Effect of MicroRNA-30e on the Behavior of Vascular Smooth Muscle Cells via Targeting Ubiquitin-Conjugating Enzyme E2I

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Background: Many microRNAs (miRNAs) have recently been shown to demonstrate critical roles in differentiation, proliferation and migration of vascular smooth muscle cells (VSMCs).

Methods and Results: In this study, a certain amount of miRNA expression in VSMCs was evaluated by real-time polymerase chain reaction, and it was found that microRNA-30e (miR-30e) was expressed more strongly than other common vascular well-expressed miRNAs in vitro. Subsequently, both a gain and loss of function study was performed in vitro and in vivo. It was found that miR-30e in VSMCs was strongly downregulated concomitantly with stimulation, and miR-30e inhibited VSMCs proliferation and migration both in vitro and in vivo. Furthermore, ubiquitin-conjugating enzyme E2I (Ube2i) was identified as the target gene of endogenous miR-30e by luciferase reporter assay, and it was confirmed that overexpression of miR-30e significantly reduced Ube2i and inhibited the phenotypic switch of VSMCs. Knockdown of Ube2i had an influence over the proliferation and migration of cultured VSMCs, as same as the miR-30e mimic did. Overexpression of miR-30e induced the apoptosis of VSMCs and deregulated the protein expression of IκBa, which is crucial for the NFκB signal pathway.

Conclusions: The results of this study indicated that miR-30e in VSMCs exerted an anti-atherosclerosis effect via inhibiting proliferation and migration, and promoting apoptosis of VSMCs. More specifically, it was demonstrated that miR-30e exhibited these effects on VSMCs partially through targeting Ube2i and downregulating the IκBa/NFκB signaling pathway.

Key Words: Apoptosis; MicroRNA-30e; Proliferation/migration; Ubiquitin-conjugating enzyme E2I (Ube2i); Vascular smooth muscle cells (VSMCs)

Cardiovascular diseases have long been recognized as the leading contributors to the death rate in both industrialized societies and developing countries.1,2 Clinically, neointima is a common pathological lesion in a multitude of cardiovascular diseases such as atherosclerosis, coronary artery diseases, postangioplasty restenosis, and transplantation arteriopathy, as a result of the migration and proliferation of vascular smooth muscle cells (VSMCs) from the media. Aberrant VSMCs are pivotal for the pathogenesis of these diseases. Contrary to other terminally differentiated cells, VSMCs can proliferate and migrate in response to the altered homeostasis of vasculature,3,4 which is normally maintained in quiescence and equilibrium, whereas the capacities of VSMCs to proliferate and migrate are markedly enhanced by various growth factors and cytokines such as platelet-derived growth factor-BB (PDGF-BB), fibroblast growth factor, insulin-like growth factor-1, and tumor necrosis factor-α (TNF-α),5,6 as well as a number of genes and cytokines that have been identified as risk factors in these processes. Intriguingly, microRNAs (miRNAs) are functional transcription factors and have been implicated in the regulation of the proliferation and migration of VSMCs.7,8 MiRNAs are small, non-coding RNA molecules and negative regulators of gene expression via the degradation or translational inhibition of their target mRNAs, which involve a variety of pathological processes of atherosclerosis, leukemias, lung cancer, colorectal cancer, diabetes mellitus, etc.11-13 The expression of miRNAs are tissue- and cell-specific, involving a panorama of cell processes such as proliferation, development, migration, differentiation and apoptosis. As a group, miRNAs directly regulate at least 30% of the genomes in cells. Many miRNAs have recently been found to be critical for VSMC differentiation, proliferation and migration.7,8 It is likely that many others are involved in controlling such processes. Our goal was to identify the miRNAs involved in regulating the behavior of VSMCs.

MiR-1 is highly expressed both in cardiac and skeletal muscles, and is encoded by 2 genes (miR-1-1 and miR-1-2),
proliferation and migration by computational analysis. The analysis suggested that miR-30e may be bound to the 3'-UTR of ubiquitin-conjugating enzyme E2I (Ube2i) mRNA. Ube2i is an E2-conjugating enzyme essential for sumoylation, which was first identified as a human homolog of yeast UBC9 in a yeast two-hybrid system, which interacted with RAD52 and RAD51.

We proposed that Ube2i was involved in the regulation mechanism of miR-30e, and we aimed to investigate the role of Ube2i in the panorama of VSMC behavior.

In this study, we were determined to elucidate the effects and the target of miR-30e in the process of the proliferation, migration, and apoptosis of VSMCs.

**Methods**

**VSMC Culture and Carotid Artery Balloon Injury in Rats**

The studies were approved by the Animal Ethics
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Oligo Transfection in Cultured VSMCs and Adenovirus Transduction in Injured Carotid Artery

The miR-30e and miR-1 mimics or inhibitors (RiboBio) were added to the complexes for overexpressing or knocking down the miRNAs in vitro. The experiments in vitro were performed in the following groups: control group (CTR group), miR-30e mimic group, miR-30e inhibitor group, miR-30e mimic control group (miR-CTR group), miR-1 mimic group, miR-1 inhibitor group. Ube2i expression was knocked down using Ube2i siRNA (RiboBio) in vitro. Cells were transfected using a RiboFect™ CP Transfection Kit (RiboBio) according to the manufacturer’s protocol.

Rats were randomly selected for the control group (CTR group), miR-30e group, miR-30e antago group and the miR-30e scramble group (miR-CTR group) (each group, n=9). Adenovirus of miR-30e, miR-30e antago and adenovirus of miR-30e scramble (Hanbio) were transduction in injured carotid artery; the protocol of this experiment is described in a previous study.29 Rats were provided with water and food provided ad libitum for 14 days.

Results

Evaluation of the RNA Expressions of the miR-30 Family and Known Vascular Well-Expressed miRNAs

The levels of the miR-30 family were detected by RT-PCR separately. miR-30e is a member of the miR-30 family and
and miRNA expression, miRNA expression levels in VSMCs were measured at different times of proliferation. The VSMCs were stimulated with 10% serum at 0, 12, 24, 48 and 96 h after synchronization by 24-h serum-starvation-induced quiescence. Their proliferation was detected by Edu dyeing. At 24 h, the proliferating VSMCs were most numerous (Figure 2A, B). At the different time points after serum stimulation, miR-30e was significantly downregulated in the proliferating VSMCs compared with their quiescent counterparts at 0 h (Figure 2C). In contrast, the miR-1 levels remained unchanged (Figure 2D). Proliferating VSMCs have very low levels of miR-30e.

MiR-30e Regulation of VSMC Migration In Vitro

To confirm whether miR-30e also influences VSMC migration, we treated VSMCs with PDGF-BB at 0, 1, 2, 4, 6 and 8 h. PDGF-BB caused a time-dependent increase in VSMC migration (Figure 2E, F). To investigate the potential link between miR-30e and VSMCs migration, the expression of miR-30e and miR-1 was determined in non-migrating and migrating VSMCs; the latter being stimulated by PDGF-BB. As shown in Figure 3, the expression of miR-30e after 8 h in PDGF-BB-treated VSMCs was significantly lower than at 0 h, and the change was time dependent (Figure 2G). In contrast, miR-1 levels remained unchanged (Figure 2D). MiR-30e Regulation in Quiescent and Proliferating VSMCs In Vitro

To investigate the potential link between VSMC proliferation and miRNA expression, miRNA expression levels in VSMCs were measured at different times of proliferation. The VSMCs were stimulated with 10% serum at 0, 12, 24, 48 and 96 h after synchronization by 24-h serum-starvation-induced quiescence. Their proliferation was detected by Edu dyeing. At 24 h, the proliferating VSMCs were most numerous (Figure 2A, B). Both miR-30a–d and 384-5p did not change among the four groups. (Figure 1C–G). As shown in Figure 1H and I, we compared miR-30e with miR-1 and other known vascular well-expressed miRNAs, including miR-221, miR-222 and miR-200b, miR-200c.
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were marked with α-SMA. We also found that the phenotypic switch of VSMCs marked with α-SMA was decreased in de-differentiated VSMCs transfected with the miR-30e inhibitor (Figure 4A, B). VSMC differentiation was increased when migration decreased. Compared with the CTR group, mRNA levels of ACTA2 was decreased while mRNA levels of MYH11 was increased, and miR-30e mimic reversed these changes after incubation with PDGF-BB in the miR-CTR group (Figure 4C, D).

Identification of the Target Gene of MiR-30e in VSMCs

In order to identify the targets of miR-30e, we used TargetScan and identified Ube2i as a potential target for regulating VSMC proliferation and migration in vitro; it is upregulated in animal models of vascular injury. We built reporter plasmids containing potential miRNA binding sites of rat Ube2i at the position of the luciferase reporter gene (wt-Ube2i) or containing a mutated sequence of the miRNA binding site (mut-Ube2i); these were transfected into VSMCs stably overexpressing miR-30e or miR-1. wt-Ube2i with overexpressing miR-30e sequences displayed significantly lower luciferase activity than the empty vector or their respective mutated constructs (Figure 5A), indicating the functionality of putative Ube2i 3'UTR miR-30e seed sequences. Ube2i protein was upregulated in balloon-injured rat carotid arteries from 0 to 14 d (Figure 5B, C). In quiescent and proliferating VSMCs, Ube2i mRNA and protein were maximally enriched at the 24-h time point (Figure 5D, E). The mRNA level of Ube2i was higher than in controls after miR-30e inhibitor transfection, and lower after miR-30e mimic transfection (Figure 5F, G). Western blotting demonstrated that inhibition of miR-30e increased,
and overexpression of miR-30e decreased Ube2i protein levels in relation to controls in vitro and in vivo (Figure 5G, K, L). Changes in Ube2i mRNA and protein levels followed a trend opposite to that of miR-30e.

Knockdown of Ube2i Downregulated the Proliferation and Migration of VSMCs With Low NF-κB Levels

The siRNA-mediated knockdown reduced Ube2i mRNA expression to below the level of controls (Figure 6A). As the results revealed, proliferation and migration of VSMCs, as detected by Edu dyeing and Transwell chambers after stimulation with 10% serum and 20ng/mL PDGF-BB, were downregulated after knock-down of Ube2i (Figure 6B, C).

The protein levels of IκBα and P65 were different after miR-30e mimic transfection in cells stimulated by PDGF-BB (20ng/mL) for 8 h. The protein levels of IκBα and P65 were lower after miR-30e transfection (Figure 7A–C); this was also detected in the groups that had a knockdown of Ube2i (Figure 7D–F).

Knockdown of MIR-30e Inhibits Apoptosis in Cultured VSMCs In Vitro and In Vivo

The apoptosis-related proteins were tested by Western blotting, and the rate of cell apoptosis was detected by Annexin-V APC/PI double staining flow cytometry. The apoptosis-related proteins did not alter after incubation with PDGF-BB in the miR-CTR (negative control) group. Both the Bcl-2 and Bax expression levels were downregulated and upregulated, respectively, in the miR-30e mimic group compared with the CTR group. However, Bcl-2 and Bax
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Our studies provide significant novel insights into the critical importance and functional complexities of miRNAs in regulating VSMCs proliferation, migration and the development of vascular diseases. Previous publications have provided little information about miR-30e regulation of VSMC behavior.

MiR-30e is a member of the miR-30 family with which it shares similar targets: miR-30a/b/c/d/e and miR-384-5p. We observed that miR-30e was the most expressed member in VSMCs and other members of the miR-30 family were not implicated in following the stimulating by PDGF-BB. This study is to illustrate the essential role of miR-30e in VSMC proliferation, migration and apoptosis in vitro, substantiating miR-30e as a novel modulator. We found that the expression level of miR-30e in VSMCs was nearly 1000-fold that of miR-1, which has already been demonstrated experimentally to have only minor effects on VSMC proliferation. Therefore, it is necessary to compare miR-30e with other known vascular well-expressed miRNA, such as miR-221/222 (which increases during proliferation). We observed that the expression level of miR-30e was approximately close to that of miR-221, which is abundantly expressed in VSMCs.

We demonstrated that miR-30e expression is distinctly downregulated in proliferating and migrating VSMCs and proliferation and migration. Our studies provide significant novel insights into the critical importance and functional complexities of miRNAs in regulating VSMCs proliferation, migration and the development of vascular diseases. Previous publications have provided little information about miR-30e regulation of VSMC behavior.

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induced cell dysregulation; their exact functions remain to be elucidated. But mechanisms of some miRNAs were elucidated recently. For example, induction of miRNAs-146b by PDGF-BB is modulated via MAPK-dependent induction of c-fos.

MicroRNA-328 was found as a prominent downregulated microRNA, displaying a specific dose- and time-dependent downregulation by PDGF-BB treatment in pulmonary artery smooth muscle cells in which proliferation and migration were inhibited by microRNA-328. The decrease of microRNA-328 by PDGF-BB might be due to the increased expression of DNA methylation transferase 1 and DNA methylation.

We speculated that miR-30e may involve in the similar mechanism by reduction of PDGF-BB.

Recently, Liu et al. reported that miR-30c and miR-30e inhibits neointimal hyperplasia by targeting Ca²⁺/calmodulin-dependent protein kinase IIδ (CaMKIIδ). While one miRNA may have several target mRNAs, while one mRNA may have several miRNA regulators. We searched for evolutionarily conserved targets of miR-30e by TargetScan. After transient cotransfection with WT-Ube2i-3'UTR and

![Figure 8. miR-30e regulation in cultured vascular smooth muscle cells (VSMCs) apoptosis in vivo and in vitro. (A–D) Represents apoptosis-related proteins, Bcl-2, Bax and cleaved caspase-3 after platelet-derived growth factor-BB (PDGF-BB) stimulation. (E,F) The apoptosis rate was increased after transfection with the miR-30e mimic, but decreased after transfection with the miR-30e inhibitor. (G–I) Apoptosis-related proteins, Bcl-2, Bax and cleaved caspase-3 in vivo. *P<0.05, **P<0.01, ***P<0.001 vs. the control (CTR); ###P<0.001 vs. the miR-30e mimic. Data are expressed as the mean±SD (n=3).]
miR-30e mimic into cultured VSMCs, fluorescence was reduced. The luciferase reporter assay result proved that Ube2i is a direct target for miR-30e. Our study indicated that miR-30e is an important regulator of Ube2i expression, and Ube2i is a direct target for this miRNA. Meanwhile, we found that the trends of Ube2i levels were diametrically opposite to those of miR-30e, including in inhibiting VSMC proliferation and migration.

Ube2i modifies key enzymes in the inhibition of target protein degradation; maintaining and adjusting target protein levels are important for many processes. The first SUMO-modified protein implicated in NF-κB regulation is IκBα. This NF-κB inhibitor plays multiple critical roles in regulating both the initial activation of NF-κB and the duration of this activity in response to extracellular signals. Ube2i interacts with IκBα, and overexpression of a catalytically inactive mutant of Ube2i delays both IκBα degradation and NF-κB activation.47 However, in the regulation of VSMC proliferation and migration, its specific mechanism needs further research. In our study, balloon-injured vessels expressed different Ube2i levels at different days; expression was higher on later days. The thicker the neointima, the greater its content. Downregulation of Ube2i in VSMCs reduced the IκBα and NF-κB levels. We found that miR-30e inhibits VSMC proliferation and migration partially through targeting the transcription factor, Ube2i, and downregulating the IκBα/NF-κB signal pathway. Ube2i has also been demonstrated to be deregulated in cardiomyocytes, which are transfected with pre-miR-199a. Hence, Ube2i may be modulated by more kinds of miRNA in several cells.

miR-30e expression was substantially downregulated in proliferating and migrating VSMCs, and restoration of its expression markedly inhibited both proliferation and migration. We examined the effect of miR-30e on these transcription factors and found that modulation of miR-30e expression significantly affected the expression of Ube2i, a major regulator of IκBα and NF-κB expression.49,50 NF-κB is one of the known SUMO targets.49 Sumoylation inactivates NF-κB by conjugation with IκBα. Carbia-Nagashima et al. reported that RWD-containing sumoylation enhancer (RSUME) increased IκBα levels by promoting the sumoylation of IκBα, leading to an inhibition of NF-κB transcriptional activity and target expression. RSUME is induced by hypoxia and enhances the sumoylation of HIF-1α, promoting its stabilization and transcriptional activity during hypoxia.

Recently, miR-146a was demonstrated as regulator of the maturation and differentiation of VSMCs via targeting NF-κB gene.7 We speculated that VSMCs were modulated by several miRNAs targeting several signaling pathways.

In summary, the present study identifies that miR-30e exerts anti-atherosclerosis effects via inhibiting proliferation and migration and promoting apoptosis in VSMCs. More explicitly, we demonstrate that miR-30e inhibits the proliferation and migration of VSMCs partially by targeting the transcription factor, Ube2i, and downregulating the IκBα/NF-κB signaling pathway.

Acknowledgments

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Disclosures

None.

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