRNA for the treatment of IDD.

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Declaration of conflicts of interest

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of this manuscript.

Informed consent

Informed consent was obtained from all participants included in this study.

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