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Regulation of microRNA-155 in Endothelial Inflammation by Targeting Nuclear Factor (NF)-κB P65

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ABSTRACT

Increasing evidences have illuminated the fundamental role of inflammation in mediating all stages of atherosclerosis. miR-155, a typical multi-functional miRNA, has recently emerged as a novel component of inflammatory signal transduction in the pathogenesis of atherosclerosis. However, little is known about whether endothelial highly expressed miR-155 can regulate endothelial inflammation-related transcription factors and the predicted role of miR-155 as a negative feedback regulator in endothelial inflammation involved in atherosclerosis. Bioinformatics analysis showed that RELA (nuclear factor-κB p65) is a potential target gene of miR-155 and this was confirmed by a luciferase reporter assay. Our results show that microRNA-155 mediate endothelial inflammation and decrease NFκB p65 and adhesion molecule expression in TNFα-stimulated endothelial cells. Transfection with miR-155 significantly inhibited TNFα-induced monocyte adhesion to endothelium. Inhibition of miR-155 enhanced p65 level and endothelial inflammatory response which was counteracted through the depletion of P65 by Si-P65. On the other hand, knockdown of eNOS, another target of miR-155, while transfecting with miR-155 inhibitor resulted in more significant inflammatory response. miR-155 is highly expressed in TNFα treated HUVECs, deprived of endogenous p65 could reverse TNFα-induced upregulation of miR-155. Thus, TNFα induced miR-155 may serve as a negative feedback regulator in endothelial inflammation involved in atherosclerosis by targeting nuclear transcription factor P65. These results provide a rationale for intervention of intracellular microRNA as possible anti-atherosclerotic targets. J. Cell. Biochem. 115: 1928–1936, 2014.

KEY WORDS: NFκB P65; microRNA 155; ENDOTHELIAL INFLAMMATION; TNFα

Atherosclerotic disease is the leading cause of death worldwide despite significant progress in the management of critical risk factors [Callow, 2006]. However, the exact mechanisms are not fully clarified. Recent researches on endothelial cells (ECs) showed that inflammation is observed at all stages of atherosclerosis. The initial stage of atherosclerosis is characterized by recruitment of leukocytes to activated ECs [Zhu et al., 2011], which is facilitated by the adhesion molecules expressed on endothelium, such as endothelial-leukocyte adhesion molecule-1 (ELAM-1 or E-selectin), vascular cell adhesion molecule-1 (VCAM-1), and intercellular adhesion molecule-1 (ICAM-1). Moreover, transcription of these adhesion molecules above is induced by the inflammatory cytokines interleukin-1β (IL-1β) and tumor necrosis factor-a (TNF-a) [Collins et al., 1995]. During which, the transcription factor family nuclear factor (NF)-κB is important mediator of this proinflammatory responses in the endothelium

TNF is pleiotropic cytokine with different effects on different cellular level including proliferation depending on cell type [Jurisic et al., 2006]. It should be noted that TNF is regulated by NF-κB, but it is also activator of the NF-κB pathway. Therefore, this cytokine may affect atherosclerosis by regulating many other NF-κB dependent genes and amplify the inflammatory response.

NF-κB family members, including RELA (P65), RelB, c-Rel, NF-κB1 (p50), and NF-κB2 (p52), bind as homodimers or heterodimers to the specific binding sites on the promoters of target genes [Oeckinghaus and Ghosh, 2009]. Activation of the NF-κB pathway

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leads to the expression of variable inflammation associated genes, including cytokines, chemokines, and adhesion molecules [Dryden et al., 2012]. Hence, the control of NF-κB activity is essential to maintain quiescence and for the resolution of the inflammatory response; whereas, in chronic inflammation the tight control on NF-κB is lost, leading to vascular diseases such as atherosclerosis [Rocha et al., 2009]. As a key component of NF-κB family, P65 plays a critical role in manipulating the transcription process of target genes. In the inactive state, P65 combines with the protein IκB in the cytoplasm without transcriptional activity. In the presence of inflammatory cytokines and other stimuli, the IκB upstream kinases IκB kinase (IKK) is activated through phosphorylation, resulting in IκB degradation. Without the shackles of IκB, P65 is transferred to the nucleus after combining with p50, and then P65 binds to the specific DNA sequences, which controls the transcription of target genes [Collins et al., 1995].

microRNAs (miRNAs) are a class of 19–25 nucleotides, non-protein-coding RNAs that repress target gene expression at the posttranscriptional levels by targeting the 3′UTR of specific mRNAs through induction of mRNA degradation or translational repression [Ambros, 2004; Bartel, 2009]. Growing body of evidences have indicated that miR-155 was highly expressed in human umbilical vein endothelial cells (HUVECs), and might be a possible therapeutic target in atherosclerosis. miR-155 may co-target angiotensin II type 1 receptor (AT1R) and E26 transformation-specific sequence (Ets)-1, which indirectly regulate the inflammatory molecules in ECs, and attenuate the adhesion of Jurkat T cells to activated HUVECs and reduce HUVECs migration [Ambros, 2004]. These findings revealed that upregulation of miR-155 in ECs may retard the initiation and progression of atherosclerosis. However, previous studies demonstrated that mitogen-activated protein kinase kinase kinase 10 (MAP3K10) and endothelial nitric oxide synthase (eNOS) is direct target of miR-155 [Sun et al., 2012; Zhu et al., 2012]. Additionally, other researchers also found that miR-155 would promote atherosclerosis by repressing Bcl6 (a transcription factor that attenuates proinflammatory NF-κB signaling) in macrophages [Nazari-Jahantigh et al., 2012a]. Taken together, miR-155, as a typical multi-functional miRNA, may play a conflicting role in the pathogenesis of atherosclerosis [Ma et al., 2013].

Early studies also indicated that, in response to *Helicobacter pylori* infection, NF-κB pathway was required for the induction of miR-155 and miR-155, in turn, repressed the target genes of NF-κB pathway [Xiao et al., 2009]. However, it has not been completely clarified about the relationship between NF-κB P65 and miR-155 in endothelial inflammation involved in atherosclerosis. Therefore, we aim to verify the inflammation-related target (NF-κB P65) of miR-155 in ECs and to explore the important role of miR-155 in suppressing ECs inflammatory cascade reaction during atherosclerosis.

MATERIALS AND METHODS

CELL CULTURE

Human umbilical vein endothelial cells (HUVECs) (ScienCell, Carlsbad, CA) were cultured in endothelial cell medium (ECM) supplemented with 10% fetal bovine serum and 1% endothelial growth factor and penicillin. HEK-293T cells (SIBS, Shanghai, China) were grown in high-glucose DMEM (Hyclone) (SHHY, Shanghai, China) supplemented with 10% FBS and 1% penicillin. The human monocytic cell line THP-1 (SIBS, Shanghai, China) was cultured in RPMI 1640 medium supplemented with 10% foetal bovine serum, 0.05 mM mercaptoethanol (Sigma, USA), 1% pen/strep solution. All cells were maintained in a humidified atmosphere of 5% CO_2 at 37°C.

HUVEC TRANSFECTION

HUVECs were transfected with 50 nM miRNA mimics, miRNA inhibitors or si-RNA using Lipofectamine RNAiMAX (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol. Control samples were transfected with an equal concentration of mimic negative control (NC) or inhibitor negative control (INC) as described previously [Chen et al., 2012]. The miR-155 and Si-P65, Si-eNOS, Si-NC (control) duplexes were synthesized by Guangzhou RiboBio siRNA/miRNA Technologies. The effects of transfections with miR-mimics/inhibitors were assessed by quantitative real-time PCR (QRT-PCR) (data not shown).

DUAL-LUCIFERASE ASSAYS

microRNA target genes prediction was performed using the online software: Targetscan, PicTar, and miRanda. A 782-bp fragment of RELA-3′UTR containing the miR-155-binding site were amplified by PCR from human genomic DNA using specific primers (see online supplement). The amplified products were restricted and ligated to the *Xho*I and *Not*I sites of psiCHECK2 vectors (Promega, Madison). Mutant RELA 3′UTR was cloned as controls lacking the miR-155 target sequence (m-P65). The region complementary to the miR-155 seed sequence in position 197–204 of the human RELA 3′UTR, AGCAUUAA, was scrambled to CATGGCGC (MP65 3′UTR). The constructed reporter plasmid was confirmed by sequencing. For the luciferase assay, psiCHECKTM-2 vectors with or without target gene 3′-UTR and 50 nM miR-155 mimics or negative control were co-transfected into HEK-293T cells with Lipofectamine 2000 (Invitrogen), respectively. Forty eight hours after transfection, cells were assayed for both firefly luciferase (internal control) and renilla luciferase using the Dual-Luciferase Reporter Assay System (Promega, Madison) according to manufacturer's instructions. All experiments were performed in triplicate.

WESTERN BLOT ANALYSIS

HUVECs were lysed in RIPA-containing protease inhibitors (Beyotime Institute of Biotechnology, Jiangsu, China). Soluble lysate was mixed with loading buffer (40% glycerol, 200 mmol/L dithiothreitol, and 0.04% bromophenol blue) and boiled for 5 min. A supernatant aliquot was separated by 8% SDS–PAGE. Following transfer to PVDF membrane and blocking with 5% nonfat milk, the membranes were incubated overnight with primary antibodies against P65, ICAM-1, VCAM-1, eNOS, and a-tubulin (Cell Signaling, 1:8,000) and then immersed in HRP-conjugated secondary antibody, followed by chemiluminescence detection.

RNA ISOLATION AND REAL-TIME PCR

Total RNA was extracted from cells using TRIzol reagent (Invitrogen) according to the manufacturer's protocol. cDNA was synthesized

from 1 μg total RNA using RevertAid^{TM} First Strand cDNA Synthesis Kit for target gene detection. cDNAs was quantified by SYBR green real-time quantitative PCR and normalized according to GAPDH expression. miR-155 reverse transcription was performed using the TaqMan microRNA Reverse Transcription kit (Life Technology, Carlsbad, CA). Real-time PCR was performed using the TaqMan universal master mix in the Light-Cycler480 qPCR detection system (Roche, Mannheim, Germany). RNU6B was used as a reference for normalization. Relative levels of miRNA were defined from threshold cycle (Ct) values calculated by the $2^{-\triangle\triangle Ct}$ method.

CELL ADHESION ASSAY

HUVECs were transfected with miR-control, miRNA 155 for 48 h and then treated with TNF-a (10 ng/ml) for 6 h. THP-1 cells were labeled with Calcein AM (fluorometric) in the culture media for 30 min at 37°C. The labeled THP-1 cells were washed and incubated with transfected HUVECs for 40 min at 37°C. After incubation with confluent HUVEC monolayers, the media containing non-attached THP-1 cells were removed, and remaining cells were washed twice with PBS in accordance with the manufacturer's protocol using Leukocyte-endothelium Adhesion Kit (Cell Biolabs, San Diego, CA). Monocyte adhesion was observed using an inverted fluorescence microscope (Zeiss, Oberkochen, Germany). The number of THP-1 cells adhering to HUVECs was counted in five randomly selected fields for each experiment.

STATISTICAL ANALYSIS

Data were reported as means ± SEM from at least three independent experiments. Comparisons between treatment groups and control groups were made based on the unpaired Student's *t*-test. Multiple comparisons were determined using one-way ANOVA. All statistical analyses were performed using SPSS 13.0 or GraphPad Prism 5.0, and *P* values <0.05 were considered statistically significant.

RESULTS

MIR-155 IS HIGHLY EXPRESSED IN TNFα TREATED HUVECs, DOWNREGULATION OF ENDOGENOUS P65 ATTENUATED THE TNFα-INDUCED UPREGULATION OF miR-155 EXPRESSION

To identify whether miR-155 is involved in certain circumstances to inflammatory stimuli and the mechanisms mediating the induction of miR-155, which further verify the negative feedback mechanism of inflammation [Suárez et al., 2010], we examined miR-155 expression in HUVECs after exposure to TNFα (10 ng/ml). miRNA expression level was measured by quantitative PCR. Our results showed that TNFα treatment significantly increased miR-155 expression in HUVECs. miR-155 expression was also induced by inflammatory cytokines LPS, which consistent with previous studies (Fig. 1A). Other researchers proposed that TNFα-induced upregulation of miR-155 was partially blocked by pyrrolidine dithiocarbamate (a NF-κB inhibitor) [Sun et al., 2012]. Here, we also found that knockdown of endogenous P65 by SI-P65 decreased TNFα-induced upregulation of miR-155 expression in HUVECs (Fig. 1B). These results implied that the expression level of endogenous P65 mediates the TNFα-induced upregulation of miR-155, at least partially.

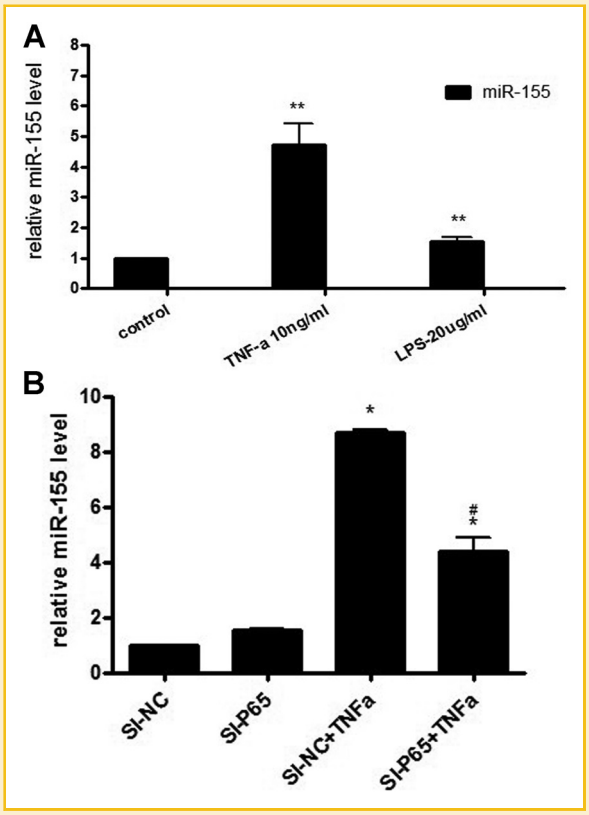

Fig. 1. Inflammatory cytokines induced miR-155 expression in human umbilical vein endothelial cells (HUVECs). Relative expression levels of miR-155 in HUVECs treated with TNFα and LPS (A) (**$P < 0.01$ vs. control). Effects of SI-P65 on TNFα-evoked miR-155 expression in HUVECs (B) (n = 4, *$P < 0.05$ vs SI-NC, #$P < 0.05$ vs. SI-NC + TNFα group).

RELA IS A TARGET OF miR-155

To determine the potential role of miR-155 in mediating cardiovascular disease, three prediction programs: Targetscan, miRanda, and picTar were combined used to improve miRNA target identification quality. Bioinformatics analysis showed that RELA 3′UTR contained miR-155 binding sites at 197–204 nucleotides (Fig. 2A). To examine whether miR-155 can repress P65 expression through direct 3′UTR interaction, we cloned P65 3′-UTR into *Xho*I and *Not*I sites of psiCHECK2 vectors, which located downstream of luciferase mRNA open reading frame. Then we performed reporter analysis in HEK-293T cells. The 293 T cells were transfected with miR-control, miR-155 together with psiCHECK^{TM}-2 empty vector (CHECK-2-miR-155), psiCHECK^{TM}-2-eNOS 3′UTR (enos-3′UTR-miR-155, as positive control), psiCHECK^{TM}-2-P65 3′UTR (P65–3′UTR-miR-155) or psiCHECK^{TM}-2-MP65 3′UTR (P65–3′UTR-mut-miR-155). Results showed that luciferase activity remained stable ($P > 0.05$ Fig. 2B, n = 6) when miR-155 were co-transfected with empty vector or psiCHECK^{TM}-2-MP65 3′UTR with a mutated miR-155 seed sequence when compared with miR-control (NC), but lowered when they were co-transfected with psiCHECK^{TM}-2-eNOS 3′UTR and psiCHECK^{TM}-2-P65 3′UTR (Fig. 2B, n = 6, *$P < 0.05$ vs. control). These data indicated that P65 is a direct target of miR-155.

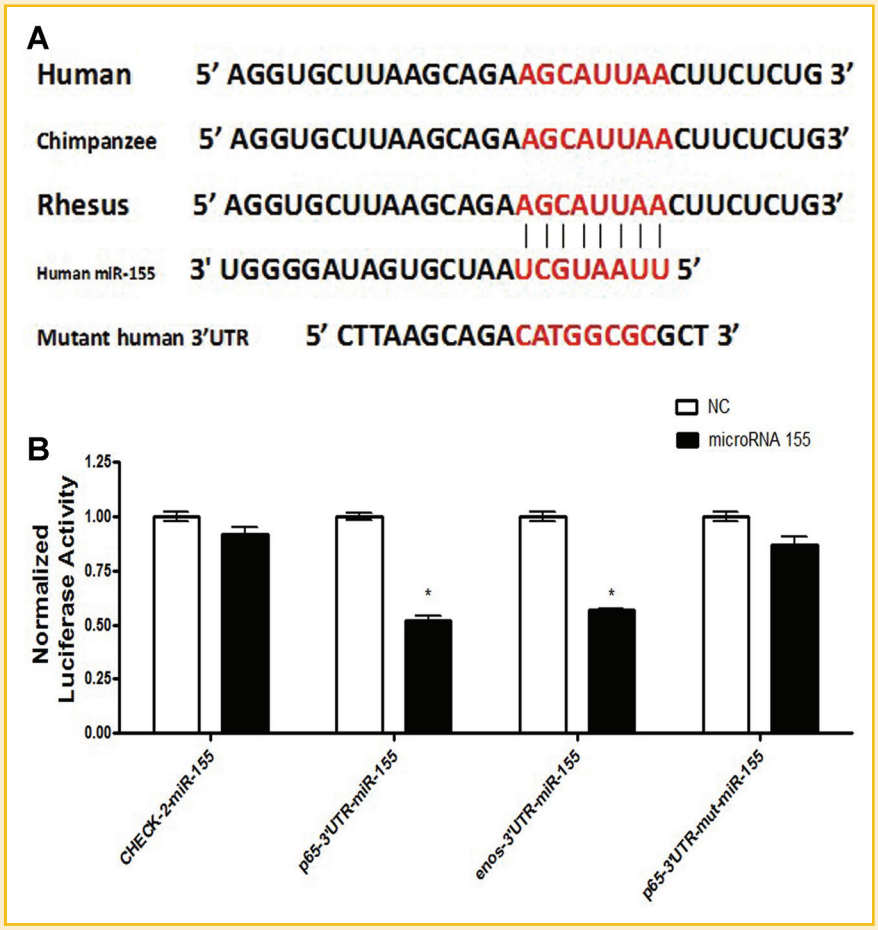

Fig. 2. RELA is a target of miR-155. The sequence of miR-155 and its potential matching sites in the P65 3′UTR, with the "seed sequence" represented in red letters. The sequence of mutant P65 3′UTR (mutant human 3′UTR) used for reporter assay is also shown (A). 293T were co-transfected with psiCHECKTM-2 empty vector, psiCHECKTM-2-eNOS 3′UTR (as positive control), psiCHECKTM-2-P65 3′UTR or psiCHECKTM-2-MP65 3′UTR (P65-3′UTR-mut-miR-155) and miR-control or miRNA 155 mimics (50 nM). Luciferase activities were measured 48 h after transfection. Firefly-luc activity was normalized to Renillaluc expression (B). Data are the mean activities ± SEM from three independent experiments (n = 6, $*P < 0.05$ vs. miR-control transfection).

OVEREXPRESSION OF miR-155 IN HUVECs DOWNREGULATED ENDOGENOUS P65 EXPRESSION AND TNFα-INDUCED UPREGULATION OF ADHESION MOLECULES

Members (in our case, P65) of the transcription factor family nuclear factor (NF)-κB are important mediators of proinflammatory responses in the ECs [Dryden et al., 2012]. To further validate whether miR-155 can downregulate P65 expression, we tested the effects of miR-155 on endogenous P65 levels in HUVECs transfected with miR-155 mimics. Overexpression of miR-155 for 48 h reduced P65 protein expression correlated with decreases of ICAM-1, VCAM-1 protein levels in HUVECs, eNOS (another known target of miR-155) were also downregulated which could as a positive control to prove the effectiveness of miR-155 transfection (Fig. 3A). Indicating that miR-155 can inhibit inflammatory response of endothelial cells under basic conditions.

To further investigate whether miR-155 plays a role in restraining endothelial inflammation in pathological cases, in another set of experiments, HUVECs were transfected with miR-155 mimics for 48 h and then stimulated with TNF-a for 6 h. Western blot showed that ICAM-1 and RELA (P65) protein expression were obviously reduced when compared with control (NC) (Fig. 3B). But VCAM-1 did not change significantly after adding TNFα (data not shown), which unlike the basic conditions (Fig. 3A).

INHIBITION OF miR-155 ENHANCED ENDOTHELIAL INFLAMMATORY RESPONSE, WHICH WAS STRENGTHENED BY REMOVING OF SIMULTANEOUSLY UPREGULATED eNOS

Inside the nucleus, RELA (P65) recognizes and binds to the specific DNA sequences then controls the transcription of inflammatory genes. Here, our results showed that P65 is the direct target of miR-155 and TNFα can increase miR-155 expression, indicating miR-155 may play a role in preventing excessive inflammation. To validate this hypothesis, we examined the effects of TNFα on adhesion molecule expression in HUVECs transfected with miR-155 inhibitor (I155). Results showed that transfection of miR-155 inhibitor (I155) but not INC enhanced P65 protein level and TNFα-induced upregulation of ICAM-1 expression (Fig. 4A). Curiously, VCAM-1 was not significant difference when adding TNFα, similar to

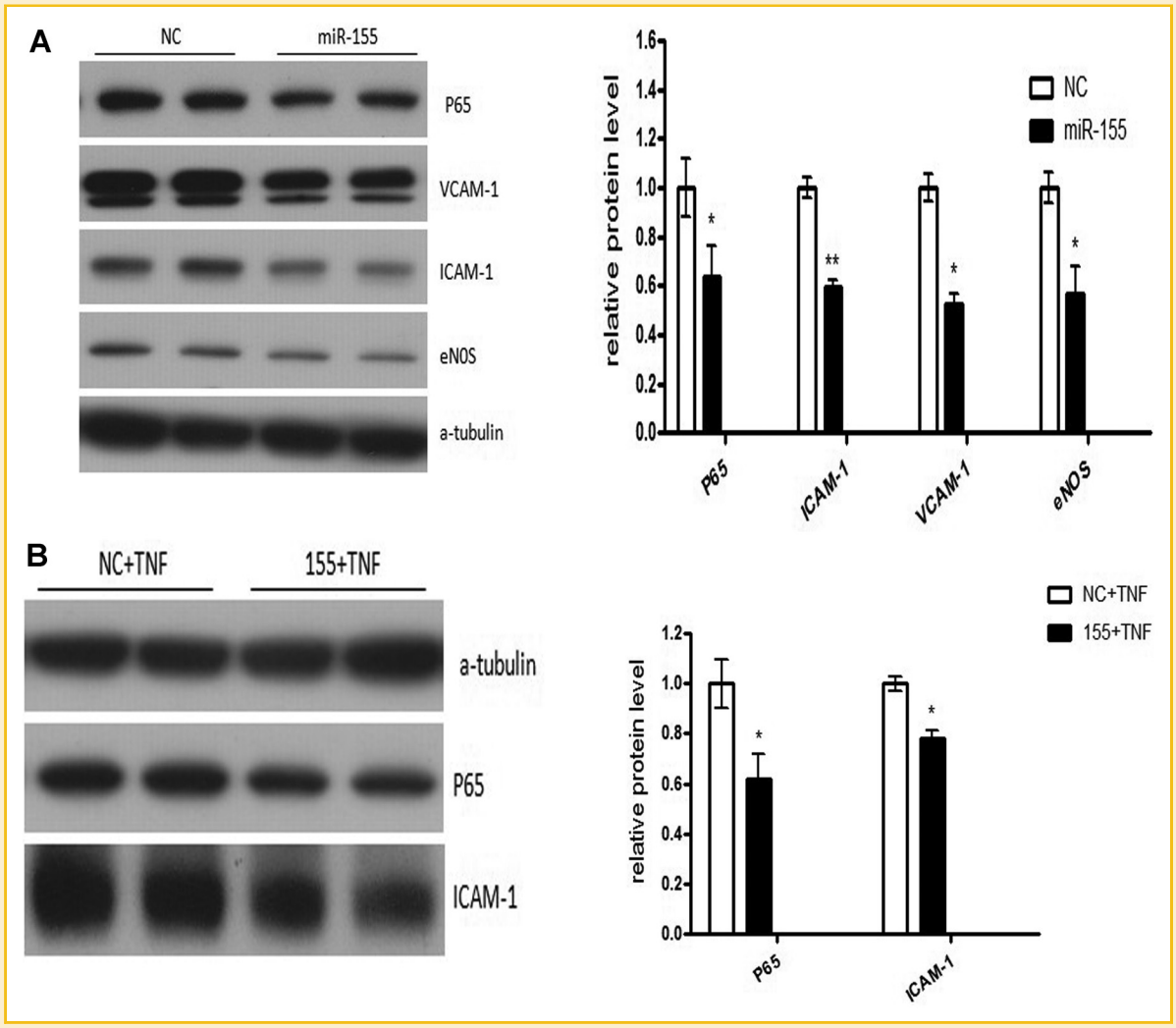

Fig. 3. miR-155 inhibited endogenous P65 expression in human umbilical vein endothelial cells (HUVECs). Overexpression of miR-155 inhibited P65 expression and adhesion molecules of ICAM-1/VCAM-1 levels. A: HUVECs were transfected with miR-155 mimics or NC at the concentration of 50 nm. Forty eight hours later, stimulated with 10 ng/ml of TNF α for 6 h, then the cell lysates were collected for Western blotting (B) ($n = 6$, $^{*}P < 0.05$, $^{**}P < 0.01$ vs. control).

transfecting with miR-155 mimics plus TNFα, there may be alternative mechanisms exist (data not shown).

Moreover, miR-155 inhibitor transfection without TNFα increased P65 expression, which further confirmed P65 as a target of miR-155 from the opposite aspect. In agreement with its effect on P65 expression, anti-miR-155 also increased ICAM-1 and VCAM-1 at the basal condition (without TNFα), but not particularly obvious by roughly observation (data not shown). We speculated that it may be affected by simultaneously upregulated eNOS (another validated target of miR-155) and tried to figure out the interaction between different targets. Then, we designed to knockdown eNOS by Si-eNOS while transfected with miR-155 inhibitor in order to exclude the interference of eNOS. Results showed that adhesion molecule expression was dramatically increased compared to SI-NC (Fig. 4B). Furthermore, the miR-155 inhibitor-mediated inflammatory response was counteracted through the inhibition of P65 by Si-P65 (Fig. 4B, column chart not shown), that means the proinflammatory effects of miR-155 inhibitor depending on its target p65. So far, by using Western blot, we confirmed that overexpression of miR-155 in HUVECs could downregulate P65 protein expression, while inhibition of miR-155 upregulated P65 protein levels, indicating that P65 is a target of miR-155, consistent with Dual-luciferase assays.

P65 AS A FUNCTIONAL TARGET INVOLVED IN THE miR-155-MEDIATED INFLAMMATORY EFFECT

Based on the above evidence of P65 regulation by miR-155, and considering the proinflammation mechanism of P65, we deduced that P65 could be a functionally important target of miR-155. To verify the biological importance of P65 as miR-155 target gene, HUVECs were depleted of P65 by siRNA. Knockdown of endogenous P65 through siRNA efficiently repressed P65 protein levels. Simultaneously, adhesion molecule expression (ICAM-1, VCAM-1) were decreased (Fig. 5A). These results corresponding to the effects of

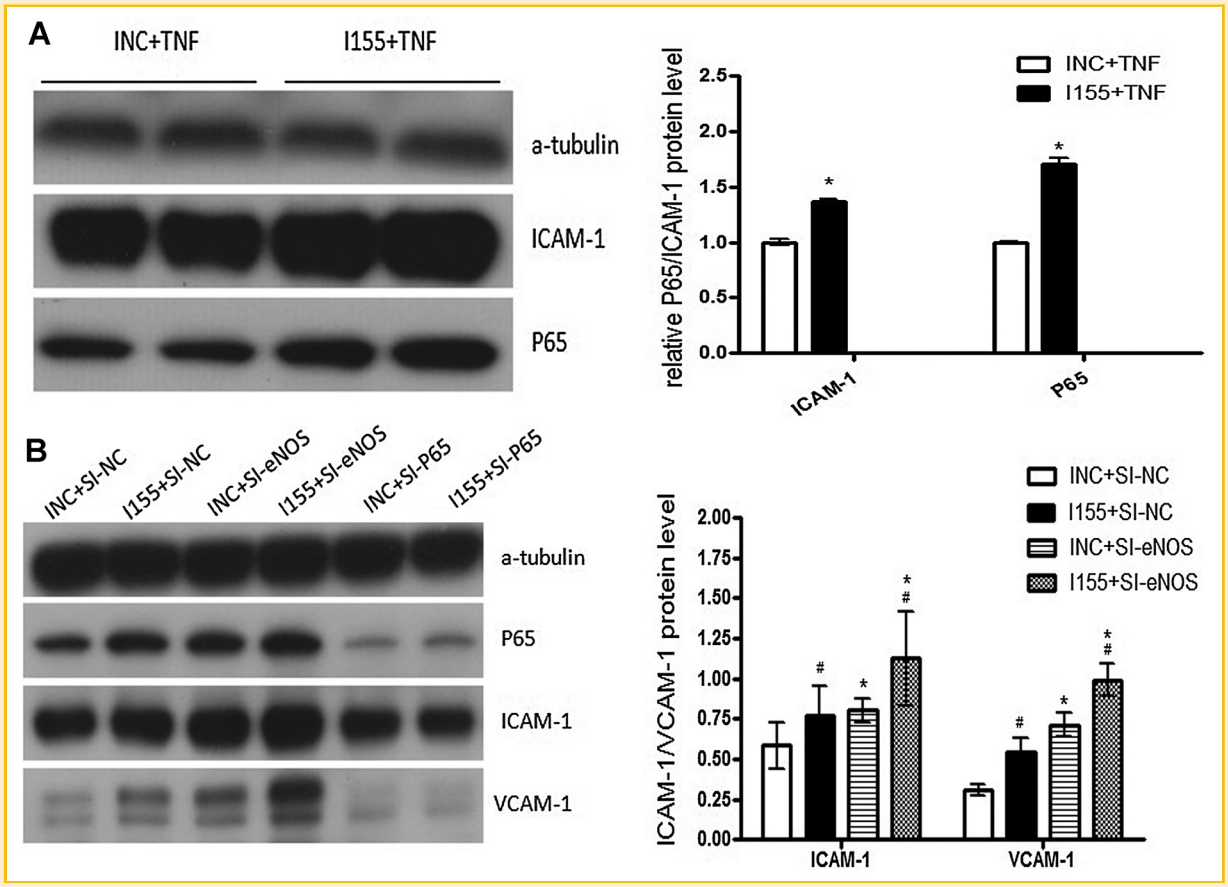

Fig. 4. miR-155 inhibitor promoted tumor necrosis factor (TNF) α-induced upregulation of adhesion molecules through increasing P65. HUVECs were transfected with miR-155 inhibitors. Twenty-four hours later, stimulated with 10 ng/ml of TNFα for 6 h. P65 and ICAM-1 protein expression were assayed by Western blot (*$P < 0.05$ vs. the control group). A: The miR-155 inhibitor-mediated inflammatory effect was rescued via small interference RNAs for P65 and enhanced by Si-eNOS (another target of miR-155). B: Data are presented as mean ± SEM ($n = 6$, *$P < 0.05$ compared with the siRNA control (SI-NC) and #$P < 0.05$ compared with the INC group) (B).

the miR-155 mimic overexpression that shown above. P65 and VCAM1, ICAM1 mRNA was downregulated markedly when Si-P65 was introduced into HUVECs compared to siRNA-control group (Fig. 5B). Accordingly, the data implied the important role for P65 as a mediator of the biological effects of miR-155 on HUVECs inflammation.

miR-155 ATTENUATED MONOCYTES ADHESION TO TNFα-STIMULATED HUVECs

Endothelium inflammation plays a key role in the initiation and progression of atherosclerosis [Hansson, 2005]. One of the crucial steps is that circulating monocytes adhere and migrate into the intima, which is mediated by adhesion molecules and chemokines [Charo and Ransohoff, 2006]. To explore the functional relevance of miR-155, HUVECs were transfected with miR-control, miRNA 155 for 48 h and then treated with TNFα (10 ng/ml) for 6 h. Subsequently, monocyte adhesion to endothelial cells was observed using an Inverted fluorescence microscope. As expected, TNFα thoroughly increased THP-1 cells adhesion to HUVECs. The introduction of miR-155 markedly decreased the number of THP-1 cells adhered to TNFα-activated or not treated HUVECs compared with transfected control (Fig. 6).

These results suggested that miR-155 is involved in the regulation of endothelial inflammation via inhibition of P65 expression, followed by reduced adhesion molecule levels. Taken together, these findings indicated that the increased expression of miR-155 induced by TNFα reduced TNFα-induced impairment of endothelial inflammation. That means miR-155 could be the negative feedback regulator of atherosclerosis inflammation.

DISCUSSION

Recent data indicate that miR-155 has distinct expression profiles and plays a crucial role in various physiological and pathological processes such as hematopoietic lineage differentiation, immunity, inflammation, cancer, and cardiovascular diseases [Faraoni et al., 2009]. Previous studies showed that the level of circulating miR-155 is remarkably reduced in CAD patients [Fichtlscherer et al., 2010]. Moreover, miR-155 is dramatically upregulated in atherosclerotic plaques and oxidized low-density lipoprotein-treated macrophages [Raitoharju et al., 2011; Nazari-Jahantigh et al., 2012b]. These findings revealed that miR-155 may be an essential regulator of atherosclerotic disease.

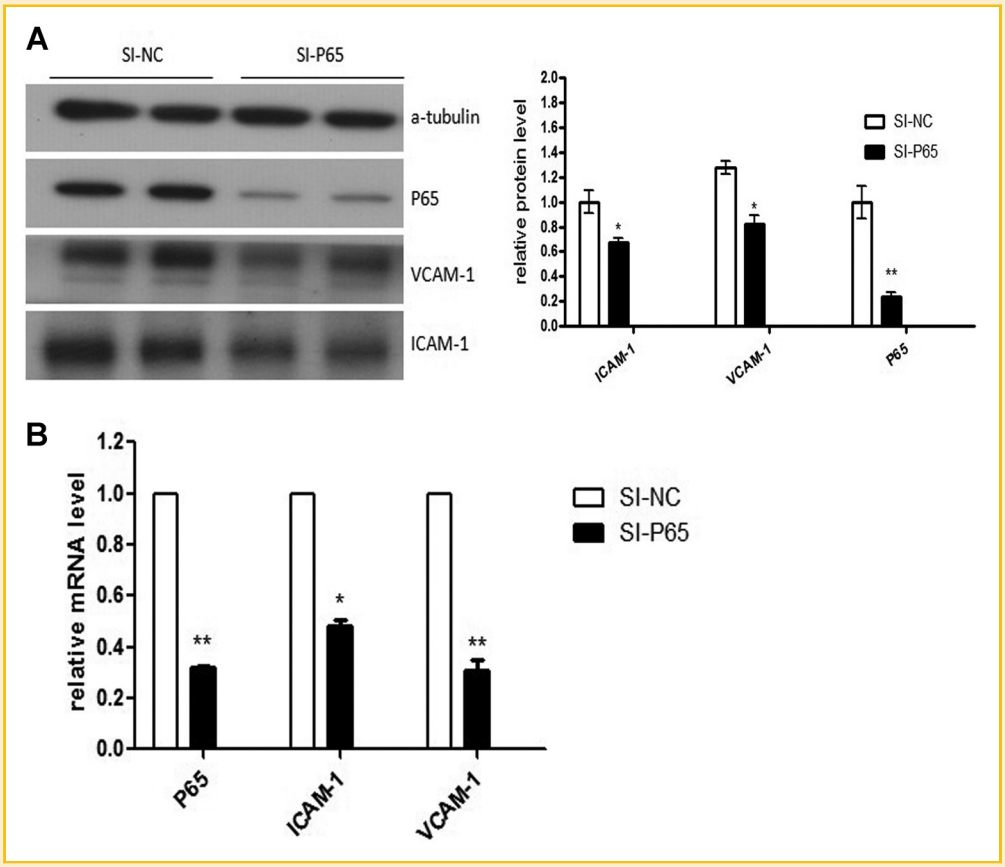

Fig. 5. RELA (P65) is a functional target gene involved in the miR-155-mediated inflammatory effect on HUVECs. After transfection of small interference RNAs for P65 (SI-P65) to knockdown endogenous P65, ICAM-1, VCAM-1, and P65 protein expression were detected by Western blot (A). Relative mRNA expression for P65, ICAM-1, and VCAM-1 after transfection of SI-P65 is detected by RT-qPCR (B). Data are presented as the mean ± SEM ($n = 6$, $^{*}P < 0.05$, $^{**}P < 0.01$ vs. control sequence (SI-NC)).

So far, a few direct targets of miR-155 have been identified by luciferase report assay. Although miR-155 targets several mRNAs in macrophages, such as SOCS1, SHIP1, IL13R α1, and SMAD2, suppression of Bcl6, which is a nuclear factor-κB antagonist, mediates the proinflammatory effects of miR-155 [Wei et al., 2013b], in endothelial cells, miR-155 is upregulated by high shear stress and targets the angiotensin II type 1 receptor and Ets-1, which reduces the proinflammatory activity of angiotensin II [Martin et al., 2007; Weber et al., 2010]. Thus, miR-155 gene as a typical multifunctional microRNA may obtain growing evidence in the regulation of cardiovascular functions.

Inflammation plays a crucial role in the initiation and progression of atherosclerosis. Compelling evidence show that the recruitment of leukocytes to the vascular wall by activated endothelial cells expressed a set of adherent molecules is the key process in this condition [Zhu et al., 2011]. Here, our data demonstrated that RELA is a direct target of miR-155. Overexpression of miR-155 decreased P65 expression and adhesion molecules production in HUVECs and attenuated TNFα-induced monocyte adhesion to endothelium. miR-155 downregulated P65 expression mainly through binding to its 3′UTR. From another side, pretreatment of miR-155 inhibitor enhanced TNF α-induced upregulation of ICAM-1 expression in HUVECs. We further confirmed that miR-155 inhibitor increased P65 expression in HUVECs in basal conditions, indicating miR-155 is implicated in regulation of P65 expression in physiological status.

The major NF-κB-regulated cytokines studied in atherogenesis include: TNF, IL-1β, IL-6, IL-10, IL-12, and interferon-γ(IFNγ). TNF is multifunctional, and one of the most important proinflammatory and immune modulatory cytokines [McDermott, 2001]. Therefore, it may have a major role in atherogenesis. TNF exerts its cellular effects such as differentiation, proliferation, and cell survival or cytotoxicity through signaling of two distinct TNF receptors, TNFR1 and TNFR2. TNF also correlated with degree of vascularization in different diseases [Jurisic et al., 2008].

Previous data demonstrated that some inflammation-stimulating factor such as ox-LDL, TNFα, LPS, or interferon-γ could increase miR-155 expression in human primary monocytes [Chen et al., 2009], macrophages, human mesangial cells and human coronary artery endothelial cells [O′Connell et al., 2007; Chen et al., 2008]. Our present results showed that miR-155 was upregulated in HUVECs after treatment with TNFα, LPS, which consistent with these studies. Sun et al. [2012] reported that the promoter of B-cell integration cluster/miR-155 gene contains NF-κB binding motifs, and inhibitors of NF-κB significantly inhibited TNFα-induced upregulation of miR-155 in HUVECs. In our data, we found that endogenous P65 knockdown by siRNA weakened the TNFα-induced increase in miR-155. This implies

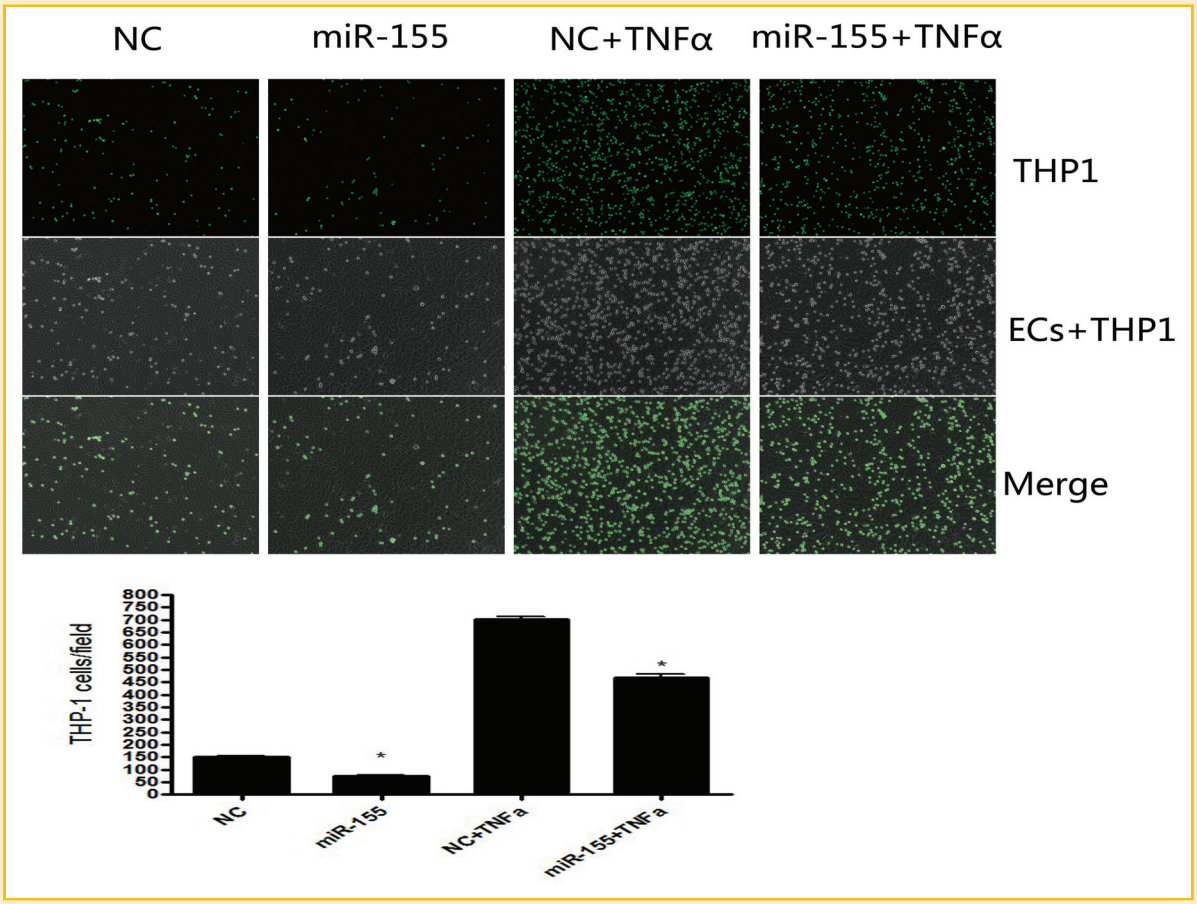

Fig. 6. miR-155 decrease the adhesion of THP-1 cells to TNFα stimulated HUVECs at a 200× magnification. HUVECs were transfected with miR-control, miRNA 155 for 48 h and then stimulated with TNFα (10 ng/ml) for 6 h. Calcein AM labeled THP-1 cells were added to confluent HUVECs, incubated at 37°C for 40 min, and washed twice in PBS. The adherent cells were photographed with an Inverted fluorescence microscope. The number of THP-1 cells adhered to HUVECs was counted in five randomly selected fields for each experiment. The results were represented as mean ± SEM of at least three separate experiments (*$P < 0.05$ vs. miR-control transfection).

that p65 subunit of NF-κB played an essential effect in the process of TNF-induced upregulation of miR-155.

Our results suggested that miR-155 may play a critical role in regulation of atherosclerosis inflammation through suppression of RELA and inflammatory factor expression. And we also certified that miR-155 was upregulated after treatment with TNFα, suggesting that TNFα-induced upregulation of miR-155 inhibits TNFα-induced excessive inflammation. Previous reports have shown that E-selectin and ICAM-1 are targets of TNFα-induced miRNAs miR-31 and miR-17-3p, respectively, and transfections with mimics of these miRNAs decreased neutrophil adhesion to endothelial cells [Suárez et al., 2010]. Combined with previous research, our experiments further demonstrated that several special miRNAs provide negative feedback control of inflammation.

It is worth mentioning that knockdown of eNOS (another target of miR-155) while transfecting with miR-155 inhibitor resulted in more significant inflammatory response by increasing the ICAM-1 and VCAM-1 expression levels. This phenomenon means that there is mutual interference between different targets of microRNA 155, which led to the ultimate expression level of adhesion molecules. Therefore, miR-155 may play a complex even diverse role in endothelial function, and it seems arbitrary to simply judge whether a specific microRNA is good or bad [Voorhoeve and Agami, 2007; Wei et al., 2013a].

Conclusively, our study demonstrated that endothelial cells highly expressed miR-155 induced by TNFα attenuated endothelial inflammation by targeting NFκB P65 and inhibited monocyte adhesion to inflammatory factors stimulated endothelial cells. These findings provide potent evidence that miR-155 may be an important intervention target in atherosclerotic inflammation.

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