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MicroRNAs expression in ox-LDL treated HUVECs: MiR-365 modulates apoptosis and Bcl-2 expression

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ABSTRACT

Endothelial cells (ECs) apoptosis induced by oxidized low-density lipoprotein (ox-LDL) is thought to play a critical role in atherosclerosis. MicroRNAs (miRNAs) are a class of noncoding RNAs that posttranscriptionally regulate the expression of genes involved in diverse cell functions, including differentiation, growth, proliferation, and apoptosis. However, whether miRNAs are associated with ox-LDL induced apoptosis and their effect on ECs is still unknown. Therefore, this study evaluated potential miRNAs and their involvement in ECs apoptosis in response to ox-LDL stimulation. Microarray and qRT-PCR analysis performed on human umbilical vein endothelial cells (HUVECs) exposed to ox-LDL identified 15 differentially expressed (4 up- and 11 down-regulated) miRNAs. Web-based query tools were utilized to predict the target genes of the differentially expressed miRNAs, and the potential target genes were classified into different function categories with the gene ontology (GO) term and KEGG pathway annotation. In particular, bioinformatics analysis suggested that anti-apoptotic protein B-cell CLL/lymphoma 2 (Bcl-2) is a target gene of miR-365, an apoptomir up-regulated by ox-LDL stimulation in HUVECs. We further showed that transfection of miR-365 inhibitor partly restored Bcl-2 expression at both mRNA and protein levels, leading to a reduction of ox-LDL-mediated apoptosis in HUVECs. Taken together, our findings indicate that miRNAs participate in ox-LDL-mediated apoptosis in HUVECs. MiR-365 potentiates ox-LDL-induced ECs apoptosis by regulating the expression of Bcl-2, suggesting potential novel therapeutic targets for atherosclerosis.

1. Introduction

Atherosclerosis is a chronic inflammatory disease of the arterial wall with enormous epidemiological relevance [1]. Under pathological conditions, endothelial cells (ECs) apoptosis is thought to play a fundamental role in the development of atherosclerosis by inducing neointima formation [2], inflammatory cell infiltration [3], lipid transport [4], and plaque rupture [5]. Oxidized low-density lipoprotein (ox-LDL), an essential atherosclerotic risk factor, contributes greatly to the development and progression of atherosclerosis. It induces the expression of adhesion molecules on ECs, reduces the synthesis of ECs-derived relaxing factor, and enhances ECs apoptosis [6]. Increased LDL oxidizability and the presence of plasma ox-LDL has been seen in hypothyroidism, metabolic syndrome, and diabetes, perturbations largely known to be related to atherogenesis [7,8]. Previous studies have shown that ox-LDL induces ECs apoptosis by decreasing the expression of anti-apoptotic gene B-cell CLL/lymphoma 2 (Bcl-2) [9,10].

MicroRNAs (miRNAs) are a family of highly conserved, small noncoding RNAs that posttranscriptionally repress gene expression via degradation or translational inhibition of their target mRNAs. There is mounting evidence suggesting that miRNAs are involved in nearly all physiological and pathological processes [11,12]. Early studies suggest a significant role of miRNAs in vascular integrity [13], vascular smooth muscle cell (VSMC) proliferation [14], and cholesterol metabolism [15]. These mechanisms are vital to the initiation and progression of atherosclerosis and other cardiovascular diseases. However, the functional role of miRNAs in ox-LDL induced apoptosis in ECs is unknown. Therefore, the aim of the present study is to analyze the miRNA expression profile in apoptotic human umbilical vein endothelial cells (HUVECs) induced by ox-LDL, and to study the possible role of miRNAs in atherosclerotic processes.

2. Materials and methods

2.1. Cell culture

HUVECs were purchased from the American Type Culture Collection (ATCC, Rockville, MD, USA) and cultured in RPMI 1640 (Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal

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bovine serum (FBS) (Invitrogen, Carlsbad, CA, USA) and 100 μg/ml penicillin/streptomycin (Invitrogen, Carlsbad, CA, USA).

2.2. Ox-LDL preparation

Human ox-LDL was isolated from fresh human plasma by sequential ultracentrifugation, as described previously [16].

2.3. Flow cytometry (FCM) analysis of apoptosis

HUVECs were cultured in 6-well plates and exposed to ox-LDL (50 μg/ml) for 0, 24, 48, and 72 h. Cells were harvested and stained with the Annexin V-FITC Apoptosis Detection Kit (Beyotime, Jiangsu, China). The number of cells undergoing apoptosis was determined by FCM.

2.4. Analysis of the expression of miRNAs by microRNA microarray assay

HUVECs were exposed to ox-LDL (50 μg/ml) or normal control condition for 48 h. RNA samples were isolated, labeled with fluorescent dyes, and hybridized to the miRCURY™ locked-nucleic acid arrays (version 14.0, Exiqon). Raw data were normalized and a *t*-test was performed to analyze the statistical significance between the two groups. Significance was determined using a cutoff of 1.5-fold change and $p < 0.05$. Hierarchical clustering was performed to distinguish expression patterns among different genes and samples.

2.5. MiRNA quantitative real-time PCR (qRT-PCR)

As determined by microarray results, four differently expressed miRNAs (miR-142-3p, miR-365, miR-590-5p, and miR-33a) were analyzed further using TaqMan MicroRNA assays (Applied Biosystems, CA, USA) in a 7900HT RT-PCR machine (Applied Biosystems, CA, USA) according to the manufacturer's protocol.

2.6. Bioinformatics analyses of miRNA-targeted genes

The miRNA-targeted genes were predicted by online-available software (TargetScan, microRNA.org and MicroCosm v5). The gene ontology (GO) database (www.geneontology.org) and KEGG pathway database (www.genome.ad.jp/kegg) were used to determine the functions of these target genes.

2.7. MiRNA inhibitor transfection

The hsa-miR-365 inhibitor or miRNA inhibitor control (Gene Pharma, Shanghai, China) were transfected into HUVECs at a final concentration of 25 and 50 nM using lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA).

2.8. mRNA qRT-PCR and Western blot analysis

Bcl-2 mRNA levels were analyzed using the SYBR® Premix Ex Taq™ II kits (TaKaRa, Dalian, China) with gene specific primers on a Applied Biosystems 7900HT RT-PCR system, according to the manufacturer's instructions. The protein extracts were denatured and the solubilized proteins (20 μg) were subjected to electrophoresis, blotted to polyvinylidene difluoride membrane, and reacted with specific antibodies. Primary antibodies against Bcl-2 and β-actin were from Sigma (St. Louis, MO, USA) and the secondary antibody was from KPL (Gaithersburg, MD, USA).

2.9. Measurement of apoptosis by fluorescence microscopy observation and FCM after transfection

HUVECs were transfected with hsa-miR-365 inhibitor or miRNA inhibitor control. After 24 h, cells were exposed to ox-LDL (50 μg/ml) for an additional 48 h. Then HUVECs were incubated with 1 μg/ml Hoechst 33342 for 20 min at 4 °C (Beyotime, Jiangsu, China). Apoptotic cells were visualized using a fluorescent microscope (excitation filter $\lambda = 360–425$ nm). After transfection and ox-LDL treatment, HUVECs were collected and a quantitative measurement of apoptosis was determined by FCM as described previously.

2.10. Statistical analysis

Data are presented as means ± SD and evaluated by analysis of variance (AVOVA) or Student *t*-test when appropriate. Significance was established at a level of $p < 0.05$.

3. Results

3.1. Ox-LDL-induced apoptosis in HUVECs

The atherogenic and apoptotic properties of ox-LDL were determined in HUVECs. Apoptosis was detected by Annexin V-FITC stain and was distinguished from necrosis by propidium iodide (PI) staining. Incubation of HUVECs with 50 μg/ml ox-LDL for 0–72 h resulted in a time-dependent induction of apoptotic cell death. Incubating with ox-LDL for 24 and 48 h did not significantly increase the necrotic percentage; however, 72 h treatment resulted in a 3-fold increase in necrotic cells (Supplementary Fig. 1A and B). HUVECs treated with ox-LDL (50 μg/ml) for 48 h significantly increased the percentage of apoptosis with little to no effect on the number of necrotic cells, therefore, the 48 h time point was used to identify apoptosis related miRNAs.

3.2. MicroRNA microarray

Using a miRNA microarray approach, we compared the miRNA expression profile in HUVECs following a 48 h treatment of ox-LDL (50 μg/ml) with those in normal control cells. The results reflect the temporal changes of miRNA expression levels during the apoptosis of HUVECs induced by ox-LDL stimulation. Ox-LDL significantly up-regulated four miRNAs as depicted by the shades of red in the heatmap (Fig. 1A and Supplementary Table 1). Additionally, ox-LDL down-regulated 11 miRNAs, which are shown as shades of green in the heatmap (Fig. 1A and Supplementary Table 1).

3.3. Validation of microarray data by qRT-PCR analysis

We selected 4 miRNAs that displayed either an increase or decrease in expression (hsa-miR-142-3p, hsa-miR-365, hsa-miR-590-5p, and hsa-miR-33a) to validate with qRT-PCR. The results confirmed the miRNA microarray data and indicated a positive correlation between the quantities of transcripts measured by both microarray and qRT-PCR assay (Fig. 1B).

3.4. Prediction of miRNA-targeted genes and their functions

We searched for potential mRNA targets of the 15 differentially expressed miRNAs using computational prediction algorithms (TargetScan, microRNA.org and MicroCosm v5). Using consensus targets selected by at least two prediction programs, we identified 5196 putative targets. These genes were submitted to the GO database and KEGG pathway database for GO annotation and pathway

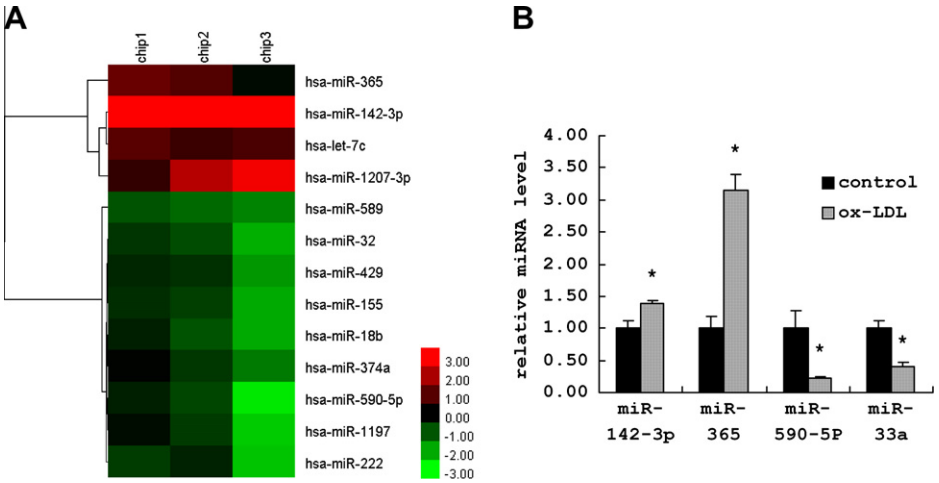

Fig. 1. Ox-LDL stimulation alters miRNA expression in HUVECs. (A) Expression analysis of miRNA using microarray in ox-LDL treated HUVECs compared to control ($n = 3$). Heatmap shows ox-LDL treated vs. control miRNA log 2 ratios with a threshold of 1.5-fold and $p < 0.05$. Red bands represent up-regulated miRNAs and green bands, down-regulated miRNAs. (B) Relative expression levels of miR-142-3p, miR-365, miR-590-5p, and miR-33a in HUVECs were analyzed using qRT-PCR. Data are expressed as means ± SD, $^*p < 0.01$ compared to control group ($n = 3$). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this paper.)

enrichment analysis. According to the results of data mining, 20 up-regulated and 20 down-regulated GO terms with the most significant *p*-values were classified on the basis of biological processes (Table 1, upper). The GO terms related to cell cycle, apoptosis, lipid oxidation, gene expression, and metabolism represented up to 75% of the top 20 significantly enriched GO terms targeted by the up-regulated and down-regulated miRNAs. A separate functional analysis by the KEGG pathway showed these target genes were highly enriched in 8 up-regulated and 15 down-regulated signaling pathways with a *p*-value less than 0.01 (Table 1, lower). Proliferative, apoptotic and survival, oncogenic and metabolic signaling pathways were abundant among the significantly enriched pathways. This was supported by the GO analysis.

3.5. MiR-365 repressed anti-apoptotic gene Bcl-2 expression in HUVECs

Previous studies have demonstrated ox-LDL induced ECs apoptosis accompanied by a decrease in Bcl-2 expression [9,10]. More importantly, Bcl-2 is a consensus target gene for miR-365 in both TargetScan 5.1 and miRanda algorithms (Supplementary Fig. 2). Our current data revealed that miR-365 was up-regulated by ox-LDL stimulation in HUVECs. To further elucidate this relationship, HUVECs were transfected with hsa-miR-365 inhibitor. Human Bcl-2 mRNA and protein levels were quantified by qRT-PCR and Western blotting. As shown in Fig. 2A, there was a significant decrease in Bcl-2 mRNA expression after exposure to ox-LDL (50 μg/ml) for 48 h compared to control, whereas miR-365 inhibitor dose-dependently attenuated the inhibitory effect of ox-LDL on the expression of Bcl-2 mRNA in HUVECs. The Bcl-2 protein expression consistently increased in the miR-365 inhibitor groups (Fig. 2B). Together, these results suggest there is an inverse correlation between miR-365 and Bcl-2 in HUVECs exposed to ox-LDL.

3.6. Effects of miR-365 inhibitor on ox-LDL induced apoptosis in HUVECs

HUVECs were exposed to ox-LDL (50 μg/ml) for 48 h and transfected with miR-365 inhibitor or miRNA inhibitor control to determine its functional role in HUVECs. Hoechst 33324 staining and FCM were used to analyze changes in apoptosis. Ox-LDL treatment produced cell shrinkage, nuclear segmentation, and chromatin condensation in the miRNA inhibitor control group (highlighted by arrows). In contrast, dose dependent transfection with miR-365 inhibitor (25 and 50 nM) reduced ox-LDL-induced apoptotic HUVECs (Fig. 3A). Double stain of Annexin V-FITC/PI showed ox-LDL stimulation increased apoptosis of HUVECs by nearly 4-fold, whereas the miR-365 inhibitor at 25 and 50 nM significantly decreased the apoptotic percentage, indicating that the miR-365 inhibitor protects HUVECs from ox-LDL induced apoptosis (Fig. 3B and C).

4. Discussion

Endothelial dysfunction is an initiative stage of atherosclerosis. A potential target in the prevention and/or treatment of atherosclerosis is maintaining ECs viability by inhibiting the induction of apoptosis. Because ox-LDL is a major risk factor in the development of atherosclerosis, we chose ox-LDL-induced apoptotic HUVECs as a model to determine miRNAs associated with apoptosis of ECs in atherosclerosis. In the present study, we revealed ox-LDL induced expression of a distinct group of miRNAs in HUVECs, and one of these miRNAs, miR-365, exerted a pro-apoptotic function through suppressing Bcl-2 expression in ox-LDL treated HUVECs.

According to published data, aberrantly expressed miRNAs identified in our study are closely related to atherosclerosis, lipoprotein metabolism, and cell apoptosis. For example, miR-222 was found to be involved in atherosclerotic intraplaque neovascularization by targeting STAT5A [17]. Silencing of miR-155 has been shown to enhance inflammatory response and lipid uptake in ox-LDL-stimulated human THP-1 macrophages [18]. MiR-33a/b were reported to target the cholesterol transporter ABCA1, with important consequences for cholesterol trafficking and HDL synthesis [19]. Additionally, many current studies focus on miRNAs and their involvement in the apoptotic process. Among these apoptomirs, let-7 regulates apoptosis via directly targeting oncogenes Ras, Myc, HMGA2, CDC25A, CDK6 [20], and anti-apoptotic protein Bcl-xl [21]. Although little is known regarding the function of miR-365, it has been shown to be up-regulated in apoptotic NIH3T3 cells after UVB irradiation [22]. It also correlates with cell cycle regulators p16 and p21, as well as PTEN in growth arrested fibroblasts [23], identifying a role of miR-365 in the regulation of cell proliferation and apoptosis. MiR-32 is another apoptomir

Table 1
GO and KEGG pathway analysis of the predicted target genes of the 15 differentially expressed miRNAs. (A) Only the top 20 GO terms representing functional significance are listed. (B) Significant pathways targeted by up-regulated and down-regulated miRNAs, respectively.

GOID	Term	Gene No.	*p* Value
(A) Gene ontology analysis			
Up-regulated GOs by GO analysis[a]			
GO:0048666	Neuron development	46	2.56E-04
GO:0006357	Regulation of transcription from RNA polymerase II promoter	83	3.22E-04
GO:0060021	Palate development	10	5.33E-04
GO:0016044	Membrane organization	48	9.77E-04
GO:0043066	Negative regulation of apoptosis	42	1.09E-03
GO:0009890	Negative regulation of biosynthetic process	66	1.14E-03
GO:0031175	Neuron projection development	35	1.38E-03
GO:0030030	Cell projection organization	46	1.50E-03
GO:0010558	Negative regulation of macromolecule biosynthetic process	63	1.50E-03
GO:0031327	Negative regulation of cellular biosynthetic process	64	1.73E-03
GO:0016192	Vesicle-mediated transport	65	2.06E-03
GO:0030182	Neuron differentiation	52	2.17E-03
GO:0030258	Lipid modification	14	2.20E-03
GO:0019395	Fatty acid oxidation	10	2.46E-03
GO:0034440	Lipid oxidation	10	2.46E-03
GO:0006325	Chromatin organization	46	2.51E-03
GO:0000122	Negative regulation of transcription from RNA polymerase II promoter	35	2.62E-03
GO:0048667	Cell morphogenesis involved in neuron differentiation	29	3.08E-03
GO:0022403	Cell cycle phase	49	3.14E-03
GO:0000087	M phase of mitotic cell cycle	30	2.56E-04
GO:0048285	Organelle fission	31	3.15E-03
Down-regulated GOs by GO analysis[b]			
GO:0006357	Regulation of transcription from RNA polymerase II promoter	181	7.06E-16
GO:0045449	Regulation of transcription	486	3.00E-14
GO:0010628	Positive regulation of gene expression	144	1.34E-12
GO:0006350	Transcription	395	8.94E-12
GO:0010558	Negative regulation of macromolecule biosynthetic process	135	1.01E-11
GO:0045941	Positive regulation of transcription	137	2.34E-11
GO:0045893	Positive regulation of transcription, DNA-dependent	120	4.44E-11
GO:0009890	Negative regulation of biosynthetic process	137	7.58E-11
GO:0051254	Positive regulation of RNA metabolic process	120	7.85E-11
GO:0010557	Positive regulation of macromolecule biosynthetic process	151	1.13E-10
GO:0009891	Positive regulation of biosynthetic process	158	1.35E-10
GO:0042981	Regulation of apoptosis	143	1.42E-10
GO:0031328	Positive regulation of cellular biosynthetic process	156	1.61E-10
GO:0010629	Negative regulation of gene expression	123	2.02E-10
GO:0031327	Negative regulation of cellular biosynthetic process	133	2.71E-10
GO:0016481	Negative regulation of transcription	114	3.30E-10
GO:0045935	Positive regulation of nucleobase, nucleoside, nucleotide and nucleic acid metabolic process	144	3.37E-10
GO:0051173	Positive regulation of nitrogen compound metabolic process	147	4.82E-10
GO:0006796	Phosphate metabolic process	203	7.48E-10
GO:0006793	Phosphorus metabolic process	203	7.48E-10
	Pathway title	Count	*p* Value
(B) KEGG pathway analysis			
Up	Glyoxylate and dicarboxylate metabolism	6	0.000746
Up	Progesterone-mediated oocyte maturation	18	0.001888
Up	Neurotrophin signaling pathway	20	0.002819
Up	Fc gamma R-mediated phagocytosis	16	0.00486
Up	Prion diseases	8	0.005999
Up	One carbon pool by folate	5	0.007158
Up	Cell cycle	19	0.008052
Up	Adherens junction	13	0.008679
Down	Notch signaling pathway	18	2.58E-05
Down	Renal cell carcinoma	23	3.79E-05
Down	Axon guidance	35	4.74E-05
Down	TGF-beta signaling pathway	26	7.64E-05
Down	Focal adhesion	47	0.000159
Down	Neurotrophin signaling pathway	33	0.000161
Down	Pathways in cancer	68	0.000463
Down	Adherens junction	22	0.00053
Down	Wnt signaling pathway	35	0.001214
Down	Ubiquitin mediated proteolysis	31	0.003311
Down	MAPK signaling pathway	53	0.004312
Down	Heparan sulfate biosynthesis	9	0.006088
Down	Progesterone-mediated oocyte maturation	24	0.007916
Down	mTOR signaling pathway	14	0.009155
Down	ECM-receptor interaction	20	0.009219

[a] GOs targeted by up-regulated miRNAs.
[b] GOs targeted by down-regulated miRNAs.

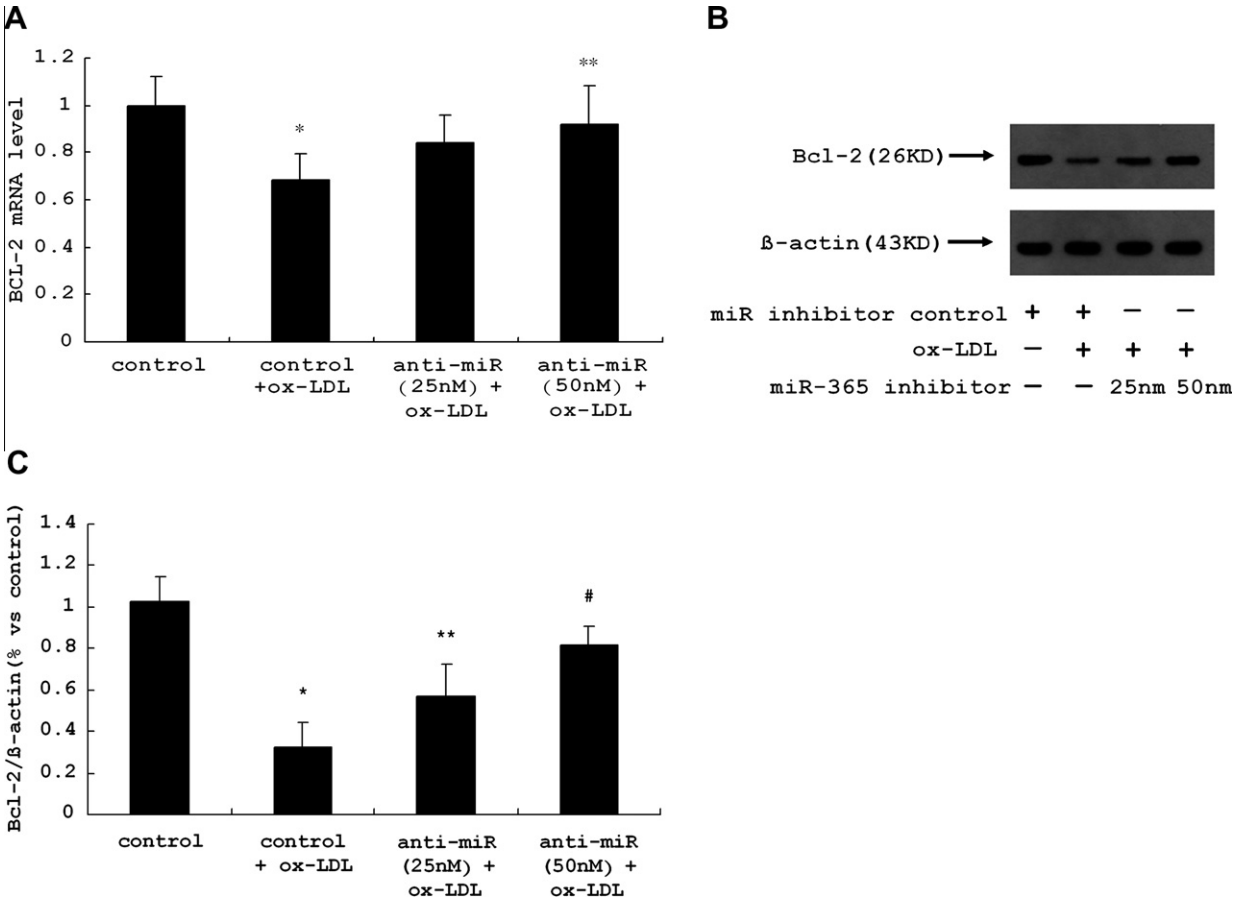

Fig. 2. Effects of miR-365 inhibitor on Bcl-2 expression in ox-LDL stimulated HUVECs. HUVECs were transfected with miR-365 inhibitor or miRNA inhibitor control followed by 50 μg/ml ox-LDL stimulation for 48 h. (A) Bcl-2 mRNA was measured by qRT-PCR. (B) Bcl-2 protein was measured by Western blot, and the β-actin expression was used for protein level normalization. (C) Graphical data of Bcl-2 protein expression showing the densitometry ratio of Bcl-2 to β-actin. Values are means ± SD ($n = 3$). $^{*}p < 0.05$ compared with miRNA inhibitor control, $^{**}p < 0.05$ compared with *marked group, $^{\#}p < 0.01$ compared with *marked group.

whose determined target genes included p53 positive regulator PCAF and pro-apoptotic gene Bim [24,25]. In keeping with previous studies, our data showed these miRNAs were dysregulated in ox-LDL-induced HUVECs apoptosis, but their exact function merits further investigation.

Our current study predicted and analyzed the biological functions of the differentially expressed miRNA-targeted genes. As a result, GO term and KEGG pathway annotation showed that these target genes are highly associated with cell apoptosis and proliferation, gene expression and cell metabolism. Some signaling pathways have already been reported to take part in ECs apoptosis and atherosclerosis. For example, signaling from the Notch pathway is associated with transplant arteriosclerosis by triggering ECs activation and apoptosis [26]. Likewise, LDL induced activation of the p38 MAPK pathway in HUVECs is important for the up-regulation of endothelial genes implicated in atherogenesis [27]. This functional identity suggests these differentially expressed miRNAs have regulatory effects on endothelial apoptosis and atherogenesis.

Bcl-2 codes for an outer mitochondrial membrane protein that blocks cytochrome *c* release from mitochondria and inhibits caspase activity, suppressing apoptosis. Previous studies have implicated Bcl-2 in sustaining morphous and function of HUVECs, and ox-LDL induced apoptosis in ECs through the down-regulation of Bcl-2 [9,10]. Both transcriptional and posttranscriptional regulations of Bcl-2 have been discovered. With regard to miRNA regulation, previous research clearly demonstrated that Bcl-2 is a direct target of miR-15 and miR-16 in chronic lymphocytic leukemia [28]. We found miR-365 was up-regulated in ox-LDL-induced HUVECs apoptosis and bioinformatics data suggested Bcl-2 maybe one of the target genes of miR-365 (Supplementary Fig. 2). To explore the relationship between up-regulated miR-365 and decreased expression of Bcl-2 we utilized miR-365 inhibitor to suppress miR-365 expression in HUVECs. We found that the inhibition of miR-365 increased the expression of Bcl-2 at both the mRNA and protein levels suggesting that miR-365 may combine with the base-paring site in the 3′-UTR of Bcl-2 and lead to mRNA degradation. To determine if Bcl-2 is a direct target of miR-365 requires further analysis. It is also possible that indirect mechanisms may increase the potency of miR-365 action. In addition, inhibition of miR-365 could partly alleviate apoptotic cell death induced by ox-LDL, indicating miR-365 promotes apoptosis in ox-LDL-treated HUVECs through attenuating Bcl-2 expression. Moreover, previous studies demonstrated Bcl-2 prevents cell necrosis as well [29–31]. In our current study, miR-365 inhibitor restored the expression of Bcl-2, but had little influence on ECs necrosis. This phenomenon may be due to the relatively low percentage of necrosis as the result of the experimental conditions, thereby limiting the impact of Bcl-2 up-regulation.

In summary, we found that 15 dysregulated miRNAs, especially miR-365, are associated with apoptosis induced by ox-LDL in HUVECs. Transfection of miR-365 inhibitor increased Bcl-2 expression and partially protected HUVECs from apoptotic death. Our findings demonstrate ox-LDL-induced miRNAs signature and the corresponding mechanisms, which provide insight into the molecular mechanisms underlying endothelial dysfunction and the atherosclerotic process. We believe that once the functional roles

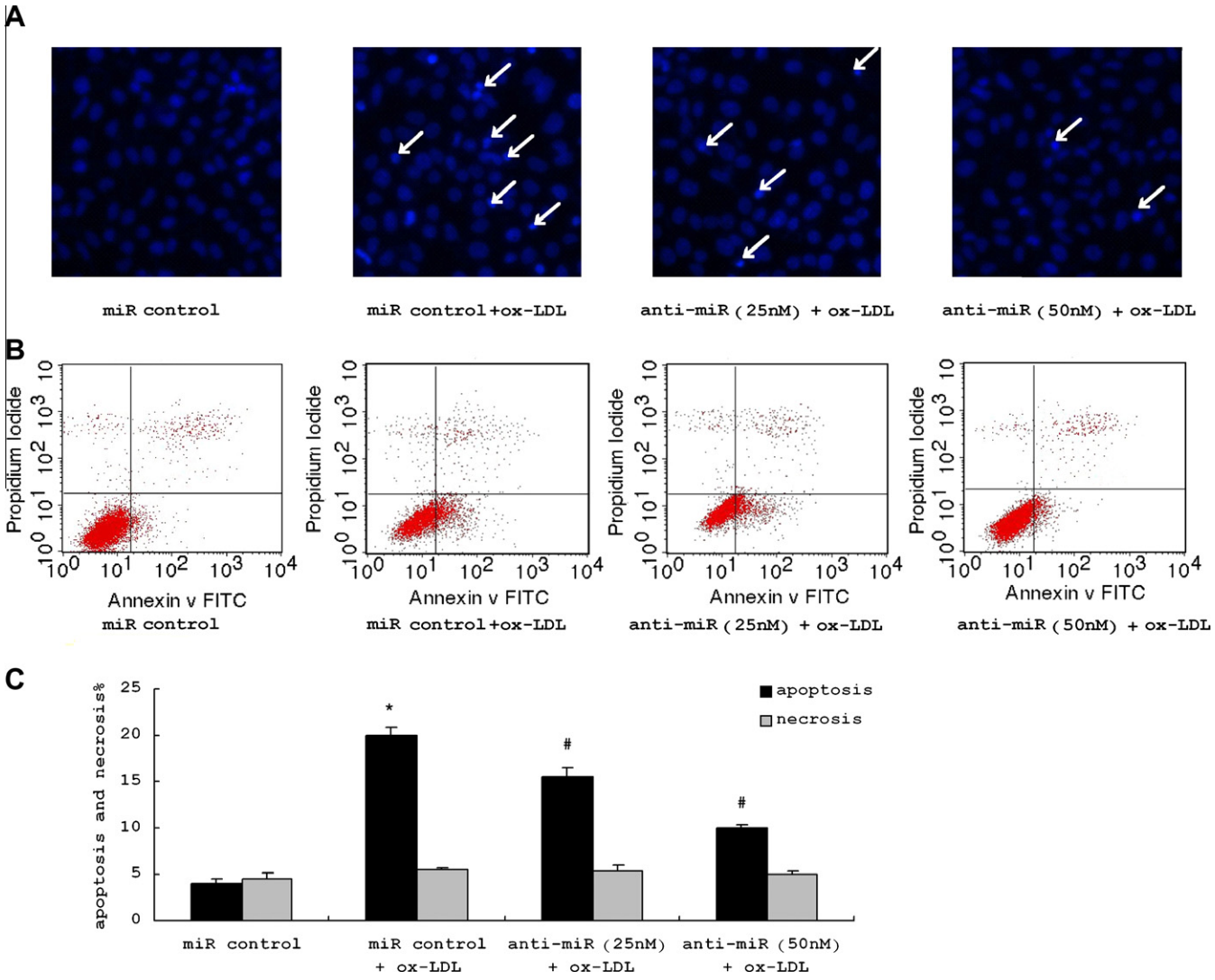

Fig. 3. Effects of miR-365 inhibitor on ox-LDL-induced apoptosis in HUVECs. HUVECs were exposed to ox-LDL (50 μg/ml) for 48 h after transfection with miR-365 inhibitor or miRNA inhibitor control. (A) Hoechst 33324 staining was employed to detect apoptotic cells. Cellular pyknosis is indicated with a white arrow. (B) Quantitative measurement of apoptosis and necrosis by FCM after AV/PI double stain. (C) Comparison of the apoptotic and necrotic death rate of HUVECs. Values are means ± SD ($n = 3$), and $^{*}p < 0.01$ compared with the miRNA inhibitor control group, $^{\#}p < 0.01$ compared with *marked group.

of miRNAs have been further clarified, they will be used as a novel strategy for treatment of endothelial apoptosis in atherosclerosis.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2011.05.118.

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