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Original Article

Down-regulation of microRNA-145 expression promote resistance to chondrocyte apoptosis in osteoarthritis

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Abstract: Objective: This study was undertaken to probe deeply into effects of microRNA-145 (miR-145) expression on chondrocyte apoptosis in osteoarthritis (OA). Methods: A group of 71 OA patients (32 males and 39 females) with age ranging from 49 to 77 years were recruited into this study. Chondrocytes isolated from their OA tissues were separated into four groups: group A (blank control, no transfection), group B (transfection with empty vector pGenesil-1), group C (miR-145 plasmid) and group D (miR-145 inhibitor). Transfection efficiency was observed under fluorescence microscope. Real-time quantitative PCR (RT-qPCR) and western blotting were used to detect expressions of miR-145 and N-cadherin (mRNA) in the chondrocytes of each group. And flow cytometry was utilized to determine cycle and apoptosis of cells in each group. Results: miR-145 expression showed relationship with gender and OA grading (both $P < 0.05$). miR-145 expression increased in the group C and significantly decreased in the group D compared with that in the group A and group B (all $P < 0.05$). Interestingly, N-cadherin expression in the group D was increased compared with that in the three groups (all $P < 0.05$). Moreover, compared with the group A and group B, cell apoptosis was significantly abated and the cell proportion was decreased at the S stage in the group D, but promoted in the group C (all $P < 0.05$). Also, the group D had lower cell proportion and cell apoptosis rate than the group C ($P < 0.05$). Conclusion: Inhibition of miR-145 can suppress chondrocyte apoptosis in OA, indicating a new direction for OA treatment.

Keywords: microRNA-145, osteoarthritis, chondrocyte, n-cadherin, apoptosis

Introduction

Osteoarthritis (OA) is a kind of cartilage-degenerative disease caused by chondrocyte exposure to biochemical or mechanical stimuli, and disturbance of osteochondral homeostasis [1]. It is the chief reason of physical deformity and deterioration of life in the developed society, particularly representing a burden to the aging population [2]. And patients with OA always bear higher risks of death than those general individuals, with an OR value of 1.54 [3]. It is noted that the incidence of OA in hand, knee and hip joints has increased to 27 million adults in 2005 from 21 million in 1995 in the United States in which lower extremity joints (knee and hip) show predisposition to OA [4]. OA can be triggered by a variety of factors which can be divided into person-level factors as well as joint-level factors. The former includes age and gender, obesity, genetics and nutritional factors, and the latter comprises injury, physical activity, muscle strength and occupation feature [5]. At present, there is no way to cure OA, and the main treatment is limited to pain regulation, anti-inflammatory drugs such as steroids, physio-therapeutics, and final joint replacement [6]. To improve the treatment, pathogenic mechanism of OA needs continuous exploration.

Intriguingly, a study reveals that altered expression of microRNAs (miRNAs) is involved in the pathogenesis of diverse types of arthritis including OA [7]. And some experts have suggested that miRNA-directed therapy for OA can be a good choice without potential side effects [8]. miRNAs consist of small non-encoded RNAs with length of nearly 22 nucleotides through modulating post-transcriptional gene expressions, which assume critical roles in cell metabolism, proliferation, differentiation and apoptosis of various cellular types [7, 9]. It is reported that miRNA-21 (miR-21), miR-25, miR-98, miR-

Table 1. Relationship of miR-145 expression with clinicopathologic parameters of OA (t test was used)

Clinicopathologic parameters	Cases	miR-145	*P*
Age			0.284
< 60	30	0.38 ± 0.11	
≥ 60	41	0.35 ± 0.11	
Gender			< 0.001
Male	32	0.27 ± 0.07	
Female	39	0.44 ± 0.06	
OA grading			< 0.001
I	21	0.26 ± 0.11	
II	19	0.34 ± 0.04	
III	16	0.41 ± 0.04	
IV	15	0.48 ± 0.06	
Obesity			0.874
Yes	29	0.37 ± 0.11	
No	42	0.36 ± 0.11	
Osteoporosis			
Yes	43	0.36 ± 0.11	0.749
No	28	0.37 ± 0.11	

137, miR-182, miR-185, miR-211, miR-299, and miR-342 all presented high expression in chondrocyte of OA, besides, suppressed expression of miR-107, miR-146, miR-148, and miR-149 in the cell were also significant in initiation and progression of OA [10-13]. Also, previous evidence demonstrated that changed level of miR-145 was associated with cartilage matrix metabolism in OA [14]. In OA, it is discovered that cellular homeostasis is always lost with relatively increased chondrocyte apoptosis [15]. And the cell apoptosis has been in positive relation to the degree of cartilage impairment and matrix exhaustion [16]. Thus, this study is carried out to investigate how expression of miR-145 in OA influences the chondrocyte apoptosis, which sets a basis for the understanding of OA pathogenesis as well as a novel molecular-targeted treatment for OA.

Materials and methods

Ethnic statement

All participants have given the informed consent, and the protocol of this study has obtained approval from the Academic Ethics Committee of Zhongnan Hospital of Wuhan University.

Subjects

OA patients (n = 71; 32 males and 39 females; age 49~77 years; mean age of 60.23 ± 5.49 years) were collected from Zhongnan Hospital of Wuhan University between January 2014 and December 2014, and all of them have received arthroscopic debridement or knee replacement. The included patients had osteoarticular pain, joint clicking and knee joint hypertrophy, while those with serious disease in brain, heart, lung and kidney, hematonosis, diabetes, tumor or rheumatoid arthritis and septic arthritis were excluded. According to the OA grading [17], there were 21 cases of grade I, 19 cases of grade II, 16 cases of grade III and 15 cases of grade IV. All cases were diagnosed based on the Guidelines for OA Diagnosis and Treatment [18].

Cell culture and transfection

OA chondrocytes were grown in RPMI-1640 medium containing 10% fetal bovine serum, and then placed at 37°C in 5% CO_2. The medium was renewed every 1~2 days. Subsequently, transfection was performed referring to instructions of Lipofectamine 2000 kit (Thermo Fisher Scientific, Shanghai, China), with group A (no transfection), group B (transfection with empty vector pGenesil-1), group C (miR-145 plasmid) and group D (miR-145 inhibitor). Within 24 hours after transfection, green fluorescent cells were observed under fluorescence microscope to determine transfection efficiency.

Real-time quantitative polymerase chain reaction (RT-qPCR)

The chondrocytes transfected after 48 hours were collected. Total RNA was isolated from the cells of each group based on TaqMan kit instructions (Thermo Fisher Scientific, Shanghai, China) and spectrophotometer was used for quantitation. Then cDNA was synthetized via reverse transcription of RNA. RT-qPCR was carried out using ABI7500 qPCR instrument (ABI., Foster City, CA, USA), with reaction system consisting of 2X All-in-One TM qPCR Mix 10 μl (Biomed Co. Ltd., Beijing, China), upstream primer and downstream primer (10 μM) 1 μl respectively, cDNA 1 μl and ddH_2O till to 20 μl. The primers adopted in reactions were as follows: miR-145 (sequence-specific), forward 5'-GTCCAGTTTTCCCAGGAAT-3' and reverse 5'-

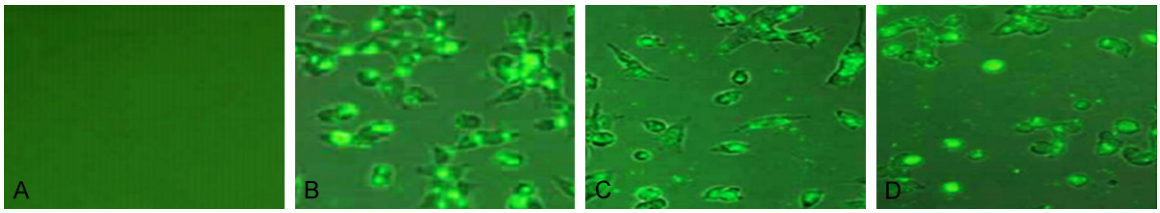

Figure 1. Observation of transfection efficiency under fluorescence microscope. Note: A: Blank control (no transfection); B: Transfection with empty vector pGenesil-1; C: Transfection with plasmid miR-145; D: Transfection with miR-145 inhibitor.

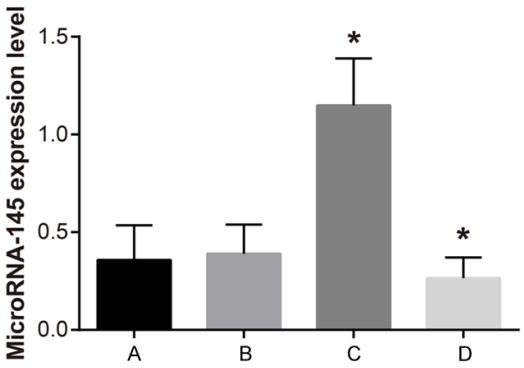

Figure 2. miR-145 expression in transfected cells of each group. Note: A: Blank control (no transfection); B: Transfection with empty vector pGenesil-1; C: Transfection with plasmid miR-145; D: Transfection with miR-145 inhibitor.

TGGTGTCGTGGAGTCG-3'; internal control U6, forward 5'-CTCGCTTCGGCAGCACA-3'; reverse 5'-AACGCTTCACGAATTTGCGT-3'; N-cadherin, forward 5'-AGGGTGGACGTCATTGTAGC-3' and reverse 5'-CTGTTGGGGTCTGTCAGGAT-3'; internal control β-actin, forward 5'-TGTTACCAACTGGGACGACA-3' and reverse 5'-GGGGTGTTGAAGGTCTCAAA-3'. The PCR condition was pre-denaturation at 95°C for 10 min, then 95°C for 15 s, 60°C for 60 s and 70°C for 1 s, with a total of 40 cycles. Finally, relative quantification (RQ) of target gene expression can be figured out according to a formula, and RQ of miR-145 was calculated. This experiment was repeated for 3 times.

Western blotting

Cell protein was extracted from each group and quantified by bicinchoninic acid (BCA) method. Through electrophoresis with 10% SDS-polyacrylamide gel, samples (60 μg protein) were transferred onto polyvinylidene fluoride (PVDF) membrane and blocked at room temperature in 5% bovine serum albumin (BSA) for 2 hours. Subsequently, rabbit-anti-human antibodies (N-cadherin and β-actin), each at 1:2000) were added and incubated at 4°C overnight. Next, horseradish peroxidase (HRP) labeled goat-anti-rabbit IgH (1:5000) was added and incubated at room temperature for 1 hour. Finally, electrochemiluminesce (ECL) assay was utilized for visualization. Gray density was analyzed with Image J software, relative expression of N-cadherin = gray density of N-cadherin band/β-actin band. This experiment was repeated for 3 times and the average value was valid.

Flow cytometry

The first-generation chondrocytes during logarithmic growth phase were collected from each group. Then, the cells were digested using trypsin and rinsed twice with phosphate buffer solution (PBS). Following cultivation in complete medium and centrifugation (1000 r/min) for 4 min, the cells were fixed in 70% ethanol overnight. With 1×10^5 chondrocytes seeded in each hole at a six-well plate, the cells were divided into four groups: miR-145 mimic group, miR-145 inhibitor group, negative control group (sequence-unrelated) and blank group. Each group had 3 auxiliary holes. Then correspondent reagent was added into each hole and the plate was placed in darkness for 30 min. In the same way, chondrocytes were collected, washed and cultured but not fixed in ethanol. Then RNaseA was added and water bath was performed for 30 min in the dark. Subsequently, the annexin V/PI double-staining was applied according to operating instructions (measuring cell apoptosis) in darkness for 10 min. This experiment was repeated 3 times.

Statistics

Statistical analyses were carried out with SPSS 20.0 system (SPSS Inc., Chicago, IL, USA). All data were displayed as mean ± standard devia-

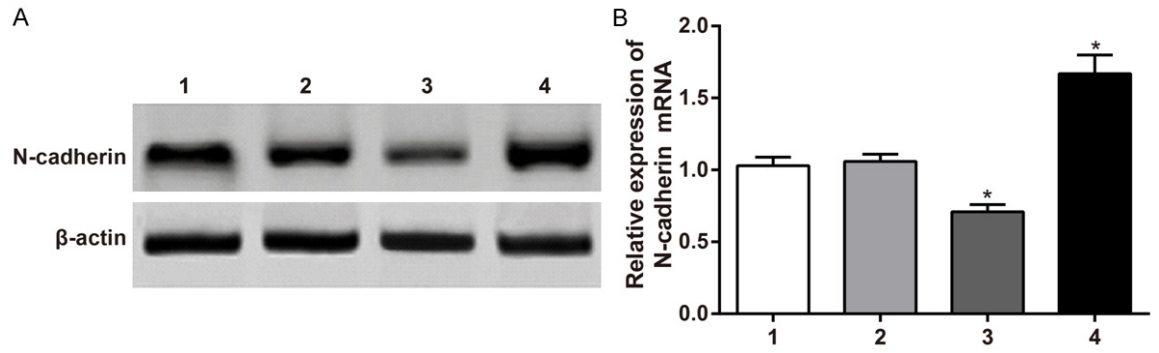

Figure 3. Expression of N-cadherin protein by Western blot (A) and expression of N-cadherin mRNA by RT-qPCR (B) in transfected cells of each group. Note: 1: blank control (no transfection); 2: transfection with empty vector pGenesil-1; 3: transfection with plasmid miR-145; 4: transfection with miR-145 inhibitor; *: $P < 0.05$ compared with the blank contro.

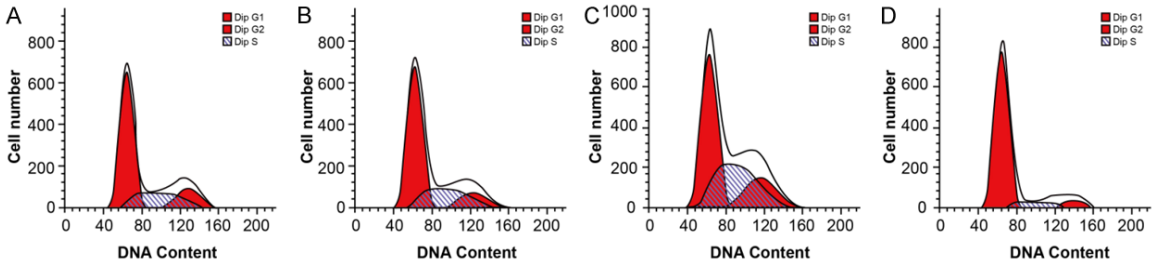

Figure 4. Changes of transfected cell cycle in each group. Note: A: Blank control (no transfection); B: Transfection with empty vector pGenesil-1; C: Transfection with plasmid miR-145; D: Transfection with miR-145 inhibitor.

tion. Sample means of three experimental groups were analyzed by one way variance analysis (ANOVA). Comparisons between groups were examined with LSD-t method. $P < 0.05$ was considered as significance.

Results

miR-145 expression and clinicopathologic parameters of OA

As revealed in **Table 1**, the miR-145 expression was related to OA grading and gender (both $P < 0.05$), but not to age, obesity or osteoporosis of patient (all $P > 0.05$).

Transfection efficiency

Transfection efficiency = green fluorescent cells/total cells. As shown in **Figure 1**, the transfection efficiency of the three groups (B, C and D) all exceeded 70%.

Expression of miR-145 after transfection

After 48 hours of transfection, the mean RQ value of miR-145 in the four groups was 0.36 ± 0.18, 0.39 ± 0.15, 1.15 ± 0.24 and 0.27 ± 0.10, in succession. There was no difference of miR-145 expression between the group A and group B ($P > 0.05$), while miR-145 expression increased in the group C and significantly decreased in the group D compared with that in the group A and group B (all $P < 0.05$) (**Figure 2**).

Expression of N-cadherin after transfection

As indicated in **Figure 3**, N-cadherin expression of the chondrocytes in the group D became significantly higher but lower in the group C than that in the group A and group B (all $P < 0.05$). And N-cadherin expression in the group D was higher than that in the group C. N-cadherin mRNA expression was in line with N-cadherin expression.

Cell cycle and apoptosis

At the S stage, the cell proportion in the four groups was (2.98 ± 0.17)%, (3.04 ± 0.12)%, (5.46 ± 0.46)% and (2.29 ± 0.11)%, in succession. No difference was found between the

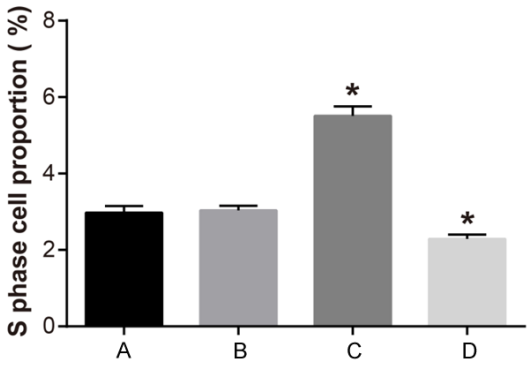

Figure 5. At the S stage, the cell proportion in each group after cell transfection. Note: A: Blank control (no transfection); B: Transfection with empty vector pGenesil-1; C: Transfection with plasmid miR-145; D: Transfection with miR-145 inhibitor (*, $P < 0.05$ compared with the group A).

group A and group B ($P > 0.05$). Compared with the group A, at the S stage, the cell proportion significantly increased in the group C while decreased in the group D (both $P < 0.05$) (**Figures 4**, **5**). Cell apoptosis rate in the four groups was (2.51 ± 0.43)%, (2.58 ± 0.37)%, (8.47 ± 0.60)% and (2.09 ± 0.31)%, in succession. There was little difference of cell apoptosis in the group A and group B ($P > 0.05$). Compared with the group A and group B, cell apoptosis was significantly abated in the group D, but promoted in the group C (all $P < 0.05$) (**Figures 6**, **7**). Also, the group D had lower cell apoptosis rate than the group C ($P < 0.05$).

Discussion

As a major health problem, OA exhibits significant impact on patients' life quality. There is no cure for OA currently, and the pathogenesis still needs to further explore. It has been known that many miRNAs play important parts in development and progression of OA, and miR-145 is one of them. Therefore, this study was undertaken to explore how miR-145 influences chondrocyte apoptosis in OA and hopes to be helpful to elucidate the pathogenic mechanism of OA.

At first, this study discovered that miR-145 expression was in significant association with gender of patient and OA grading, which further proved that miR-145 expression contributed to OA evolvement. Consistent with our result, Tonelli et al. announced that OA affected a greater population of females than males and clinically females suffered stronger pain intensity and greater functional defects [19]. And it was reported that miR-145 overexpression occurred in OA [20], which also verified the other outcome. To clarify the potential mechanism, chondrocytes with high and low expression of miR-145 were obtained through cell transfection. Then it was demonstrated that N-cadherin expression in chondrocytes with high expression of miR-145 significantly decreased but increased in chondrocytes with low expression of miR-145 compared with the blank control, revealing that miR-145 expression is in adverse relationship with N-cadherin expression in OA. Cadherins are defined as a multi-gene family constituted with calcium-dependent transmembrane proteins mediating cell-cell adhesion [21]. N-cadherin was revealed to push on chondrogenesis (gathering chondroprogenitor cells into precartilage condensation) and chondrocyte differentiation (PMID: 20735983). Furthermore, Chang et al. have found cadherin-11 involving in modulation of synovial fibroblasts which can lead to inflammation reactions in rheumatoid arthritis [22]. And miR-145 has been proved to be able to target N-cadherin directly [23]. Thus, on basis of this study it was speculated that miR-145 probably participated the ignition and progression of OA through negative regulation of N-cadherin.

This study also showed that at the S stage, the cell proportion and cell apoptosis in chondrocytes with high expression of miR-145 was significantly promoted, predicting that high expression of miR-145 may exacerbate the OA condition. Ryu et al. and Zhang et al. have revealed that HIF-2α enhances Fas-mediated cell apoptosis in OA cartilage destruction, and miR-145 can target HIF-2α [24, 25]. Yang et al. also revealed that miR-145 can affect Smad3 (an important factor for chondrocyte homeostasis) expression directly as well as IL-1β-induced extracellular matrix degradation of OA cartilage [14]. Besides, at the S stage, the cell proportion and cell apoptosis in chondrocytes was significantly reduced with blocking of miR-145 expression, predicting that miR-145 can be a point to suppress chondrocyte apoptosis and suspend development of OA disease. Martinez-Sanchez et al. have suggested that via target-

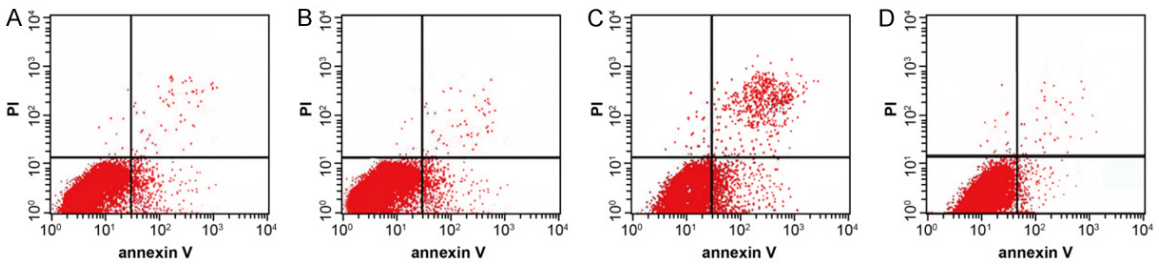

Figure 6. Cell apoptosis in each group after cell transfection. Note: A: Blank control (no transfection); B: Transfection with empty vector pGenesil-1; C: Transfection with plasmid miR-145; D: Transfection with miR-145 inhibitor.

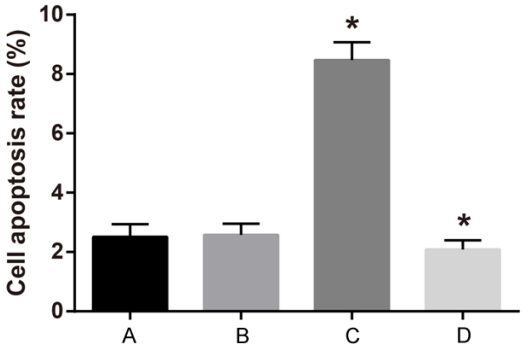

Figure 7. Apoptosis rate of cell in each group after transfection. Note: A: Blank control (no transfection); B: Transfection with empty vector pGenesil-1; C: Transfection with plasmid miR-145; D: Transfection with miR-145 inhibitor (*represents $P < 0.05$ when compared with the group A).

ing SOX9 (through a specific binding site in the 3'-UTR of miR-145) alteration of miR-145 expression in chondrocytes may provide a potential and promising therapy for cartilage impairment as well as the chronic disease of OA [20].

In summary, overexpression of miR-145 may deteriorate OA situation and blocking of miR-145 expression can abate cell apoptosis and delay the OA development, which guides a novel molecular-targeted therapy for OA disease. To our best knowledge, this is the first study about miR-145 effects on cell apoptosis in OA. More efforts would be made to further confirm whether N-cadherin is the target gene of miR-145 and delve into the correlation between miR-145 and genes implicated in the apoptosis of cartilage cells. With deepening research, miR-145 is expected to become a new strategy for the diagnosis and prognosis of OA.

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Disclosure of conflict of interest

None.

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