



# miR-17 regulates the proliferation and apoptosis of endothelial cells in coronary heart disease via targeting insulin-like-growth factor 1

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## ABSTRACT

Coronary heart disease (CHD) is one of the main risks of death, which is mainly caused by coronary arteries arteriosclerosis. The present study aims to investigate the potential roles of miR-17 in CHD. In the present study, Human umbilical vascular endothelial cells (HUVECs) were treated with oxidized low density lipoprotein (ox-LDL). qRT-PCR and western blot were used to examine the mRNA and protein levels, respectively. CCK-8 and flow cytometry were conducted to determine the proliferation and apoptosis of ox-LDL treated HUVECs. Moreover, luciferase assay was performed to confirm whether insulin-like Growth Factor-1 (IGF-1) was a target of miR-17. The results showed that miR-17 was upregulated in ox-LDL treated HUVECs, while IGF-1 was downregulated. The luciferase activity of ox-LDL treated HUVECs was decreased after the treatment of miR-17 mimics and IGF-1 3'UTR WT. Moreover, overexpressed miR-17 promoted the cell viability and inhibited the apoptosis of ox-LDL treated HUVECs, which was more potent after the treatment of IGF-1 siRNA. Furthermore, the expression of Bax and Caspase3 was decreased, and Bcl-2 was increased in ox-LDL treated HUVECs transfected with miR-17 mimics, which was further decreased after transfection with IGF-1 siRNA. Taken together, miR-17 may regulate the proliferation and apoptosis of ox-LDL treated HUVECs. miR-17 may be a promising biomarker for CHD.

## 1. Introduction

Coronary heart disease (CHD) is one of the main threats to human health worldwide [1]. CHD is characterized with coronary arteries arteriosclerosis, which contribute to 80% of sudden cardiac death [2,3]. The accumulation of oxidized low-density lipoprotein (ox-LDL) is reported to be involved in the initiation and development of atherosclerosis (AS) [4]. Therefore, ox-LDL plays an crucial roles in the progression of coronary heart disease [5]. It is showed that ox-LDL participates in the development of coronary heart disease via its cytotoxic roles in human umbilical vascular endothelial cells (HUVECs) [6]. Endothelial dysfunction or abnormal proliferation induces the progression of CHD via promoting the functions of circulating blood monocytes and macrophages at the endothelial-injured part [7]. Thus, it is of vital importance to maintain the normal endothelial functions [8]. However, the possible underlying mechanisms has not been fully elucidated.

MicroRNAs (miRNAs) are a family of small endogenous noncoding RNA at the length of ~22 nucleotides [9]. miRNAs are deemed as post-transcriptional via regulating the 3'-UTR of the targets [10]. Increasing evidence report that miRNAs play an important role in physiological or

pathological processes, including cell growth, differentiation, and apoptosis [11]. Furthermore, mRNAs participate in the progression of AS [12]. miR-23 promotes the development of CHD via targeting A20 [13]. miR-17 was upregulated in patients with CHD and may serve a promoter in the development of CHD [14]. However, the molecular mechanisms underlying are still unclear.

In the present study, we found that miR-17 was upregulated in ox-LDL models. Moreover, upregulated miR-17 promoted the proliferation and inhibited the apoptosis of ox-LDL models. This may lay a basis for further study.

## 2. Materials and methods

### 2.1. Cell culture

Human umbilical vascular endothelial cells (HUVECs) were obtained from American Type Culture Collection (ATCC, USA). HUVECs were incubated in DMEM medium containing HUVECs 10% fetal bovine serum (FBS, Gibco, USA) and 1% penicillin and 100 mg/ml streptomycin (Invitrogen, USA) in the atmosphere humidified with 5% CO<sub>2</sub>.

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Oxidized low density lipoprotein (ox-LDL) was purchased from AngYuBio (Shanghai, China). Cells were cultured with 100 mg/l of ox-LDL for 24 h to construct CHD model.

## 2.2. Transfection

miR-17 mimics and the scrambled control (NC) were provided by GenePharma (Shanghai, China). Cells were transfected with 1.25  $\mu$ L (20  $\mu$ mol) of miR-17 mimics and the negative control (NC) using 5  $\mu$ L Lipofectamine 2000 (Invitrogen, USA) according to the manufacturer's instructions. After 48 h transfection, cells were used for further study. The sequences of miR-17 mimics and its NC were as follows: miR-17 mimics, 5'-ACUGCAGUGAAGGCACUUGUAG-3'; NC, 5'-UUCUCCGAA CGUGUCAGU-3'.

As for the transfection with insulin-like Growth Factor-1 (IGF-1) siRNA, IGF-1 siRNA and IGF-1 NC siRNA were purchased from GenePharma (Shanghai, China). Cells were transfected with IGF-1 siRNA and IGF-1 NC siRNA at 37 °C for 24 h. Then cells were cultured with 100  $\mu$ L complete culture medium. 48 h later, cells were harvested and used for the following experiment. The sequences of IGF-1 siRNA and NC siRNA were: IGF-1 siRNA, forward: 5'-CUGUUCACCAAAUUGUGA A-3', reverse: 5'-UUCACAAUUGUGAACAG-3'; NC siRNA, forward: 5'-UUCUCCGAAACGUGUCAGU-3', reverse: 5'-ACGUGACAGUUCGGA GAA-3'.

## 2.3. Quantitative reverse transcription-polymerase chain reaction (qRT-PCR)

Total RNA was refined from HUVECs with TRIzol reagent (Life Technologies, USA). Equal amount of RNA (1  $\mu$ g) was reversely transcribed into cDNA using Taqman miR Reverse Transcription kit (Applied Biosystems, USA). TaqMan miR assay system (Applied Biosystems, USA) was used for the analysis of miRNA and SYBR Green RT-qPCR assay (Applied Biosystems, USA) for the expression of mRNA. U6 and GAPDH served as the loading control. The PCR were performed under the following thermocycling conditions: 95 °C for 5 min; 40 cycles of 95 °C for 10 s, 60 °C for 20 s and 72 °C for 20 s. The relative expression level was measured with  $2^{-\Delta\Delta Ct}$  [15]. The sequences of the primers were listed in Table 1.

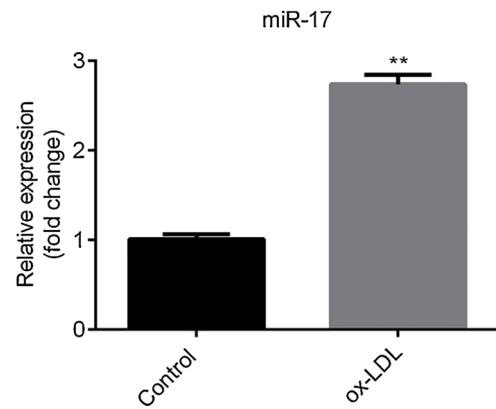
## 2.4. Western blot

Total protein was extracted from HUVECs using RIPA buffer (Beyotime, China). Then the cells were centrifugated at 12,000  $\times$  g for 15 min. The concentration of protein was calculated with BCA Kit (Thermo Fisher Scientific, USA). Later, total protein was isolated with 10% SDS-PAGE. Then cells were moved onto polyvinylidene difluoride (PVDF) membrane (EMD Millipore, USA). The membrane was blocked

**Table 1**

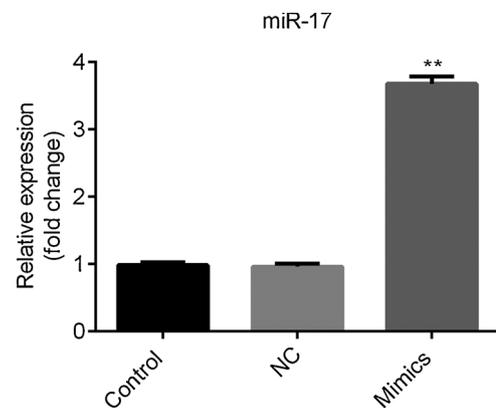
Oligonucleotide primers used for Q-PCR.

Name	Primer sequence
miR-17 U6	Forward 5'-CGGCGCAAAGTGTCTACAG-3' Reverse 5'-GTGCAGGGTCCGAGGT-3' Forward 5'-CTCGCTTCGGCAGCACA-3' Reverse 5'-AACGCTTCACGAATTTGCGT-3'
IGF-1	Forward 5'-GAGGGGTGCGCAATACATCT-3' Reverse 5'-CGGCCCTTCAGGTTCACTT-3'
Bax	Forward 5'-CACCAGCTCTGAACAGATCATGA-3' Reverse 5'-TCAGCCCATCTTCTCCAGATGT-3'
Caspase 3	Forward 5'-AACTGGACTGTGGCATTGAG-3' Reverse 5'-ACAAAGCGACTGGATGAACC-3'
Bcl-2	Forward 5'-CACCCCTGGCATCTTCTCCTT-3' Reverse 5'-AGCGTCTTCAGAGACAGCCAG-3'
GAPDH	Forward 5'-GGAGCGAGATCCCTCCAAAT-3' Reverse 5'-GGCTGTGTGCATCTTCTCATGG-3'



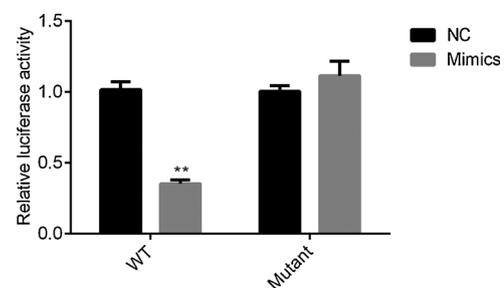
**Fig. 1. miR-17 was upregulated in ox-LDL treated cells.**

Compared with control group, the expression of miR-17 in cells treated with ox-LDL was significantly increased. \*\*P < 0.01 vs. Normal HUVECs. ox-LDL: oxidized low-density lipoprotein; HUVECs: human umbilical vascular endothelial cells.



**Fig. 2. The expression of miR-17.**

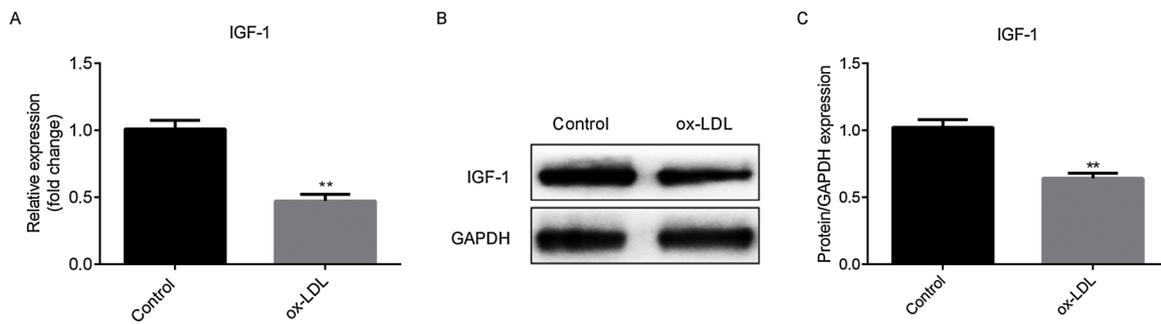
The expression of miR-17 was significantly increased after the treatment of miR-17 mimics compared with NC group. \*\*P < 0.01 vs. NC mimics. NC mimics, cells treated with miR-NC mimics; mimics, cells treated with miR-17 mimics.



**Fig. 3. IGF-1 was a target of miR-17.**

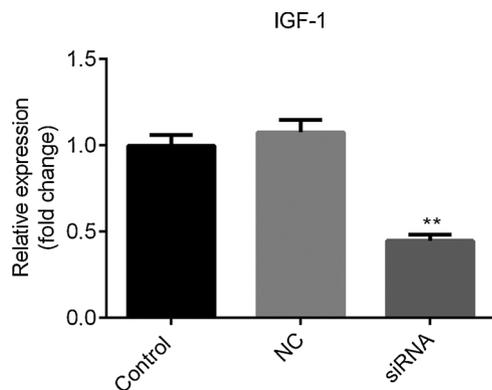
The luciferase activity of cells was significantly decreased after cotransfected with miR-17 mimics and IGF-1 3'UTR WT. \*\*P < 0.01 vs. NC mimics. NC mimics, cells treated with miR-NC mimics; mimics, cells treated with miR-17 mimics; IGF-1: Insulin-like growth factor 1.

with 5% skimmed milk. After 2 h, cells were incubated with the following primary antibodies: anti-BAX (ab32503, 1:1000, Abcam, USA), -Bcl-2 (ab32124 1:1000, Abcam, USA), -Caspase3 (ab13847, 1: 500, Abcam, USA) and -GAPDH (ab9485, 1: 2500, Abcam, USA). Subsequently, the membrane was incubated with goat anti-rabbit immunoglobulin G (IgG)-horseradish peroxidase (ab97051, 1: 2000, Abcam, USA). The protein bands were captured with enhanced chemiluminescence system (Bio-Rad Clarity Western ECL, USA) and



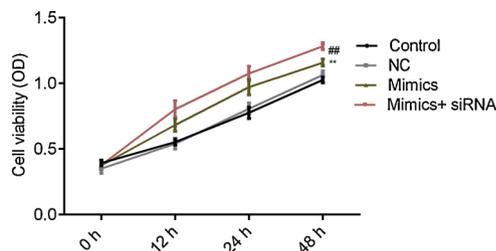
**Fig. 4.** IGF-1 was downregulated in ox-LDL treated cells.

A: Compared with control group, the mRNA level of IGF-1 in cells treated with ox-LDL was significantly decreased. B: The protein level of IGF-1 was significantly decreased in ox-LDL treated cells. C: Quantification analysis of B. \*\* $P < 0.01$  vs. Normal HUVECs. ox-LDL: oxidized low-density lipoprotein; HUVECs: human umbilical vascular endothelial cells; IGF-1: insulin-like growth factor 1.



**Fig. 5.** The expression of IGF-1.

The expression of miR-17 was significantly decreased after the treatment of IGF-1 siRNA compared with NC group. \*\* $P < 0.01$  vs. NC siRNA. NC siRNA, cells treated with IGF-1 NC siRNA; siRNA, cells treated with IGF-1 siRNA. IGF-1: Insulin-like growth factor 1.



**Fig. 6.** Overexpressed miR-17 increased the cell viability of ox-LDL treated cells.

miR-17 significantly increased the cells viability of ox-LDL treated cells compared with NC group, which was more potent after the transfection with IGF-1 siRNA. \*\* $P < 0.01$  vs. NC mimics. ## $P < 0.01$  vs. mimics. NC mimics, cells treated with miR-NC mimics; mimics, cells treated with miR-17 mimics; siRNA, cells treated with IGF-1 siRNA. ox-LDL: oxidized low-density lipoprotein. IGF-1: Insulin-like growth factor 1.

calculated with Gel Doc 2000 (BioRad, USA).

Cell Counting Kit-8 (CCK-8) assay

Cell viability was determined with CCK-8 kit (Dojindo, Japan). Cells were plated in 96-well plate ( $3 \times 10^3$  cells/well). Then each well was supplemented with 10  $\mu$ L of CCK-8 solutions. After 2 h, the optical density (OD) at the wavelength of 450 nm was determined with ELx808 absorbance reader (BioTek Instruments, USA).

## 2.5. Flow cytometry

The apoptosis of HUVECs were determined with Annexin V-FITC

Early Apoptosis Detection kit (Cell Signaling Technology, USA). Cells ( $1 \times 10^6$ ) were trypsinized, and resuspended with binding buffer. Then cells were stained with 10  $\mu$ L of Annexin V-fluorescein isothiocyanate (FITC) and 5  $\mu$ L of propidium iodide (PI) for 10 min in shade. The cell apoptosis was calculated with flow cytometer (BD Biosciences, USA) and analyzed with WinMDI software 2.5 (Purdue University Cytometry Laboratories, USA).

## 2.6. Luciferase assay and bioinformatics analysis

We predicted the target of miR-17 using TargetScan ([http://www.targetscan.org/vert\\_71/](http://www.targetscan.org/vert_71/)). The wild type 3'UTR of IGF-1 containing the binding site of miR-17 was amplified. The binding site of miR-17 was mutated by site-directed mutagenesis kit (Agilent Technologies, USA). Then cells were plated into 24-well plates ( $5 \times 10^3$  cells/well). To determine the luciferase activity, cells were co-transfected with miR-17 mimics or miR-NC mimics, and pGL3-IGF-1-3'UTR-WT or pGL3-IGF-1-3'UTR-MUT with Lipofectamine 2000. At 48-h post transfection, cells were harvested with Dual Luciferase Assay system (Promega, USA). Firefly luciferase served as internal control.

## 2.7. Statistical analysis

Data were expressed as mean  $\pm$  SD (standard deviation) and evaluated with SPSS 19.0 (SPSS, USA). The difference between two groups were analyzed using student *t*-test ANOVA with Turkey's post hoc test was applied to analysis the difference among multigroups.  $P < 0.05$  was deemed as statistical significance.

## 3. Results

### 3.1. miR-17 was upregulated in ox-LDL treated cells

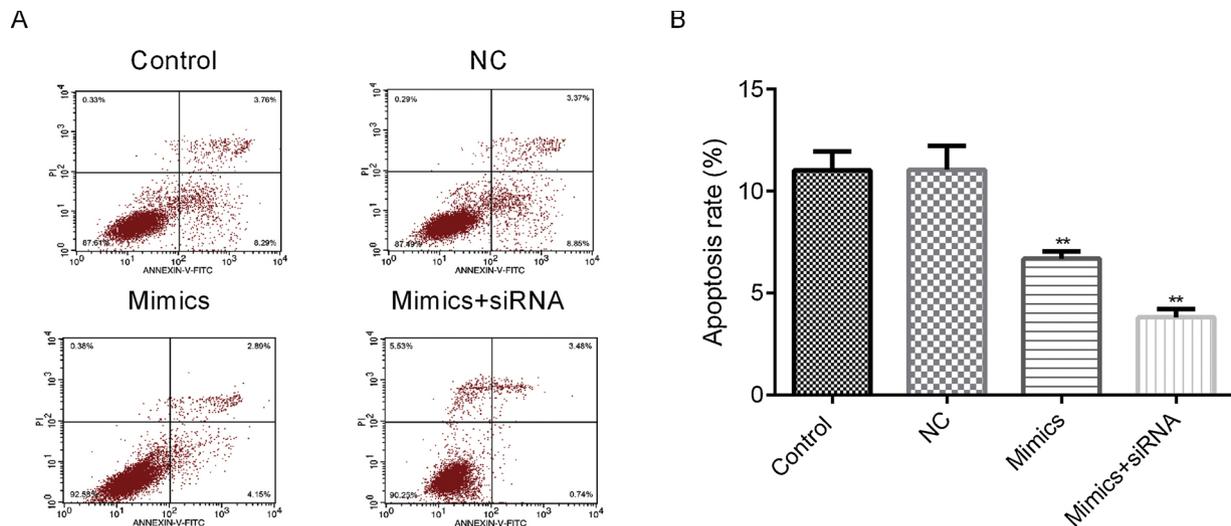
HUVECs were treated with ox-LDL. After 24 h treatment, the results showed that the expression of miR-17 in ox-LDL treated cells were significantly higher than that in control group (Fig. 1).

### 3.2. The expression of miR-17

ox-LDL treated cells were transfected with miR-17 mimics and miR-NC mimics. As showed in Fig. 2, the expression of miR-17 in cells treated with miR-17 mimics was significantly increased compared with NC group, while the difference between control group and NC group was of no statistical significance.

### 3.3. IGF-1 was a target of miR-17

To determine the potential roles of miR-17 in CHD. TargetScan was applied to predict the target of miR-17 and the binding site (as showed



**Fig. 7. Upregulated miR-17 decreased the apoptosis of ox-LDL treated cells.**

A-B: The apoptosis rate was significantly decreased after the treatment of miR-17 mimics, and miR-17 mimics + IGF-1 siRNA was more efficacious in mediating the apoptosis rate. \*\* $P < 0.01$  vs. NC mimics. ## $P < 0.01$  vs. mimics. NC mimics, cells treated with miR-NC mimics; mimics, cells treated with miR-17 mimics; siRNA, cells treated with IGF-1 siRNA. IGF-1: Insulin-like growth factor 1.

in Fig. 3A). Luciferase assay was performed to verify whether IGF-1 was a target of miR-17. The results suggested that the luciferase activity of cells treated with miR-17 mimics and pGL3-IGF-1-3'UTR-WT was significantly decreased, while there was no significant difference in MUT groups (Fig. 3B).

### 3.4. IGF-1 was downregulated in ox-LDL treated cells

qRT-PCR and western blot were applied to determine the mRNA and protein level. As showed in Fig. 4A–C, the mRNA and protein level of IGF-1 in ox-LDL treated cells was significantly decreased compared with control group.

### 3.5. The expression of IGF-1

qRT-PCR was performed to examine the expression of IGF-1. As showed in Fig. 5, the expression of IGF-1 was significantly decreased after the treatment of IGF-1 siRNA.

### 3.6. Overexpressed miR-17 increased the cell viability of ox-LDL treated cells

CCK-8 assay was conducted to determine the cell viability of ox-LDL treated cells. The results showed that upregulated miR-17 significantly increased the cells viability of ox-LDL treated cells compared with NC group, which was more potent after the transfection with IGF-1 siRNA (Fig. 6).

### 3.7. Upregulated miR-17 mediated the apoptosis of ox-LDL treated cells

Flow cytometry was conducted to determine the apoptosis rate of ox-LDL treated cells. The results showed that the apoptosis rate was significantly decreased after the treatment of miR-17 mimics, and miR-17 mimics + IGF-1 siRNA was more efficacious in mediating the apoptosis rate (Fig. 7).

### 3.8. miR-17 regulated the Bcl-2, BAX and Caspase 3 signaling pathways

qRT-PCR and western blot were performed to examine the mRNA and/or protein level. After the transfection with miR-17 mimics, the mRNA level of BAX and Caspase 3 was significantly decreased compared with NC group, which was more apparent after transfected with

IGF-1 siRNA. Moreover, miR-17 significantly upregulated the protein level of Bcl-2, and downregulated Caspase 3 and BAX. Meanwhile, co-transfection with miR-17 mimics and IGF-1 siRNA were more efficient (Fig. 8).

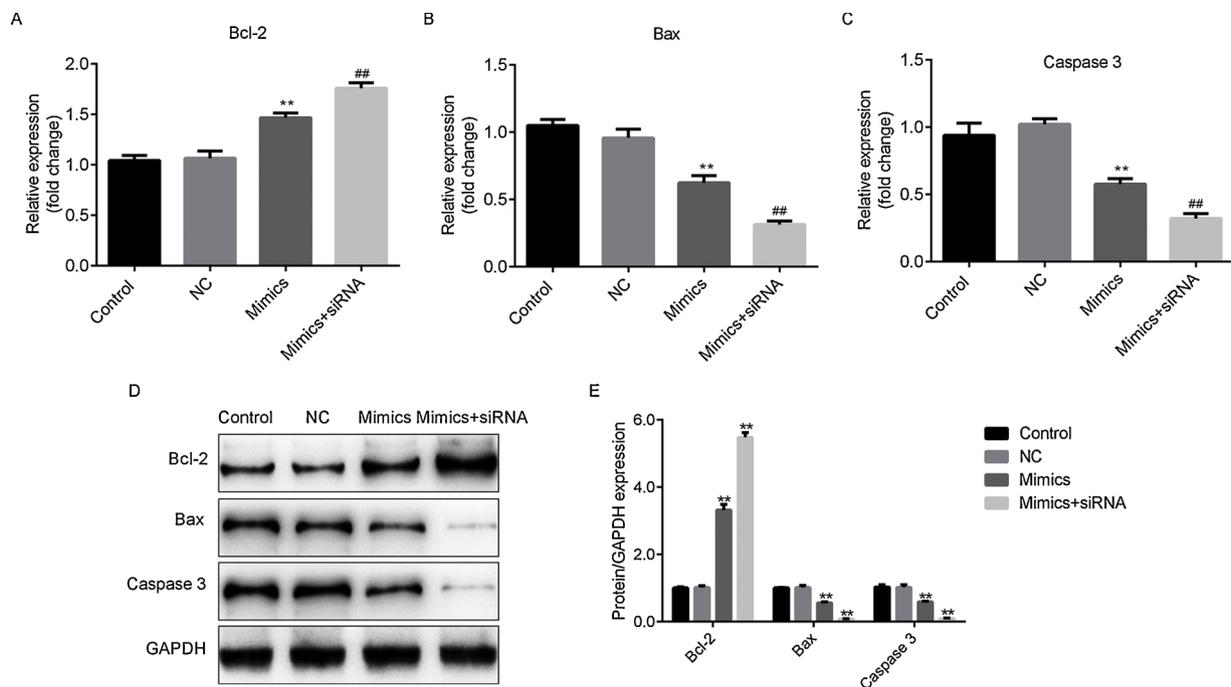
## 4. Discussion

Augmenting evidence revealed that the progression of CHD is a complex process [16]. Recently, increasing reports focus on the potential roles of miRNA in CHD [17]. miR-17 is associated with dyslipidemia and participates in the progression of CHD [14]. However, the underlying mechanism has not been fully elucidated. In the present study, miR-17 was upregulated in ox-LDL treated cells. miR-17 may contribute to the development of CHD, which is consistent with Liu et al study [14]. Nevertheless, the potential mechanisms is still unclear.

CHD is generally induced by coronary arteries arteriosclerosis [2,3]. EC dysfunction induced pathogenesis of atherosclerosis [18], which play a crucial role in the coronary heart disease. In the present study, HUVECs cells were treated with ox-LDL to construct AS model. We examined the effects of increased miR-17 on CHD. The results showed that upregulated miR-17 promoted the proliferation and inhibited the apoptosis of ox-LDL treated cells, which was paralleled with Tang et al study [18].

Of note, the roles of miR-17 in development of CHD are contradictory and alluring. Fichtlscherer et al study reveals that circulating miR-17 was downregulated in patients with coronary artery disease [19]. However, the underlying mechanism is unknown. Interestingly, the expression of miR-17-5p is negatively correlated with fibronectin, Dnmt3a, and Stat3, the upregulation of which increases cell adhesion, migration, proliferation, survival, and wound repair, which promotes the activation of endothelial cells and further aggravates the development of heart diseases [20–22]. Moreover, upregulated miR-17 was positively correlated with TC, LDL-C, and ApoB, which attributes as a detrimental role in patients with CHD [14]. The present study showed that upregulated miR-17 induced the proliferation of ox-LDL treated HUVECs, which revealed that miR-17 is positively associated with the abnormality of HUVECs and the development of CHD. This further proved the findings of Loyer's and Liu's study [14].

Insulin-like growth factor 1 (IGF-1) is a peptide hormone that plays an crucial role in promoting cell proliferation and growth and inhibiting inflammation [23]. Moreover, the anti-inflammatory and pro-repairing properties of IGF-1 endow antiatherogenic function, while



**Fig. 8.** miR-17 regulated the Bcl-2, BAX and Caspase 3 signaling pathways.

A-C: Overexpressed miR-17 significantly increased the mRNA level of Bcl-2 and decreased the mRNA level of BAX and Caspase 3, which was more apparent after transfected with IGF-1 siRNA. D: miR-17 significantly upregulated the protein level of Bcl-2, and downregulated Caspase 3 and BAX, which was more potent in miR-17 mimics and IGF-1 siRNA group. E: quantification analysis of D. \*\* $P < 0.01$  vs. NC mimics. ## $P < 0.01$  vs. mimics. NC mimics, cells treated with miR-NC mimics; mimics, cells treated with miR-17 mimics; siRNA, cells treated with IGF-1 siRNA. IGF-1: Insulin-like growth factor 1.

partial deficiency of IGF-1 induces decrease in contractility and angiotensin II sensitivity, and converted the expression pattern of genes involved in cardiac structure and function, and proliferation and apoptosis of endothelial cells [24,25]. Meanwhile, Bcl-2/Bax and Caspase 3 are involved in the proliferation and apoptosis of oxLDL treated cells [26,27]. In the present study, the combination of upregulated miR-17 and downregulated IGF-1 was more potent in promoting the proliferation and inhibited the apoptosis of HUVECs. Moreover, overexpressed miR-17 and decreased IGF-1 were more efficacious in decreasing the expression of Bax/Bcl-2 and Caspase 3. Taken together, miR-17 may promote the proliferation and inhibited the apoptosis of HUVECs via targeting IGF-1.

However, the present study still have some limitations. The study is lack of in vivo assay. A miRNA may target several genes and a gene may be targeted by various miRNA, which needs further study.

In conclusion, miR-17 was upregulated in HUVECs. Overexpressed miR-17 promoted the proliferation and inhibited the apoptosis of HUVECs via targeting IGF-1. Our study may provide a novel target for CHD at the gene level.

Conflict of interest: No.

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