

# MicroRNA-195 induced apoptosis in hypoxic chondrocytes by targeting hypoxia-inducible factor 1 alpha

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**Abstract.** – **OBJECTIVE:** The chondrocytes, the resident cells of cartilage, are maintained and take effects in the whole life upon chronic hypoxic exposure, which hypoxia-inducible factor 1 alpha (HIF-1 $\alpha$ ) play pivotal roles in response to. Dysregulation of some microRNA (miRNAs) have also been identified to be involved in hypoxia-related physiologic and patho-physiologic responses in some tissues or cell lines. However, the mechanism of miRNAs response to hypoxia remain largely unknown in chondrocytes, including the microRNA-195 (miR-195). AIM To investigate the effects of microRNAs (miRNAs) and hypoxia-inducible factor 1 alpha (HIF-1 $\alpha$ ) on chondrocytes in physiologic environment.

**MATERIALS AND METHODS:** We compared the expression of miR-195 and HIF-1 $\alpha$  mRNA on hypoxia with that on normoxia in ATDC 5 cells by qRT-PCR. Further experiments was performed to confirmed the relationships of miR-195 and HIF-1 $\alpha$  by bioinformatics analysis and dual reporter gene assay. we also assessed the effect of miR-195 on apoptosis in hypoxic ATDC 5 cells by transfect with miR-195 mimics.

**RESULTS:** It was found the downregulated miR-195 and upregulated HIF-1 $\alpha$  were present in hypoxic ATDC 5 cells. miR-195 negatively regulated HIF-1 $\alpha$  by targeting its 3'-untranslated region. Moreover, the founding indicated miR-195 greatly increased apoptosis and downregulated HIF-1 $\alpha$  mRNA occurred simultaneously in hypoxic chondrocytes.

**CONCLUSIONS:** We concluded that miR-195 induced apoptosis in hypoxic chondrocytes by directly targeting HIF-1 $\alpha$ .

*Key Words:*

MicroRNA, MicroRNA-195, Hypoxia-inducible factor 1 alpha, Chondrocytes, ATDC 5, Apoptosis, Hypoxia.

## Introduction

Chondrocytes, the resident cells of cartilage, are indispensable in human, since they take important effects on not only articulation of joints but also joint homeostasis<sup>1</sup>. The degradation of articular cartilage has been characterized to be one of the most important factors resulting in osteoarthritis, which is the most prevalent joint disorder worldwide<sup>2</sup>. Notwithstanding vast reports are involved in mechanisms of cartilage degradation, these mechanisms remain largely lacking.

The chondrocytes are specific cell type in the tissues as they are maintained and take effects in the whole life upon chronic hypoxic exposure. Hypoxia-inducible factor 1 alpha (HIF-1 $\alpha$ ) plays pivotal roles in mediating the ubiquitous hypoxia responses in cells and tissues, especially in chondrocytes<sup>3</sup>. The functions of HIF-1 $\alpha$  refer to multifunctional roles in skeletal development and cartilage. The expression of HIF-1 $\alpha$  was detectable in normal chondrocytes or osteoarthritic chondrocytes under hypoxic condition, and the HIF-1 $\alpha$  is higher in osteoarthritic chondrocytes than that in normal chondrocytes<sup>4</sup>. Inflammatory factor such as TNF $\alpha$  can stimulate the HIF-1 $\alpha$ , which sustains ATP levels and matrix synthesis in chondrocytes adaptive to the inflammation<sup>4-6</sup>. It is certain HIF-1 $\alpha$  is a essential factor in chondrocytes response to hypoxia. yet the understanding of HIF-1 $\alpha$  is still largely unknown in chondrocytes.

MicroRNAs (miRNAs), consist of about 22 nucleotides, modulated abundant gene expression by targeting the 3'-untranslated regions (3'-UTRs) to cause negative regulation of relevant

mRNAs for expression<sup>7</sup>. Some of the major functions of miRNAs are regulation of cellular biology and pathophysiological regulatory pathways<sup>8,9</sup>. In chondrocytes, it is found miR-140 takes important functions of specific to cartilaginous tissues and influences the growth plate cartilage of long bones in zebrafish and mouse<sup>10-12</sup>. There have been lots of dysregulated miR-675 and miR-145 to be discovered in primary chondrocytes<sup>13-15</sup>. Owing to the multifunctional effects of HIF-1 $\alpha$  and miRNAs on physiological and pathological regulation, more reports on the mechanisms of their interactions take place in cancer rather than in chondrocytes. Interactions of miRNAs and HIF-1 $\alpha$  in chondrocytes are important and need to be understood.

In this research, we compared the expression of miR-195 and HIF-1 $\alpha$  mRNA on hypoxia with that on normoxia in ATDC 5 cells. The dysregulation of miR-195 and HIF-1 $\alpha$  mRNA was present on hypoxic environment. Further study indicated HIF-1 $\alpha$  might be a direct target of miR-195. Moreover, we investigated the effect of miR-195 and HIF-1 $\alpha$  on apoptosis in hypoxic chondrocytes. And we revealed the MiR-195 induced apoptosis in hypoxic chondrocytes by targeting HIF-1 $\alpha$ . It is the first report on the mechanism of miR-195/HIF-1 $\alpha$  interaction and the regulatory effect of miR-195 on apoptosis in hypoxic chondrocytes.

## Materials and Methods

### Cell Culture

ATDC 5 cells were propagated in maintenance medium (1:1 Dulbecco's modified Eagle's medium (DMEM): Ham's F-12 mix (Gibco, Invitrogen, Carlsbad, USA), 1% antibiotic-antimycotic (AB) (Gibco), 5% fetal bovine serum (FBS) (Gibco) containing 10  $\mu$ g/ml human transferrin and 30 mM sodiumselenite (Sigma-Aldrich, St Louis, MO, USA) for 12 h, 24 h and 48 h, and maintained in Anoxomat chambers (Mart Microbiology, Lichtenvoorde, Netherlands) for physiological hypoxia (5% O<sub>2</sub>) or normoxia (21% O<sub>2</sub>) at 37°C. human embryonic kidney epithelial cells (293 T cells) were grown in DMEM with 10% FBS in a humidified incubator containing 5% CO<sub>2</sub> at 37°C.

### RNA Extraction and Quantitative Real-Time PCR

RNA were extracted from cells by using RNeasy kits (Qiagen, Valencia, CA, USA). miR-

NAs was isolated using mirVANA miRNA isolation kit (Ambion, Austin, TX, USA). The expression of miR-195 was detected by the mirVana qRT-PCR miRNA Detection Kit (Ambion, Austin, TX, USA) and qRT-PCR Primer Sets. qRT-PCR was performed on Light Cycler 2.0 (Roche, Darmstadt, Germany). Data were analyzed by using  $\Delta\Delta$ Ct method as relative quantification. The U6 small nuclear RNA was used as an endogenous reference of expression of miR-195. The expression of HIF-1 $\alpha$  mRNA were assayed by using the  $\beta$ -actin as the internal control. The used primers were: HIF-1 $\alpha$  F 5'-CCT ATG ACC TGC TTG GTG CTG-3', R 5'-CTG GCT CAT ATC CCA TCA ATT CG-3'.  $\beta$ -actin F 5'-CTG GCA CCA CAC CTT CTA CAA TG-3', R 5'-CCT CGT AGA TGG GCA CAG TGT G-3'.

### Transient Transfection

ATDC 5 cells were transfect with synthetic miR-195 mimics or miR-control (Ambion/Applied Biosystems, Foster City, CA, USA) at 50 nM concentration by using Lipofectamine 2000 (Invitrogen). ATDC 5 cells were harvested after transfection as indicated time. 293T cells was co-transfect with the plasmid or reporter constructs and miR-195 mimics or miR-control by using Lipofectamine 2000.

### Luciferase Reporter Assays

The reporter constructs was performed as reference<sup>16</sup>. Briefly, the seed sequence(GCC AAT AT 370-377 as shown in Figure 2a) of mouse HIF-1 $\alpha$  3'-UTR (Untranslated Region) which was analyzed by bioinformatics and its flanking sequences (302-455) was cloned into pGL3 basic luciferase expression vector (Promega, Madison, WI, USA) named as pUTR-wt. The seed sequence were mutated from GCC AAT AT to GCG TAT GT. the construct containing mutant seed sequence was designated as pUTR-mut. The constructs and the internal control vector pRL-TK (Promega) were co-transfected into 293T cells. the cells were transfect with 50 nM of miR-195 mimics or miR-control 6 h later. Luciferase reporter assays were performed after 24 h transfection by the Dual-Luciferase Assay System (Promega) according to manufacturer's instructions.

### Apoptosis Assays

Cell apoptosis was determined with annexin V-FITC apoptosis detection kit (Sigma-Aldrich, St. Louis, MO, USA). Briefly, 1-5 $\times$ 10<sup>5</sup> ATDC 5 cells were resuspended in 0.5 ml of binding buffer and

incubated with annexin V-FITC and propidium iodide (PI) for 10 min in the dark at room temperature. AFACScan flow cytometer (BD Biosciences, San Jose, CA, USA) equipped with a FITC (fluorescein isothiocyanate) signal detector FL1 (excitation 488 nm, green) and a phycoerythrin emission signal detector FL3 (excitation 585 nm, red) was used to analyze cellular apoptosis. The results were calculated using the CellQuest<sup>TM</sup> Pro software (BD Biosciences) and expressed as the percentage of apoptotic cells from the total cells.

### Statistical Analysis

Student t-test was used to analyze the quantitative data.  $p < 0.05$  was considered as statistically significant.

## Results

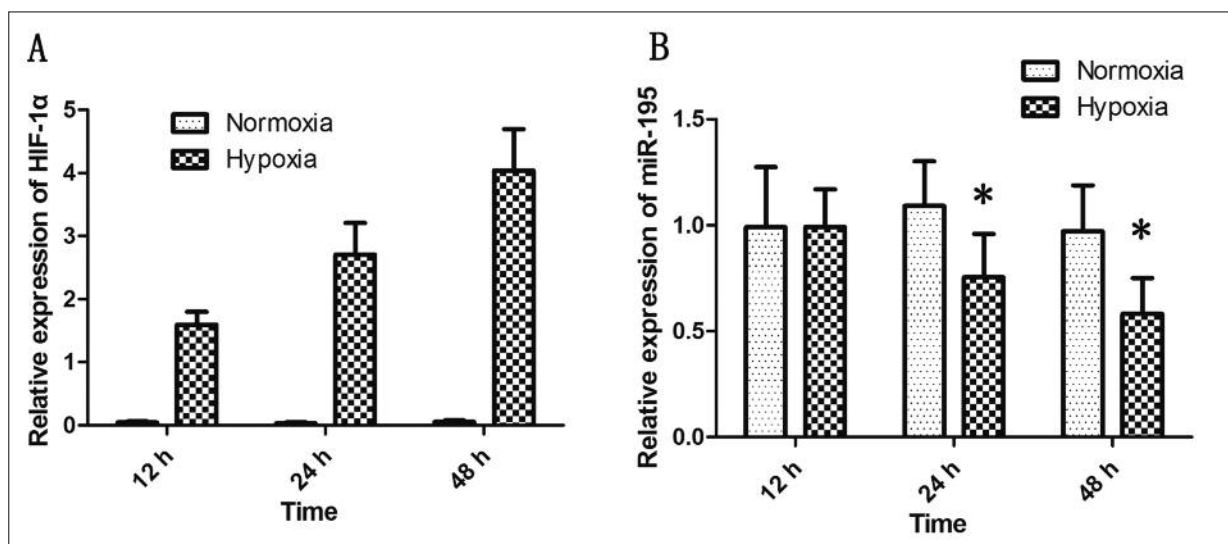
### Hypoxia Stimulated the Expression of Decreased miR-195 and Upregulated HIF-1 $\alpha$ in Chondrocytes ATDC 5 Cells

To show the chondrocytes response to the hypoxia, we assessed the expression of HIF-1 $\alpha$  gene in ATDC 5 cells on hypoxic exposure at 12 h, 24 h, 48 h. And we found there was dramatically low expression of HIF-1 $\alpha$  mRNA in ATDC 5 cells upon normoxia. In addition, Time course

of HIF-1 $\alpha$  mRNA expression displayed the expression of HIF-1 $\alpha$  mRNA was significantly higher on hypoxia than that on normoxia in ATDC 5 cells (Figure 1A). Corresponding with the increased HIF-1 $\alpha$  expression, experiments were conducted to detect the expression of miR-195 by qRT-PCR. Time course of miR-195 expression showed a progressive reduction on hypoxia, generating significant differences compared to that on normoxic exposure at 24 h ( $p = 0.033$ ) and 48 h ( $p = 0.013$ ) as shown in Figure 1B. All these results indicated hypoxia caused an increased HIF-1 $\alpha$  expression and attenuated miR-195 expression in hypoxic ATDC 5 cells.

### HIF-1 $\alpha$ was a Direct Target of miR-195

Owing to the miRNAs negative modulation of relevant mRNAs for expression<sup>7</sup>, it was supposed if HIF-1 $\alpha$  was a direct target of miR-195. In order to investigate the mechanism underlying the miR-195 downregulation and upregulated HIF-1 $\alpha$ , the putative targeting sites (seed sequence) of HIF-1 $\alpha$  3'-UTR by miR-195 were analyzed by bioinformatics analysis and were cloned into luciferase reporter constructs, in which the seed sequence was mutated simultaneously (Figure 2A). With artificially adding the miR-195 by transfect with miR-195 mimics, luciferase reporter gene activity was decreased by 31% in the pUTR-wt. Nevertheless, there was no significant change of



**Figure 1.** Hypoxia induced decreased miR-195 and upregulated HIF-1 $\alpha$  in chondrocytes ATDC 5 cells. The relative expression of HIF-1 $\alpha$  mRNA was assayed by qRT-PCR on normoxia or hypoxia in ATDC 5 cells at 12 h, 24 h and 48 h (A). The relative expression of miR-195 was investigated by qRT-PCR in ATDC 5 cells on hypoxia also, by contrast to that on normoxia (B). Error bars indicated the standard error of the mean of five individual experiments. The data was normally distributed and was statistically analyzed using the Student's *t* test. The asterisk denoted significant differences ( $p < 0.05$ ).

luciferase reporter gene activity in lacking seed sequence. Interestingly, the mutant type of seed sequence inverted the effect of putative targeting sites on attenuated luciferase reporter gene activity. Luciferase reporter gene activity in pUTR-mut was no significantly different from that in lacking putative targeting sites, as shown in Figure 2B. all this data identified the HIF-1 $\alpha$  might be a direct target of miR-195.

### Over-Expression of miR-195 Increased Apoptosis via HIF-1 $\alpha$ in Hypoxic ATDC 5 Cells

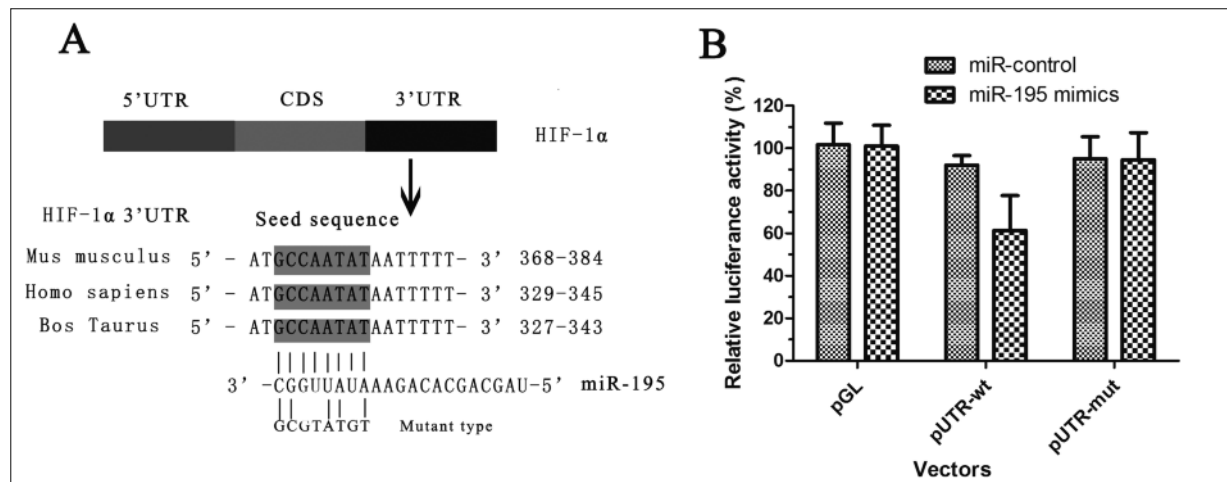
We investigated the effect of miR-195 on apoptosis on hypoxic ATDC 5 cells. Apoptosis of ATDC 5 cells was tested post 12 h, 24 h, and 48 h culture. It was found overexpression of miR-195 increased apoptosis of ATDC 5 cells in a time dependent manner on hypoxia. The apoptotic rate enhanced in ATDC 5 cells transfect with miR-195 mimics with time going on, significantly at 48 h ( $p = 0.036$ ) compared to that with miR-control on hypoxia as showed in Figure 3A. And the average maximum apoptotic rate was 19.2% at 48 h. To investigate the effects of miR-195 on HIF-1 $\alpha$ , we detected the expression of HIF-1 $\alpha$  mRNA in hypoxic ATDC 5 cells transfect with miR-195 mimics or miR-control. We discovered significantly downregulated HIF-1 $\alpha$

mRNA in hypoxic ATDC 5 cells transfect with miR-195 mimics at 24 h ( $p = 0.042$ ), 48 h ( $p = 0.009$ ). All these founding illustrated overexpression of miR-195 increased the apoptosis of ATDC 5 cells depending on HIF-1 $\alpha$  on hypoxia.

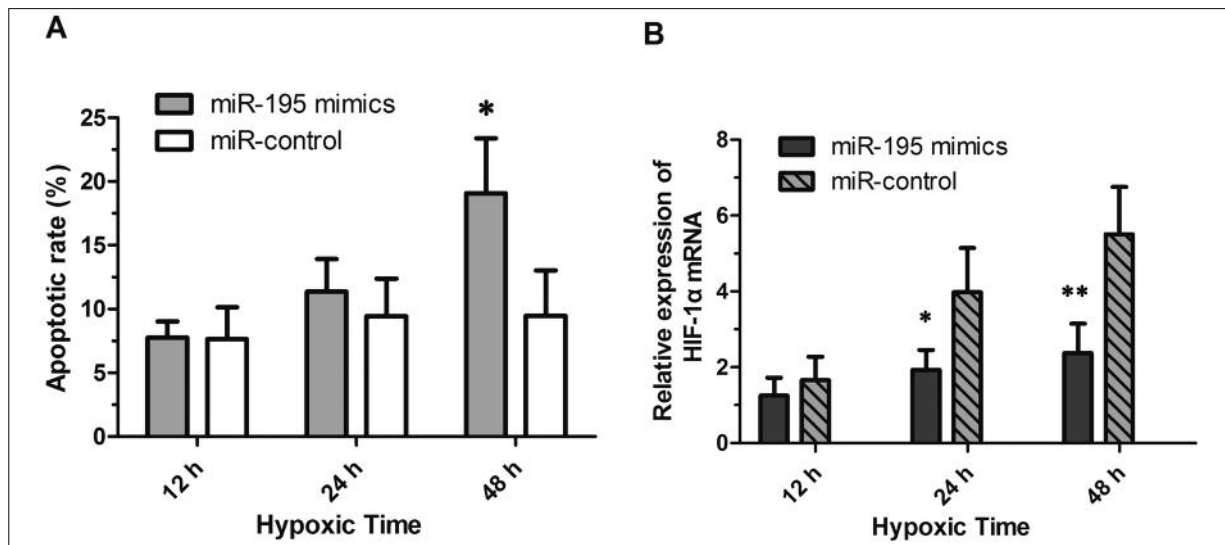
## Discussion

HIF-1 $\alpha$ , a key regulator of the transcriptional response to hypoxia, takes functions on normal chondrocytes or osteoarthritic chondrocytes<sup>4,17</sup>. miRNAs have multifunctional regulation on cartilaginous tissues, also<sup>10-12</sup>. Up to date, the interaction of miRNAs and HIF-1 $\alpha$  refer to cancer cells, epithelial cells<sup>18,19</sup>. However, there is no report on mechanisms of miRNAs/HIF-1 $\alpha$  interaction in chondrocytes.

In this research, to investigate physiological mechanism of chondrocytes response to hypoxia, the expression of miR-195 and HIF-1 $\alpha$  was assayed by qRT-PCR in ATDC 5 cells upon hypoxia or normoxia. Subsequently, we found the decreased miR-195 and increased HIF-1 $\alpha$  mRNA upon hypoxic environment at the same time, contrasted by that on normoxia (Figure 1). It indicated that hypoxia might be the reason to result in overexpression of HIF-1 $\alpha$  as well as the down regulated miR-195 in ATDC 5 cells.



**Figure 2.** Identification of the relationships of miR-195 and HIF-1 $\alpha$  by bioinformatics analysis and dual reporter gene assay. Bioinformatics analysis was performed to investigate the putative target gene sites (seed sequences) of miR-195 in HIF-1 $\alpha$  3'-UTR. The seed sequence was GCC AAT AT that was conservative across species such as in *Mus musculus*, *Homo sapiens*, *Bos Taurus* (A). The seed sequence and its mutant type (GCG TAT GT) was cloned into pGL3 basic luciferase expression vector (pGL) named as pUTR-wt and pUTR-mut. The constructs (pGL, pUTR-wt or pUTR-mut) and the internal control vector pRL-TK (Promega) were co-transfected into 293T cells. the cells were transfect with 50 nM of miR-195 mimics or miR-control 6 h later. Luciferase reporter assays were performed after 24 h transfection by the Dual-Luciferase Assay System. Relative luciferase activity in cells transfected with miR-195 mimics were compared that with miR-control (B). All experiments was performed separately in triplicate.



**Figure 3.** Over-expression of miR-195 increased apoptosis via HIF-1 $\alpha$  in hypoxic ATDC 5 cells. Apoptosis of ATDC 5 cells transfected with miR-195 or miR-control was measured by flow cytometry after 12, 24 and 48 h upon hypoxia (**A**). The expression of HIF-1 $\alpha$  mRNA was assayed at the same time by qRT-PCR (**B**). Three individual experiments were conducted. The significant differences were denoted as \* $p < 0.05$  and \*\* $p < 0.01$ .

HIF-1 $\alpha$  plays an essential role in hypoxia-related physiologic and pathophysiologic responses and some miRNAs have been identified to be induced or modulated by hypoxia<sup>20,21</sup>. It is confirmed that there could be interactions of miRNAs and HIF-1 $\alpha$  directly or indirectly upon hypoxia, according to the links between oxygen-specific stress factors and gene expression regulation<sup>19,22,23</sup>. MiR-210, directly targeting HRE (hypoxia responsive element) is regulated by HIF-1 $\alpha$  which has been verified as proximal miR-210 promoter<sup>24</sup>. Furthermore, hypothesis has been made miR-210, HIF-1 $\alpha$  and their relevant genes constitute HIF-1 $\alpha$ /miR-210 Signaling Pathway, which is activated in response to hypoxia in various cancer cell lines<sup>25-27</sup>. Besides miR-210, miR-29c is upregulated by HIF- $\alpha$  activation which attenuates renal interstitial fibrosis<sup>28</sup>. Up-regulated miR-183, inducing HIF-1 $\alpha$  activation, takes effect on glioma biology<sup>29</sup>. These reports make the proof of the positive effects of miRNAs on HIF-1 $\alpha$  activation. Conversely, some miRNAs play negative roles in HIF-1 $\alpha$  activation. Hypoxia-induced downregulation of miR-206 promotes pulmonary hypertension by targeting the HIF-1 $\alpha$ /Fhl-1 pathway in pulmonary artery smooth muscle cells<sup>30</sup>. miR199b negatively regulates HIF-1 $\alpha$  by targeting its 3'-untranslated region and reduces cellular proliferation and promotes cellular death in prostate cancer<sup>16</sup>. HIF1 $\alpha$ -mediated inhibition of miR-503 takes functions on tu-

morigenesis by targeting FGF2 and VEGFA rather than by direct targeting HIF-1 $\alpha$ <sup>31</sup>. So it is uncertain the roles of miRNAs in regulation of HIF-1 $\alpha$ . And the functions of miRNAs on HIF-1 $\alpha$  are highly dependent on the cells, tissues and micro-environment.

In our research, we discovered the upregulated HIF-1 $\alpha$  and decreased miR-195 simultaneously in ATDC 5 cells upon hypoxia. We postulated the negative regulatory effect of miR-195 on HIF-1 $\alpha$  further. To make sure the hypothesis and verify the direct targets of the miR-195, we supposed the negative regulation of miR-195 by direct targeting HIF-1 $\alpha$  through bioinformatics analysis (Figure 2A). We experimentally illustrated that miR195 negatively regulated HIF-1 $\alpha$  by targeting its 3'-untranslated region (Figure 2B). It is the first report on the negative and direct regulatory roles of miR-195 in modulation of HIF-1 $\alpha$  expression until now.

MiR-195, an important member of the miR-15/16/195/424/497 family, is activated in multiple diseases, such as cancers, heart failure, and schizophrenia in which miR-195 takes effect on the cell cycle, apoptosis, proliferation<sup>32</sup>. Overexpression of miR-195 induces apoptosis in human embryonic stem cell-derived neural progenitor cells<sup>33</sup>, cancer cells<sup>34</sup> and podocytes<sup>35,36</sup>. In our study, we discovered the miR-195 played the role as a positive regulator in apoptosis in hypoxic chondrocytes (Figure 3A). Furthermore we de-

tect expression of HIF-1 $\alpha$  in hypoxic chondrocytes with upregulated miR-195 (Figure 3B). The results showed there was significant decreased expression of HIF-1 $\alpha$  in hypoxic chondrocytes with upregulated miR-195, compared to that with miR-control. However, the expression of HIF-1 $\alpha$  is still higher on hypoxia than that on normoxia. These findings further supported the negative regulatory effect of miR-195 on HIF-1 $\alpha$  and revealed overexpression of miR-195 might induce the apoptosis in hypoxic chondrocytes by targeting HIF-1 $\alpha$ .

### Conclusions

We reported the hypoxia inducing downregulated miR-195 and increased HIF-1 $\alpha$  in chondrocytes. We also revealed HIF-1 $\alpha$  might be a direct target of miR-195. Further study demonstrated miR-195 induced apoptosis in hypoxic chondrocytes by targeting HIF-1 $\alpha$ . All these findings was a new beginning to understand the life cycles of chondrocytes and mechanisms of its degradation in physiological environment.

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### Conflict of Interest

The Authors declare that there are no conflicts of interest.

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