

MiR-15a-5p regulates viability and matrix degradation of human osteoarthritis chondrocytes *via* targeting VEGFA

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Summary

Previous studies demonstrated that miR-15a-5p was probably associated with human hepatocellular carcinoma, while the function of miR-15a-5p in OA (Osteoarthritis) still remains unknown. Here, we uncovered the potential role of miR-15a-5p on OA pathogenesis and confirmed its predicted target VEGFA (Vascular Endothelial Growth Factor A). Measured by RT-PCR, miR-15a-5p expression increased remarkably while VEGFA expression was significantly decreased in OA chondrocytes compared with normal conditions. According to Luciferase activity assay, miR-15a-5p directly targeted the 3'-UTR of VEGFA to inhibit its expression. Functional analysis including CCK-8 assay and flow cytometry revealed that overexpression of VEGFA or inhibition of miR-15a-5p promoted cell proliferation, suppressed cell apoptosis and reduced matrix degradation in OA chondrocytes. Moreover, rescue assays carried out with both expression of VEGFA and miR-15a-5p demonstrated that miR-15a-5p contributes to cell apoptosis and matrix degradation via inhibiting VEGFA. We further provided evidence that multiple proteins related to matrix synthesis were regulated by miR-15a-5p and VEGFA using Western blot and ELISA assays. Taken together, our findings elucidated an underlying mechanism by which miR-15a-5p regulates viability and matrix degradation of OA and indicated a new target for OA diagnosis and therapy.

Keywords: Osteoarthritis, miR-15a-5p, VEGFA

1. Introduction

Osteoarthritis (OA), the most prevalent disease of the articulating joints affecting millions of people worldwide is a chronic degenerative joint disease that is characterized by deterioration in the integrity of cartilage and is coupled with pain, tenderness, disability and inflammation without systemic effects (1). Although multiple factors are included in OA etiology, chondrocytes are crucial to tissue function and dominate the degenerative process of cartilage if the genes are expressed inappropriately when compared with adjacent tissues.

Small non-coding RNAs, namely MicroRNAs, are

highly conserved and prevalent in human cells. They are important in especially down-regulating target gene expression by binding to the 3' untranslated region of corresponding mRNAs. Meanwhile, they control significant basic biological functions, including cell cycle, cellular differentiation proliferation, apoptosis and so forth. Several recent studies demonstrated their importance in maintaining cartilage homeostasis and their effects on promoting the pathogenesis of OA. For instance, it was claimed in 2016 by Xu Cui and his group that by targeting PIK3R1, overexpression of miR-634 can suppress survival and matrix synthesis of human osteoarthritis chondrocytes (2). It is illustrated by Tadahiro Sakai and his colleagues that miR-125b plays a pivotal role in regulating expression of aggrecanase-1 (ADAMTS-4) in human osteoarthritic chondrocytes (3).

VEGFA (Vascular endothelial growth factor A) is important in regulating growth plate endochondral ossification. The VEGFA family consists of three different isoforms, including VEGFA120, VEGFA164, and VEGFA188, which possess different domains.

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VEGFA120 and VEGFA 164 control heparin-binding domains, which permit interactions with heparin sulfate, whereas VEGFA120 only exists in mice. VEGFA seems to have a few functions during the process of bone formation. On the one hand, VEGFA is crucial in the early as well as late stages of cartilage angiogenesis, since VEGFA conditional knockout (CKO) mice illustrated both delayed blood vessel invasion and a delayed shift of terminal hypertrophic chondrocytes. On the other hand, VEGFA is important for chondrocyte survival, as the joint and epiphyseal regions of VEGFA CKO endochondral bones suffer substantially from abundant cell death (4). Given its important role in keeping the normal construction of the growth plate and endochondral ossification, VEGFA would be a potential target for remedies of Osteoarthritis (5).

MicroRNA signatures have been recently reported as useful diagnostic tools applied to the treatment of Osteoarthritis. Just take miR-15a-5p as an example. According to current research achievements, attention has been paid to the role of miR-15a-5p in human hepatocellular carcinoma, and the findings have shown that by targeting BDNF, miR-15a-5p suppresses cancer proliferation and division in human hepatocellular carcinoma (6). Little is known about the effect and function of miR-15a-5p in other diseases. For the first time, this article proposes to illustrate the impact of miR-15a-5p on cell proliferation and matrix synthesis of the OA chondrocyte and demonstrate the target-relationship between miR-15a-5p and VEGFA. It has been illuminated that by suppressing VEGFA, miR-15a-5p is able to promote degradation of matrix in OA chondrocytes. At the same time, miR-15a-5p also helps to inhibit the activities of the cell and finally leads to the apoptosis of OA chondrocytes.

2. Materials and Methods

2.1. Patients

Six normal cartilage tissues from patients without OA or RA (rheumatic arthritis) history and 15 cartilage tissues from OA patients were collected from Yiwu Central Hospital Affiliated to Wenzhou Medical University.

2.2. Immunohistochemistry (IHC)

Human cartilage tissues were fixed overnight with 4%

paraformaldehyde, decalcified, dehydrated, and then embedded in paraffin. Tissues were cut into sections 6 mm thick and deparaffinized with xylene, serially dehydrated in ethanol and washed in PBS. Endogenous peroxidases was blocked with 5% hydrogen peroxide in methanol and then digested with 100 mg/mL hyaluronidase. Nonspecific protein binding was blocked using a serum blocking solution. Sections were then incubated with primary antibodies against VEGFA (ab31745, Abcam, UK), Bcl-2 (ab59348, Abcam, UK), MMP13 (ab75606, Abcam, UK), TIMP1 (ab38978, Abcam, UK) and TIMP2 (ab180630, Abcam, UK) overnight at 4°C, followed by biotinylated secondary antibodies (ZSJB-BIO, China) joined with 3,3'-diaminobenzidine (Sigma-Aldrich, St. Louis, USA) for 15 min at room temperature. The immune stained sections were counterstained briefly with hematoxylin. Negative controls included the use of IgG isotype controls and secondary antibody only (omission of primary antibody) controls.

2.3. Cell culture

HEK293T cell lines were purchased from the Chinese Academy of Sciences (Shanghai, China). Human articular cartilage tissues were digested with 0.2% collagenase II in DMEM. Chondrocytes were cultured in DMEM with 10% FBS at 37, 5% CO₂ atmosphere.

2.4. Cell transfection

Lentivirus with human mature miR-15a-5p mimics (Lenti-miR-mimics), inhibitor (Lenti-miR-in) and negative control (Lenti-null), were obtained from SunBio (SunBio Medical Biotechnology, China). Recombinant eukaryotic expression vector pEGFP-VEGFA as well as its negative control (pEGFP-null) was constructed specifically.

2.5. Real-time Quantitative PCR (RT-PCR)

Total RNA was extracted with Trizol (Thermo Fisher Scientific, MA, USA). RNA extracts were reverse-transcribed into cRNA and then RT-PCR was performed (Related primers are listed in Table 1). The expression of VEGFA mRNA and miR-15a-5p was normalized with GAPDH mRNA and RNU6B respectively as endogenous control. Relative RNA levels were determined with the 2- $\Delta\Delta$ Ct method.

Table 1. Primer sequences

Items	Forward sequences	Reverse sequences
miR-15a-5p	5'-TAAGGCACGCGGTGAATGCC-3'	
VEGFA	5'-CGAGGGCCTGGAGTGTGT-3'	5'-GATCCGCATAATCTGCATGGT-3'
RNU6B	5'-ACGCAAATTCGTGAAGCGTT-3'	
GAPDH	5'-GGAAGGTGAAGGTCGGAGTCA-3'	5'-GTCATTGATGGCAACAATATCCA-3'

2.6. Luciferase reporter assay

MiRDB and TargetScan were employed to predict miR-15a-5p targets. The wild-type sequence of VEGFA-3'UTR containing miR-15a-5p binding sites was inserted into the pmiR-RB-Reporter vector (Ribobio, Guangzhou, China) and a mutant was used as control (named as pmiR-VEGFA-wt and pmiR-VEGFA-mut respectively). HEK293T cells were co-transfected with the pmiR-VEGFA-wt or pmiR-VEGFA-mut and Lenti-miR-mimics or Lenti-null using Lipofectamine 2000 (Invitrogen, CA, USA). Luciferase activity was measured 48 h after transfection.

2.7. Cell proliferation analysis

Cell vitality was evaluated with Cell Counting Kit-8 (CCK-8, Sigma-Aldrich, MO, USA) after cells were incubated for various periods of time (0 d, 3 d, 6 d, and 9 d). The OD values at 450 nm were measured with spectrophotometry.

2.8. Cell apoptosis assay

Forty eight hours after transfection, cells were collected and washed with PBS, and then re-suspended in binding buffer. Cell apoptosis was evaluated by flow cytometry with the Annexin V: FITC Apoptosis Detection Kit I (BD Biosciences, NJ, USA) according to the manufacturers' protocol.

2.9. Western blot analysis

Forty eight hours after transfection, cells were washed with cold PBS and lysed with the cell lysis buffer. Protein extracts were subjected to SDS-PAGE, transferred to PVDF membranes and blocked with 5% nonfat milk in TBST. The membranes were subsequently incubated with primary VEGFA (ab31745, Abcam, UK), Bcl-2 (ab59348, Abcam, UK), COL2A1 (ab21291, Abcam, UK), MMP13 (ab75606, Abcam, UK), β -actin (ab8227, Abcam, UK), and GAPDH (ab9483, Abcam, UK) antibodies overnight at 4°C.

Then, the secondary antibodies (ZSJB-BIO, China) were added and co-incubated with the membranes for 1 h. Chemiluminescent detection was performed using an ECL kit (Pierce Chemical, Rockford, IL, USA).

2.10. MMP13 ELISA assay

The MMP13 protein level in extracellular matrix was analyzed with the human MMP13 kit (Sciencell, CA, USA) according to the procedure supplied by the manufacturer. The OD values at 450 nm were measured with spectrophotometry.

2.11. Statistical analysis

The results were represented as means and differences between two groups were analyzed using the Student's *t*-test, otherwise by the One-Way ANOVA method. $p < 0.05$ indicates significant variation between groups.

3. Results

3.1. The expressions of miR-15a-5p, VEGFA and related cytokines in OA chondrocytes

According to the RT-PCR assay, Figure 1A showed that the expression level of miR-15a-5p in OA chondrocytes was significantly higher than that in normal chondrocytes ($*p < 0.05$). On the contrary, the expression of VEGFA mRNA was suppressed in OA chondrocytes (Figure 1B-C, $*p < 0.05$). Based on IHC results (Figure 1D), the numbers of cells that stained positively for VEGFA, Bcl-2, TIMP1 and TIMP2 respectively were significantly lower in OA chondrocytes compared with normal chondrocytes while the numbers of cells that are stained positively for MMP13 showed an opposite trend which is consistent because OA chondrocyte cells possessed a weak viability and a remarkable pathological apoptosis.

3.2. MiR-15a-5p directly targeted VEGFA

VEGFA 3'UTR contained the miR-15a-5p binding sites (Figure 2A). To make sure that miR-15a-5p actually

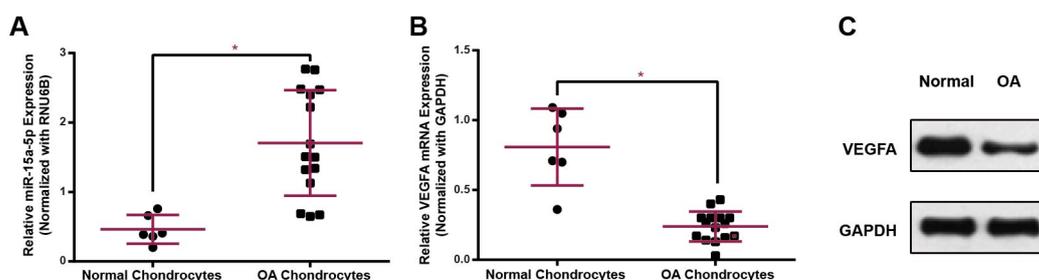


Figure 1. The expression of miR-15a-5p, VEGFA and related cytokines in OA chondrocytes were compared with normal chondrocytes. (A) MiR-15a-5p in normal and OA chondrocytes measured by RT-PCR. It was normalized by RNU6B. (B) VEGFA mRNA was detected with RT-PCR in normal and OA chondrocytes. It was normalized using GAPDH mRNA. (C) The VEGFA expression levels in normal and OA chondrocytes analyzed by Western blot with GAPDH as control. (D) Immunohistochemical activities of VEGFA, Bcl-2, MMP13, TIMP1 and TIMP2 in normal and OA chondrocytes. Scale bar = 50 μ m.

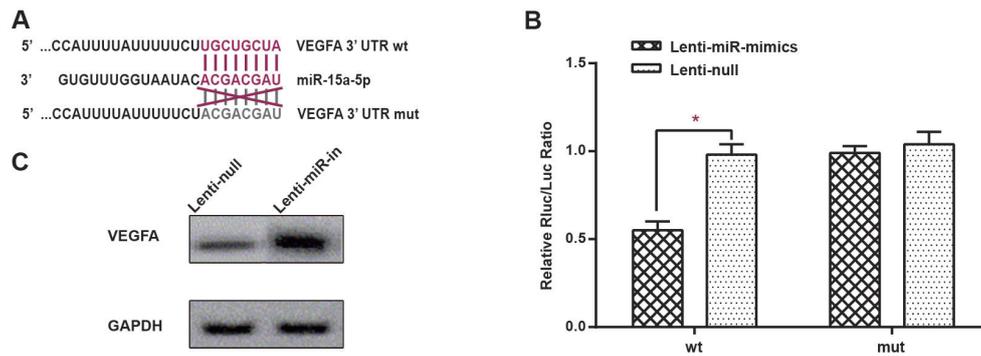


Figure 2. MiR-15a-5p targets VEGFA in human OA chondrocytes. (A) Schematic of the VEGFA 3'UTR containing the miR-15a-5p binding sites. **(B)** The results of Luciferase reporter assay. **(C)** VEGFA protein levels in OA chondrocytes evaluated by Western blot analysis with GAPDH as control.

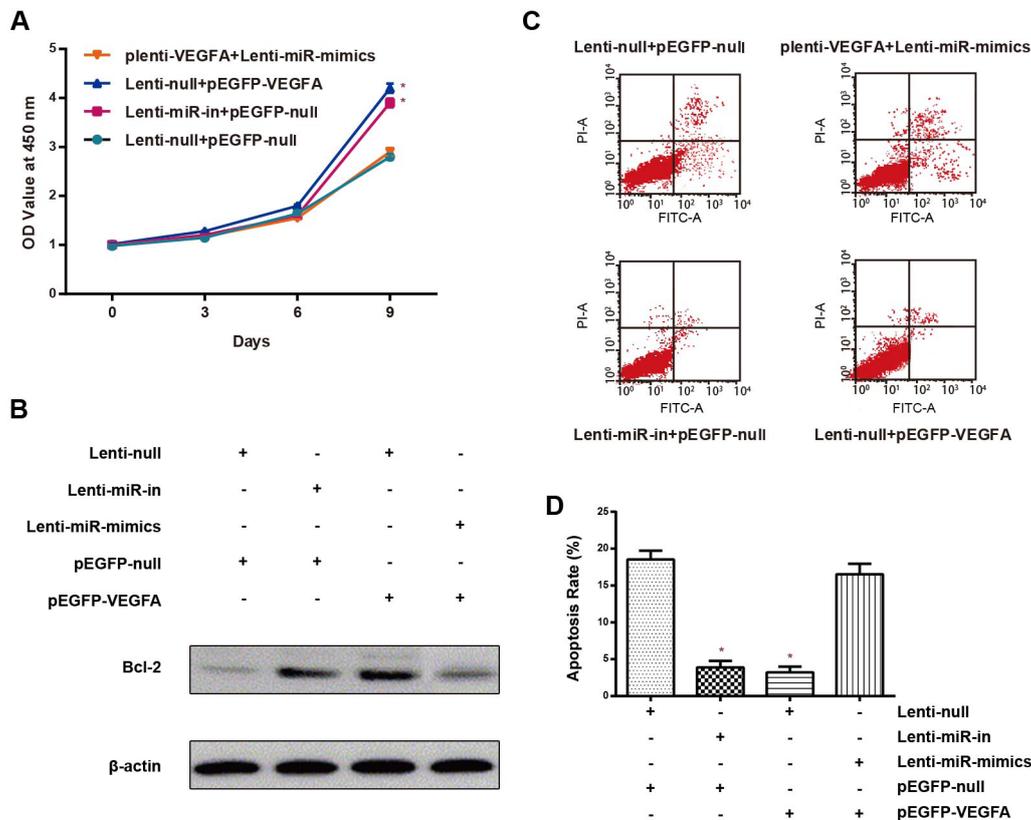


Figure 3. MiR-15a-5p suppressed OA chondrocytes vitality and anti-apoptosis. (A) The proliferation rate of cells measured by CCK8 assay. **(B)** The protein level of Bcl-2 was measured by Western blot with β -actin as control. **(C-D)** Apoptosis measured by flow cytometry.

targets VEGFA, dual-luciferase analysis was performed 48 h after HEK293 cells were transfected with pmir-VEGFA-wt or pmir-VEGFA-mut reporter vectors and Lenti-miR-mimics or Lenti-null, respectively. As presented in Figure 2B, cells co-transfected with miR-15a-5p and pmir-VEGFA-wt vector showed a significant decrease of luciferase activity in comparison with NC (negative control), showing that miR-15a-5p directly targeted VEGFA ($*p < 0.05$, versus NC). Furthermore, the protein levels of VEGFA were analyzed with Western blot analysis. Figure 2C displayed that miR-15a-5p inhibitor up-regulated VEGFA protein level. Consequently, miR-15a-5p

could directly bind the VEGFA mRNA 3'UTR region and regulate the VEGFA protein level, indicating that VEGFA could be one of miR-15a-5p targets.

3.3. MiR-15a-5p suppressed viability of OA chondrocytes by inhibiting expression of VEGFA

The effect of miR-15a-5p on chondrocyte viability was evaluated with CCK8 assays. The results suggested that miR-15a-5p inhibitor promoted cell viability of OA chondrocytes and overexpression of VEGFA exerted a similar effect (Figure 3A, $*p < 0.05$, versus NC). Moreover, the expression level of anti-apoptotic

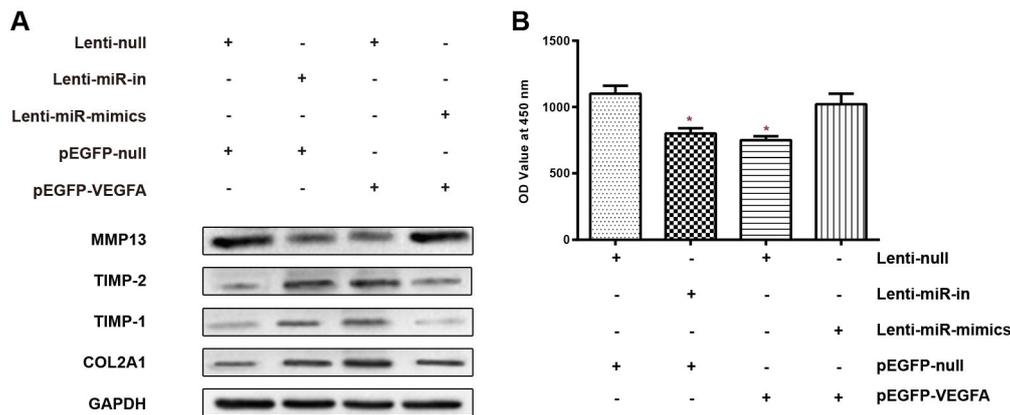


Figure 4. MiR-15a-5p promoted matrix degradation of OA chondrocytes. (A) The protein levels of MMP13, TIMP-2, TIMP-1 and COL2A1 were analyzed with Western blot (GAPDH as control). (B) The MMP13 protein level in extracellular matrix in OA chondrocytes was measured by ELISA.

protein Bcl-2 was analyzed by Western blot analysis to investigate the effect of miR-15a-5p on OA chondrocytes apoptosis. Figure 3B shows that compared with NC, Bcl-2 is overexpressed in OA chondrocytes transfected with miR-15a-5p inhibitor or pEGFP-VEGFA respectively while those transfected with both miR-15a-5p mimics and pEGFP-VEGFA appeared to have little difference. Meanwhile, the results of cell apoptosis assays by flow cytometry were consistent with the Western blot results (Figure 3C-D, $*p < 0.05$, versus NC). Both inhibition of miR-15a-5p and overexpression of VEGFA significantly reduced the apoptosis rate compared with NC. Overall, miR-15a-5p could suppress the viability of OA chondrocytes and induce cell apoptosis *via* targeting VEGFA.

3.4. MiR-15a-5p promoted matrix degradation of OA chondrocytes

The expression of matrix synthesis biomarkers COL2A1, MMP13, TIMP-1 and TIMP-2 in each group of cells were analyzed via Western blot analysis to look for the effect of miR-15a-5p on OA chondrocytes matrix synthesis (Figure 4A). The results indicated that both inhibition of miR-15a-5p and overexpression of VEGFA dramatically down-regulated MMP13 protein level, but on the contrary increased TIMP-1, TIMP-2 and COL2A1 expression, indicating suppression of matrix degradation. In addition, ELISA results also showed a decrease in MMP13 protein levels in the extracellular matrix of cells in the Lenti-miR-in group and pEGFP-null group (Figure 4B, $*p < 0.05$, versus NC). Together, we could draw a conclusion that miR-15a-5p promoted matrix degradation of OA chondrocytes by regulating VEGFA expression.

4. Discussion

OA is one of the most common joint diseases among senior citizens, which is characterized by degenerative

alteration of cartilage. Because the joint dysfunction interferes with daily living and work ability, OA is considered to be an enormous threat to human function (7). Recent studies demonstrated that abnormal expression of miRNAs played a crucial role during the development of OA, for example, Park *et al.* (2014) found that miR-127-5p could regulate MMP13 in human OA chondrocytes and might promote the progression of OA (8). Kang *et al.* (2016) demonstrated that miR-23a-3p was up-regulated in OA chondrocytes and could suppress ECM synthesis through targeting SMAD3, which accelerated the development of OA (9).

The aberrant expression of miR-15a has been reported in several types of human cancers. For example, abnormal overexpression of miR-15a-5p was reported to suppress cancer proliferation, induce cell cycle arrest in human HCC cells, and have a specific and negative regulating effect on BDNF (6). In addition, the protein levels of p27, GSK-3beta, Bax, procaspase3, and active caspase 3 were upregulated by the overexpression of miR-15a, which inhibited proliferation and induced apoptosis of CNE1 cells (10). There were other studies that investigated the role of miR-15a in OA. In our study, the results of RT-PCR indicated that miR-15a-5p was up-regulated in OA chondrocytes compared with normal chondrocytes.

VEGFA is a strong angiogenic protein with a selective mitogenic influence on vascular endothelial cells. It is an important cytokine in angiogenesis, and its circulating levels were associated with cell proliferation, migration and organization (11). As a pivotal element, VEGFA has been identified to act as an essential characteristic substance in many human cancers, for example, breast cancer, brain tumor, and cutaneous melanoma, to name just a few (12-14). On the other hand, VEGFA is a necessary factor for chondrocyte growth during skeletal development (4). Brew *et al.* (2010) found that VEGFA was downregulated in OA chondrocytes (15), which was consistent with the results of our study. The results of RT-PCR and Western blot

assays indicated that VEGFA was down-regulated in OA chondrocytes compared with normal chondrocytes.

The protein encoded by the Bcl-2 gene has been widely implicated in the prolongation of cell survival by blocking apoptosis and necrosis (16,17). Many observations confirmed that Bcl-2 in human articular chondrocyte prevents their apoptosis in osteoarthritis (18,19). A time-dependent relationship between p53, Bcl-2 and VEGF protein has been revealed in esophagus cancer cells (20). In this study, it was found that the association of VEGFA and Bcl-2 in osteoarthritis suggests a potential understanding of VEGFA's function on chondrocyte apoptosis.

Previous studies confirmed that MMP13 is a major enzyme targeting cartilage for degradation of type II, IV, IX collagen, proteoglycan, osteonectin and perlecan (21,22). Overexpression of MMP13 contributes to cartilage destruction among in both human OA patients and mice models (23,24). TIMP1 and TIMP2 are two members of Tissue inhibitors of metalloproteinases (TIMPs) and act as inhibitors of MMP13. A balance between MMPs and TIMPs is necessary for the physiological processes of OA (25). A relationship of VEGF and TIMP1 has been reported in the diagnosis of patients with breast cancer (26). To the best of our knowledge, it's a novel finding that overexpression of VEGFA promoted the expression of TIMP1 and TIMP2 and repressed the level of MMP13 which supported the idea that VEGFA fortified the viability of chondrocyte cells.

The results of RT-PCR and Western blots suggested that miR-15a-5p was up-regulated in the OA cells compared with adjacent normal cells, whereas VEGFA was down-regulated, which inspired us to think that there was probably a targeting relationship between miR-15a-5p and VEGFA. Previously, an analogous outcome has been confirmed in the study of Yang *et al.* (27). In our study, the targeting relation was further verified using luciferase reporter assays and Western blot assays. The results of the following experiments demonstrated that miR-15a-5p could significantly suppress the viability, promote apoptosis, suppress the synthesis of matrix and promote the degradation of matrix of the OA chondrocytes through targeting VEGFA, which was consistent with Nagata *et al.* (28) in miR-15a function.

Despite the fact that it is innovative to investigate the relationship between miR-15a-5p and VEGFA, as well as their roles in OA, there are still a few limitations and controversies. Whether there are other microRNAs that have the same function as miR-15a-5p of inhibiting VEGFA *via* a targeted relationship remains questionable, further investigation should be conducted. Also Li *et al.* (2012) found that miR-146a might promote OA pathogenesis by upregulating VEGF and by impairing the TGF-beta pathway through inhibiting Smad4 in chondrocytes (27), which are different views compared to our study of VEGF function. This needs

further research to explore the complicated mechanism. In addition, the possibility that miR-15a-5p targets other growth factors that are also essential for the formation of the matrix in OA chondrocytes can't be ruled out.

In a nutshell, on the basis of our newly-produced data, it has been revealed that an excess of miR-15a-5p can lead to the inhibition of VEGFA, resulting in OA progression and inhibiting apoptosis of OA chondrocytes. Therefore, miR-15a-5p may serve as a new target for OA therapy in the future to relieve the suffering of patients.

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