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Original Article

Silent information regulator 1 (*SIRT1*) promotes the migration and proliferation of endothelial progenitor cells through the PI3K/Akt/eNOS signaling pathway

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Abstract: Silent information regulator 1 (*SIRT1*) mediates many effects of caloric restriction (CR) on an organism's lifespan and metabolic pathways. Recent reports have also emphasized its role in vascular function. The present study was designed to investigate the effects of *SIRT1* on the properties of mouse spleen derived endothelial progenitor cells (EPCs). *SIRT1* in EPCs was significantly increased by serum and by vascular endothelial growth factor (VEGF). Moreover, an adenovirus (Ad) vector expressing *SIRT1* (Ad-*SIRT1*)-mediated overexpression of *SIRT1* directly enhanced migration and proliferation of EPCs, whereas silencing of endogenous *SIRT1* in EPCs inhibited cell functions. In addition, LY294002 (a PI3K inhibitor), sc-221226 (an Akt inhibitor), and L-NAME (an NOS inhibitor) abolished Ad-*SIRT1*-induced migration and proliferation of EPCs, and prevented nitric oxide (NO) production. Phosphorylation of Akt, PI3K, and endothelial nitricoxide synthase (eNOS) were up-regulated by Ad-*SIRT1*, which was attenuated by LY294002, sc-221226, and L-NAME. Together, the results suggested that through the PI3K/Akt/eNOS signaling pathway, *SIRT1* plays an important role in the biological properties of EPCs.

Keywords: Silent information regulator 1, endothelial progenitor cells, migration, proliferation

Introduction

Enhancement of re-endothelialization plays an important role in the repair of injured blood vessels. Endothelial progenitor cells (EPCs) have the capacity to proliferate and differentiate into mature endothelial cells, which facilitate repair of injured blood vessels [1, 2]. EPCs can migrate to sites of injury and differentiate into endothelial cells (ECs), to eventually participate in the re-endothelialization after vascular injury [3-5]. However, the regulatory mechanisms of the migration and proliferation of EPCs in re-endothelialization after vascular injury remain unclear. It was reported that one of the sirtuins, silent information regulator 1 (*SIRT1*), is responsible for maintenance of vascular endothelial cell homoeostasis [6-8], and was shown to exert anti-atherosclerotic effects against EPC dysfunction [9, 10].

The sirtuins are a highly conserved family of NAD^+-dependent histone deacetylases that help regulate the lifespan of diverse organisms. It has been reported that sirtuins are associated with age-related diseases, obesity-associated metabolic diseases, and cardiac aging [11]. The human genome encodes seven different sirtuins (*SIRT1*-7), which share a common catalytic core domain, but possess distinct N-terminal and C-terminal extensions. Of the seven mammalian sirtuin proteins, *SIRT1* has been the most extensively characterized. *SIRT1* regulates a variety of physiological functions, such as metabolism, senescence, and differentiation in multiple cell types. It is highly expressed in the vasculature during blood vessel growth and controls the angiogenic activity of EPCs. Recently, *SIRT1* has been shown to be a key component of EPC dysfunction in metabolic syndrome and re-endothelialization after vascular injury [12], although, the signaling mechanisms responsible for these *SIRT1*-mediated EPC functions have not been determined.

Activations of the survival signal PI3K/Akt pathway and the endothelial specific eNOS/NO pathway are closely associated with vascular

remodeling and angiogenesis [13-15]. PI3K/Akt activation induced by pro-angiogenic factors has been shown to participate in the proliferation and migration of EPCs [16]. NO plays critical roles in EPC migration and proliferation. It is mainly produced by endothelial nitricoxide synthase (eNOS), and endothelial dysfunction is characterized by a loss of NO bioavailability. Furthermore, VEGF increased EPC survival and angiogenesis by promoting Akt-dependent eNOS phosphorylation and NO production [14].

In this study, we identified the effects of *SIRT1* on the migration and proliferation of EPCs, in cultured mouse spleen-derived EPCs. In addition, we provided evidence that the biological properties of EPCs are mediated through the PI3K/Akt/eNOS pathway.

Materials and methods

Ethics statement

All experimental procedures were approved by the Ethics Committee of the 305 Hospital of PLA (Beijing, P. R. China).

Isolation and characterization of EPCs

Culture and characterization of EPCs were done as previously described by Werner N et al [5]. Spleens were explanted from C57BL/6 mice (6 to 8 weeks of age, 20 to 25 g of weight, Beijing, P. R. China). Total spleen-derived mononuclear cells were isolated using a Ficoll gradient (Lympholite-M, Cedarlane). After three washing steps, 4×10^6 spleen-derived mononuclear cells were seeded on fibronectin-coated cell culture flasks and re-suspended in 6 ml endothelial basal medium (Cell Systems) supplemented with 1 μg/ml hydrocortisone, 3 μg/ml bovine brain extract, 30 μg/ml gentamicin, 50 μg/ml amphotericin B, 10 μg/ml human endothelial growth factor, and 20% fetal calf serum (FCS). The harvested cells were cultured at 37°C under an atmosphere of 5% CO_2. Forty-eight hours later, non-adherent cells were removed, and the adherent cells were cultured continuously. Only adherent cells were used in further experiments. The medium was changed every second day. For characterization, after 4 days in culture, the cells were incubated with 10 mg/ml acetylated low-density lipoprotein/binding (Dil-Ac-LDL, Invitrogen, CA, USA) for 4 h, fixed with 4% paraformaldehyde and then incubated with 10 mg/ml fluorescein isothiocyanate-Ulex Europeaus lectin-1 (UEA-1, Sigma-Aldrich, St Louis, MO, USA) for 1 h. Finally, the cells were incubated with 1 μg/ml 4',6-diamidino-2-phenylindole (DAPI, Life Technologies, NY, USA) for 5 min. Triple-stained cells positive for Dil-Ac-LDL, lectin, and DAPI, were identified as EPCs. Additionally, fluorescence activated cell sorting (FACS) analysis was performed using the following monoclonal antibodies: FITC conjugated anti-Sca-1 (Abcam, Cambridge, MA, USA), PE conjugated anti-VEGFR-2 (Biosciences, San Diego, CA, USA), or their corresponding isotype controls (Biosciences, San Diego, CA, USA).

Recombinant adenoviral vectors expressing SIRT1

In order to evaluate the role of *SIRT1*, adenovirus vector expressing *SIRT1* was generated using the pAd-Easy system. Briefly, full-length murine *SIRT1* cDNA was first TA-cloned into pMD19-T simple vector and then subcloned into pAdTrack-CMV, resulting in pAdTrack-*SIRT1*. The shuttle vector was used to generate recombinant adenovirus Ad-*SIRT1* according to the manufacturer's protocol. All PCR-amplified fragments and cloning junctions were verified by DNA sequencing (Sangon, Shanghai, China). An adenovirus encoding green fluorescent protein (GFP; Ad-GFP) was used as control. All adenoviruses were replication deficient and used at 20 multiplicity of infection (mois) for 24 h without apparent cytotoxicity.

Small interfering RNA-mediated silencing of SIRT1 expression

Transient silencing of *SIRT1* was accomplished by transfection with small interfering RNAs (si-*SIRT1*). The selected siRNA duplex sequences specifically targeted mouse *SIRT1* (GenBank accession number NM_019812.2), and showed no homology to any other sequences, as determined by a blast search. A non-silencing control (si-CON) sequence was designed to be used as a negative control. Transfection of si-*SIRT1* used the Lipofectamine 2000 reagent with a molar ratio between DNA and lipid of approximately 1:3. Forty-eight hours after transfection, cells were collected and used for functional assays.

Reverse transcription-PCR (RT-PCR)

Total RNA was extracted from EPCs using TRIzol (Life Technologies, NY, USA), followed by cDNA synthesis using oligo (dT) and M-MLV reverse

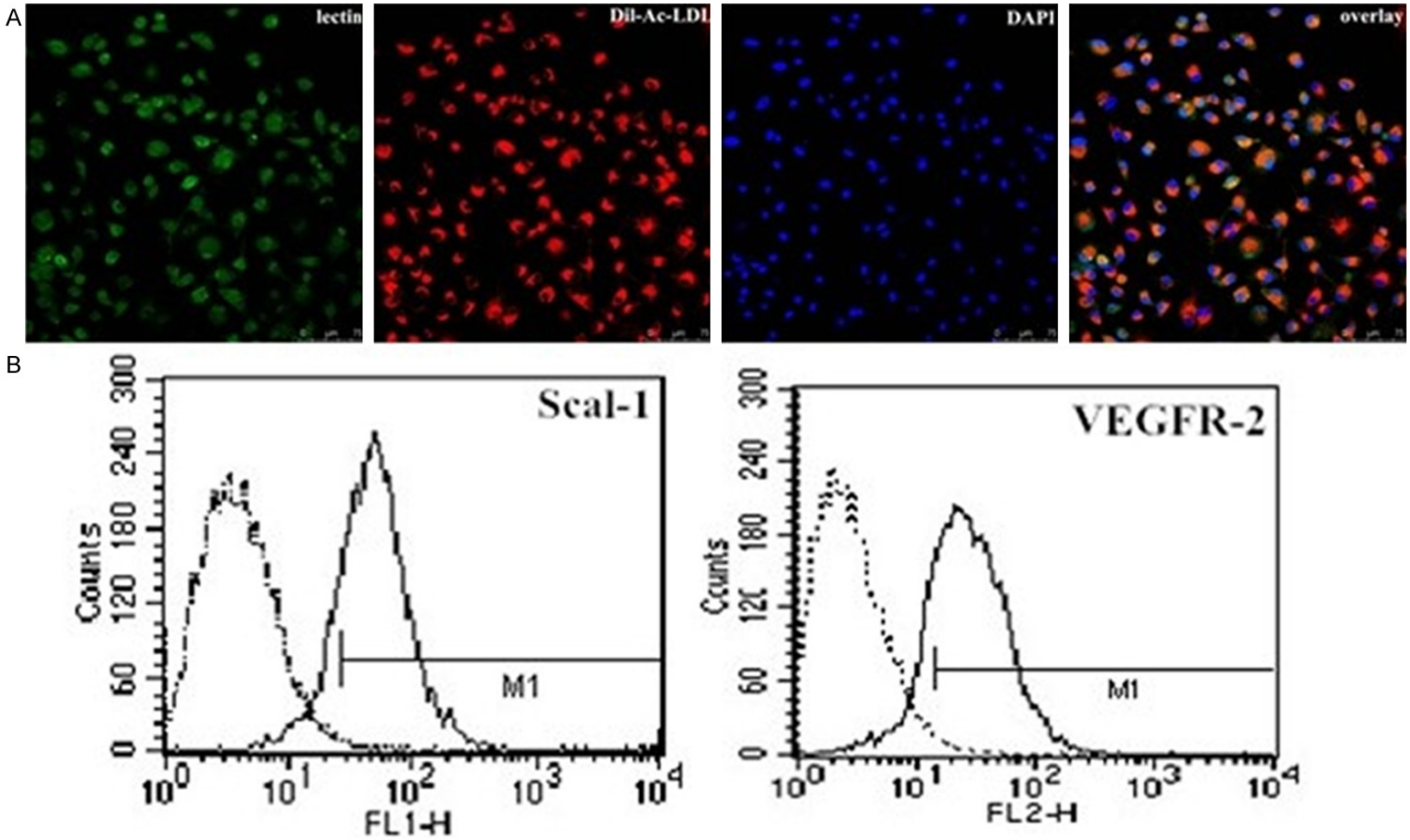

Figure 1. The isolation and characterization of EPCs. A. EPCs stained with Dil-Ac-LDL, lectin, and DAPI. B. FACS analysis of primary EPCs cultured for 5-7 days. FACS analysis of cultured EPCs for FITC-Sca-1 and VEGFR2, representing a stem/progenitor cell marker and endothelial cell marker, respectively. Positive cells were 81.53 ± 3.96% (n = 3) for Sca-1 expression and 49.72 ± 2.58% (n = 3) for VEGFR2 expression. The left peak in each box denotes corresponding negative isotype control labeling, and the positive gate M1 is shown.

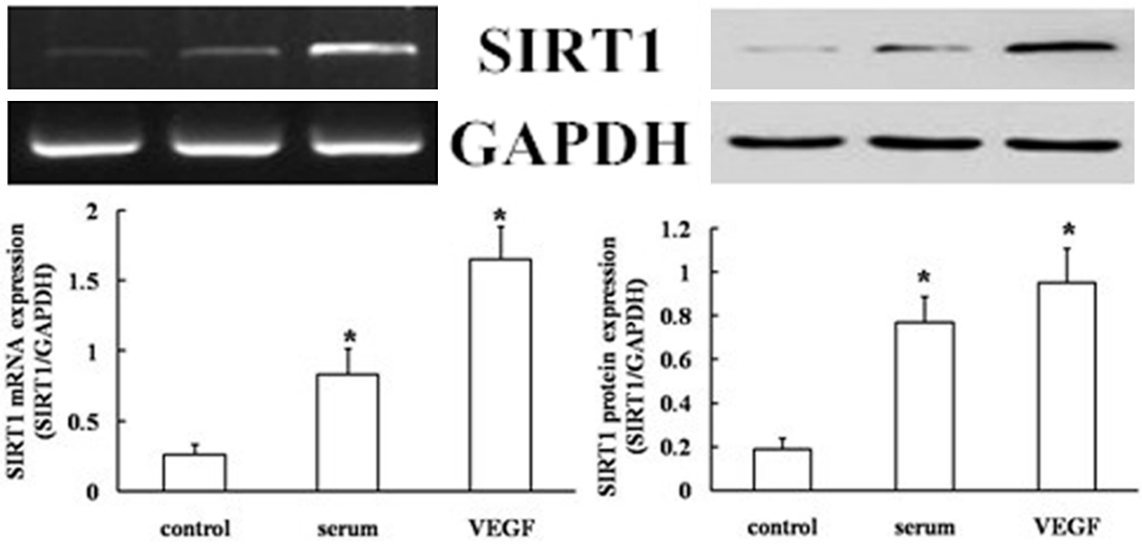

Figure 2. *SIRT1* expression in EPCs. Protein and mRNA levels of *SIRT1* in EPCs using western blotting and RT-PCR analysis. Values are the percentage of GAPDH (serum- and VEGF-free, null treatment). Representative images from semi-quantitative RT-PCR and western blots. Data are expressed as mean ± SD of three independent experiments done in triplicate, with $*P < 0.05$ compared with the controls.

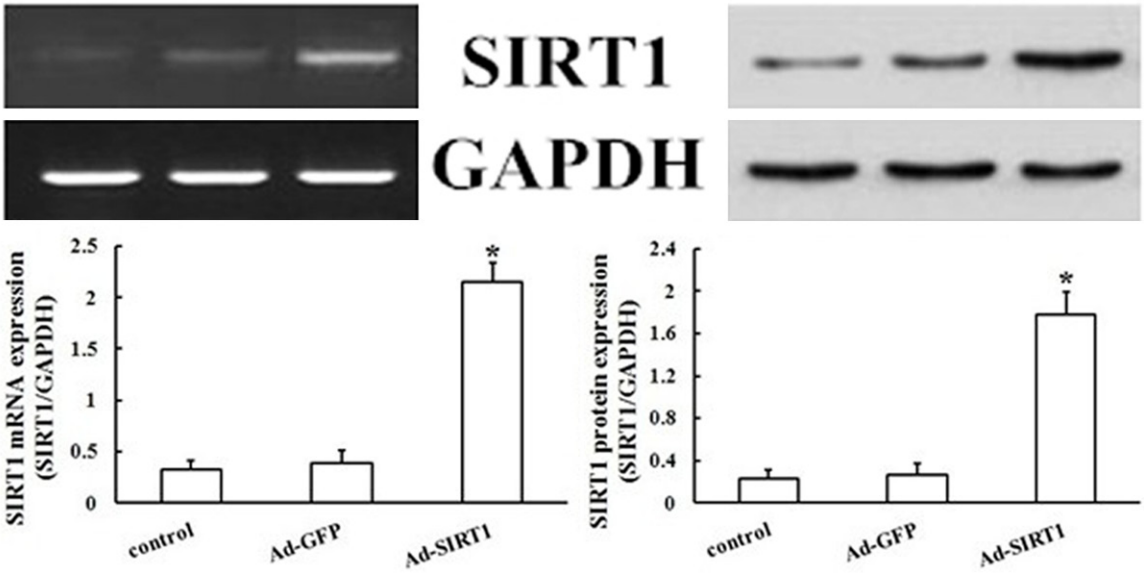

Figure 3. Effect of *SIRT1* overexpression on levels of *SIRT1*. Changes in levels of protein and mRNA of *SIRT1* were detected by overexpression of *SIRT1* in EPCs using western blotting and RT-PCR analysis. Values are the percentage of GAPDH. *SIRT1* gene and protein levels were increased by *SIRT1* overexpression, whereas Ad-GFP levels were not affected ($*P < 0.05$ vs. Ad-GFP).

transcriptase (Takara, Dalian, China), according to the manufacturer's instructions. For quantitative RT-PCR analyses, the ABI PRISM 7000 Sequence Detection System (Applied Biosystems, Foster City, CA, USA) and SYBR Green PCR Master Mix (Takara, Dalian, China) were used with the following primers: SITR1 sense: 5'-ACTGCAGAAACTTTTAGCCTTTCAA-3'; *SIRT1* antisense: 5'-GGCAATGTTCCAAAGAAGTCTGT-3'; GAPDH sense: 5'-TGAACGGGAAGCTCACTGG-3'; GAPDH antisense: 5'-GCTTCACCACCTTCTTGATGTC-3'. All primers were synthesized by Invitrogen (Shanghai, China) and were the highest available purity.

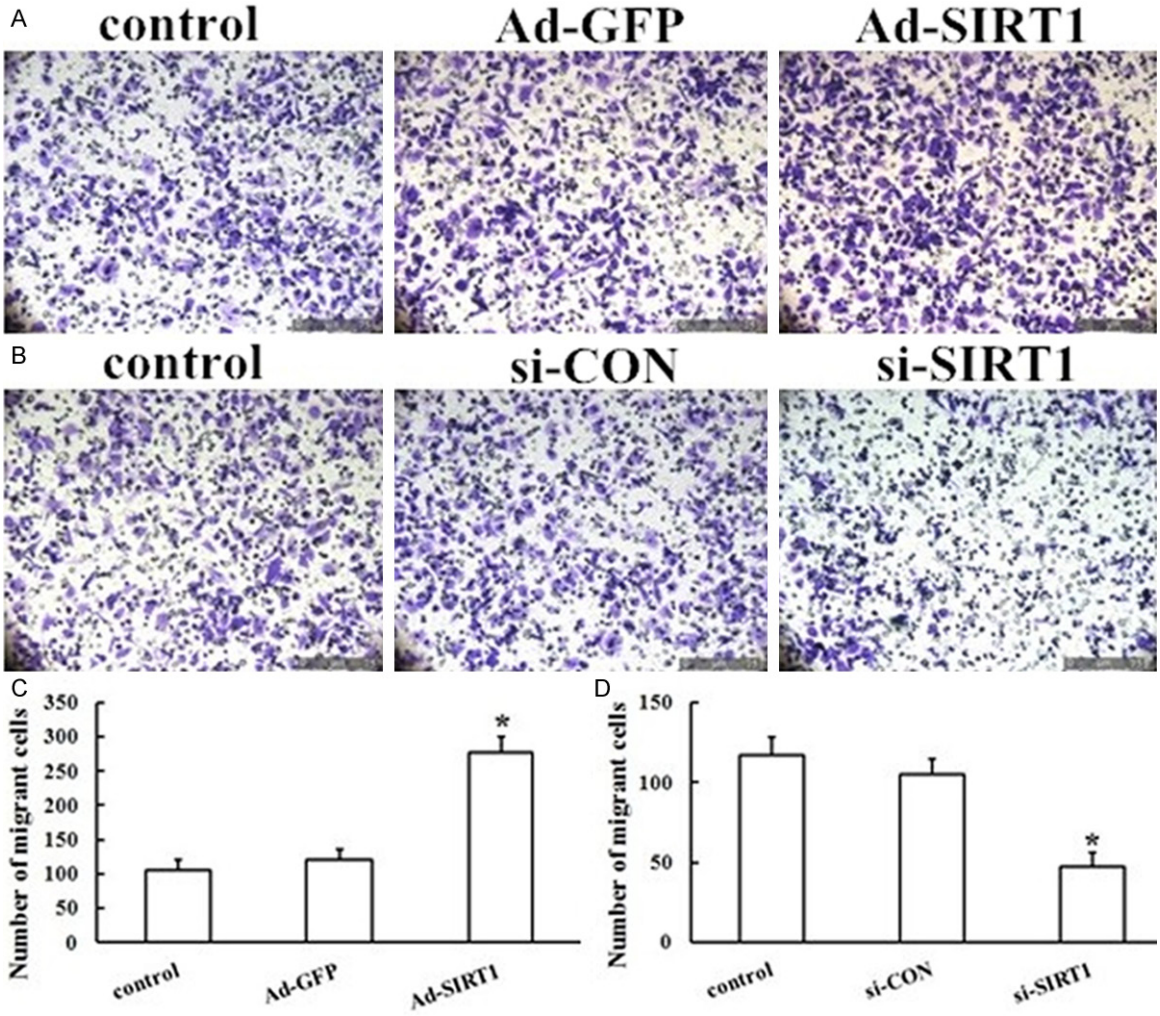

Figure 4. Effect of *SIRT1* overexpression and silencing on the migration of EPCs. A, B. Representative photographs of *SIRT1* overexpression and silencing on the migration of EPCs. EPC migration in response to Ad-*SIRT1* and si-*SIRT1* was detected using the Transwell system. C, D. The migration of EPCs transfected with Ad-*SIRT1* was enhanced as compared with that of Ad-GFP-transfected EPCs, but knockdown of endogenous *SIRT1* significantly reduced the migration of EPCs compared with the Ad-GFP group. The results are expressed as the mean ± SD (**P* < 0.05 vs. Ad-GFP).

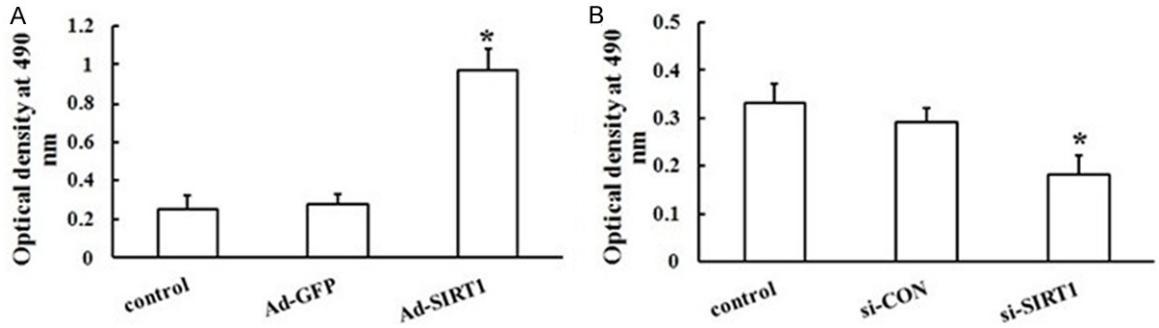

Figure 5. Effects of *SIRT1* overexpression and silencing on the proliferation of EPCs. Proliferation of EPCs was examined using the MTS assay. A. EPCs were transfected with or without Ad-GFP, and Ad-*SIRT1*. Three separate experiments were done in triplicate. B. EPCs were transfected with or without negative control of siRNA or si-*SIRT1*. The effect was decreased in the presence of si-*SIRT1*. The results are expressed as the mean ± SD (**P* < 0.05 vs. Ad-GFP or si-CON).

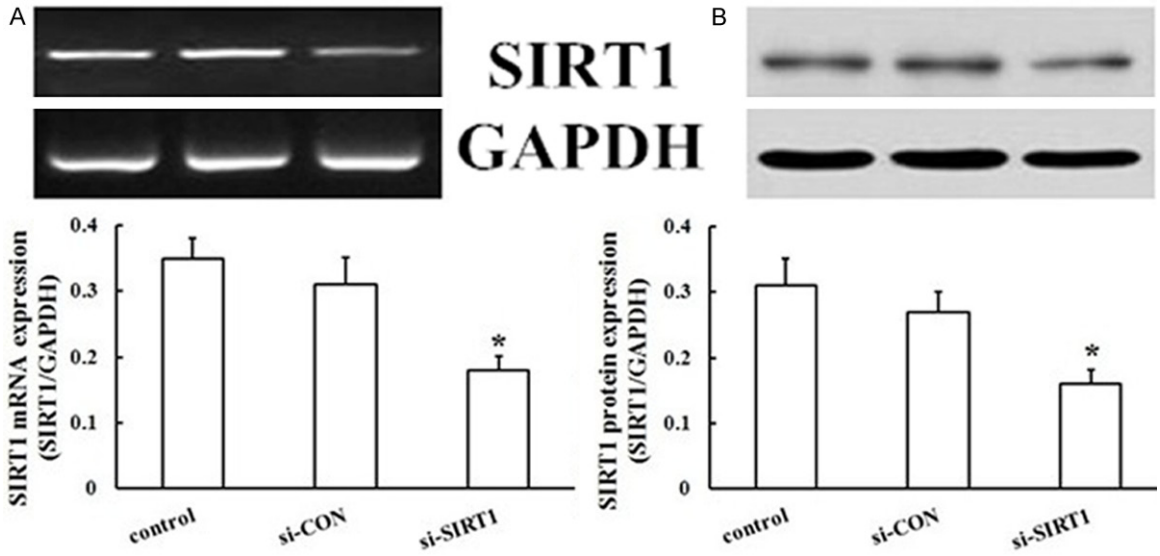

Figure 6. Influence of *SIRT1* silencing on changes in levels of *SIRT1*. Changes in levels of protein and mRNA of *SIRT1* were detected using western blotting and RT-PCR analysis after silencing of *SIRT1* in EPCs. Values are the percentage of GAPDH. *SIRT1* gene and protein levels were decreased by *SIRT1* silencing, but si-CON was not affected (**P* < 0.05 vs. control).

Western blot analysis

After treatment, cells were lysed in lysis buffer. The protein concentration of cell lysates was determined using the Bradford method. The same amounts of protein were separated by SDS-PAGE and electrophoretically transferred onto a polyvinylidene fluoride membrane. Membranes were blocked with 5% non-fat milk solution in TBS, with 0.5% Tween-20. Membrane-bound proteins were probed with primary antibodies against *SIRT1* (1:200) and GAPDH (1:500), followed by probing with secondary horseradish peroxidase-conjugated antibodies. Protein bands were visualized by chemiluminescent detection (Amersham Pharmacia Biotech, UK), and quantified using Quantity One software (Bio-Rad, USA). Anti-GAPDH monoclonal antibody was used to test for equal protein loading.

EPC migration assay

The migration of EPCs was assayed using a Transwell system (Corning Costar, USA) containing 8 μm polycarbonate filter inserts in 24-well plates. EPCs (2×10^5) in 100 μl of serum-free DMEM were placed in the upper chamber. DMEM containing 10% FCS (500 μl) was placed in the lower chamber. After 6 h in culture, cells on the bottom of the Transwell membrane were fixed with 4% paraformaldehyde at 37°C for 20 min and stained with 1% crystal violet at 37°C for 5 min. Migration activity was determined as the mean number of migrated cells in six random high-power fields (× 200) per chamber.

EPC proliferation assay

The EPCs were harvested from the cultures and placed, in triplicate, into fibronectin-coated 96-well plates (2×10^6 cells/ml). Cell proliferation was measured using the MTS assay (Cell Titer 96 Aqueous, Promega, USA) according to the manufacturer's protocol. Before reading the optical density at 490 nm, 20 μl of MTS solution was added to each well. All groups of experiments were performed in triplicate.

Concentration of NO in media

The concentration of NO released from EPCs was determined using a NO assay kit (Nanjing Jiancheng Institute of Biological Engineering, China) according to the manufacturer's instructions. EPCs were pretreated with LY294002 (30 μM), sc-221226 (30 μM), or L-NAM (200 μM) for 1 h, then treated with Ad-*SIRT1* or Ad-GFP for 24 h. The concentration of NO in 100μl of supernatant in media from different groups was detected at 550 nm. The total protein in every group was quantified using the BCA method. The NO-releasing ability of EPCs was calculated as the ratio of NO concentration and total protein.

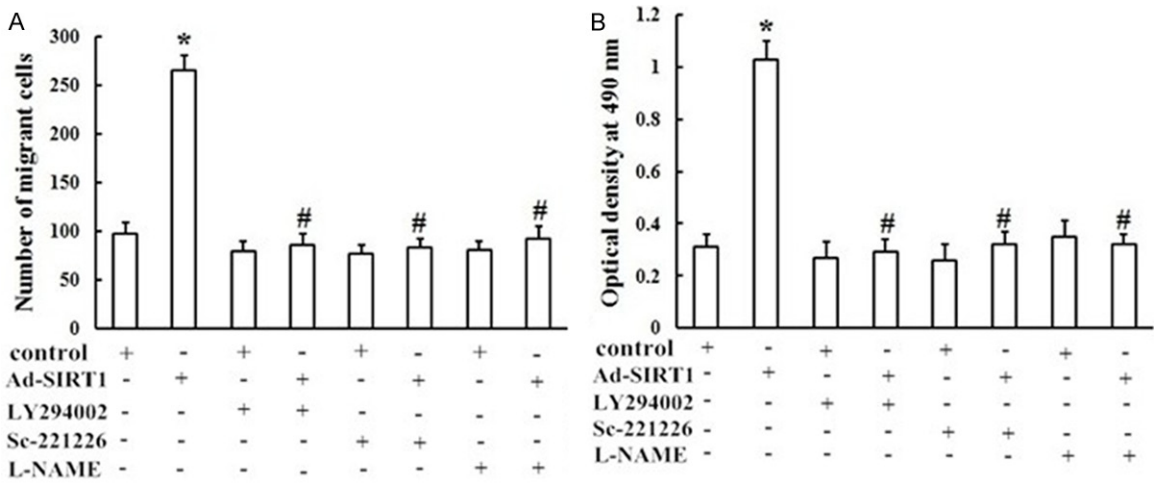

Figure 7. Role of the PI3K/Akt/eNOS signaling pathway in *SIRT1*-induced migration and proliferation of EPCs. Cells in the control group and the Ad-*SIRT1* group with or without pretreatment with LY294002 (30 μM), sc-221226 (30 μM), or L-NAM (200 μM). Ad-*SIRT1*-induced migration (A) and proliferation (B) of EPCs were significantly inhibited by pretreatment with LY294002, sc-221226, or L-NAME (*$P < 0.05$ vs. control, #$P < 0.05$ vs. Ad-*SIRT1*).

Statistical analysis

Data from at least three independent experiments were expressed as the mean ± S.D. SPSS 18.0 software was used for statistical analysis. Comparisons between multiple groups were performed using Multi-Way ANOVA or One-Way ANOVA. Comparisons between groups were performed using Fisher's LSD test. P values < 0.05 were considered to be statistically significant.

Results

Characterization of spleen-derived EPCs

After 4-7 days of culture (typical culture period before coculture and further experiments), adherent EPCs were characterized by immunofluorescence and fluorescence activated cell sorting (FACS) analysis. The majority of cells (>90%) stained positively for Dil-Ac-LDL, lectin, and DAPI (**Figure 1A**). In addition, 81.53 ± 3.97% of these cells expressed mouse stem-cell marker Sca-1, and 56.32 ± 2.18% expressed endothelial cell marker VEGFR-2 (**Figure 1B**).

SIRT1 expression and localization in EPCs

SITR1 was present at low levels in quiescent EPCs, but was up-regulated upon stimulation with serum and VEGF, as determined using either mRNA levels as determined by RT-PCR, or by protein levels as determined by western blotting (**Figure 2**).

Overexpression of SIRT1 enhances migration and proliferation of EPCs

To determine if *SIRT1* was involved in the regulation of migration and proliferation of EPCs, an adenoviral vector was constructed that expressed *SIRT1* exogenously using the pAd-Easy system. The transfection efficacy was approximately 60-70% as assessed by western blot analysis. Increased levels of *SIRT1* after adenovirus-mediated overexpression of *SIRT1* was confirmed by RT-PCR and western blot analysis (**Figure 3**). The parental adenoviral vector or Ad-GFP was used as a transfection control.

EPCs transfected with Ad-*SIRT1* or control Ad-GFP were subsequently subjected to separate assays to examine their migration and proliferation. The Transwell system was used to examine the effects of *SIRT1* overexpression on EPC migration. As shown in **Figure 4A** and **4C**, transfection of EPCs with Ad-*SIRT1* increased the number of migrating cells compared with Ad-GFP cells ($P < 0.05$). The MTS assay was used to examine how *SIRT1* overexpression affected EPC proliferation. The proliferation of EPCs transfected with Ad-*SIRT1* was enhanced approximately 300% compared with Ad-GFP transfected cells and control cells ($P < 0.05$) (**Figure 5A**). Taken together, and as

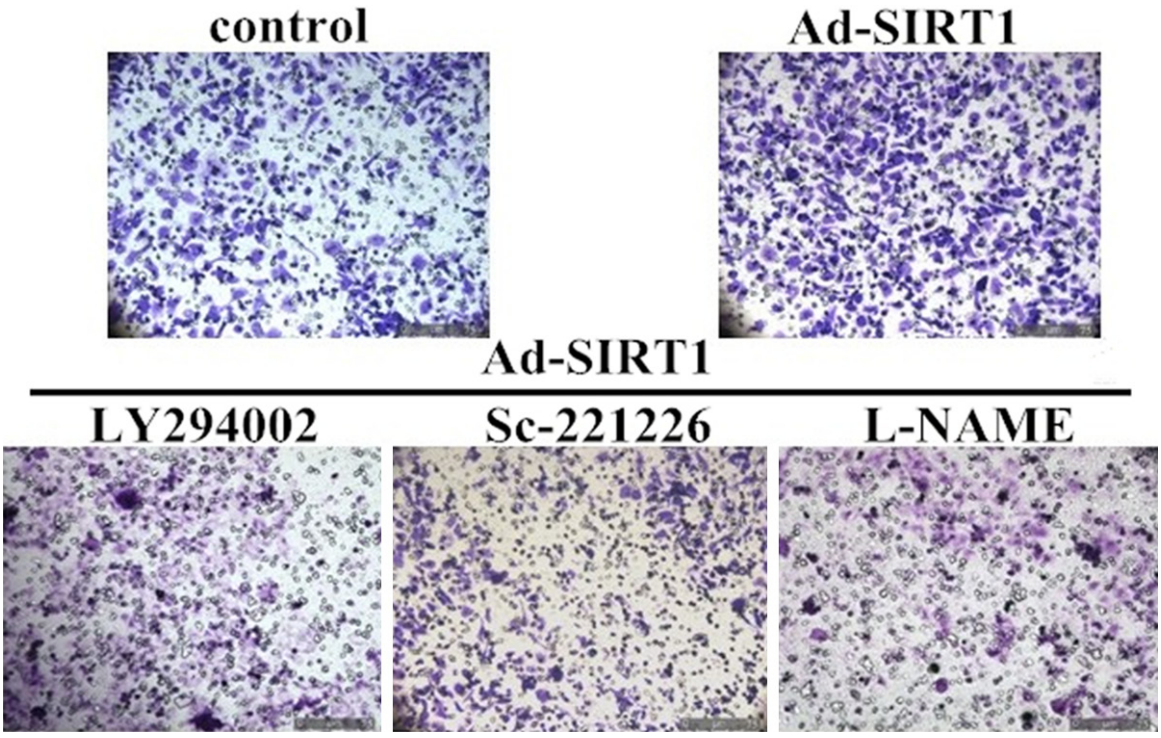

Figure 8. The role of the PI3K/Akt/eNOS signaling pathway in *SIRT1*-induced migration of EPCs. Representative photographs of EPC migration.

expected, the results showed that Ad-*SIRT1* promoted EPCs migration and proliferation in vitro. *SIRT1* therefore is an important component in the regulation of migration and proliferation of EPCs.

EPC migration and proliferation are inhibited by siRNA-mediated knockdown of SIRT1

Although overexpression of exogenous *SIRT1* directly enhanced the migration and proliferation of EPCs, the role of endogenous *SIRT1* was not determined. To determine whether endogenous *SIRT1* affected the migration and proliferation of EPCs, siRNA fragments were used to knockdown *SIRT1* protein levels. Forty-eight hours after transfection, si-*SIRT1* caused a significant loss of *SIRT1* in EPCs as measured by RT-PCR and western blot (**Figure 6**) (all, $P < 0.05$). Importantly, EPCs exhibited a decrease in cell migration (**Figure 4B**, **4D**) and proliferation (**Figure 5B**) compared to si-CON cells (all $P < 0.05$). The results were reproducible in at least three independent experiments. Thus, knockdown of endogenous *SIRT1* significantly reduced the migration and proliferation formation of EPCs, suggesting an important role of endogenous *SIRT1* in EPCs.

The role of the PI3K/Akt/eNOS pathway in SIRT1-induced migration and proliferation of EPCs

To evaluate the functional roles of the PI3K/Akt/eNOS signaling pathway in *SIRT1*-induced migration and proliferation, EPCs approximately 7 days old were pretreated with LY294002 (a PI3K inhibitor, 30 μM), sc-221226 (an Akt inhibitor, 30 μM), and L-NAME (an NOS inhibitor, 200 μM) for 1 h, before migration and proliferation assays. Pretreatment of EPCs with LY294002 almost completely blocked Ad-*SIRT1*-induced EPC migration (**Figures 7A** and **8**) and proliferation (**Figure 7B**). Simultaneously, Ad-*SIRT1*-induced migration (**Figures 7A** and **8**) and proliferation (**Figure 7B**) were all significantly inhibited by pretreatment with sc-221226. To further study the molecular mechanisms of Ad-*SIRT1*-mediated migration and proliferation, EPCs at 7 days were pretreated with eNOS inhibitor L-NAME. As shown in **Figures 7** and **8**, L-NAME significantly attenuated Ad-*SIRT1*-induced EPC migration (**Figures 7A** and **8**) and proliferation (**Figure 7B**). Taken together, these results demonstrated that the PI3K/Akt/eNOS signal transduction pathway plays an important

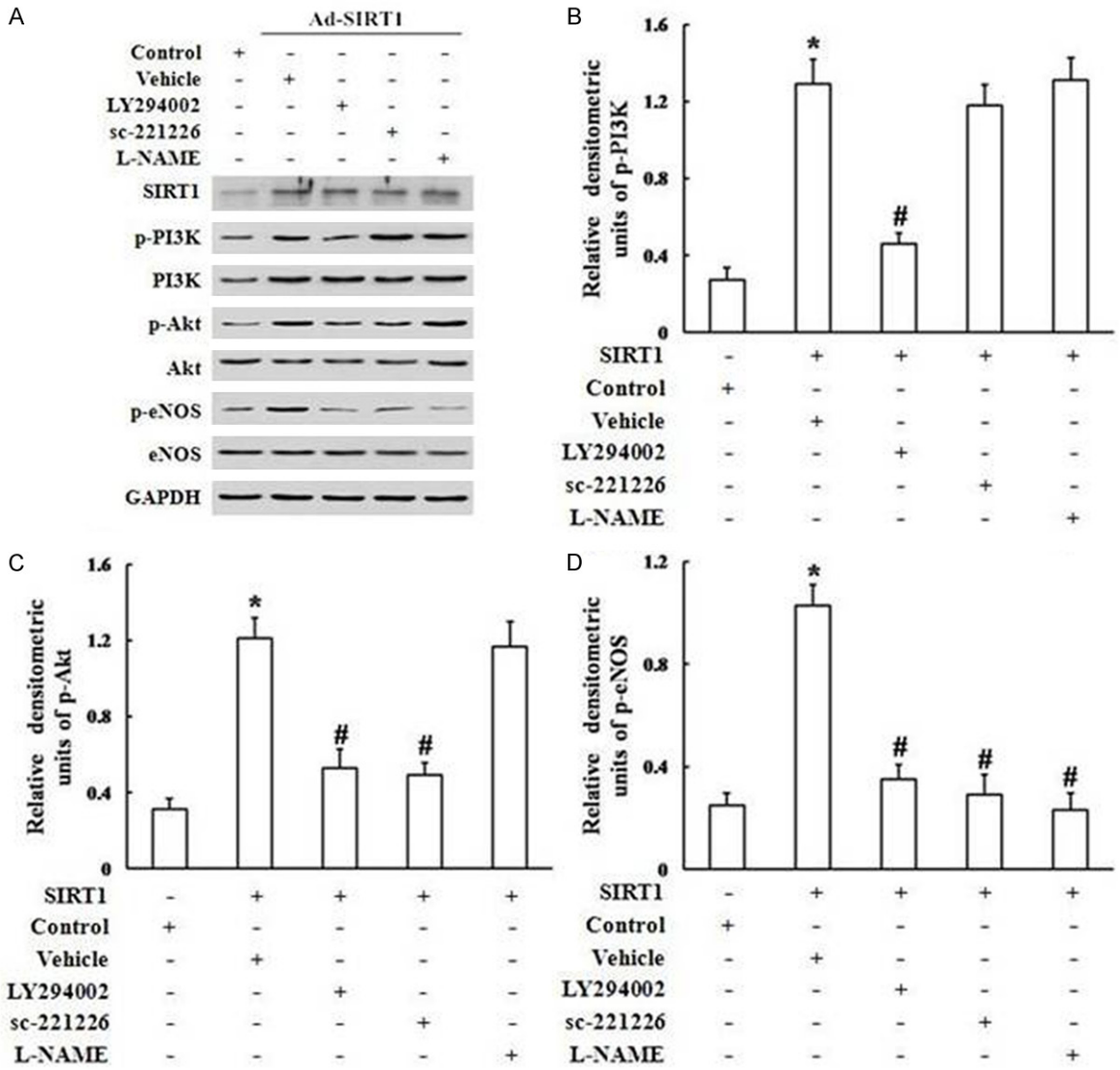

Figure 9. Blockage of the PI3K/Akt/eNOS signaling pathway abrogated *SIRT1*-induced phosphorylation of PI3K, Akt, and eNOS. (A) Western blot demonstrating that blockade by the PI3K inhibitor LY294002, the Akt-specific inhibitor sc-221226, and the NOS inhibitor L-NAME, reduced p-PI3K, p-Akt, and p-eNOS in EPCs transfected with Ad-*SIRT1*. Ad-*SIRT1*-induced phospho-PI3K (B), phospho-Akt (C), and phospho-eNOS (D) were normalized to total PI3K, Akt, or eNOS, respectively (**P* < 0.05 vs. control, #*P* < 0.05 vs. Ad-*SIRT1*).

role in *SIRT1*-induced EPCs migration and proliferation.

Ad-SIRT1 treatment activated the PI3K/Akt/eNOS pathway

Because *SIRT1*-induced migration and proliferation of EPCs were regulated by the PI3K/Akt/eNOS signaling pathway, we examined the effect of Ad-*SIRT1* on PI3K, Akt, and eNOS phosphorylation in EPCs. Exogenous stimulation with Ad-*SIRT1* significantly up-regulated the phosphotyrosine levels of PI3K (**Figure 9B**), Akt (**Figure 9C**), and eNOS (**Figure 9D**) (all $P < 0.05$), confirming the activation of the PI3K/Akt/eNOS signaling pathway.LY294002 (30 μM), sc-221226 (30 μM), and L-NAM (200 μM) were further used to determine the effects of Ad-*SIRT1* on PI3K, Akt, and eNOS phosphorylation in EPCs. LY294002, a highly selective inhibitor of PI3K, prevented the Ad-*SIRT1*-induced phosphorylation of PI3K (**Figure 9B**), Akt (**Figure 9C**), and eNOS (**Figure 9D**). As shown in **Figure 9**, treatment with sc-221226 attenuated levels of *SIRT1*-induced p-Akt (**Figure 9C**) and p-eNOS (**Figure 9D**) expression

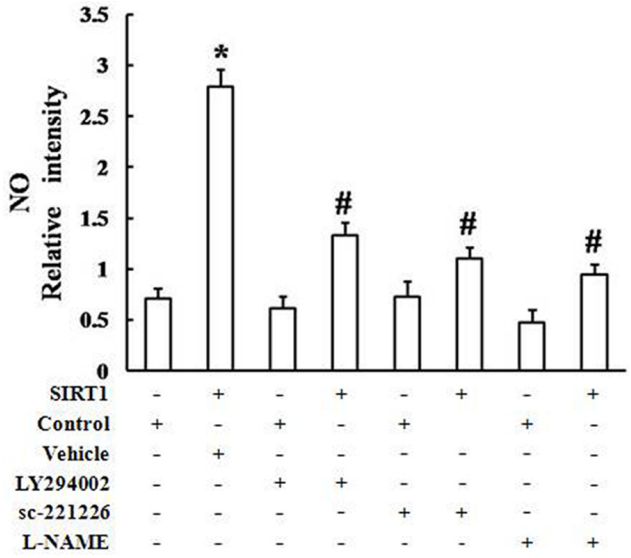

Figure 10. Ad-*SIRT1* induced NO production via the PI3K/Akt/eNOS signaling pathway. The intracellular NO level was elevated after treatment with Ad-*SIRT1*. Pretreatment of EPCs with LY294002 (30 μM), sc-221226 (30 μM), or L-NAM (200 μM) downregulated Ad-*SIRT1*-induced NO production in EPCs, in comparison with the controls (*$P < 0.05$ vs. control, #$P < 0.05$ vs. Ad-*SIRT1*).

in EPCs transfected with Ad-*SIRT1*, but not p-PI3K (**Figure 9B**). *SIRT1*-induced p-eNOS expression was abrogated by L-NAME as determined by western blot analysis (**Figure 9A**, **9D**). Together, the results suggested that eNOS activation was mediated through the PI3K/Akt signaling pathway.

Ad-SIRT1 regulated intracellular NO levels via the PI3K/Akt/eNOS signaling pathway in EPCs

The eNOS/NO signaling pathway is recognized as an important mediator of the angiogenic processes. However, the effect of *SIRT1* on NO production is unknown. As shown in **Figure 10**, the intracellular NO levels were elevated after treatment with Ad-*SIRT1*. However, in comparison with controls, pretreatment of EPCs with LY294002 (30 μM), sc-221226 (30 μM), and L-NAM (200 μM) downregulated Ad-*SIRT1*-induced NO production in EPCs (**Figure 10**). Taken together, the results provided direct evidence that Ad-*SIRT1* enhanced NO production via the PI3K/Akt/eNOS pathway.

Discussion

Damage of endothelial cells following percutaneous coronary interventions such as angioplasty and stenting is an important pathophysiological event during atherosclerosis and restenosis. EPCs have been found to be the main endogenous repair mechanism that responds to endothelium repair, and contributes to re-endothelialization by reducing neointima formation after vascular injury [17]. This mechanism is regulated by various processes and signals [18-23]. However, the regulatory mechanisms for the biological properties of EPCs remain unclear. Recent studies demonstrated that endothelial *SIRT1* may serve as an anti-atherosclerosis factor [9, 10], which may be a key component of EPC dysfunction.

The present study offers novel insights into the relationship between the *SIRT1* pathway and PI3K/Akt/eNOS signaling in EPCs during migration and proliferation. The results demonstrated that *SIRT1* stimulated EPCs migration and proliferation; upregulated the phosphotyrosine levels of PI3K, Akt, and eNOS; and enhanced NO production via the PI3K/Akt/eNOS pathway. These effects were abrogated in the presence of the PI3K-specific inhibitor LY294002, the Akt inhibitor sc-221226, and the NOS inhibitor L-NAME. In addition, knockdown of endogenous *SIRT1* significantly reduced the migration and proliferation of EPCs. These results demonstrated that the migration and proliferation of EPCs are all mediated through the *SIRT1*/PI3K/Akt/eNOS signaling pathway.

SIRT1 is a member of a protein class known as sirtuins, belonging to the Sir2 family, which has been identified as NAD^+-dependent deacetylases [24, 25]. *SIRT1* affects the activity of many proteins, resulting in the regulation of a number of proteins and their translation, which have important roles in biological processes such as metabolism, oxidative stress, and cell proliferation [26-29]. *SIRT1* has been implicated in cancer, aging, metabolic diseases, and cardiovascular dysfunctions [30-33]. Direct application of the *SIRT1* activator resveratrol has been shown to protect cardiomyocytes against H_2O_2- and hypoxia-induced apoptosis [34-37]. The antioxidant ability of resveratrol is *SIRT1*-dependent, because knockdown of *SIRT1* resulted in the loss of resveratrol-mediated reduction of reactive oxygen species and cell protection [37, 38]. Endothelial *SIRT1* may

also serve as an anti-atherosclerosis factor. *SIRT1* can protect endothelial cells from oxidative stress, and oxidative low-density lipoprotein-induced apoptosis [39, 40]. In endothelial cell-specific *SIRT1* transgenic mice, high fat-induced impairment in endothelium-dependent vasorelaxation decreased, accompanied by less atherosclerotic lesions [41], suggesting that *SIRT1* improved endothelial function to prevent atherosclerosis. *SIRT1* is highly expressed in endothelial cells and controls their angiogenic function. It is involved in vascular growth of cultured endothelium, in the formation of the vascular network of the developing zebrafish, and even in ischemia-induced neovascularization of the adult mouse [42-44].

The PI3K/Akt pathway provides essential signaling for cell survival and proliferation. Signaling from different eNOS agonists, such as VEGF, insulin, estrogen, and platelet-derived lipid mediators, can affect eNOS activity through the PI3K/AKT pathway. It has been reported that activations of the survival signal PI3K/Akt pathway and the endothelial specific eNOS/NO pathway were closely associated with vascular remodeling and angiogenesis [13, 14, 45]. Recent studies also reported that activation of *SIRT1* improved endothelium relaxation through up-regulating endothelial nitric oxide synthase (eNOS) expression and production of nitric oxide [46, 47]. However, whether *SIRT1* affects the biological properties of EPCs, and the role of PI3K/Akt/eNOS signaling pathway in *SIRT1*-induced migration and proliferation, have remained poorly understood.

In the present study, we determined the effects of the *SIRT1*/PI3K/Akt/eNOS signaling pathway on EPC migration and proliferation. The results showed that *SIRT1* induced the activation of the PI3K/Akt/eNOS signaling pathway during these processes. *In vitro* transfection of EPCs with Ad-*SIRT1* induced phosphorylation of Akt via PI3K, the phosphorylation of eNOS via AKt, and increased the expression of NO via eNOS. In addition, knockdown of endogenous *SIRT1* reduced migration and proliferation of EPCs. Together, the results suggested that the migration and proliferation of EPCs are attributable to the up-regulation of *SIRT1*, p-PI3K, p-Akt, and p-eNOS, as well as the production of NO. In addition, blockage of the PI3K/Akt/eNOS signaling pathway by the PI3K inhibitor LY294002, the Akt-specific inhibitor sc-221226, and the NOS inhibitor L-NAME, abrogated *SIRT1*-induced EPC migration and proliferation. Furthermore, treatment with the NOS inhibitor L-NAME decreased phosphorylation of eNOS, but did not affect PI3K/Akt activity, suggesting that PI3K/Akt is upstream of eNOS. These findings, therefore demonstrated the existence of a *SIRT1*/PI3K/Akt/eNOS signaling pathway during EPC migration and proliferation.

In conclusion, by mediating EPC proliferation and recruitment, the present study demonstrated important roles of *SIRT1* in neovascularization and re-endothelialization; In addition, the *SIRT1*/PI3K/Akt/eNOS signaling pathway may also play an important role during these same processes. Future studies should therefore be directed towards characterization of the complex mechanisms and therapeutic potentials of*SIRT1*, PI3K, Akt, and eNOS in the angiogenesis and tissue regeneration process mediated by EPCs.

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Disclosure of conflict of interest

None.

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