Analysis of cardiovascular disease-related NF-κB-regulated genes and microRNAs in TNFα-treated primary mouse vascular endothelial cells[*][#]

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Abstract: Activated nuclear factor-κB (NF-κB) plays an important role in the development of cardiovascular disease (CVD) through its regulated genes and microRNAs (miRNAs). However, the gene regulation profile remains unclear. In this study, primary mouse vascular endothelial cells (pMVECs) were employed to detect CVD-related NF-κB-regulated genes and miRNAs. Genechip assay identified 77 NF-κB-regulated genes, including 45 upregulated and 32 down-regulated genes, in tumor necrosis factor α (TNFα)-treated pMVECs. Ten of these genes were also found to be regulated by NF-κB in TNFα-treated HeLa cells. Quantitative real-time PCR (qRT-PCR) assay confirmed the up-regulation of *Egr1*, *Tnf*, and *Btg2* by NF-κB in the TNFα-treated pMVECs. The functional annotation revealed that many NF-κB-regulated genes identified in pMVECs were clustered into classical NF-κB-involved biological processes. Genechip assay also identified 26 NF-κB-regulated miRNAs, of which 21 were upregulated and 5 downregulated, in the TNFα-treated pMVECs. Further analysis showed that nine of the identified genes are regulated by seven of these miRNAs. Finally, among the identified NF-κB-regulated genes and miRNAs, 5 genes and 12 miRNAs were associated with CVD by miRWalk and genetic association database analysis. Taken together, these findings show an intricate gene regulation network raised by NF-κB in TNFα-treated pMVECs. The network provides new insights for understanding the molecular mechanism underlying the progression of CVD.

Key words: Cardiovascular disease; Nuclear factor-κB (NF-κB); MicroRNA; Endothelial cell

1 Introduction

Cardiovascular disease (CVD) that involves heart and systemic blood vessels accounts for about half of global deaths and prevalence is increasing (Estruch, 2014; Mangge et al., 2014). As a barrier between the blood and surrounding tissue, endothelial cells (ECs) are extremely important for the maintenance of vascular function and homeostasis, and their dysfunction is mainly responsible for the progression of CVD (Widlansky et al., 2003; Tousoulis et al., 2010; Zhu et al., 2018). Endothelial dysfunction (ED) is characterized by changes in endothelial function

and homeostatic control in response to extensive noxious stimuli (Madamanchi et al., 2005; Murdaca et al., 2012; Steyers and Miller, 2014). Endothelium undergoes a phenotypic modulation from normal to non-adaptive, which often manifests with impaired vascular reactivity, reduced nitric oxide (NO) availability, increased oxidative stress, and increased cellular adhesion molecules, such as vascular adhesion molecule-1 (VCAM-1) and intercellular adhesion molecule-1 (ICAM-1) (Madamanchi et al., 2005; Murdaca et al., 2012; Steyers and Miller, 2014). Investigation of altered gene expression in the response of ECs to external stimuli may provide clues for CVD research. The ED that is frequently found in atherosclerosis, hypertension, heart failure, and myocardial infarction leads to increased endothelial permeability and infiltration of inflammatory cells into the arterial wall (Boulanger, 1999; Lerman and Zeiher, 2005; Tousoulis et al., 2010). This process ultimately results in atherosclerosis (Zampetaki et al., 2013). Therefore, ED is a key step in the development of CVD events (Vanhoutte, 2009; Steyers and Miller, 2014).

Nuclear factor-κB (NF-κB) is a key inducible transcription factor family that contains five members including p65/RelA, p52, p50, c-Rel, and RelB (Zhou et al., 2017). NF-κB participates in physiological and pathological processes by regulating target genes (Zhou et al., 2017). Under stimulation by inducers (e.g. tumor necrosis factor α (TNFα)), the inhibitor-blocked NF-κB (e.g. IκBα) is liberated and subsequently moves into the cell nucleus to regulate its target genes (Zhou et al., 2017; Song Y et al., 2018). Many well-known NF-κB target genes such as *TNF*, *E-selectin*, *ICAM-1*, and *VCAM-1* play pivotal roles in the progression of CVD (Vanhoutte, 2009; Murdaca et al., 2012; Steyers and Miller, 2014). Selective inhibition of the NF-κB upregulating endothelial-specific target genes, *E-selectin* and *VCAM-1*, attenuated chronic intermittent hypoxia-induced atherosclerosis in mice (Song DM et al., 2018). NF-κB-mediated upregulation of cell adhesion molecule 1 (*CADM1*), a recently identified target, has also been identified as a mechanism of TNFα-induced rat endothelial progenitor cell migration (Prisco et al., 2016).

MicroRNAs (miRNAs) are a class of short (approximately 22 nucleotides (nt)) endogenous noncoding RNAs that repress their target gene expression at the post transcriptional level by binding to 3' untranslated regions (3' UTRs) (Ma et al., 2011; Menghini et al., 2014). Reports about the crucial role of miRNA in the progression of CVD are increasing (Menghini et al., 2014). For example, the silence of miR-15 family members, including miR-497, miR-195, miR-16-2, miR-16-1, miR-15b, and miR-15a, reduces myocardial infarction size in mouse (Hullinger et al., 2012). Endogenous miR-221, increased by high-level glucose, ultimately results in ED including reduction of human umbilical vein endothelial cell (HUVEC) migration and c-kit expression, which are antagonized by antagomirs of miR-221 (Li et al., 2009). The five CVD-related miRNAs, miR-497 (Wei et al., 2016), miR-195 (Wei et al., 2016), miR-15a (Yang et al., 2015), miR-15b (Zhu et al., 2016), and miR-221 (Galardi et al., 2011), are all transcriptionally regulated by NF-κB. In this study, we have identified CVD-related NF-κB-regulated genes and miRNAs using primary mouse vascular endothelial cells (pMVECs) as approximate representations of the endothelial monolayer in blood vessels. The exposure of pMVECs to TNFα was used to cause the expression of potential NF-κB regulatory genes and miRNAs. *RelA* small interfering RNA (siRNA) was introduced to identify the genes and miRNAs specifically regulated by NF-κB.

2 Materials and methods

2.1 Mouse primary endothelial cell culture

The mouse experiments were approved by the Animal Care and Use Committee of the Southeast University (Nanjing, China). After sacrifice by cervical dislocation, the dead mice were soaked in 75% (v/v) ethanol for 1 min. Under sterile conditions, aorta was removed from the chest and immersed in sterile phosphate-buffered saline (PBS). Fats and fibrous tissues were removed from vascular adventitia by ophthalmic scissors and tweezers. The internal and external surfaces of blood vessels were washed with PBS containing antibiotics. Blood vessels were longitudinally cut open and cut into 1.5 mm×1.5 mm artery explants, and then placed into a petri dish coated with 1% (0.01 g/mL) gelatin at the density of 1 cm^{-2}. The intima of the artery explant was in contact with the gelatin. The petri dish was then placed into a 5% (v/v) CO_2 incubator (100% humidity) at 37 °C for

2 h. Primary endothelial cell special culture medium (PriCells, Wuhan, China) with 10% (v/v) fetal bovine serum (TransGen, Beijing, China) was then added to the dish without causing the artery explant to float. The medium was replaced after 72 h and thereafter every 48 h. The integrated pMVECs were dissociated into single cell suspensions using 0.25% (2.5 g/L) trypsin (Amresco, Boise, USA) containing 0.025% (0.25 g/L) ethylene diamine tetraacetic acid (EDTA). The pMVECs were used for experiments between the 4th and 7th passages.

2.2 Construction of the pMVEC model with different NF-κB activity

The NF-κB activator (TNFα) and NF-κB inhibitor (*RelA* siRNA) were used to construct pMVEC models as follows. pMVECs with about 50% confluency were first transfected with 30 nmol/L NF-κB *RelA* siRNA (Cell Signaling Technology, Danvers, MA, USA) by Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) according to the instructions. After 48 h, the transfected cells were stimulated with 30 ng/mL TNFα (Sigma, Shanghai, China) in the serum-free primary endothelial cell special culture medium at 37 °C for 30 min. Simultaneously, pMVECs were subjected to the same amount of Lipofectamine 2000 and treated with or without TNFα. From this, we created three pMVEC groups: *RelA* siRNA and TNFα co-treated pMVECs (thereafter depicted as siRNA-treated pMVECs), TNFα-treated pMVECs, and control pMVECs.

2.3 Genechip assay

TRIzol reagent (Invitrogen, Carlsbad, CA, USA) was used to extract total RNA from pMVECs. The extracted RNA samples underwent Genechip assay as in a previous study (Liu and Wang, 2013), to detect the global gene expression profile. Biotin-labeled complementary RNA (cRNA) generated by in vitro transcription reactions was fragmented and hybridized with Affymetrix, MOUSE 2.0T Genechip microarrays (Affymetrix, USA) at 45 °C for 16 h. The hybridized arrays were then reacted with streptavidin-phycoerythrin, and scanned by an Affymetrix Genechip Scanner 3000 7G (Thermo Fisher Scientific, Waltham, USA). Image analysis and quantification were performed with Genechip Operating Software (Thermo Fisher Scientific). After normalization and

background filtration, the differentially expressed genes (DEGs) were analyzed in the siRNA-treated pMVECs, TNFα-treated pMVECs, and control pMVECs. The gene ontology (GO) and Kyoto encyclopedia of genes and genomes (KEGG) analyses on these DEGs were performed by Database for Annotation, Visualization and Integrated Discovery (DAVID) online servers (http://david.abcc.ncifcrf.go).

Similarly, the miRNAs isolated from the pMVECs were hybridized with Affymetrix MICRORNA 3.0 Genechip (Affymetrix) to detect the global miRNA expression profile. After normalization and background filtration, the differentially expressed miRNAs (DEMs) were analyzed in the siRNA-treated pMVECs, TNFα-treated pMVECs, and control pMVECs.

2.4 qRT-PCR assay

Total RNA was extracted from pMVECs and mouse hepatoma Hepa1-6 cells and 1 μg of the extracted RNA was reversely transcribed into cDNA by PrimeScript™ RT Master Mix (TaKaRa, Dalian, China). The cDNAs were then used to detect gene expression by quantitative real-time PCR (qRT-PCR) on an ABI StepOne™ real-time PCR system (Applied Biosystems, Life Technologies, Grand Island, NY, USA) with SYBR Green Mix (Roche, Shanghai, China). The primers (5'→3') of the determined genes were as follows: *RelA*, AGACACAGA TGATCGCCACC and GGCTTGGGGACAGAAGT TGA; *Cxcl10*, CCACGTGTTGAGATCATTGCC and GAGGCTCTCTGCTGTCCATC; *Btg2*, GCACTGA CCGATCATTACAA and GGATCAACCCACAG GGTCAG; *Egr1*, CAATTGATGTCTCCGCTGC and AAAAGGACTCTGTGGTCAGGTG; *Tnf*, CGTCGT AGCAAACCACCAA and ATAGCAAATCGGCTG ACGGT; *Icam1*, CCTCCGGACTTTCGATCTT and CTGGTCCGCTAGCTCCAAAA; *Vcam1*, GGAGG TCTACTCATTCCCTGA and GGTGGGGATGAA GGTCGTTT; *GADPH*, TGGCAAAGTGGAGATTG TT and CTCGCTCCTGGAAGATGG. The expression of *GADPH* was used as an internal control. The melting curve was used to analyze the specificity of amplification. Relative expression was calculated by the comparative C_T method. At least three biological replications for each gene were performed and the data were shown as mean±standard deviation (SD). Significant differences were assessed by one-way analysis of variance (ANOVA) using SPSS 13.0

(SPSS Inc., Chicago, IL, USA) for windows. *P*<0.05 was regarded as statistically significant.

2.5 Prediction of the target gene of NF-κB-regulated miRNA

The target genes of NF-κB-regulated miRNAs were identified according to the method used in our previous study (Zhou et al., 2014) with the online miRWalk program (Dweep et al., 2011) being used initially to predict the genes. The miRNA binding sites on 3' UTR of mRNA were predicted by setting the following parameters: the longest transcript, minimum seed length of 7 nt, and *P*-value of 0.05. The comparative platform of the miRWalk web was used to analyze the output result. An miRNA target that was predicted by at least six programs was regarded as a high-confidence nominated target. We then identified the targets of NF-κB-regulated miRNA from the nominated targets according to the previous criterion that miRNA downregulates its targeted mRNA (Zhou et al., 2014). In other words, the change in their expression level is inversely correlated to that of miRNA.

2.6 Analyses of CVD-associated NF-κB target genes and miRNAs

Five thousand and eleven disease-related mouse genes are collected in the genetic association database

(GAD) (Becker et al., 2004). The collected genes were compared with the detected DEGs to find those related to CVD. The miRWalk database contains 324 disease-related mouse miRNAs (Dweep et al., 2011). The collected miRNAs were compared with the DEMs to find out the CVD-related miRNAs. Finally, these genes and miRNAs were visualized by the Cytoscape program (Shannon et al., 2003).

3 Results

3.1 Qualitative evaluation of pMVECs

The pMVECs were isolated and cultured as described in Section 2. As shown in Fig. 1a, the cultured pMVECs presented adherent growth patterns and polygons. When pMVECs grew into a confluent monolayer, they showed a typical cobblestone-like arrangement. *Tie-2*, *VE-cadherin*, *Vcam1*, and *Icam1* were endothelial-specific markers (Lim et al., 2003; Sun et al., 2014). Expression of these four genes was therefore detected by qRT-PCR to qualify isolated pMVECs. As a result, the expression of the four genes was significantly increased in pMVECs compared with Hepa1-6 cells (Fig. 1b). Of note, the expression change of *Tie-2* increased 25 294-fold in pMVECs compared with Hepa1-6 cells (Fig. 1b).
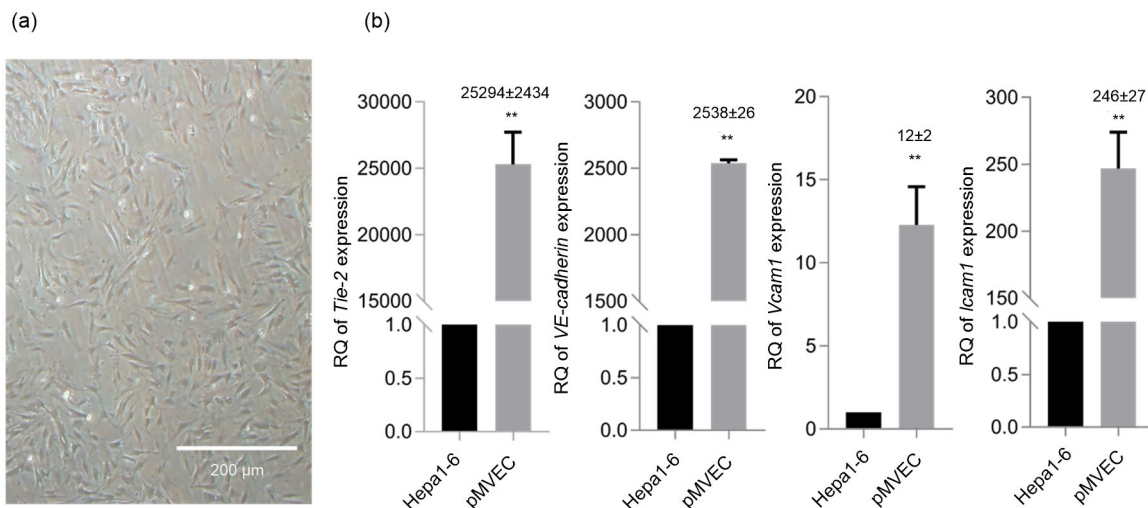

Fig. 1 Quality evaluation of cultured pMVECs

(a) Morphology of pMVECs. (b) Expression of *Tie-2*, *VE-cadherin*, *Vcam1*, and *Icam1* in pMVECs compared with Hepa1-6. Data are shown as mean±standard deviation. Detection for *Tie-2* and *VE-cadherin* expression, *n*=4; detection for *Vcam1* and *Icam1* expression, *n*=3. The significant differences were assessed by one-way analysis of variance (ANOVA). ** *P*<0.01. Numbers over the stars represent the fold change of gene expression in pMVECs compared with Hepa1-6. pMVECs, primary mouse vascular endothelial cells; RQ, relative quantitation

3.2 Qualitative evaluation of the pMVEC model with different NF-κB activity

To identify CVD-related NF-κB-regulated genes and miRNAs, pMVEC models with different NF-κB activity were constructed by treating pMVECs with NF-κB activator (TNFα) and its inhibitor (*RelA* siRNA). The models were then evaluated by qRT-PCR assay. The result showed that the expression of *RelA* was significantly increased by TNFα and repressed by *RelA* siRNA (Fig. 2). In general, the activated NF-κB was transported into the nucleus to regulate expression of target genes (Zhou et al., 2017). To further evaluate the cell models, we used the qRT-PCR assay to detect the expression of two well-known NF-κB-regulated genes, *Cxcl10* (Hein et al., 1997; Varley et al., 2003) and *Vcam1* (Iademarco et al., 1992). We found that both were significantly increased by TNFα and repressed by *RelA* siRNA, which is consistent with the NF-κB levels (Fig. 2). These findings validate the pMVEC model for different NF-κB activity.

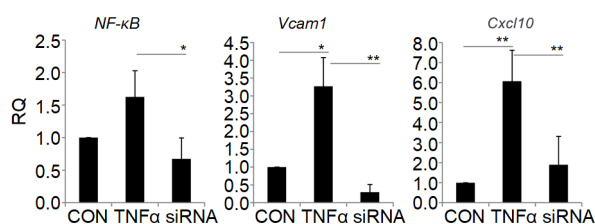**Fig. 2 Expression of NF-κB (*RelA*), *Cxcl10*, and *Vcam1* in pMVECs**

RQ was calculated with the comparative C_T method and normalized against *GADPH*. The data are shown as mean±standard deviation (*n*=4). The significant differences were assessed by one-way analysis of variance (ANOVA). [*] *P*<0.05, [**] *P*<0.01. RQ, relative quantitation; pMVECs, primary mouse vascular endothelial cells; CON, control pMVECs; TNFα, TNFα-treated pMVECs; siRNA, siRNA-interfered pMVECs

3.3 Identification of NF-κB-regulated genes

In order to identify NF-κB-regulated genes, the RNA samples from pMVECs with different NF-κB activity were analyzed by Genechip assay. Upon stimulation by TNFα, the expression of NF-κB potentially-regulated genes changed. Ninety were markedly upregulated and eighty-five downregulated compared with control cells (fold change ≥1.5) (Fig. 3a). To find reliable NF-κB-regulated genes, the gene expression profile of the *RelA* siRNA-interfered pMVECs was

compared with that of the TNFα-treated pMVECs. As shown in Fig. 3, the expression of 45 TNFα-increased and 32 TNFα-inhibited genes was markedly antagonized by *RelA* siRNA (fold change ≥1.2). The fold changes of expression of the 77 genes that responded to the TNFα and *RelA* siRNA treatments are shown in Table S1. These 77 genes are regarded as NF-κB-regulated genes in TNFα-treated pMVECs. By comparing these with the NF-κB-regulated gene profile identified in similarly treated HeLa cells (Xing et al., 2013), we found ten NF-κB-regulated genes, including *Egr1*, *Zfp36*, *Nfkbia*, *Tnf*, *Nr4a1*, *Btg2*, *Jun*, *Ier3*, *Cldn1*, and *Fosb*, that are also regulated by this factor in the TNFα-treated HeLa cells (Fig. 4a). To further confirm these NF-κB-regulated genes, the expression of *Egr1*, *Tnf*, and *Btg2* was detected by qRT-PCR assay. The results demonstrated that their expression was significantly enhanced by TNFα and then blocked by *RelA* siRNA (Fig. 4b), which is consistent with the Genechip assay. To explore the biological processes influenced by the identified NF-κB-regulated genes, 77 NF-κB-regulated genes were submitted to the DAVID database online for GO annotation. All the GO terms with *P*-value of <0.05 are presented in Table 1. The results indicate that the NF-κB-regulated genes are mainly clustered into GO terms of signal transduction, immunity and defense, stress response, intracellular signaling cascade, and mesoderm development, all of which are well-documented, classical biological processes involving NF-κB (Xing et al., 2013). It was also noted that four new NF-κB-regulated genes belonging to the *Olfr* gene family, *Olfr480*, *Olfr490*, *Olfr1109*, and *Olfr1463*, are clustered under the GO signal transduction term. To better understand the molecular function of these NF-κB-regulated genes, we analyzed the KEGG pathway influenced by the NF-κB-regulated genes and found ten NF-κB-regulated genes clustered into other important pathways such as the Toll-like receptor signaling pathway (Table 2).

3.4 Identification of NF-κB-regulated miRNAs

To explore NF-κB-controlled miRNAs in pMVECs, we analyzed miRNAs isolated from pMVECs with different NF-κB activity (Fig. 2) using the Genechip assay. The results show that expression of 41 and 15 miRNAs was markedly increased and inhibited in TNFα-treated pMVECs, respectively. To increase the

Table 1 GO analysis of NF-κB-regulated genes by the DAVID online server

GO term	Count	*P*-value	Fold enrichment	Clustered gene
BP00148: immunity and defense	15	0.0005	2.7545	*Ier3*, *Ccl3*, *Tnf*, *Nfkbia*, *Nr4a1*, *Il11ra1*, *Itpr3*, *Fos*, *Gstp1*, *Antxr1*, *Dusp8*, *Cd200*, *Oas1E*, *H2-M10.5*, *H2-T10*
BP00178: stress response	5	0.0042	7.4293	*Ier3*, *Nr4a1*, *Nfkbia*, *Itpr3*, *Dusp8*
BP00111: intracellular signaling cascade	9	0.0060	3.1588	*Zfp36*, *Spry1*, *Btg2*, *Dgkg*, *Jun*, *Il11ra1*, *Nfkbia*, *Itpr3*, *Dusp8*
BP00248: mesoderm development	6	0.0320	3.2951	*Bmp4*, *Fos*, *Spry1*, *Il11ra1*, *Myom1*, *Angpt2*
BP00102: signal transduction	20	0.0426	1.4984	*Zfp36*, *Ccl3*, *Tnf*, *Il11ra1*, *Nfkbia*, *Nr4a1*, *Itpr3*, *Spry1*, *Wisp1*, *Vmn1R94*, *Btg2*, *Dgkg*, *Jun*, *Cd200*, *Dusp8*, *Angpt2*, *Olfr480*, *Olfr490*, *Olfr1109*, *Olfr1463*

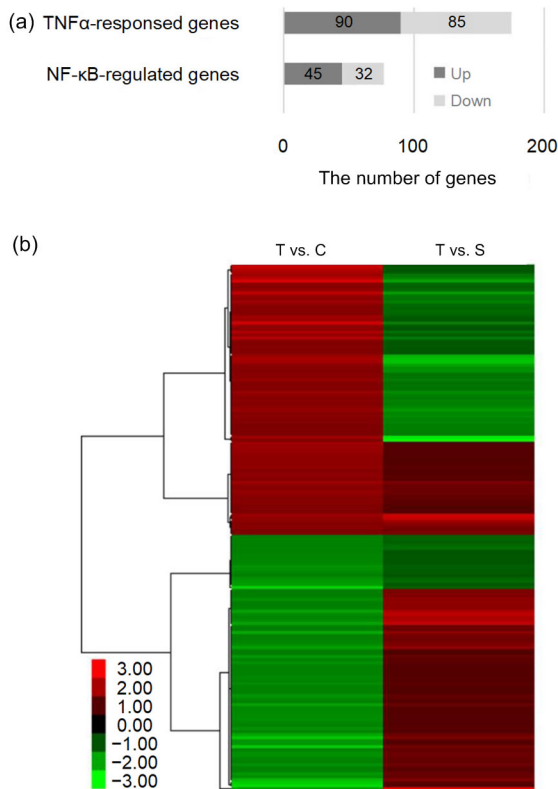GO, gene ontology; NF-κB, nuclear factor-κB; DAVID, Database for Annotation, Visualization and Integrated Discovery

Fig. 3 NF-κB-regulated genes in TNFα-treated pMVECs
(a) Number of NF-κB-regulated genes. (b) Hierarchical cluster analysis of NF-κB-regulated genes. The horizontal axis shows comparison groups of gene expression change. T vs. C group represents the fold change of gene expression in the TNFα-treated pMVECs compared with control pMVECs. T vs. S group represents the fold change of gene expression in the TNFα-treated pMVECs compared with siRNA-interfered pMVECs. The left vertical axis shows clusters of NF-κB-regulated genes. Red indicates upregulated and green downregulated genes. pMVECs, primary mouse vascular endothelial cells (Note: for interpretation of the references to color in this figure legend, the reader is referred to the web version of this article)
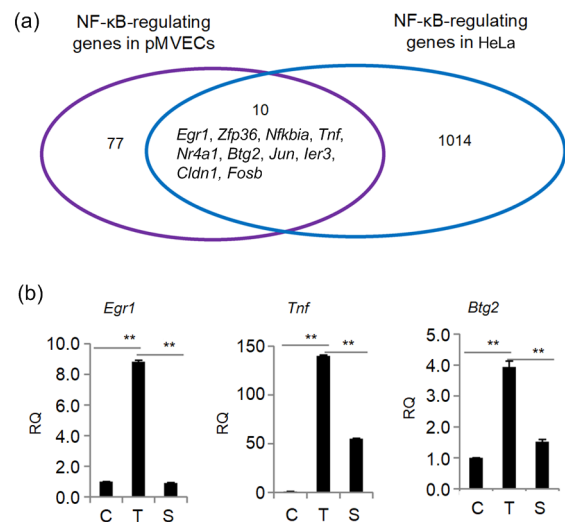

**Fig. 4 Verification of NF-κB-regulated genes in TNFα-treated pMVECs**
(a) Comparison of the NF-κB-regulated gene profile in TNFα-treated pMVECs with TNFα-treated HeLa cells. (b) Expression of *Egr1*, *Tnf*, and *Btg2* in TNFα-treated pMVECs. Data are shown as mean±standard deviation (*n*=3). The significant differences were analyzed by one-way analysis of variance (ANOVA). ** *P*<0.01. pMVECs, primary mouse vascular endothelial cells; RQ, relative quantitation. C, control pMVECs; T, TNFα-treated pMVECs; S, siRNA-interfered pMVECs

reliability of our findings, we compared the miRNA expression profile of *RelA* siRNA-interfered pMVECs with that of TNFα-treated pMVECs and found that the expression of 21 TNFα-increased and 5 TNFα-inhibited miRNAs was antagonized by the siRNA with fold change more than 1.2 (Fig. 5). Therefore, 26 miRNAs are regarded NF-κB-regulated in TNFα-treated pMVECs.

Table 2 KEGG pathway analysis of NF-κB-regulated genes by the DAVID online server

KEGG pathway	Count	P-value	Fold enrichment	Clustered gene
mmu04620: Toll-like receptor signaling pathway	5	0.0012	9.9930	*Fos, Ccl3, Tnf, Jun, Nfkbia*
mmu04660: T cell receptor signaling pathway	4	0.0191	6.7072	*Fos, Tnf, Jun, Nfkbia*
mmu05330: allograft rejection	3	0.0321	10.2342	*Tnf, H2-M10.5, H2-T10*
mmu05332: graft-versus-host disease	3	0.0321	10.2342	*Tnf, H2-M10.5, H2-T10*
mmu04940: type I diabetes mellitus	3	0.0373	9.4220	*Tnf, H2-M10.5, H2-T10*
mmu04010: MAPK signaling pathway	5	0.0380	3.7332	*Fos, Tnf, Jun, Nr4a1, Dusp8*

KEGG, Kyoto encyclopedia of genes and genomes; NF-κB, nuclear factor-κB; DAVID, Database for Annotation, Visualization and Integrated Discovery; MAPK, mitogen-activated protein kinase
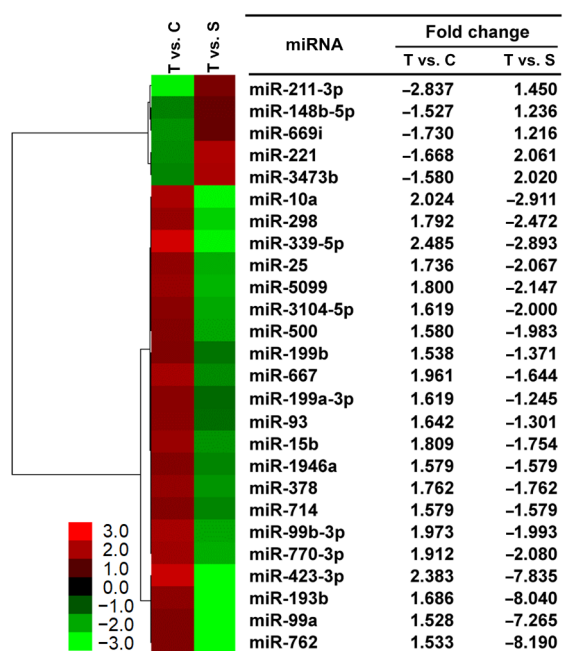
Fig. 5 NF-κB-regulated miRNAs in TNFα-treated pMVECs

Hierarchical cluster analysis of NF-κB-regulated miRNAs. The horizontal axis shows comparison groups of expression change of miRNAs. T vs. C group represents the fold changes of miRNA expression in TNFα-treated pMVECs compared with control pMVECs. T vs. S group represents the fold change of miRNA expression in the TNFα-treated pMVECs compared with siRNA-interfered pMVECs. The left vertical axis shows clusters of NF-κB-regulated miRNAs whose fold change is listed in the right-hand table. Red indicates upregulated and green downregulated miRNAs. pMVECs, primary mouse vascular endothelial cells (Note: for interpretation of the references to color in this figure legend, the reader is referred to the web version of this article)

3.5 Analysis of target genes of NF-κB-regulated miRNAs

We submitted the identified NF-κB-regulated miRNAs to the miRWalk server in order to predict their target genes, according to the parameters and criteria in Section 2. Any miRNA target predicted by at least six miRWalk programs was regarded with high confidence as a nominated target of NF-κB-regulated miRNAs. From this computational approach, we found high-confidence nominated targets for a total of 19 NF-κB-regulated miRNAs. Of these, miR-423-3p had the fewest target genes (i.e. three), while miR-298 had the most (i.e. 754) (Fig. 6a). We also found that one miRNA had multiple nominated target genes and that several miRNAs had the same nominated target gene. Thus, a total of 3205 nominated target genes were hit by these NF-κB-regulated miRNAs. The Cytoscape program showed that these miRNAs and their target genes construct a complex regulatory network in the TNFα-treated pMVECs (Fig. 6b). The above nominated target profile was subsequently compared with the NF-κB-regulated gene profile to find expression changes for several of them. According to the criterion that changes in expression of the target gene are inversely correlated with that of miRNA (Zhou et al., 2014), we found that changes in expression of nine nominated target genes were inversely correlated to those of the seven corresponding NF-κB-regulated miRNAs in pMVECs treated with TNFα and *RelA* siRNA (Table 3). The nine genes were therefore considered as targets of the seven NF-κB-regulated miRNAs. For example, when pMVECs were subjected to TNFα stimulation and *RelA* siRNA-interference, the expression of *Btg2* and *Fos* changed contrary to that of miR-221. Therefore, *Btg2* and *Fos* were regarded as the target genes of miR-221 (Table 3). Accordingly, we identified the target genes of miR-15b, miR-25, miR-298, miR-339-5p, miR-500, and miR-93, as well as miR-221 (Table 3).
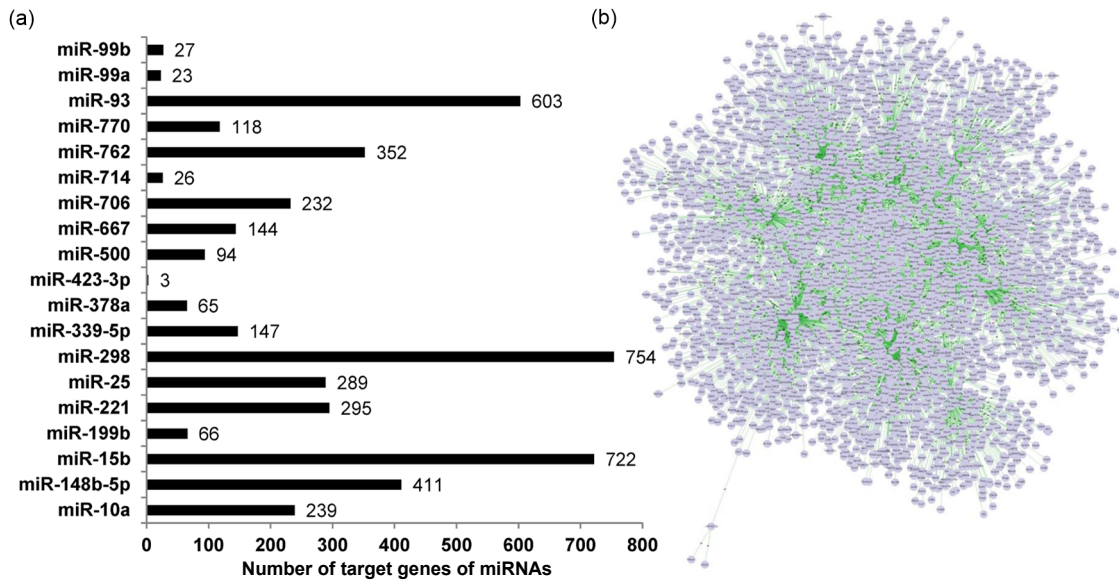
(a)

(b)

Fig. 6 Nominated targets of NF-κB-regulated miRNAs and their constructed regulatory network
(a) Prediction of target genes of NF-κB-regulated miRNAs. (b) Regulatory network formed by NF-κB-regulated miRNAs and their predicted target genes

Table 3 Expression changes of NF-κB-regulated miRNAs and their predicted target genes in pMVECs

miRNA	Target gene (*n*=9)	Fold change	
		TNFα vs. control	TNFα vs. siRNA
miR-15b		1.809	−1.754
	Wisp1	−1.500	1.632
	Myom1	−1.719	1.261
miR-221		−1.668	2.061
	Fos	1.959	−2.302
	Btg2	1.612	−1.383
miR-25		1.736	−2.067
	Btg2	1.612	−1.383
miR-298		1.792	−2.472
	Grhl2	−1.745	1.335
	Fem1a	−1.803	1.276
miR-339-5p		2.485	−2.893
	Cadm4	−1.586	1.692
miR-500		1.580	−1.983
	Myom1	−1.719	1.261
miR-93		1.642	−1.301
	Grhl2	−1.745	1.335

NF-κB, nuclear factor-κB; pMVECs, primary mouse vascular endothelial cells; TNFα vs. control, TNFα-treated pMVECs compared with control pMVECs; TNFα vs. siRNA, TNFα-treated pMVECs compared with siRNA-interfered pMVECs

3.6 Analyses of CVD-related NF-κB-regulated genes and miRNAs

A series of disease-related mouse genes and miRNAs were collected in the GAD (Becker et al., 2004) and miRWalk (Dweep et al., 2011) databases. To fully understand the involvement of NF-κB in CVD, the identified NF-κB-regulated genes and miRNAs were analyzed by these databases. The GAD analysis found five NF-κB-regulated genes related to

CVD (Fig. 7). For example, *Zfp36*, *Tnf*, *Jun*, *Il11ra1*, and *Bmp4* were related to the cardiovascular system phenotype (Table 4). miRWalk analysis identified twelve NF-κB-regulated miRNAs related to CVD (Fig. 7). For example, miR-10a was related to atherosclerosis and plaque atherosclerotic (Table 5). Therefore, 5 genes and 12 miRNAs were identified as CVD-related NF-κB-regulated genes and miRNAs in the TNFα-treated pMVECs.
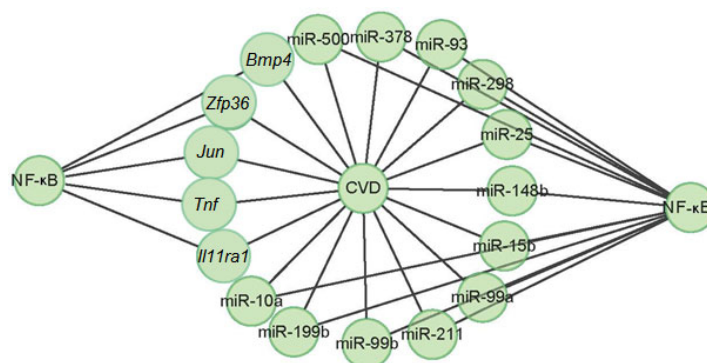
Fig. 7 Involvement of NF-κB in the process of cardiovascular disease (CVD) via its regulated genes and miRNAs

Table 4 CVDs-related NF-κB-regulated genes in TNFα-treated pMVECs

Gene	Cardiovascular disease (CVD)-related phenotype
Zfp36	Heart inflammation; cardiovascular system phenotype
Tnf	Cardiovascular system phenotype
Jun	Vasculature congestion; thin ventricular wall; cardiovascular system phenotype
Il11ra1	Abnormal vascular development; cardiovascular system phenotype
Bmp4	Abnormal heart development; abnormal aorta morphology; abnormal ventricular septum morphology; abnormal atrial septum morphology; double outlet right ventricle; abnormal endocardial cushion morphology; cardiovascular system phenotype

NF-κB, nuclear factor-κB; TNFα, tumor necrosis factor α; pMVECs, primary mouse vascular endothelial cells

Table 5 CVDs-related NF-κB-regulated miRNAs in TNFα-treated pMVECs

miRNA	Cardiovascular disease (CVD)
miR-10a	Atherosclerosis; plaque atherosclerotic
miR-148b	Stroke
miR-15b	Hypertension; reperfusion injury; stroke
miR-199b	Cardiomegaly; cardiomyopathies; heart failure
miR-211	Vascular diseases
miR-25	Cardiomegaly; heart diseases; heart septal defects ventricular; stroke; inflammation artery; infarction middle cerebral
miR-298	Brain injuries; hemorrhage; infarction middle cerebral artery; intracranial hemorrhages
miR-378	Myocardial ischemia; heart diseases
miR-500	Cardiomyopathies; cardiomyopathy dilated; heart failure
miR-93	Heart diseases; heart septal defects ventricular
miR-99a	Heart defects congenital; infarction middle cerebral artery
miR-99b	Infarction middle cerebral artery

NF-κB, nuclear factor-κB; TNFα, tumor necrosis factor α; pMVECs, primary mouse vascular endothelial cells

4 Discussion

NF-κB-regulated genes and miRNAs are involved in the pathogenesis of cardiovascular events. For example, upregulation of NF-κB-regulated genes, such as *ICAM-1*, *VCAM-1*, *TNF*, and *IL6*, contributes to the process of ED in CVD patients (Vanhoutte, 2009; Murdaca et al., 2012; Steyers and Miller, 2014).

Overproduction of metalloproteinase-1 (*MMP-1*), *MMP-3*, and *MMP-9*, which are target genes of NF-κB (Bond et al., 1998; Vincenti et al., 1998; Borghaei et al., 2004), promotes atherosclerotic plaque rupture and myocardial infarction (Newby, 2008). The silence of NF-κB-regulated miRNAs, including miR-497, miR-195, miR-15b, and miR-15a (Yang et al., 2015; Wei et al., 2016; Zhu et al., 2016), reduces

myocardial infarction size (Hullinger et al., 2012). Therefore, NF-κB plays a molecular role in the development of CVD. The NF-κB-regulated genes and miRNAs identified in this study provide new insights into cardiovascular events.

We used qualified pMVECs to represent the endothelial monolayer in blood vessels. The treatment of pMVECs with TNFα was used to detect a series of genes and miRNAs potentially regulated by NF-κB. *RelA* siRNA-interference was used to identify genes and miRNAs specifically regulated by NF-κB in the TNFα-treated pMVECs. A total of 77 NF-κB-regulated genes, of which 45 were upregulated and 32 downregulated, were identified. Among these, 10 NF-κB-regulated genes in the TNFα-treated pMVECs were also regulated in TNFα-treated HeLa cells (Xing et al., 2013). Among the genes we identified, some were well-known NF-κB target genes, such as *TNF* (Collart et al., 1990) and *NFKBIA* (Sun et al., 1993). The regulation of *Egr1*, *Tnf*, and *Btg2* by NF-κB was also confirmed by qRT-PCR assay in TNFα-treated pMVECs. These results demonstrate the reliability of the NF-κB-regulated genes identified in TNFα-treated pMVECs.

We identified a total of 26 NF-κB-regulated miRNAs, 21 of them upregulated and 5 downregulated. It was noted that several miRNAs such as miR-199a (Chen et al., 2008) and miR-221 (Galardi et al., 2011; Santhekadur et al., 2012) have been documented as NF-κB-regulated miRNAs in human cell lines or tissues. This supports the reliability of the findings in our study. We also identified several new NF-κB-regulated miRNAs, such as miR-15b, a member of miR-15 family, and nine target genes of the seven identified NF-κB-regulated miRNAs. For example, both *Btg2* and *Fos* were identified as target genes of miR-221, and *Btg2* was identified as a target gene of miR-25.

As expected, we found 5 CVD-related NF-κB-regulated genes and 12 CVD-related NF-κB-regulated miRNAs by informatics analysis. For example, *Zfp36*, identified as a NF-κB target gene, was highly expressed in ECs overlying atherosclerotic lesions and exerted an anti-inflammatory effect by attenuating inflammatory cytokine/chemokine expression in ECs (Zhang et al., 2013). miR-15b, miR-221, and miR-199a, identified as NF-κB-target miRNAs, were also documented to take part in the CVD process. Silence of miR-15b reduced myocardial infarction size in

mouse (Hullinger et al., 2012). Upregulation of the miR-221/222 cluster in initial atherogenic stages increased ED and EC apoptosis (Chistiakov et al., 2015). miR-221/222 also indirectly contributed to cardiovascular pathology by participating in fat and glucose metabolism in non-vascular tissues (Chistiakov et al., 2015). Overexpression of miR-199a suppressed cardiomyocyte autophagy (Chen et al., 2017; Li et al., 2017) and induced cardiac hypertrophy in miR-199a-transgenic mice (Li et al., 2017). The underlying mechanism of miR-199a-impaired cardiomyocyte autophagy was achieved by silencing its target genes *Hspa5* and *Gsk3b* (Chen et al., 2017; Li et al., 2017). As mentioned above, NF-κB-regulated miRNAs actually regulate their target genes. Therefore, NF-κB and its downstream molecular consequences give rise to a complex regulatory network, which may provide useful clues for uncovering the underlying mechanism in the development and progression of CVD.

In GO annotation, four new NF-κB-regulated genes, *Olfr480*, *Olfr490*, *Olfr1109*, and *Olfr1463*, were clustered into GO terms of signal transduction. Olfactory receptors encoded by the four genes interact with odorant molecules in the nose to initiate a neuronal response that triggers the perception of smell (Shiao et al., 2012). Therefore, NF-κB may play a role in the process of olfaction through its regulated *Olfr* family genes. This finding extends the understanding of the biological role of NF-κB. KEGG analysis indicates that NF-κB cross-talk with other CVD-related pathways, such as the Toll-like receptor signaling pathway, contributes to the development and progression of CVD (Frantz et al., 2007).

In conclusion, we have revealed a complex regulatory network constructed by NF-κB-regulated genes, NF-κB-regulated miRNAs, and their target genes in TNFα-stimulated pMVECs. This network suggests that NF-κB plays a variety of molecular roles in the development of CVD events. The finding provides new insights into understanding the molecular mechanisms underlying CVD events including CVD-related ED.

Contributors

WANG helped in the data analysis and revised the manuscript. All authors read and approved the final manuscript. Therefore, all authors have taken part in the study and take responsibility for the integrity and security of the data.

Compliance with ethics guidelines

Hui ZHU, Yun LI, Mao-xian WANG, Ju-hong WANG, Wen-xin DU, and Fei ZHOU declare that they have no conflict of interest.

All institutional and national guidelines for the care and use of laboratory animals were followed.

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List of electronic supplementary materials

Table S1 NF-κB-regulated genes in the TNFα-treated pMVECs

中文概要

题 目：基于 TNFα 处理的小鼠原代血管内皮细胞鉴定心血管疾病相关的 NF-κB 调控的基因和 microRNA

目 的：鉴定与心血管疾病相关的核因子 κB（NF-κB）调控的基因和小 RNA（microRNA），探讨疾病发生发展的机制。

创新点：构建心血管疾病相关的 NF-κB 调控网络。

方 法：基于 NF-κB 转录活性差异的小鼠原代血管内皮细胞模型，采用基因芯片（Genechip）检测 NF-κB 调控的基因和 microRNA。再通过实时荧光定量聚合酶链反应（qRT-PCR）和生物信息学方法进行差异基因和 microRNA 的筛选、验证、功能注释，从而发现与心血管疾病相关的 NF-κB 调控的基因和 microRNA。

结 论：在肿瘤坏死因子 α（TNFα）处理的小鼠原代血管内皮细胞中：NF-κB 调控 77 个基因，其中 45 个基因上调，32 个基因下调。NF-κB 还在 TNFα 处理的 HeLa 细胞中调控其中 10 个基因。通过 qRT-PCR 验证了 NF-κB 上调 *Egr1*、*Tnf* 和 *Btg2* 的表达。基因功能注释表明，许多 NF-κB 调节的基因聚类到经典的 NF-κB 参与的生物学过程。在 TNFα 处理的小鼠原代血管内皮细胞中还发现：NF-κB 调控 26 个 microRNA，其中 21 个上调，5 个下调。进一步研究发现，7 个 NF-κB 调控的 microRNA 还可能调控 9 个 NF-κB 调控的基因。最后通过检索数据库发现，5 个 NF-κB 调控的基因和 12 个 NF-κB 调控的 microRNA 与心血管疾病相关。因此，本研究提升了对心血管疾病进展分子机制的理解。

关键词：心血管疾病；核因子 κB；小 RNA；内皮细胞