Original Article

MiR-21 suppresses endothelial progenitor cell proliferation by activating the TGFβ signaling pathway via downregulation of WWP1

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Abstract: Endothelial damage is strongly associated with cardiovascular diseases such as atherosclerosis. Bone marrow-derived endothelial progenitor cells (EPCs) play an important role in the maintenance of endothelial homeostasis and contribute to re-endothelialization of injured vessels as well as revascularization of ischemic tissues. MicroRNAs (miRNAs) have been reported to regulate EPC biological functions. In this study, we found that EPCs of atherosclerosis patients and EPCs exposed to hypoxia have increased expression of miRNA-21 (miR-21) as well as diminished ability to proliferate. MiR-21 knockdown rescued hypoxia-induced growth arrest in EPCs. Next, we used a luciferase reporter assay to demonstrate that miR-21 downregulates the expression of WW domain-containing protein 1 (WWP1), a negative regulator of TGFβ signaling, by directly targeting the 3’-UTR of WWP1. Finally, miR-21 overexpression or WWP1 knockdown in EPCs significantly activates the TGFβ signaling pathway and inhibits cell proliferation. Taken together, our results indicate that miR-21 suppresses EPC proliferation by activating the TGFβ signaling pathway via downregulation of WWP1. These findings may help the development of strategies to enhance the vitality of EPCs for therapeutic applications.

Keywords: Atherosclerosis, endothelial progenitor cells (EPCs), microRNA-21 (MiR-21), WWP1, TGFβ signaling pathway

Introduction

Endothelial damage is strongly associated with cardiovascular diseases such as atherosclerosis, thrombosis, and hypertension [1]. Bone marrow-derived endothelial progenitor cells (EPCs) play an important role in the maintenance of endothelial integrity and homeostasis, and contribute to re-endothelialization of injured vessels as well as revascularization of ischemic tissues [2]. The number of EPCs is inversely associated with cardiovascular risk factors and vascular function, and independently predicts clinical outcomes of cardiovascular diseases [3]. The therapeutic potential of EPC transplantation in rescue of tissue ischemia has been demonstrated in a few small-scale trials; however, the relative scarcity of circulating EPCs limits the ability to expand these cells in sufficient numbers for some therapeutic applications [4, 5]. Therefore, the development of strategies to enhance proliferation and improve the function of EPCs is key to the success of EPC therapy.

Transforming growth factor-β (TGFβ) is a multifunctional cytokine that controls growth, differentiation, and other functions in most cells. Activation of TGFβ type I receptor leads to phosphorylation of Smads 2 and 3, which, together with Smad4, are translocated into the nucleus to regulate gene transcription. The inhibitory Smad (Smad7) negatively regulates TGFβ signaling by binding to activated type I receptor to cause its ubiquitination and degradation [6]. TGFβ is highly expressed in human atherosclerotic and vascular restenosis lesions [7, 8]. Experimental results indicate that TGFβ exerts both protective and harmful effects on the cardiovascular system through its functional regu-
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Figure 1. MiR-21 is upregulated in EPCs of atherosclerosis patients. A. EPCs under a phase-contrast microscope (100 ×). B. Expression of stem cell markers CD34 and CD133 and the endothelial lineage marker KDR by flow cytometric analysis. C. Cell proliferation by MTT assay (n = 3). D. MiR-21 expression by quantitative real-time PCR (n = 3). *P < 0.05, **P < 0.01 vs. normal controls.

lation of endothelial cells, vascular smooth muscle cells, macrophages, and T lymphocytes [9]. A few recent studies suggest that TGFβ also regulates the function of circulating EPCs. In EPC-seeded tissue engineering scaffolds, TGFβ1 promotes endothelial to mesenchymal cell type transformation as well as extracellular matrix production and tissue formation [10]. In isolated EPCs in culture, TGFβ1 antagonizes pioglitazone-induced cell adhesion and differentiation [11]. However, the effects of TGFβ on the proliferation of circulating EPCs are largely unclear.

WW domain-containing protein 1 (WWP1), an E3 ubiquitin ligase, interacts with Smad7 and enhances the binding of Smad7 to TGFβ type I receptor. Thus WWP1 inhibits TGFβ signaling in
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Figure 2. Sequestration of miR-21 attenuates hypoxia-induced growth inhibition of EPCs. Rat EPCs were transfected with 20 nM anti-miR-control or anti-miR-21 and cultured under normoxic (20% O₂, control) or hypoxic (1% O₂, hypoxia) conditions. (A) MiR-21 expression by quantitative real-time PCR (n = 3), *P < 0.05. (B) Cell proliferation by MTT assay (n = 3), *P < 0.05 vs. all other groups.

cooperation with Smad7 [12]. WWP1 is a putative target of microRNA-21 (miR-21) [13], an miRNA overexpressed in many cancers and associated with tumor growth and metastasis [5]. Studies have indicated cell type-specific effects of miR-21 on neovascularization. While miR-21 expression in cancer cells promotes tumor-associated angiogenesis in vivo [14, 15], miR-21 expression in endothelial cells reduces endothelial cell proliferation, migration, and in vitro angiogenesis [16]. Interestingly, a recent study links miR-21 to aging-associated senescence in EPCs [17]. MiR-21 overexpression in young EPCs causes cell senescence while miR-21 silencing rejuvenates EPCs and improves EPC angiogenesis in vitro and in vivo [17].

In this study, we examined miR-21 expression in EPCs of atherosclerosis patients and EPCs exposed to hypoxia. We then investigated the regulatory relationship between miR-21 and WWP1. Finally, we studied the role of miR-21, TGFβ signaling, and their interplay in the regulation of EPC proliferation. Our results may help the development of new strategies to enhance EPC vitality for therapeutic applications.

Materials and methods

Patients

Blood samples were collected from atherosclerosis patients admitted to Shanghai Tenth People’s Hospital and healthy volunteers. All study protocols were approved by the Research Ethics Board of the Shanghai Tenth People’s Hospital. All patients and healthy volunteers gave informed consent.

Isolation of EPCs

Human and rat EPCs were isolated as previously described [18]. Briefly, human EPCs were isolated using a Becton-Dickinson FACS Aria cytometer (BD Biosciences, America). Rat EPCs were obtained from rat bone marrow mononuclear cells (BMMCs) as follows: Rat BMMCs were isolated from bone marrow cell suspension by density gradient centrifugation utilizing Ficoll-Isopaque (Sigma, USA) according to the manufacturer’s instructions. The BMMCs obtained were seeded in six-well plates and cultured in M199 medium supplemented with 10 ng/ml vascular endothelial growth factor (VEGF, PeproTech, USA), 2 ng/ml basic fibroblast growth factor (bFGF, PeproTech), and 10% fetal bovine serum (FBS, Hyclone, USA) at 37°C, 5% CO₂ for 3 days. Nonadherent cells were removed by washing with phosphate-buffered saline (PBS), and adherent cells were collected and cultured in fresh supplemented M199 medium for 7 days. The identity of EPCs was confirmed by the expression of stem cell markers CD34 and
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Figure 3. MiR-21 negatively regulates WWP1 expression in EPCs by directly targeting the 3'-UTR of WWP1 gene. A. The luciferase reporter construct containing the wild type or mutant 3'-UTR of WWP1. WWP1-WT-UTR, sequence of the putative miR-21 binding site; WWP1-MUT-UTR, sequence of the mutant miR-21 binding site. B. Luciferase reporter activity in rat EPCs 2 days post transfection (n = 3). C. MiR-21 expression in rat EPCs (control) and rat EPCs transfected with miR-21 or miR-control (n = 3). D. WWP1 mRNA expression in rat EPCs (control) and rat EPCs transfected with miR-21 or miR-control (n = 3). E. WWP1 protein expression in rat EPCs (control) and rat EPCs transfected with miR-control, miR-21, siNC, or siWWP1 by western blotting. **P < 0.01 vs. miR-control.

Cell proliferation under hypoxic and normoxic conditions

Rat EPCs were seeded in 96-well plates and transfected with 20 nM scramble microRNA (miR-control) or 20 nM anti-miR-21 (Ambion, USA) using Lipofectamine 2000 (Invitrogen, USA) according to the manufacturer’s instructions. After the transfection, cells were cultured in supplemented M199 medium at 37°C under normoxic (20% O2) or hypoxic (1% O2) conditions for up to 7 days. To determine cell viability, cells were incubated for 4 h with 5 mg/ml MTT (Sigma) solution. The MTT solution was removed, and 150 µl of sterile DMSO (Sigma) was added to each well to dissolve the formazan crystals by incubation at 37°C for 15 min. Absorbance at 570 nm was recorded on an Easy Reader 340 AT (SLT-Lab Instruments), and a growth curve for the cells was established. Experiments were performed in triplicate.

RNA extraction and quantitative real-time PCR

Total RNA was extracted from EPCs using Trizol reagent (Invitrogen) according to the manufacturer’s instructions. RNA was reversely transcribed to cDNA using stem-loop primers and the TaqMan MicroRNA Reverse Transcription Kit (Applied Biosystems, USA). MiR-21 and WWP1 expressions were determined by quantitative real-time PCR using the SYBR Green Master Mix kit (Roche Diagnostics, USA) on an ABI PRISM 7300 system (Applied Biosystems). U6 and GAPDH were used as internal controls for miR-21 and WWP1 expression, respectively. Primers for hsa-miR-21 and U6 were purchased from Applied Biosystems. Primers for WWP1 and GAPDH were designed as follows: rat WWP1: sense, 5'-ACGATACCTCTATTCGGCTT-3';
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anti-sense, 5'-CGGGACACATTGATCTTTAC-3'. Rat GAPDH: sense, 5'-CCATCACTGCCACTCAG-3’; antisense, 5'-ATACATTGGGGGTAGGAACAC-3’.

Luciferase reporter assay

The full-length 3’-UTR of rat WWP1 gene containing the putative miR-21 binding site (WWP1-WT-UTR) was amplified from rat genomic DNA by PCR. The 3’-UTR of rat WWP1 containing a mutant miR-21 binding site (WWP1-MUT-UTR) was created using the QuikChange Site-Directed Mutagenesis Kit (Stratagene, USA). WWP1-WT-UTR and WWP1-MUT-UTR were subcloned into the pGL3-Basic plasmid (Promega, America) at the XbaI site (WWP1-WT and WWP1-MUT, respectively). Rat EPCs were seeded in 96-well clusters and co-transfected with 10 nM miR-21 mimic or miR-control mimics (Ambion) and 10 ng of WWP1-WT or WWP1-MUT using Lipofectamine 2000. Luciferase activity was detected 48 h after transfection using a dual luciferase reporter assay system (Promega) and normalized to Renilla activity.

MiR-21 overexpression and WWP1 silencing

To study the effects of miR-21 and WWP1 on TGFβ signaling and cell proliferation of EPCs, rat EPCs were plated into 6-well plates (2.5 × 10^5 cells/well), incubated overnight, and transfected with 10 nM miR-21 mimic or scramble siRNA (siNC) or WWP1 siRNA (siWWP1) using Lipofectamine 2000 (Invitrogen). A scramble microRNA (miR-control) and a scramble siRNA (siNC) were used as negative controls. MiR-21 and miR-control were purchased from Ambion, and siWWP1 and siNC were from OriGene Technologies, Inc., USA.

Western blotting

Cells were washed, collected, and lysed on ice for 30 min in modified radioimmune precipitation assay buffer (Applygen Technologies Inc., Beijing, China) containing a protease inhibitor cocktail (Fermentas, USA). After brief sonication, cell lysates were centrifuged at 15,000 g, 4°C for 10 min. The supernatant was collected, and the protein concentration was determined using the BCA Protein Assay kit (Pierce, USA). Samples (50-80 μg total protein) were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (10-15%) and transferred to nitrocellulose membranes (Millipore, USA). After blocking with 5% nonfat dry milk in TBST (10 mM Tris-Cl, pH 7.5, 150 mM NaCl, 0.05% Tween 20) for 1 h, the membranes

Figure 4. MiR-21 overexpression or WWP1 knockdown inhibits EPC proliferation and activates TGFβ signaling. Rat EPCs were transfected with miR-21 mimic (miR-21), scramble microRNA (miR-control), WWP1 siRNA (siWWP1) or scramble siRNA (siNC). A. Cell proliferation by MTT assay (n = 3). *P < 0.05 vs. miR-control. B. Levels of key proteins involved in TGFβ signaling by western blotting.
were probed with antibodies against WWP1, TGFβ receptor 1 (TβR1), Smad4, Smad2, p15, and GAPDH, respectively, by overnight incubation at 4°C. After washing with TBST, the membranes were incubated with HRP-conjugated secondary antibodies (Zhongshan Biotechnologies Inc., China) at room temperature for 1 h. Protein bands were visualized using chemiluminescent substrate (Millipore, USA). The antibody against WWP1 was purchased from Abnova, USA. The antibodies against TβR1 and Smad4 were from Cell Signaling, USA. The antibodies against Smad2, p15, and GAPDH were from Santa Cruz Biotechnology, USA.

**Statistical analysis**

All data are presented as mean ± standard derivation (SD). The Student’s t-test was used to compare results from different groups. Differences with a P value less than 0.05 were considered statistically significant.

**Results**

**MiR-21 expression is upregulated in EPCs of atherosclerosis patients and EPCs exposed to hypoxia**

Circulating EPCs were isolated from peripheral blood of atherosclerosis patients and healthy controls using a Becton-Dickinson FACS Aria cytometer. EPCs of patients and control subjects displayed similar cell morphology under a microscope (Figure 1A). In addition, both EPCs expressed stem cell markers CD34 and CD133, and the endothelial lineage marker KDR (Figure 1B). However, compared with EPCs of healthy controls, EPCs of atherosclerosis patients showed significantly higher expression of miR-21 (P < 0.01, Figure 1D) as well as decreased growth rate in culture (P < 0.05, Figure 1C), suggesting that miR-21 may have a regulatory role in the ability of EPC to proliferate. Hypoxia is an important microenvironmental factor influencing atherosclerosis progression. We subsequently studied the effects of hypoxia treatment on EPCs isolated from rat BMMCs. We found that exposure to hypoxia significantly increased miR-21 expression and decreased cell proliferation of rat EPCs (Figure 2A, 2B). Sequestration of miR-21 in rat EPCs reversed hypoxia-induced growth inhibition (Figure 2B). Taken together, these data suggest that increased miR-21 expression suppresses EPC proliferation under hypoxic conditions.

**MiR-21 directly regulates WWP1 expression by targeting the 3′-UTR of WWP1**

WWP1, a negative regulator of TGFβ signaling, is a putative target of miR-21 [19]. WWP1 is downregulated in human pulmonary artery smooth muscle cells overexpressing miR-21, and is implicated in the regulation of hypoxia-induced pulmonary vascular remodeling by miR-21 [20]. To test whether WWP1 is a direct target of miR-21 in EPCs, we prepared a luciferase reporter expression system using the 3′-UTR of rat WWP1 containing the putative miR-21 binding site (WWP1-WT-UTR) (Figure 3A). We also created a luciferase expression system using 3′-UTR of rat WWP1 containing a mutant miR-21 binding side (WWP1-MUT-UTR) (Figure 3A). Co-transfection of the luciferase reporter system containing WWP1-WT-UTR (WWP1-WT) with miR-21 in rat EPCs resulted in approximately 50% loss of luciferase reporter expression compared with miR-control (P < 0.01, Figure 3B). However, luciferase expression was not affected by co-transfection with miR-21 when WWP1-WT-UTR was replaced with WWP1-MUT-UTR in the luciferase reporter system (Figure 3B). Further, miR-21 overexpression in rat EPCs significantly suppressed mRNA and protein expression of WWP1, similar to the results obtained with siWWP1 transfection (Figure 3C-E). Taken together, these results indicate that miR-21 negatively regulates WWP1 expression in EPCs by directly targeting the 3′-UTR of the WWP1 gene.

**MiR-21 inhibits EPC proliferation by activating the TGFβ signaling pathway**

Having shown that sequestration of miR-21 reversed hypoxia-induced growth inhibition in rat EPCs, we investigated the effects of miR-21 overexpression on EPC growth under normoxic conditions. We found that rat EPCs transfected with miR-21 proliferated at a significantly slower rate than EPCs transfected with miR-control (P < 0.05, Figure 4A). Meanwhile, siWWP1 transfection, which resulted in similar loss in WWP1 expression, caused similar decrease in EPC growth rate (Figure 4A). These data suggest that the inhibitory effects of miR-21 on EPC proliferation are mediated by WWP1 downregulation. WWP1 is a negative regulator of TGFβ signaling. WWP1 works with Smad7 to cause degradation of TβR1 and inhibits TGFβ-induced phosphorylation of Smad2 [12]. TGFβ can induce antiproliferative gene responses in...
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many cell types including endothelial cells [21]. Therefore, we speculated that the inhibitory effects of miR-21 on EPC growth are mediated by activation of the TGFβ signaling pathway following WWP1 downregulation. To test this hypothesis, we determined the level of key proteins involved in TGFβ signaling by western blotting. Our results showed that miR-21 or siWWP1 transfection of EPCs led to increased protein levels of TβR1, Smad2, Smad4, and the CDK inhibitor p15, a downstream target molecule of TGFβ (Figure 4B). Taken together, our findings indicate that the inhibitory effects of miR-21 on EPC proliferation are mediated by WWP1 downregulation and subsequent activation of the TGFβ signaling pathway.

Discussion

In this study, we found that EPCs of atherosclerosis patients and EPCs exposed to hypoxia have increased expression of miR-21 as well as diminished ability to proliferate. MiR-21 knockdown rescued hypoxia-induced growth arrest in EPCs. These findings indicate that miR-21 likely plays a significant role in diminished vitality of EPCs under pathologic cardiovascular conditions such as atherosclerosis. Next, we examined the regulatory relationship between miR-21 and WWP1, a negative regulator of TGFβ signaling, using a luciferase reporter assay. Co-transfection of miR-21 with a luciferase reporter system containing the 3'-UTR of WWP1 resulted in suppression of luciferase expression; however, the luciferase activity was not affected when the wild type 3'-UTR was replaced with a 3'-UTR containing mutations at the putative miR-21 binding site. Further, the mRNA and protein expression of WWP1 was downregulated in EPCs overexpressing miR-21. These data indicate that WWP1 is a direct target of miR-21 in EPCs. Finally, miR-21 overexpression or WWP1 knockdown in EPCs significantly activates the TGFβ signaling pathway and inhibits cell proliferation. Taken together, our results suggest that the inhibitory effects of miR-21 on EPC proliferation are mediated by downregulation of WWP1 and subsequent activation of the TGFβ signaling pathway.

A number of studies have reported the regulation of EPC function by miRNAs. For example, several angiogenesis-related microRNAs are dysregulated in EPCs of patients with coronary artery diseases [22]. MiR-107 expression is increased in EPCs exposed to hypoxia, and upregulation of miR-107 antagonizes hypoxia-induced EPC differentiation by targeting hypoxia-inducible factor-1 [23]. MiR-221 expression is significantly upregulated in EPCs of patients with coronary artery disease, and overexpression of miR-221 decreases EPC proliferation by downregulating Cdc42/Rac1-activated kinase 1 (PAK1) [18]. Specifically, miR-21 and miR-10A* have been reported to modulate EPC senescence via suppressing high-mobility group A2 [17]. Our findings suggest that upregulation of miR-21 possibly contributes to the reduced availability of EPCs in people with cardiovascular diseases. In addition, our results indicate that miR-21, TGFβ, and their interplay have a significant role in the regulation of EPC proliferation. The effects of miR-21 and TGFβ on other functions of EPC such as adhesion and differentiation remain to be investigated.

Studies have shown that the number of EPCs can be increased by priming with specific growth factors and genetic modifications, as well as lifestyle modifications and pharmaceutical therapies [24, 25]. In this study, we identified a new mechanism regulating the proliferation of EPCs, which may be exploited to enhance the vitality of EPCs for therapeutic applications.

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Disclosure of conflict of interest

None.

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References

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[22] Zhang Q, Kandic I and Kutryk MJ. Dysregulation of angiogenesis-related microRNAs in endo-
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