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MicroRNA-365 functions as a mechanosensitive microRNA to inhibit end plate chondrocyte degeneration by targeting histone deacetylase 4

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ABSTRACT

End plate chondrocyte degeneration is a major cause of intervertebral disc degeneration. Mechanical biophysical forces, including intermittent cyclic mechanical tension (ICMT), exacerbate end plate chondrocyte degeneration. However, the underlying molecular mechanism of mechanical stretch-induced end plate chondrocyte degeneration is still unclear. This study sought to determine whether microRNAs (miRNAs) respond to mechanical stretch and play a role in regulating mechanically-induced end plate chondrocyte degeneration. We identified miR-365 as a mechanoresponsive miRNA in primary human end plate chondrocytes after ICMT application by miRNA microarray analysis. The expression of miR-365 was down-regulated in the disc samples obtained from patients with disc degeneration. We also found that the miR-365 stimulates chondrocyte proliferation but does not promote end plate chondrocyte death. Using bioinformatic analyses and subsequent confirmation by real-time RT-PCR, we identified multiple candidate target genes of miR-365 that responded to *in vitro* mechanical stimulation; among them, HDAC4 was fully characterized. Mutation of putative miR-365 binding sites in HDAC4 mRNA abolished miR-365 mediated repression of HDAC4 3′-untranslated region (3′UTR) luciferase reporter activity, suggesting that miR-365 binds to the HDAC4 3′UTR. Overexpression of miR-365 significantly decreased the HDAC4 protein level, suggesting that miR-365 acts as an endogenous attenuator of HDAC4 in human end plate chondrocytes. Further, perturbation of miR-365 expression also had a significant effect on the expression of *COL2A* and *ACAN* and on matrix degeneration. Overexpression of HDAC4 abolished miR-365 rescued end plate chondrocyte degeneration during ICMT application. Furthermore, we found that the wnt/β-catenin signal pathway was related to HDAC4 and promoted end plate chondrocyte degeneration. Overall, our results suggest that miR-365 is a mechanosensitive miRNA that regulates human chondrocyte degeneration by directly targeting HDAC4. We propose that therapeutic regulation of miR-365 may be an efficient anabolic strategy for inhibiting end plate chondrocyte degeneration.

1. Introduction

Intervertebral disc degeneration (IVDD) is one of most common causes of lower back pain and spinal compression nerve pain symptoms [1]. Multiple interdependent factors are considered to be associated with IVDD, such as mechanical loading, aging, reduced nutrient supply, and hereditary factors [2–4]. End plate cartilage plays a pivotal role in transporting nutrients to the nucleus pulposus, and it is also a mechanical interface between vertebral and resilient intervertebral discs [5]. Several studies have defined end plate cartilage degeneration as the most important factor that initiates and enhances IVDD [6–8].

Mechanical loading has previously been reported to play an essential role in modulating cartilage development and function through mechanosignal transduction pathways [9–11]. Some studies indicate that mechanical stress had no therapeutic effects on normal articular cartilage, but moderate biomechanical stress could reduce sensitization

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to the inflammatory response of articular cartilage and protect it against chondrocyte degeneration [12]. However, excessive pathological mechanical loading promotes cartilage damage [13,14]. High frequency and dynamic mechanical loading may lead to intervertebral disc degeneration and end plate morphology changes [15,16]. Our previous study showed that intermittent cyclic mechanical tension (ICMT) induced endplate chondrocyte degeneration by decreasing the expression of *Sox9*, chondrocyte extracellular matrix type II collagen (*COL-2A*), and aggrecan (*ACAN*) [17,18]. However, the detailed molecular signaling mechanisms of mechanical force-induced intervertebral disc and endplate cartilage degeneration have not been explored.

MicroRNAs (miRNAs) are a family of short, single-stranded noncoding RNAs that are regulate in numerous biological processes. Functioning at the posttranscriptional level, miRNAs repress gene expression *via* degradation or translational inhibition of target mRNAs by binding to the 3′-untranslated regions (3′UTRs) [19,20]. It is estimated that miRNAs regulate the expression of 30% of human protein-coding genes [19,21]. Multiple miRNAs have been identified as playing roles in chondrogenesis by regulating the expression of chondrocyte-marker genes [22–24]. However, the detailed molecular signaling mechanisms of mechanical tension stimuli on the expression of miRNAs and their functional roles in mechanotransduction in end plate chondrocytes have not been well characterized and remain to be elucidated.

In this study we screened for mechanosensitive miRNAs in ICMT-induced human end plate chondrocyte degeneration and determined that miR-365 was negatively correlated with end plate chondrocyte degeneration in response to cyclic mechanical loading. Specifically, miR-365 participates in the inhibition of chondrocyte degeneration and extracellular matrix degradation by directly targeting the 3′UTR of HDAC4, which encodes a histone deacetylase that is the master regulator of chondrocyte degeneration [25–27]. Our findings further show that therapeutic overexpression of miR365 may partly rescue the chondrocyte degeneration caused by mechanical loading. This study provides a novel mechanism and potential therapeutic target for intervertebral disc degeneration caused by mechanical loading.

2. Materials and methods

2.1. Patient samples

Endplate cartilage samples were obtained from 38 patients who underwent open vertebrae surgery in our department (the IVDD group), and from 20 patients (mean age 34 years; 8 females and 12 males) who underwent vertebrae surgery for cervical vertebrae fracture or dislocation (the normal disc control group, NC). The IVDD group included 18 patients (mean age 58 years; 10 females and 8 males) who underwent vertebrae surgery for cervical spondylosis. None of the subjects had tumors, tuberculosis, diabetes, infectious diseases, or bone metabolic diseases. MRI examination was performed routinely before surgical intervention. The MRI and pathological examinations revealed that all the disc samples in the IVDD group were classified as grades II–III, according to the Miller and Thompson classification [28,29]. All the disc samples in the NC group had no evident degeneration in macroscopic or imaging tests (Fig. 1A). This study was approved by the Ethics Committee of the Luan Affiliated Hospital of Anhui Medical University and the Yijishan Hospital of Wannan Medical College, and consent was obtained from all the participants.

2.2. Chondrocyte isolation and mechanical tensile strain application in vitro

Primary chondrocytes were isolated from the cervical intervertebral disc samples from the patients in the NC group. The end plate cartilage was extracted and the nucleus pulposus and annulus fibrosus tissue surrounding the cartilage were removed. The cartilage was cut into small pieces in PBS and digested with 10 ml trypsin (0.25%, EDTA free) for 30 mins at 37 °C to remove fibroblasts. After washing with PBS, the cartilage pieces were further digested with 5–10 ml 0.2% collagenase II solution (DMEM/F12, serum free) for 4–6 h (in an incubator at 37 °C with 5% CO2 and 100% humidity). Undigested cartilage and debris were removed by filtering. The digest was then washed with PBS and centrifuged repeatedly (37 °C, 2000 rpm × 2). The cells were counted using the trypan method then cultured in an incubator at 37 °C with 5% CO2 and 100% humidity. The caps were loosely fitted and the tubes were stood vertically. The first passage cells were used in the experiments. End plate chondrocytes were plated at a density of 2×10^5 cells per well in 2 ml growth medium containing Dulbecco's Modified Eagle Medium (DMEM) (Gibco), 10% fetal bovine serum, 100 U/ml penicillin, 100 mg/ml streptomycin (all from Hyclone) on 6-well flexible silicone rubber BioFlex™ plates coated with collagen type I (Flexcell International Corporation, Hillsborough, NC). The cells were cultured for 48 h to reach 80%–90% confluency. Then acyclic mechanical strain at 0.5 Hz sinusoidal curve at 10% elongation was applied for 8 h a day for 3 days using an FX-5000 T Flexercell Tension Plus unit (Flexcell International Corporation, Hillsborough, NC). The cultures were incubated in a humidified atmosphere at 37 °C and 5% CO2 under normoxia during the mechanical stretching and unloaded period. Cells maintained under the same culture conditions without ICMT application were used as the control group (Mock).

2.3. RNA isolation and miRNA microarray

Total RNA, which included miRNAs, was extracted from the collected cells using Trizol reagent (Invitrogen, USA) in accordance with the manufacturer's instructions. The miRNA microarray assay was performed using the Affymetrix Human miRNA Microarray v4.0 (USA). Screening was performed using the first passage proliferating end plate chondrocytes with or without ICMT application for 3 days. Total RNA was pooled from three individual samples from the Mock and ICMT groups, each sample was analyzed in triplicate. The Mock and ICMT samples were hybridized to the same microarray for dual-sample analysis. Six repeats were performed from the same sample to ensure the consistency of hybridization. The miRNAs with significant differences in expression that were highly and stably expressed were analyzed in subsequent experiments. TargetScan and miRDB software were used to predict the miRNA target genes and mRNA binding sites and to analyze the conserved binding sites [25,30].

2.4. Cells proliferation and apoptosis assay

End plate chondrocyte proliferation was assessed by Alamar Blue assay (Invitrogen) according to the manufacturer's instructions. The absorbance at 0, 12, 24, 48, and 72 h was measured to reflect the proliferation of the end plate chondrocytes with or without ICMT treatment. For apoptosis, chondrocytes were seeded and grown to about 80% confluence, then treated with or without 10% ICMT for 3 days. After this treatment, the cells were incubated with 10 μl propidium iodide and 10 μl annexin V (Invitrogen) at room temperature with protection from light for 15 min, then analyzed by flow cytometry using a FACSCalibur system (Becton Dickinson Biosciences, San Diego, CA). Propidium iodide-positive and annexin V-positive cells were considered to be apoptotic cells. The data were analyzed using Flow-Jo software.

2.5. Expression plasmid and miRNA mimic transfection

For transfection of the miR-365 mimic (Guangzhou RiboBio Co, Ltd), HDAC4 expression plasmids and small interfering (siRNA) (Guangzhou RiboBio Co, Ltd) were transfected into cells by lipofectamine 2000 (Invitrogen) in accordance with the manufacturer's instructions. The miR-365 mimic was transfected at a concentration of 200 nM, and the HDAC4 expression plasmids and HDAC4 siRNA were transfected at concentrations of 50 nM.

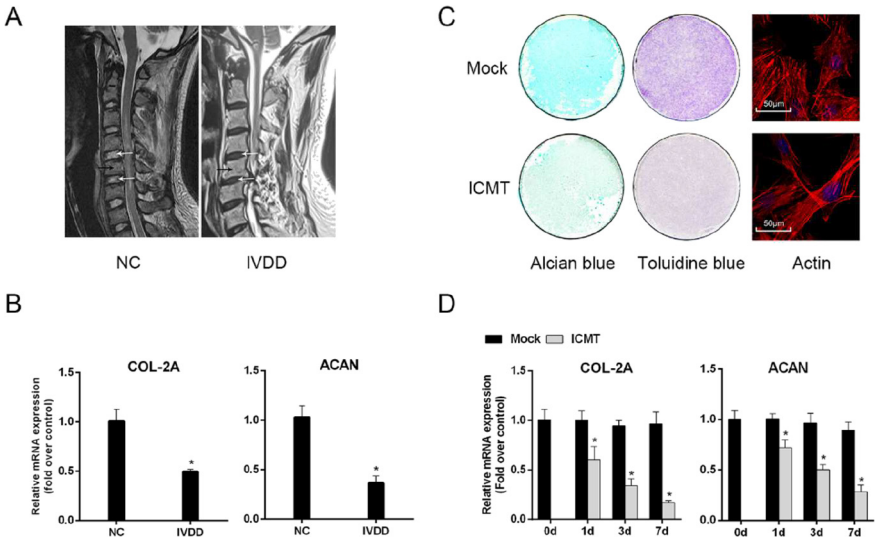

Fig. 1. Intermittent cyclic mechanical tension (ICMT) induced human end plate chondrocyte degeneration. (A) Human end plate cartilage was obtained from patients with cervical vertebrae fracture (NC, control) and intervertebral disc degeneration (IVDD). MRI signal of subjects. Black arrows indicate the location of the operation; white arrows indicate the site of the obtained material. (B) Expression of *COL-2A* and *ACAN* in the NC and IVDD groups obtained by real-time RT-PCR. (C) Alcian blue and Toluidine Blue staining shows that the chondrocyte matrix decreased after ICMT application for 3 days. Immunostaining of F-actin shows the change in the chondrocyte cytoskeleton after ICMT treatment for 3 days. Red, F-actin; blue, DAPI-stained nuclei. Original magnification: 400×. (D) Changes in *COL-2A* and *ACAN* expression in the end plate cartilage after ICMT application *in vitro* measured by real-time RT-PCR. Data are shown as fold changes over negative control group and are presented as mean ± SD ($n \geq 3$). *statistically significant difference ($P < 0.05$). All experiments were repeated three times. NC, normal disc group; IVDD, intervertebral disc degeneration group; Mock, control group; ICMT, intermittent cyclic mechanical tension, tension-loading group. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

2.6. Real-time RT-PCR

Total RNA was extracted from the chondrocytes using Trizol reagent (Invitrogen) in accordance with the manufacturer's instructions. After the reverse transcription reaction, real-time PCR was performed using a Light Cycler480 system (Roche) with SYBR1 Premix Ex Taq™ (Takara, Dalian, China) in accordance with the manufacturer's instructions. No nonspecific amplification was present as determined by the dissociation curve. *GAPDH* was used as the internal control. Data were analyzed using the comparison (Ct $2^{-\triangle\triangle Ct}$) method [31] and changes in gene expression were measured as fold changes between the ICMT group and the corresponding control group. Each sample was analyzed in triplicate. The primer sequences, optimized PCR conditions, and NCBI accession numbers are available from the corresponding author.

2.7. Western blotting

For western blot analysis, 20 μg of sample was resolved on a 12% SDS–PAGE and electro-transferred onto nitrocellulose membranes (Whatman, Piscataway, NJ). The primary antibody used was HDAC4 monoclonal antibody (Cell Signaling Technology Inc.), β-catenin monoclonal antibody (Sigma-Aldrich) at a dilution of 1:1000. For normalization of protein loading GAPDH (Cell Signaling Technology Inc.) antibody was used at a 1:5000 dilution. Horseradish peroxidase-conjugated polyclonal goat anti-rabbit antibody was added to bind to the primary antibody. The antigen–antibody complexes were visualized using the enhanced chemiluminescence detection system (Millipore, Billerica, MA) as recommended by the manufacturer. Immunoreactive bands were analyzed quantitatively in triplicate using the integrated intensities obtained with Alpha Image® software.

2.8. HDAC4 3′UTR cloning and luciferase assay

To identify the miR-365 target region in the *HDAC4* mRNA, we constructed a HDAC4 3′UTR luciferase reporter that contained mutant sequences of the candidate miR-365 binding sites (MUT HDAC4 3′UTR reporter), and then cotransfected it with miR-365 oligonucleotides into human end plate chondrocytes. The HDAC4 mRNA 3′UTR containing the miR-365-binding sequences for human HDAC4 was amplified by real-time RT-PCR from human genomic DNA. The following primer sequences were used: HDAC4-3′UTR-F: 5′-GCCCACGUGUUUUAUGGGCAUUG-3′; HDAC4-3′UTR-R: 5′-UAUUCCUAAAAAUCCCCGUAAU-3′. The PCR product was subcloned into the *Xho*I–*Not*I site downstream of the stop codon in the pmiR-RB-REPORT empty vector (Guangzhou Ribobio Co, Ltd). Binding region mutations were achieved using a QuikChange Site-Directed Mutagenesis Kit (Stratagene) following the manufacturer's instructions. Transient transfection of chondrocytes was carried out in 6-well plates with lipofectamine 2000 (Invitrogen) according to the manufacturer's instruction. The cells were cotransfected with 200 ng of the luciferase constructs and 50 ng of the Renilla luciferase (pRL-TK) plasmid (Promega, USA). Luciferase assays were performed with the dual-luciferase reporter assay system (Promega) in accordance with the manufacturer's instructions. Luminescent signals were quantified using a luminometer (Glomax; Promega), and each value from the Renilla luciferase construct was normalized using firefly luciferase.

2.9. Statistical analysis

Statistical comparisons were made using the Student's 2-tailed *t*-test or by one-way ANOVA with SPSS19.0 (SPSS, Inc., Chicago, IL, US, version 18.0). All quantitative data are expressed as the mean with standard deviation (SD). Data were obtained from three independent experiments and tested for normality using the Shapiro–Wilk test prior to parametric analyses. Statistical significance was defined as *P* values < 0.05.

2.10. Key resources table

Resource	Source	Identifier
Antibodies		
HDAC4 monoclonal	Cell Signaling Technology	HDAC4 (D15C3) Rabbit mAb #7628
ß-catenin monoclonal	Sigma-Aldrich	ABE208
Chemical		
EDTA	Brandt Chemcial	60–00-4
lipofectamine	Invitrogen	11,668,027
ProteinPeptide		
collagen	Canspecsci	C20–200110
collagenase	Qcbio s&t	40508ES6040508ES6040508ES60

3. Results

3.1. ICMT promoted endplate chondrocyte degeneration in vitro

Intervertebral disc degeneration is often correlated with end plate cartilage degeneration in patients. Therefore, we examined end plate chondrocyte-related gene expression in patients with cervical vertebrae fracture (NC control group) or cervical spondylosis (IVDD group). We found that *COL-2A* and *ACAN* expression was lower in the end plate cartilage obtained from patients in the IVDD group compared with their expression in the NC group (Fig. 1B). This means intervertebral disc degeneration was accompanied by significant end plate cartilage degeneration.

To confirm that ICMT induced endplate cartilage degeneration, we developed an *in vitro* model of end plate chondrocytes loading by intermittent mechanical tension. Toluidine Blue and Alcian blue staining showed that the chondrocyte matrix was obviously decreased and the F-actin cytoskeleton of end plate chondrocytes was obvious changed after ICMT treatment for 3 days (Fig. 1C). The real-time RT-PCR results showed that *COL-2A* and *ACAN* expression levels in the end plate chondrocytes were reduced after ICMT loading *in vitro* (Fig. 1D). These results indicate that ICMT induced end plate chondrocyte degeneration.

3.2. Identification of mechanoresponsive miRNAs

To identify mechanoresponsive miRNAs, we obtained the expression profiles of miRNAs by microarray in human end plate chondrocytes with or without ICMT loading. A total of 518 miRNAs were detected. After excluding miRNAs that were expressed below significant signal levels, we identified 9 up-regulated and 16 down-regulated miRNAs (Fig. 2A). We selected three up-regulated and four down-regulated miRNAs with the most significant fold changes and validated them by real-time RT-PCR. We found that miR-365 had the most significant change in expression in the end plate chondrocytes after ICMT application *in vitro* (Fig. 2B). The changes in miRNA expression were similar in the IVDD group endplate chondrocytes (Fig. 2C). These results indicate that miR-365 expression may be mechanosensitive during ICMT loading on human end plate chondrocytes and is associated with end plate chondrocyte degeneration.

3.3. MiR-365 promotes end plate chondrocyte proliferation but does not influence cell apoptosis

To determine the impact of miR-365 on end plate chondrocyte proliferation or apoptosis, we detected the proliferation and apoptosis of endplate chondrocytes after exposure to miR-365. MiR-365 expression was significantly increased after miR-365 mimic transfection (Fig. 3A). The Alamar Blue assay results showed that miR-365 promoted proliferation of end plate chondrocytes (Fig. 3B). Flow cytometry with annexin V-FITC and propidium iodide double staining assay indicated there was no significant difference in percentages of apoptotic cells after miR-365 mimic transfection (Fig. 3C). Thus, ICMT loading under certain conditions induced the degeneration of endplate chondrocytes and stimulated proliferation of end plate chondrocytes, but did not influence cell apoptosis.

3.4. HDAC4 is a direct target of miR-365

We identified target genes of miR-365 using TargetScan and miRanda. Both algorithms consistently identified HDAC4, a negative regulator of chondrocyte differentiation, as a putative target of miR-365. The predicted miRNA binding site was in the HDAC4 3′UTR (Fig. 4A), which is evolutionarily conserved across different species. The luciferase reporter assay demonstrated that mimic-365 decreased the activity of the wild-type HDAC4 3′UTR luciferase reporter but not the activity of the MUT HDAC4 3′UTR reporter (Fig. 4B). Overexpression of miR-365 only slightly changed the expression of the HDAC4 mRNA, but decreased the HDAC4 protein level (Fig. 4C, D). These results demonstrate that miR-365 directly targets HDAC4 by interacting with the 3′UTR in ICMT treated human end plate chondrocytes.

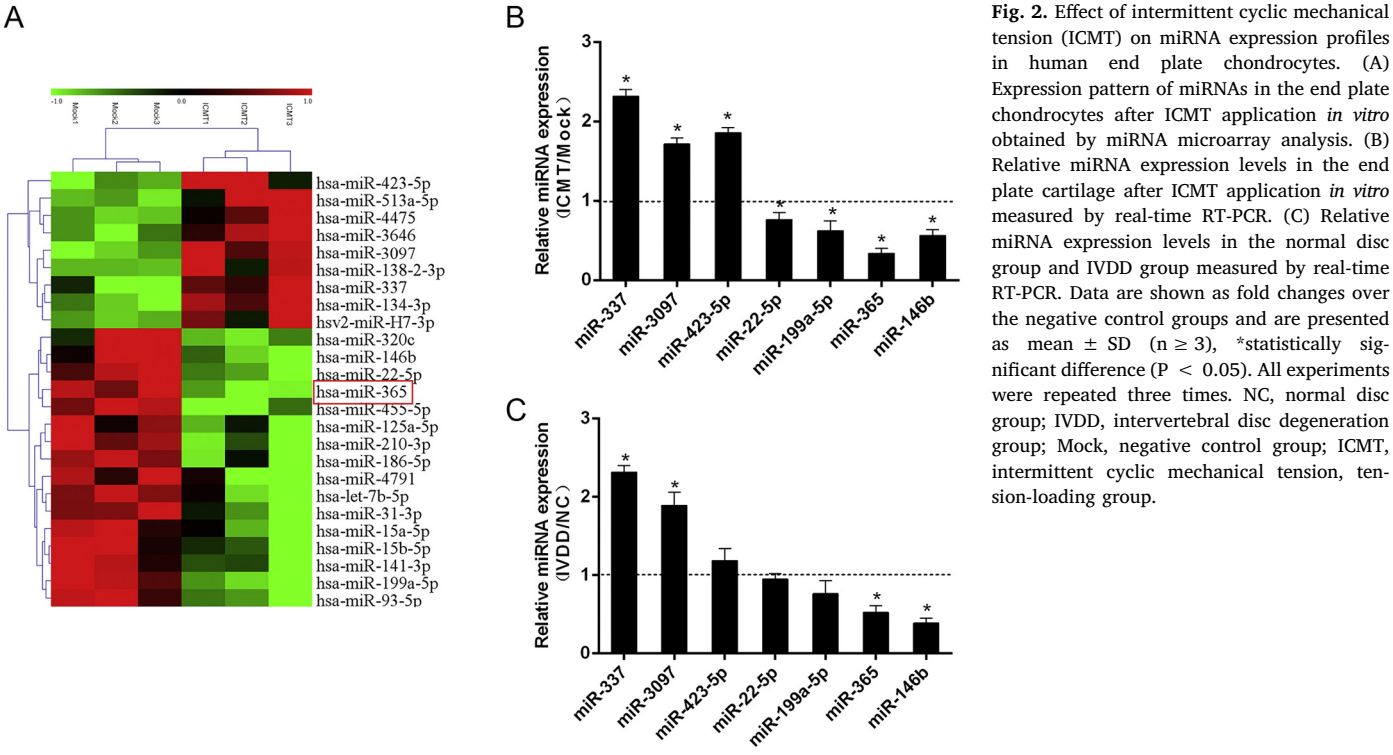

Fig. 2. Effect of intermittent cyclic mechanical tension (ICMT) on miRNA expression profiles in human end plate chondrocytes. (A) Expression pattern of miRNAs in the end plate chondrocytes after ICMT application *in vitro* obtained by miRNA microarray analysis. (B) Relative miRNA expression levels in the end plate cartilage after ICMT application *in vitro* measured by real-time RT-PCR. (C) Relative miRNA expression levels in the normal disc group and IVDD group measured by real-time RT-PCR. Data are shown as fold changes over the negative control groups and are presented as mean ± SD ($n \geq 3$), *statistically significant difference ($P < 0.05$). All experiments were repeated three times. NC, normal disc group; IVDD, intervertebral disc degeneration group; Mock, negative control group; ICMT, intermittent cyclic mechanical tension, tension-loading group.

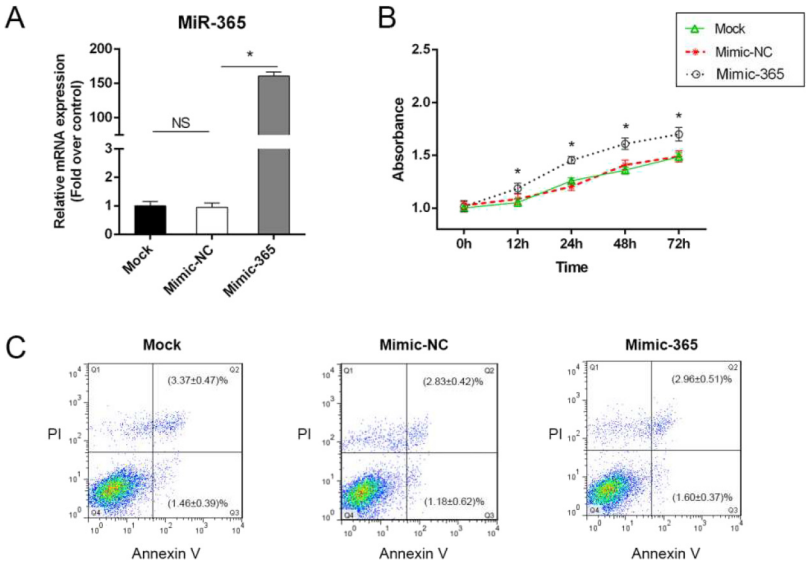

Fig. 3. MiR-365 stimulates chondrocyte proliferation but does not induce end plate chondrocytes apoptosis. (A) MiR-365 relative expression levels in human end plate chondrocytes after treatment with mimic-365 or the negative control (mimic-NC) measured by real-time RT-PCR. (B) Cell viability was examined by Alamar Blue assays after treatment with mimic-365 or mimic-NC for 72 h compared with the Mock control group. (C) End plate chondrocytes were treated with mimic-365 or mimic-NC for 36 h. Cell apoptosis was examined by propidium iodide-annexin V fluorescence in the fluorescence-activated cell sorting analysis. Data are shown as fold changes over the negative control group and presented as mean ± SD ($n \geq 3$). * statistically significant difference ($P < 0.05$). NS, no significant difference. All experiments were repeated three times. Mock, negative control group; mimic-NC, transfection of negative control mimic; mimic-365, transfection of miR-365 mimic.

3.5. MiR-365 relieved ICMT-induced end plate chondrocyte degeneration by regulating HDAC4 expression

Because miR-365 showed a correlation with endplate chondrocyte degeneration after application of ICMT, we examined whether over-expression of miR-365 could relieve the degeneration induced by ICMT in endplate chondrocytes. The down-regulated expression of *COL-2A* and *ACAN* detected in the ICMT group was partially restored by miR-365 mimic treatment (Fig. 5A). These data suggest that over-expression of miR-365 in endplate chondrocytes suppressed the ICMT-induced end plate chondrocyte degeneration. We also found that overexpression of miR-365 decreased the HDAC4 protein levels, but did not significantly alter the mRNA levels (Fig. 5B, C). To further explore the role of HDAC4 in ICMT-induced endplate chondrocyte degeneration, we constructed a HDAC4 expression plasmid and examined its effect on miR-365-regulated ICMT-induced human end plate chondrocyte degeneration. We found that HDAC4 expression was significantly increased after HDAC4 plasmid transfection (Fig. 5D, E). To determine the effect of ICMT on the endplate chondrogenic phenotype, chondrocytes were pre-transfected with miR-365 mimic or HDAC4 plasmid before ICMT stimulation. After 3 days of ICMT, HDAC4 overexpression abolished the effect of miR-365 rescued ICMT-induced chondrocyte degeneration (Fig. 5F, G). Overall, these results suggest that miR-365 alleviated the ICMT-induced loss of the endplate chondrogenic phenotype, most likely by targeting HDAC4 and suppressing its expression.

3.6. HDAC4 expression activated the Wnt/β-catenin signaling pathway in endplate chondrocytes

To investigate the specific signaling pathway downstream of HDAC4, we examined the relationship between HDAC4 and the Wnt/β-catenin signaling pathway in the endplate chondrocytes. Because HDAC4 was up-regulated after ICMT stimulation, we used specific siRNA to inhibit HDAC4 expression. The results showed the HDAC4 protein level was significantly decreased after siRNA transfection (Fig. 6A). We also found that both mRNA and protein levels of β-catenin were significantly increased in the end plate chondrocytes after ICMT application, and interestingly the β-catenin mRNA and protein levels were down-regulated when HDAC4 was blocked (Fig. 6B, C). Immunofluorescence analysis showed similar changes in β-catenin protein levels where overexpression the HDAC4 significantly promoted β-catenin protein accumulation in the nuclei after ICMT application (Fig. 6D). Taken together, these data demonstrate that ICMT activated the Wnt/β-catenin signaling pathway in endplate cartilage and its

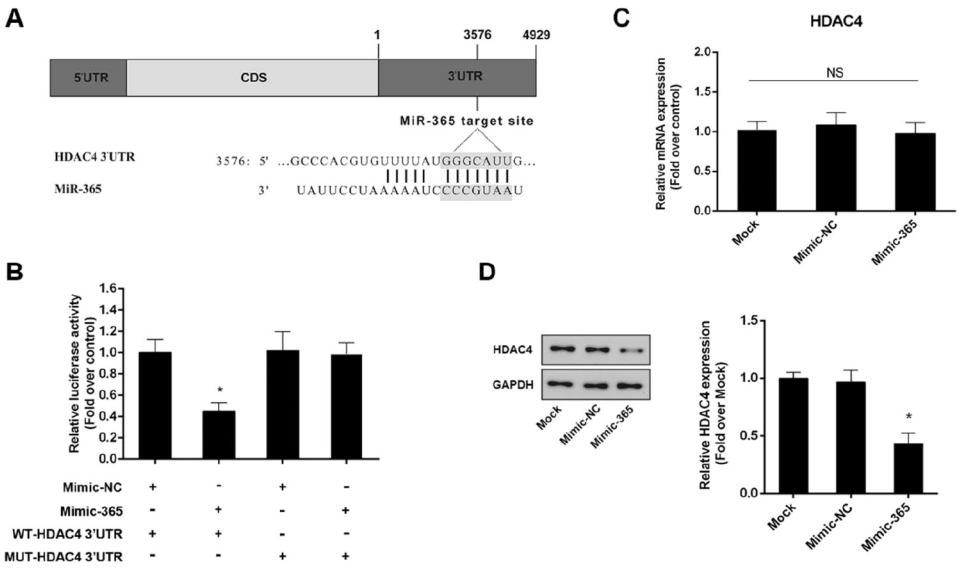

Fig. 4. HDAC4 is a direct target of miR-365. (A) Schematic diagram of the design of luciferase reporters using the wild-type HDAC4 3′UTR. (B) Effect of mimic-365 or mimic-NC on the luciferase activity of the wild-type HDAC4 3′UTR and MUT HDAC4 3′UTR reporters in human end plate chondrocytes. (C) Effect of mimic-365 or mimic-NC on HDAC4 mRNA levels in human end plate chondrocytes measured by real-time RT-PCR. (D) Western bolt analysis of HDAC4 protein expression in the human end plate chondrocytes after mimic-365 or mimic-NC treatment. Data are shown as fold changes over negative control group and are presented as mean ± SD ($n \geq 3$). *statistically significant difference ($P < 0.05$). NS, no significant difference. All experiments were repeated three times. Mock, negative control group; mimic-NC, transfection of negative control mimic; mimic-365, transfection of miR-365 mimic.

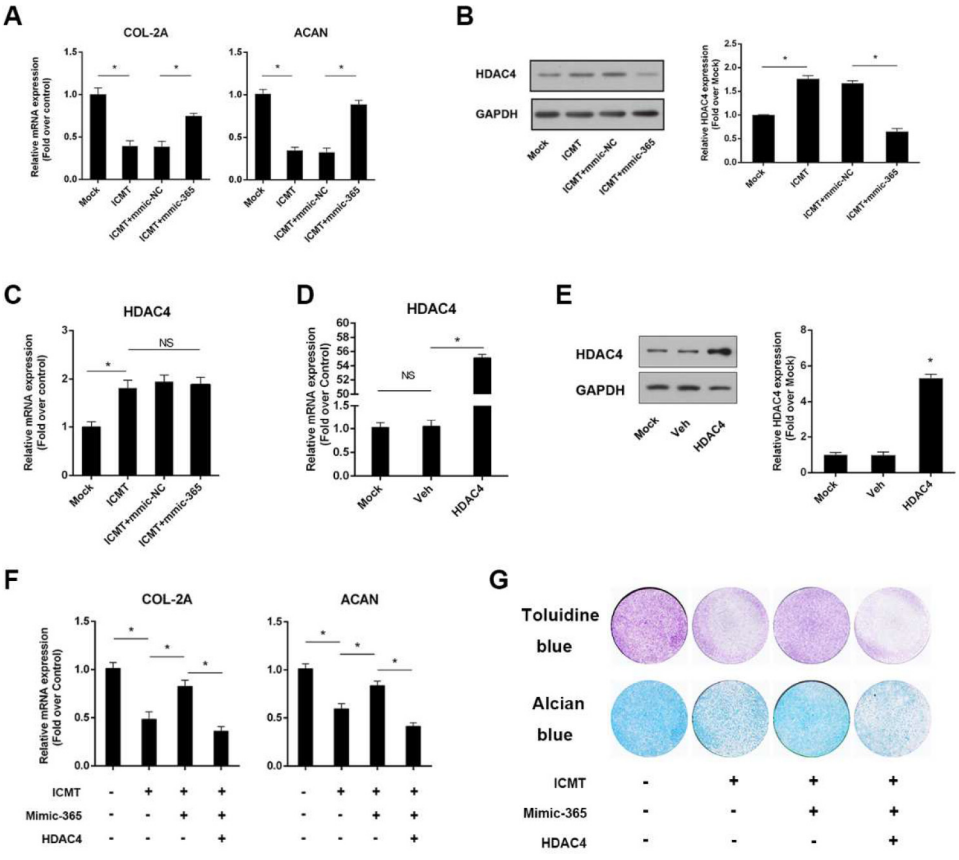

Fig. 5. MiR-365 relieved ICMT-induced end plate chondrocyte degeneration by regulating HDAC4 expression. (A) Expression levels of *COL-2A* and *ACAN* after ICMT and/or mimic-365 treatment measured by real-time RT-PCR. (B and C) HDAC4 protein (B) and HDAC4 mRNA (C) levels in human end plate chondrocytes with or without mimic-365 treatment during ICMT application obtained by western blot and real-time RT-PCR. (D and E) Overexpression of HDAC4 in human end plate chondrocytes was validated by real-time RT-PCR (D) and western blot (E). (F) Relative expression levels of *COL-2A* and *ACAN* in human end plate chondrocytes obtained by real-time RT-PCR after ICMT, mimic-365, or HDAC4 treatment. (G) Alcian blue and Toluidine Blue staining shows changes in the chondrocyte matrix after ICMT, mimic-365, or HDAC4 treatment. Data are shown as fold changes over negative control group and are presented as mean ± SD ($n \geq 3$). *statistically significant difference ($P < 0.05$). NS, no significant difference. All experiments were repeated three times. Mock, negative control group; ICMT, intermittent cyclic mechanical tension, tension-loading; mimic-NC, transfection of negative control mimic; mimic-365, transfection with miR-365 mimic. Veh, transfection with vehicle; HDAC4, transfection with HDAC4 expression plasmids. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

activation was regulated by HDAC4.

4. Discussion

The intervertebral disc (IVD) is an avascular tissue that bears and distributes the mechanical load of the spine. Nutrient deficiency is regarded as an important factor for IVDD. Endplate cartilage is a thin layer hyaline cartilage between the vertebral body and IVD and is an important pathway in nutrition supply to the IVD[2]. Cartilage-specific endplate chondrocyte matrix molecules, including Col-2A and ACAN,

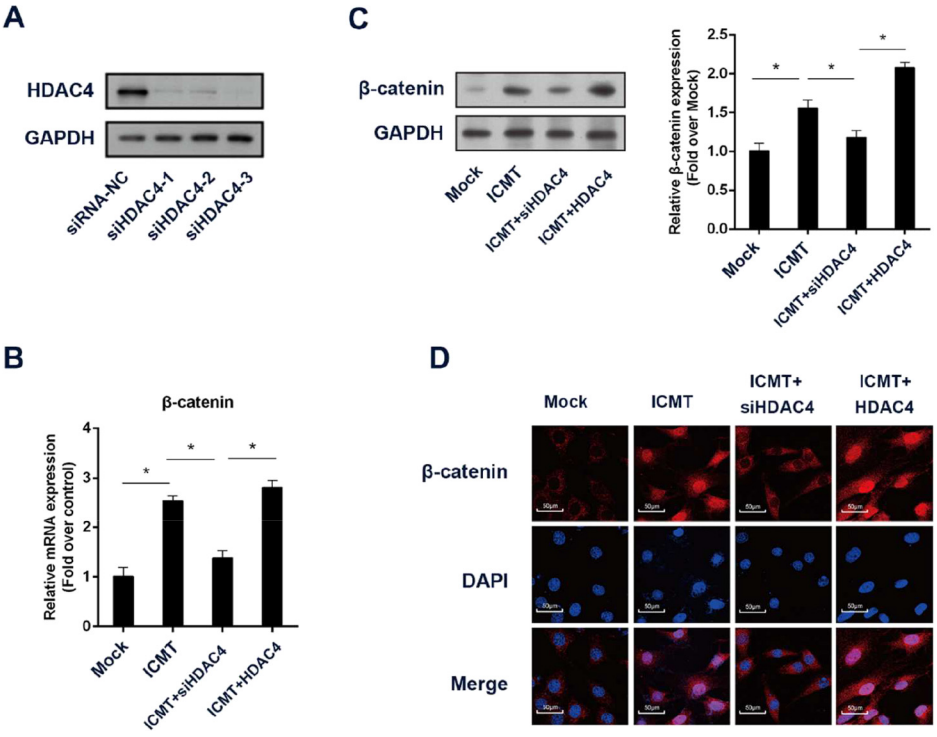

Fig. 6. HDAC4 expression activated the Wnt/β-catenin signaling pathway in endplate chondrocytes. (A) Knockdown efficiency of siRNA-HDAC4 was confirmed by comparison with a scrambled control siRNA (siRNA-NC). (B and C) The β-catenin protein (B) and mRNA (C) levels after knockdown or overexpression of HDAC4 in human end plate chondrocytes during ICMT application measured by western blot and real-time RT-PCR. (D) Expression and distribution of β-catenin protein in end plate chondrocytes treated with ICMT, siRNA-HDAC4, or HDAC4 expression plasmids detected by immunofluorescence. Original magnification: 200×. Data are shown as fold changes over negative control group and are presented mean ± SD ($n \geq 3$), *statistically significant difference ($P < 0.05$). All experiments were repeated three times. Mock, negative control group; ICMT, intermittent cyclic mechanical tension, tension-loading; siRNA-NC, transfection of negative control siRNA; siHDAC4, transfection with HDAC4 siRNA; HDAC4, transfection with HDAC4 expression plasmids.

are responsible for the maintenance of cartilage anabolism [32]. Some studies have shown that end plate cartilage degeneration, such as the destruction of matrix molecules, results in a reduction in IVD nutrition supply, which can accelerate degeneration of the IVD [33]. Mechanical stress plays an important role in the destruction of end plate cartilage in IVDD diseases. Excessive mechanical loading of human and animal IVD directly promotes endplate cartilage damage and IVD degeneration [34]. Furthermore, although long-term ICMT stimulation caused end plate chondrocyte degeneration, it did not affect cell viability in the early stages of stimulation [17,35,36]. Therefore, tension stimulation does not directly cause the death of endplate chondrocytes but rather induces endplate chondrocyte degeneration through other signal changes.

In recent years, miRNAs have been shown to be important regulators of cartilage development and maintenance of function. For example, miR-455 was found to regulate TGF-β signaling and suppress the Smad2/3 pathway to regulate chondrogenesis and degradation in articular cartilage [37]. Park et al. found that the expression of miR-127-5p and miR-27b was significantly down-regulated in human degenerative cartilage tissue and that they promoted cartilage degeneration by regulating the expression of matrix metalloproteinases [38,39]. Several studies indicated that mechanical force, including shear stress and cyclic stretch, modulated the expression of miRNAs that are involved in the cellular response to mechanical force in different cell lines [30,40–42]. However, the function of specific mechanosensitive miRNAs in ICMT-induced end plate chondrocyte degeneration has not been well characterized and is poorly understood. In this study, we used miRNA microarray technology to analyze the expression profiles of miRNAs in human endplate chondrocytes under ICMT loading and identified miR-365 as a candidate mechanosensitive miRNA. It was reported that miR-365 expression was markedly suppressed in cartilage in osteoarthritis compared with normal cartilage, and miR-365 was shown to regulate catabolic factors in chondrocytes [43]. Similarly, we found that miR-365 expression was obviously down-regulated in human end plate chondrocytes that were degraded by ICMT loading. Thus, miR-365 may be affected by ICMT stimuli during changes in the biological phenotypes of end plate chondrocytes. Furthermore, during ICMT application, overexpression of miR-365 by mimic-365 suppressed the inhibitive effect of ICMT on *COL-2A* and *ACAN* expression. Therefore, we concluded that ICMT-induced endplate cartilage degeneration is mediated by reduced miR-365 expression.

Histone deacetylases (HDACs) modulate cell growth and differentiation by regulating chromatin structure and repressing pivotal transcription factor activity. HDAC4 plays a critical role in transcriptional regulation, cell cycle progression, and developmental events. It is expressed in prehypertrophic chondrocytes, regulating chondrocyte hypertrophy, and endochondral ossification [44,45]. HDAC4 expression levels were found to be higher in degeneration cartilage explants and chondrocytes than in normal cartilage [46,47]. Interestingly, our data also confirmed HDAC4 expression was up-regulated in end plate chondrocytes by ICMT loading. Bioinformatics predictions and the luciferase reporter assay confirmed there was a specific binding relationship between miR-365 and HDAC4. We found that overexpression of miR-365 directly inhibited HDAC4 expression during ICMT application in end plate chondrocytes, and that miR-365 rescued ICMT-induced endplate chondrocyte degeneration was abolished by HDAC4 overexpression. These findings convincingly indicate that miR-365 functions as a mechanosensitive miRNA to inhibit end plate chondrocyte degeneration by targeting HDAC4 and, to a certain extent, ICMT suppresses miR-365 and promotes HDAC4 expression, ultimately leading to degeneration of endplate chondrocytes.

The Wnt/β-catenin signaling pathway plays a fundamental role in embryonic skeletal development and cartilage growth and homeostasis [48,49]. The involvement of this pathway in the stimulation of chondrocyte degeneration is well known. ICMT promotes endplate cartilage degeneration by activating the Wnt/β-catenin signaling pathway and suppresses the formation of the E-cadherin/β-catenin complex [17]. Ko et al. found that HDAC4 regulated the Wnt/β-catenin signaling pathway by mediating β-catenin deacetylation [50]. Similarly, in this study, we found that HDAC4 promoted β-catenin expression and increased nuclear β-catenin levels during ICMT-induced end plate chondrocyte degeneration.

In summary, we demonstrate that ICMT-induced human end plate chondrocyte degeneration is mediated by miR-365. MiR-365 functions by inhibiting the expression of HDAC4 at the post-transcription level, which further modulates activation of the Wnt/β-catenin pathway. In clinic trails, it is important to know how mechanical strain regulates end plate chondrocyte degeneration, thus the intrinsic regulatory mechanisms should be investigated further. Our findings provide new insights into mechanotransduction signaling pathways in end plate chondrocytes and raise the intriguing possibility of studying gene regulation by miRNAs to develop gene therapy for treating human IVDD.

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Author contributions

All authors were involved in drafting the article or revising it critically for important intellectual content, and all authors approved the final version to be published. Dr. XU had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

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Declaration of competing interests

The authors have declared that no competing interests exist.

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