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Association of SIRT1 Expression With Shear Stress Induced Endothelial Progenitor Cell Differentiation

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ABSTRACT

Shear stress imposed by blood flow is crucial for differentiation of endothelial progenitor cells (EPCs). Histone deacetylase SIRT1 has been shown to play a pivotal role in many physiological processes. However, association of SIRT1 expression with shear stress-induced EPC differentiation remains to be elucidated. The present study was designed to determine the effect of SIRT1 on EPC differentiation induced by shear stress, and to seek the underlying mechanisms. Human umbilical cord blood-derived EPCs were exposed to laminar shear stress of 15 dyn/cm^2 by parallel plate flow chamber system. Shear stress enhanced EPC differentiation toward endothelial cells (ECs) while inhibited to smooth muscle cells (SMCs). The expressions of phospho-Akt, SIRT1 and histone H3 acetylation (Ac-H3) in EPCs were detected after exposure to shear stress for 2, 6, 12, and 24 h, respectively. Shear stress significantly activated Akt phosphorylation, augmented SIRT1 expression and downregulated Ac-H3. SIRT1 siRNA in EPCs diminished the expression of EC markers, but increased the expression of SMC markers, and resulted in upregulation of Ac-H3. Whereas, resveratrol, an activator of SIRT1, had the opposite effects on both EPC differentiation and histone H3 acetylation. Wortmannin, an inhibitor of PI3-kinase, suppressed endothelial differentiation of EPCs, decreased SIRT1, and upregulated Ac-H3 expression. In addition, SIRT1 promoted tube formation of EPCs in matrix gels. These results provided a mechanobiological basis of shear stress-induced EPC differentiation into ECs and suggest that PI3k/Akt-SIRT1-Ac-H3 pathway is crucial in such a process. J. Cell. Biochem. 113: 3663–3671, 2012. © 2012 Wiley Periodicals, Inc.

KEY WORDS: SHEAR STRESS; ENDOTHELIAL PROGENITOR CELLS; DIFFERENTIATION; SIRT1; HISTONE H3 DEACETYLATION; PI3K/AKT PATHWAY; VASCULOGENESIS

The mobilization and recruitment of circulating or tissue-resident endothelial progenitor cells (EPCs) has been shown to contribute to vasculogenesis in postnatal neovascularization. EPCs are a heterogeneous population of cells and there are numerous studies on the phenotypic definitions of EPCs [Hur et al., 2004; Ingram et al., 2004; Fadini et al., 2008]. Increasing evidences show that EPCs home to sites of ischemic, incorporate into endothelial cells (ECs) and promote vascular reparative processes [Szmitko et al., 2003; Chen et al., 2009; Kirton and Xu, 2010]. They contribute as much as 25% of ECs in newly formed blood vessels [Kirton and Xu, 2010]. The endothelial monolayer in a vein graft was completely lost 3 days after surgery and subsequently replaced by circulating endothelial progenitors [Xu et al., 2003]. In the process of endothelial differentiation of stem/progenitor cells and vascular development, shear stress, a mechanical force generated by blood flow, has been recognized as an important modulator. Experimental evidences indicate that shear stress exerts a morphogenetic function during cardiac development of mouse and zebrafish embryos [Hove et al., 2003; Illi et al., 2005]. The laminar flow can enrich both adult and embryonic stem cell populations for endothelial progenitors [Yamamoto et al., 2003; Rossig et al., 2005; Wang et al., 2005]. Exposure to a shear stress of 12 dyn/cm^2 induces expression of endothelial-lineage genes in placenta-derived multipotent cells [Wu et al., 2008]. Similar study demonstrates endothelial differentiation

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Bin-Bin Cheng and Zhi-Qiang Yan contributed equally to this work.

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in a mesenchymal progenitor CH3H/10T1/2 cell line resulting from shear exposure [Wang et al., 2005]. Also it has been shown that EPCs can differentiate into endothelial phenotypes when shear stress is applied [Yamamoto et al., 2003].

Mechanisms of shear stress-induced stem/progenitor cell differentiation seem to via several signal initiators and transducers [Zeng et al., 2006], of which inhibition of histone deacetylases (HDACs) blocks the endothelial differentiation of adult progenitor cells [Rossig et al., 2005]. Shear stress upregulates HDAC3, one of class I HDACs, mediates p53 deacetylation and p21 activation, leading to EC differentiation in vitro [Zeng et al., 2006]. Thus, we hypothesized that SIRT1, a class III HDACs, might be involved in shear stress-induced EPC differentiation. SIRT1 regulates a variety of physiological functions, such as metabolism, senescence, and differentiation in multiple cell types [Zu et al., 2010; Ou et al., 2011]. It is highly expressed in the vasculature during blood vessel growth and controls the angiogenic activity of ECs [Potente et al., 2007]. Cigarette smoke-mediated oxidants/carbonyl stress decreases SIRT1 expression and causes endothelial nitric oxide synthase (eNOS) acetylation, which has ramifications in endothelial dysfunction [Edirisinghe and Rahman, 2010]. Besides, the roles of SIRT1 have been reported to be important in EPCs [Xu, 2008]. During alteration of glucose metabolism, SIRT1 is a critical modulator of EPC dysfunction [Chen et al., 2007]. SIRT1 has also been implicated in the regeneration and proliferation of EPCs [Zhao et al., 2010] and Mthfr deficiency impairs EPC formation and increases its senescence by eNOS uncoupling and downregulation of SIRT1 [Lemarie et al., 2011]. However, the effect of SIRT1 on EPC differentiation remains unclear despite there are reports about SIRT1 on mouse mesenchymal stem cell differentiation [Tseng et al., 2011] as well as other cell types such as human normal keratinocytes [Blander et al., 2009].

To explore the possible mechanism of SIRT1 on EPC differentiation, we focused on histone H3, because SIRT1 exerts its function usually by deacetylating histone or nonhistone proteins. And previous reports suggest that the global deacetylation of histones is necessary for differentiation of embryonic stem cells in vitro [Lee et al., 2004]. Indeed, the acetylation of histones is part of the complex epigenetic regulatory process for determining lineage-specific gene expression and cell fate decisions by altering the local structure of chromatin [Jenuwein and Allis, 2001].

The laminar flow increases SIRT1 level and activity, mitochondrial biogenesis, and expression of SIRT1-regulated genes in cultured ECs [Chen et al., 2010]. However, it is entirely unknown the effect of shear stress on SIRT1 expression in EPCs and the following mechanism of SIRT1 on EPC differentiation. In the present study, we found that shear stress induced EPC differentiation toward ECs in vitro via upregulated SIRT1, deacetylating histone H3, followed by an activation of the PI3k/Akt pathway.

MATERIALS AND METHODS

CELL CULTURE

Blood samples from normal full-term deliveries were obtained with informed consent and approved in accordance with the procedures of the institutional Ethics Committee. Culture of EPCs was performed as described previously in Ye et al. [2008]. Briefly, EPCs were isolated from human umbilical cord blood by density gradient centrifugation using Ficoll-Paque Plus (Cedarlane, Canada) according to the manufacturer's protocol. After two washing steps, mononuclear cells (MNCs) were plated onto collagen I-coated 6-well plates and maintained in M199 (Gibco-BRL, Grand Island, NY) essential medium supplemented with 20% fetal bovine serum (FBS, Gibco-BRL), fibroblast growth factor-basic (bFGF, Pepro Tech, Rocky Hill, NJ), epidermal growth factor (EGF, Pepro Tech, Rocky Hill, NJ), heparin (Sigma, St. Louis, MO), HEPES, 100 U/ml penicillin, 100 μg/ml streptomycin. Cells were cultured at 37°C in 5% CO_2 in a humidified atmosphere. Three days after seeding, nonadherent cells were removed by washing with PBS and fresh medium was added. Seven days after seeding, EPCs were characterized by double-fluorescence stain of 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate-labeled acetylated LDL (Dil-acLDL) and FITC-labeled *Ulex europeus* agglutinin (lectin).

SHEAR STRESS LOADING

EPCs were digested by 0.25% trypsin/EDTA solution by Day 5 and seeded at a density of 5×10^5 cells/cm^2 onto the outer side of a porous polyethylene terephthalate (PET) membrane (Becton Dickinson Labware, NJ). The inner side of the membrane was kept unseeded. Confluent monolayers of cells were exposed to a laminar flow generated by the pressure difference between the upper and lower reservoirs, with the effluent M199 medium contained 1% FBS circulated back to the upper reservoir through a peristaltic pump [Ye et al., 2008]. The flow path is 28 mm in width (w) and 200 μm in height (h). Shear stress (τ, dyn/cm^2) intensity was calculated by the formula $\tau = 6\,\mu Q/wh^2$, where μ is the viscosity of the medium (poise) and Q is the flow rate (ml/s). A normal laminar shear stress applied in the present study was 15 dyn/cm^2 (dyn/cm^2 = 10^{-1} Pa [N/m^2]). Thus, EPCs were subjected to the shear stress for 2, 6, 12, and 24 h, respectively. Flow-loading experiments were performed at 37°C in a CO_2 incubator.

FLOW CYTOMETRY

The expression of stem cell markers CD133 and CD34, endothelial lineage markers vWF and CD31 of EPCs were determined by flow cytometry. EPCs cultured by Day 7 were incubated with one of the following antibodies, a polyclonal rabbit anti-CD133 (Abcam), a monoclonal mouse anti-CD34 (Abcam), a polyclonal rabbit anti-vWF (DukoCytomation), a FITC-conjugated monoclonal mouse anti-CD31 (Millipore), or each corresponding isotype control in 1% BAS at 37°C for 1 h. Then cells of CD133, CD34, vWF groups, and corresponding isotype controls were incubated with FITC-conjugated immunoglobulin G (IgG, Cell Signaling) at 37°C for 1 h. Quantitative analysis was performed by flow cytometry (CellQuest, Becton Dickinson, CA). The data were presented as the percentage of positive cells corresponding to the gated cells and mean fluorescence intensity (MFI) in each experiment.

RNA EXTRACTION AND REAL-TIME RT-PCR

Total RNA was extracted using TRIZOL reagent (Invitrogen, Carlsbad, CA). Real-time RT-PCR was performed using Quanti-Tect SYBR Green PCR kit (Bio-Rad) with 1 μl of cDNA on an iCycler (Bio-Rad). Each transcript of target genes or the reference gene

(GAPDH) was amplified in duplicate/triplicate, and PCR products were verified via melt curve analysis and agrose gel electrophoresis. Gene-specific primers and annealing temperatures (T_m) are listed in Table I. The results were normalized to GAPDH expression levels and relative gene expression was measured by the $2^{-\Delta\Delta CT}$ method.

WESTERN BLOT

For the detection of protein expression levels, EPCs lysates were subjected to electrophoretic separation by 12% SDS–PAGE and transferred to nitrocellulose membranes (Hybond, Amersham). Western blots were performed using antibodies directed against SIRT1 (Cell Signaling Technologies), GAPDH (Santa Cruz Technologies), total-Akt (Cell Signaling Technologies), phospho-Akt (Ser 473; Cell Signaling Technologies), histone H3 (Cell Signaling Technologies), Ac-H3K9 (Bioworld Technology), α-SMA (Sigma), and sm22α (Abcam).

RNA INTERFERENCE AND ACTIVATOR/INHIBITOR TREATMENT

For small interfering RNA (siRNA) mediated gene knockdown, SIRT1 siRNA or a negative control (NC) siRNA (Shanghai GenePharma Co., Ltd.) was transfected into cells (100 nM for 10 cm^2 culture area) using Lipofectamine 2000 (Invitrogen) according to the manufacturer's protocol. The siRNA exhibited maximum gene-knockdown effectiveness at 48 h after transfection and then cells were harvested for further analysis. The target sequences for the SIRT1 were forward: 5′-GAUGAAGUUGACCUCCUCATT-3′; reverse: 5′-UGAGGAGGUCA ACUUCAUCTT-3′. The sequences of NC were forward: 5′-UUCUCCGAACGUGUCACGUTT-3′; reverse: 5′-ACGUGACACGUUCGGAGAATT-3′.

To activate SIRT1 expression, cells cultured in 6-well plates were treated with 75 μM resveratrol in 1% FBS for 24 h with its dissolvent dimethyl sulphoxide (DMSO, Sigma) as control. Similarly, to block the PI3-kinse, cells were incubated with 200 nM wortmannin and 50 μM LY294002, respectively (Sigma) in 1% FBS/M199 for 24 h with DMSO presented at equal concentration in all groups.

TABLE I. List of Primer Sequences and Annealing Temperatures for RT-PCR*

Gene	Primer sequences, 5′–3′	T_m (°C)
SIRT1	Fwd: GCCACCAAATCGTTACAT; Rev: CATCAGTCCCAAATCCAG	56
KDR	Fwd: AGACCAAAGGGGCACGATTC; Rev: AGCAAAACACCAAAAGACCAGAC	62
VE-cadherin	Fwd: GCTGAAGGAAAACCAGAAGAAGC; Rev: CGTGATTATCCGTGAGGGTAAAG	62
vWF	Fwd: TGCTGACACCAGAAAAGTGC; Rev: GTCCCCAATGGACTCACAG	60
CD31	Fwd: ATGATGCCAGTTTGAGGTC; Rev: GACGTCTTCAGTGGGGTTGT	60
α-SMA	Fwd: TTTCCGCTGCCCAGAGAC Rev: GTCAATATCACACTTCATGATGCTGT	60
sm22α	Fwd: CCGGTTAGGCCAAGGCTC Rev: GCGGCTCATGCCATAGGA	60
GAPDH	Fwd: CGCGGGCTCCAGAACATCAT; Rev: CCAGCCCCAGCGTCAAAGGTG	65.5

Fwd, forward; Rev, reverse; T_m, annealing temperature.
*Primers for all tested genes were designed via the Primer Premier5.0 (Premier Biosoft, CA).

IN VITRO TUBE FORMATION ASSAY

Matrix gels were formed by adding Biocoat Matrigel (Becton Dickinson) into 24-well plates (400 μl/well) and incubating in a CO_2-free incubator at 37°C for 30 min. EPCs were pretreated with SIRT1 siRNA for 48 h or resveratrol for 24 h, and then the cells under these conditions and respective control were seeded on Matrigel and incubated for 24 h. The images of four representative fields were photographed (IX-71, Olympus), and the averages of the total length of complete tubes per unit area among groups were measured and compared using Image Pro-Plus 4.5.1 software.

STATISTICAL ANALYSIS

Each experiment was performed at least in triplicate, and all values are expressed as mean ± SD. Significant differences between groups were assessed with a two-tailed paired *t*-test. *P* values lower than 0.05 were accepted as statistically significant.

RESULTS

IDENTIFICATION OF EPCS

Human cord blood MNCs presented sequential morphological changes during different culture days, with a typical cell colonies and cord-like structure at Day 7 (Fig. 1A). To confirm EPC phenotype, attached MNCs were incubated with Dil-acLDL and FITC-labeled *U. europeus* agglutinin (lectin). Cells demonstrating double-positive fluorescence were identified as differentiating EPCs. Nearly all adherent cells (>95%) were shown to simultaneously endocytose DiI-acLDL and bind fluorescein isothiocyanate UEA-1 (lectin) as presented before in our lab [Ye et al., 2008]. In addition, cell characterization by flow cytometry analysis revealed that cells were positive for stem cell markers CD133 (17.08 ± 1.58%), CD34 (25.08 ± 2.25%), and EC marker vWF (59.4 ± 3.85%) CD31 (67.12 ± 4.20%; Fig. 1B).

SHEAR STRESS INDUCED EPC DIFFERENTIATION INTO EC LINEAGE

EPCs were subjected to the shear stress of 15 dyn/cm^2 for 24 h, and cells kept in the static condition were used as a control. Thereafter, EC- and SMC-specific markers were detected in gene or protein levels. Real time RT-PCR showed that the shear stress induced a notable increase in the EC markers, KDR (VEGF receptor 2), VE-cadherin, vWF (von willebrand factor), and CD31 (Fig. 2A). Whereas, SMC markers, α-SMA and sm22α, were significantly decreased, as shown by both real time RT-PCR and Western blot analysis compared with the static cells (Fig. 2B,C). Taken together, the results indicate that the normal shear stress promotes differentiation of human umbilical cord blood derived-EPCs into the vascular EC lineage and inhibits it into SMC lineage.

EFFECT OF SHEAR STRESS ON SIRT1 EXPRESSION IN EPCS

In order to examine whether SIRT1 is involved in shear stress-induced EPC differentiation, the effect of shear stress on the expression of SIRT1 in EPCs was examined by Western blot analysis. After EPCs were exposed to the shear stress for 2, 6, 12, and 24 h, SIRT1 protein expressions were measured and compared with the static control, respectively. SIRT1 expression had no significant change after 2 h-exposure. However, it was up-regulated by 66% at

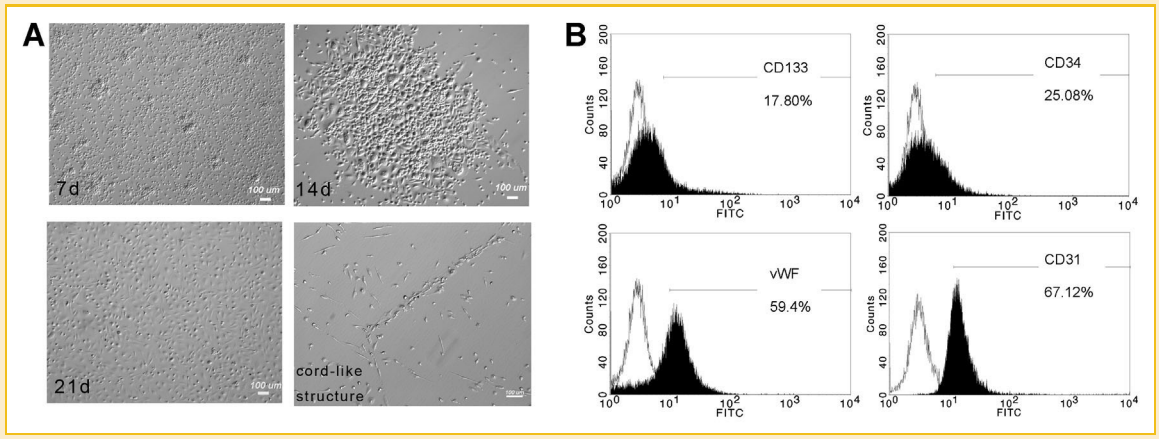

Fig. 1. Morphology and characterization of EPCs. Human umbilical cord blood MNCs were isolated and plated on colleagen I-coated 6-well plates. A: Phase-contrast microscopy of the morphological changes of EPCs after 7–21 days of culture. Cells on 7 days of culture displayed a representative cell colony and cord-like structure. Scale bar = 100 μm. B: Flow cytometric analysis of cell surface markers expression in EPCs at 7 days of culture in vitro. Cells were positive for hematopoietic stem cell markers CD133 (17.08 ± 1.58%), CD34 (25.08 ± 2.25%), and EC marker vWF (59.4 ± 3.85%) CD31 (67.12 ± 4.20%). Isotype controls (open areas) were overlaid on the histogram of each surface antigen (filled areas) tested. Data are mean ± SD percentage of positive cells from three samples.

12 h and started to decline slightly at 24 h, but was still significantly higher than the static (Fig. 3).

The PI3k/Akt signaling pathway is one of the essential pathways mediating EPC differentiation. Thus, Akt phosphorylation at serine 473 was examined to demonstrate the upstream signal pathways of SIRT1. Level of Akt phosphorylation was increased after the application of shear stress to EPCs for 2 h, reached a peak at 6 h, and kept higher than the static even at 24 h although it was found to decline (Fig. 3).

Genetic ablation of the SIRT1 gene or pharmacological inhibition of SIRT1 activity led to significant disturbances in acetylation of histone H3 [Nakahata et al., 2008]. As one of SIRT1 substrates,

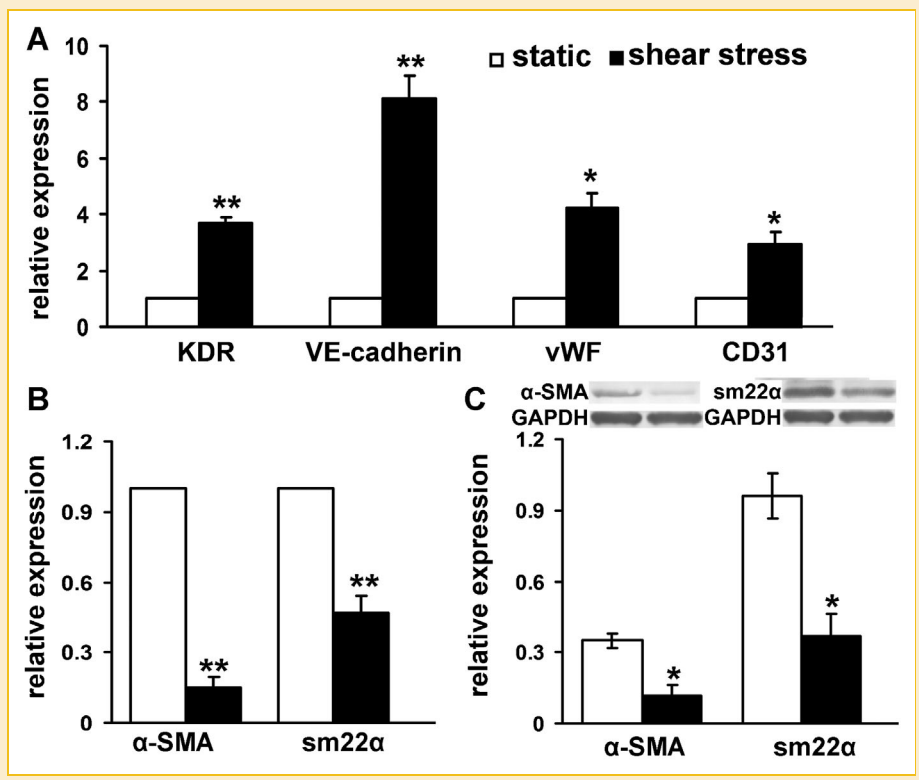

Fig. 2. Effects of shear stress on the expressions of EC and SMC markers in EPCs. Confluent monolayers of EPCs were exposed to the shear stress of 15 dyn/cm^2 for 24 h or kept as the static condition. Shear stress increased the mRNA levels of EC markers, KDR, VE-cadherin, vWF, and CD31 (A), but decreased the SMC markers, α-SMA and sm22α (B,C), at both mRNA and protein levels. Values are means ± SD averaged from at least three independent experiments, $^{*}P < 0.05$, $^{**}P < 0.01$ versus the static, $n \geq 3$.

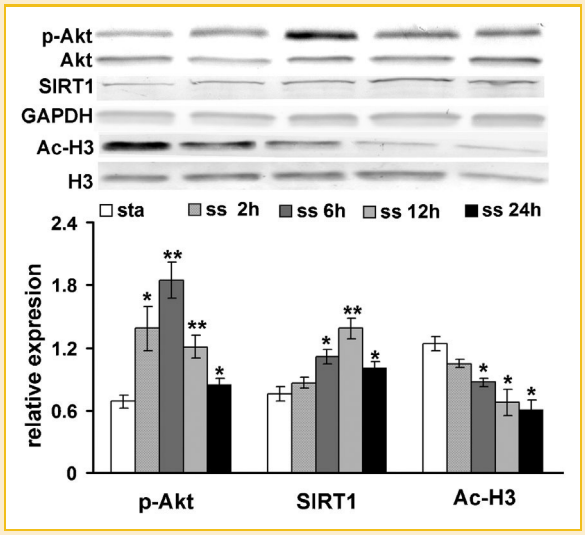

Fig. 3. Effects of shear stress on expression levels of p-Akt, SIRT1, and Ac-H3 in EPCs for different time duration. Confluent monolayers of EPCs cultured on the insert were exposed to the shear stress of 15 dyn/cm^2 for 2, 6, 12, and 24 h or kept as the static condition. Western blot analysis showed that shear stress activated Akt phosphorylation, upregulated SIRT1 expression, and downregulated the histone H3 acetylation status in a time-dependent manner. Akt phosphorylation reached a peak at 6 h and then began to decline to the basic level. SIRT1 was increased to the highest level at 12 h and kept higher than the static even at 24 h. Acetylation of histone H3 reached the lowest level at 24 h. The bar graphs in all panels are mean ± SD averaged from three independent experiments, $^{*}P < 0.05$, $^{**}P < 0.01$, versus the static, n = 3.

acetylation of histone H3 may be involved in shear stress-induced EPC differentiation, and we focused on Ac-H3K9. As shown in Figure 3, the application of shear stress decreased H3 acetylation levels in EPCs in a time-dependent manner.

CONTRIBUTION OF SIRT1 TO EPC DIFFERENTIATION

To evaluate the possible role of different expressed SIRT1 on EPC differentiation induced by shear stress, knocking down the SIRT1 expression by its target siRNA or activating SIRT1 protein by resveratrol were performed. Then, their effects on mature EC and SMC markers expression were examined, respectively. As histone H3 acetylation was down-regulated by the shear stress of 15 dyn/cm^2, accompanied with the increase of SIRT1, we next examined whether histone H3 is a downstream of SIRT1 in the process of EPC differentiation. The Ac-H3 expressions were examined after different treatments.

First, by transfecting with SIRT1 siRNA, SIRT1 expression was remarkably decreased at both mRNA level and protein level (Fig. 4A). The mRNA levels of KDR, VE-cadherin, vWF, and CD31 were significantly reduced (Fig. 4B), while mRNA and protein levels of α-SMA and sm22α were upregulated (Fig. 4C,D), as compared to the NC siRNA. Western blot analysis showed that the SIRT1 knockdown led to augmented histone H3 acetylation in EPCs (Fig. 4E). These results indicate that SIRT1 is required in endothelial differentiation of EPCs.

Subsequently to further confirm the impact of SIRT1 in EPC differentiation, SIRT1 was activated in EPCs by resveratrol.

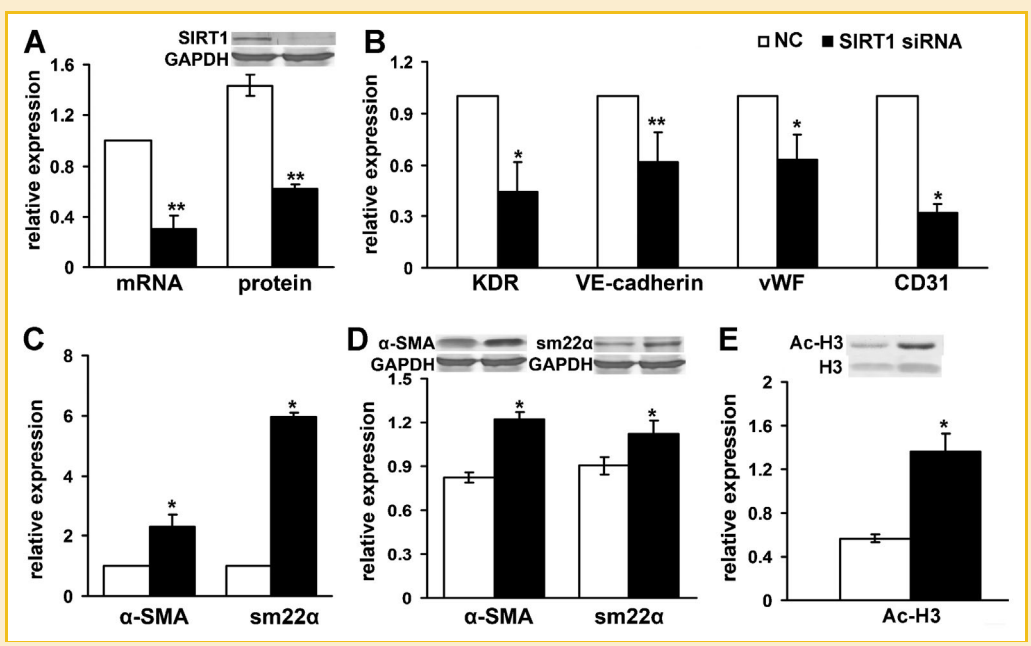

Fig. 4. Effects of SIRT1 siRNA on EPC differentiation and Ac-H3 protein expression. SIRT1 siRNA downregulated SIRT1 expression both at mRNA and protein levels compared with the negative control (NC) siRNA (A), decreased mRNA levels of EC markers, KDR, VE-cadherin, vWF, and CD31 (B) and increased SMC markers, α-SMA and sm22α, at both mRNA and protein levels (C,D). SIRT1 siRNA upregulated histone H3 acetylation (E). The bar graphs in all panels are mean ± SD averaged from at least three independent experiments, $^{*}P < 0.05$, $^{**}P < 0.01$, versus the corresponding controls, n ≥ 3.

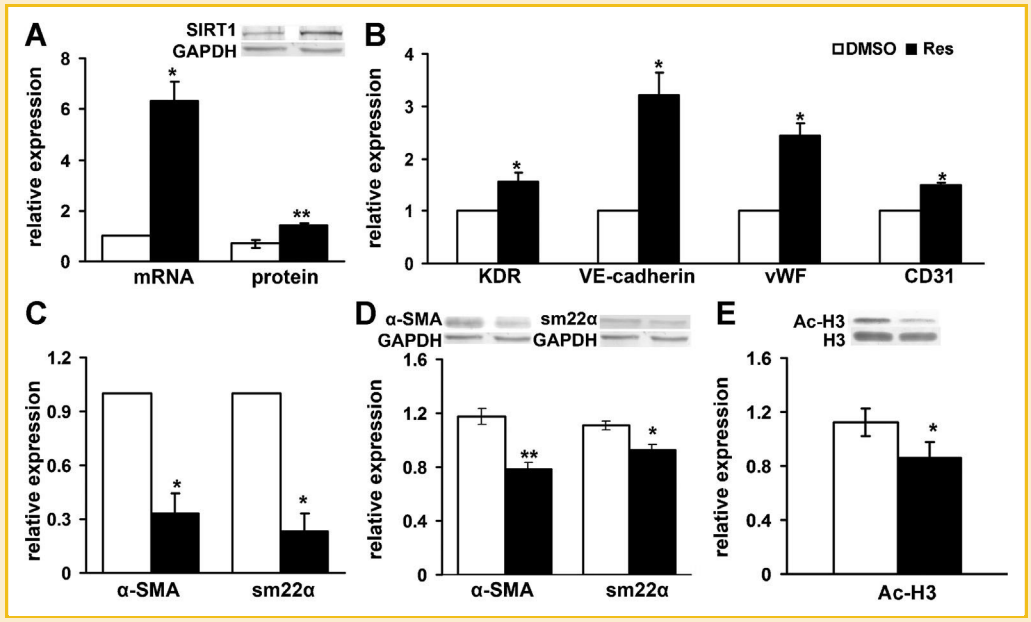

Fig. 5. Effects of SIRT1 activator resveratrol on EPC differentiation and Ac–H3 protein expression. Resveratrol (Res) upregulated SIRT1 expression both at mRNA and protein levels compared with the control (DMSO; A), increased mRNA levels of EC markers, KDR, VE-cadherin, vWF and CD31 (B) and increased SMC markers, α–SMA, and sm22α, at both mRNA and protein levels (C,D). Resveratrol down-regulated histone H3 acetylation (E). The bar graphs in all panels are mean ± SD averaged from at least three independent experiments, $^{*}P < 0.05$, $^{**}P < 0.01$, versus the corresponding controls, $n \geq 3$.

Resvertrol treatment for 24 h significantly increased SIRT1 expression on both mRNA and protein levels (Fig. 5A). Then, the expressions of EC and SMC markers in EPCs were assessed in the presence or absence of resveratrol. Quantitative RT-PCR analysis revealed a significant increase of EC markers, KDR, VE-cadherin, vWF, and CD31 (Fig. 5B) in the presence of the resveratrol. In the same conditions, the SMC markers, α-SMA and sm22α, were found to be downregulated at both mRNA and protein levels (Fig. 5C,D). Resveratrol down-regulated histone H3 acetylation at the same time (Fig. 5E). The results demonstrate that SIRT1 promotes endothelial differentiation of EPCs.

INVOLVEMENT OF PI3K/AKT SIGNAL PATHWAYS IN EPC DIFFERENTIATION

To confirm the upregulation of SIRT1 is followed by a PI3k/Akt signaling pathway, EPCs were treated with wortmannin, a specific inhibitor of PI3-kinase, for 24 h, and phosphorylation of Akt was effectively inhibited (Fig. 6A). The wortmannin treatment decreased mRNA levels of EC markers, KDR, VE-cadherin, vWF, and CD31, in EPCs (Fig. 6B) and increased SMC markers, α-SMA and sm22α, at both mRNA and protein levels (Fig. 6C,D) as compared to the control (DMSO). Western blot analysis showed that wortmannin treatment resulted in SIRT1 downregulation and the upregulating acetylation of histone H3 in EPCs (Fig. 6E). Besides, another specific inhibitor of PI3-kinase, LY294002, was used to confirm the role of PI3K/Akt on EPC differentiation. Phosphorylation of Akt and expression of EC markers, that is, KDR, VE-cadherin, vWF, and CD31, were remarkably inhibited after LY294002 treatment (Supplementary Fig. 1A,B), which was similar as that after wortmannin treatment. These results show that PI3k/Akt blocking significantly attenuates EPC differentiation toward ECs in vitro.

SIRT1 PROMOTED TUBE FORMATION OF EPCS IN MATRIX GELS

To investigate whether SIRT1 affects the ability of EPCs to form capillary-like tubes, EPCs treated by SIRT1 siRNA and resveratrol, respectively were seeded in Matrigel and examined the tube formation microscopically. As shown in Figure 7A, EPCs treated with NC siRNA had small cellular aggregates at 24 h. In contrast, SIRT1 siRNA transfected EPCs were present in the form of individual cells. Figure 7B showed that EPCs treated with resveratrol appeared to be interconnected, and had formed tightly adherent cords of cells at 24 h, significantly prominent than DMSO treated control. Quantitative analysis showed the total length in SIRT1 siRNA transfected group was significantly lower than that in the control group while it was greater in resveratrol treated group than in the DMSO control. These findings suggest that SIRT1 may induce functional capabilities of EPCs toward ECs.

DISCUSSION

Our present work show that the shear stress of 15 dyn/cm^2, which is in the range of physiologic levels, facilitates EPC differentiation to the EC lineage along with suppression of SMC differentiation as demonstrated by the elevated expression of EC markers, KDR, VE-cadherin, vWF, CD31, and decreased expression of SMC markers, α-SMA and sm22α. The positive effect of shear stress on endothelial differentiation of EPCs is consistent with previous reports [Yamamoto et al., 2003; Wang et al., 2005]. Our results reveal,

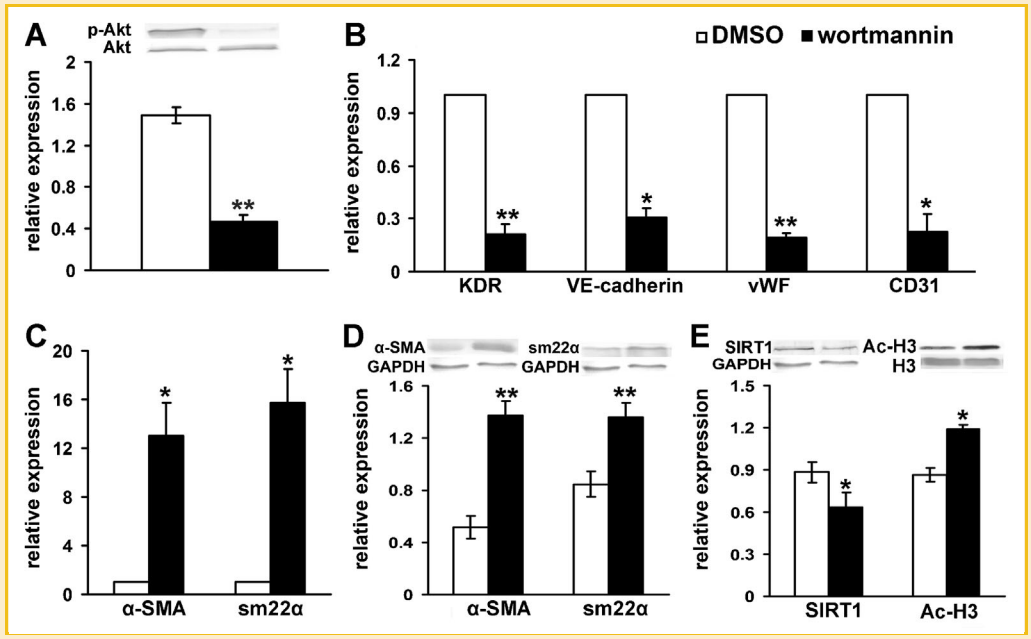

Fig. 6. Effects of PI3k/Akt inhibitor wortmannin on EPC differentiation, SIRT1, and Ac-H3 protein expressions. Wortmannin inhibited Akt phosphorylation effectively (A), decreased mRNA levels of EC markers, KDR VE-cadherin, vWF, and CD31 (B) and increased SMC markers, α-SMA, and sm22α, at both mRNA and protein levels (C,D). Wortmannin downregulated SIRT1 expression while upregulated histone H3 acetylation status (E). The bar graphs in all panels are mean ± SD averaged from at least three independent experiments, $^{*}P < 0.05$, $^{**}P < 0.01$ versus the corresponding controls, $n \geq 3$.

for the first time, that the shear stress increases SIRT1 expression in EPCs with the highest level at 12 h, which subsequently leads to acetylation of histone H3 and differentiation of EPCs. We also determine that the shear stress up-regulate SIRT1 expression by the PI3k/Akt signal pathways. The results demonstrate the association of SIRT1 expression with shear stress-induced EPC differentiation.

The endothelial differentiation of EPCs under shear stress, as shown in this study, was accompanied with the increase of SIRT1

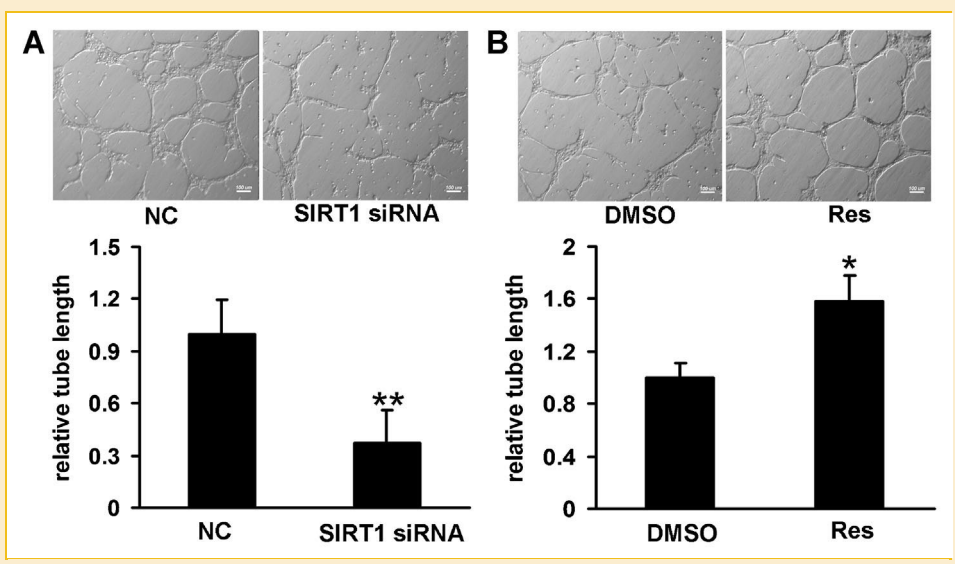

Fig. 7. Effect of SIRT1 on tube formation of EPCs on Matrigel. After treatment under different conditions, EPCs were seeded onto matrix gels formed with Biocoat Matrigel for 24 h. Representative microscopic view of tube formation in EPCs transfected with SIRT1 siRNA or negative control (NC) siRNA (A, upper), treated with resveratrol (Res) or DMSO (B, upper). Tube-like formation was not found in EPCs transfected with SIRT1 siRNA. Resveratrol treated EPCs were more active to form tubular-like structure compared with the DMSO controls. Scale bar = 100 μm. Image Pro-Plus software was used to quantitative analysis the tube-like structures, and the relative lengths of SIRT1 siRNA versus the control siRNA (A, bottom) and the resveratrol versus the DMSO (B, bottom) were presented. Values are mean ± SD of five images from three separate experiments, $^{*}P < 0.05$, $^{**}P < 0.01$ versus the corresponding controls, $n \geq 3$.

expression. Activation of SIRT1 using resveratrol and isonicotinamide stimulated osteoblastic differentiation in a dose-dependent manner, as assessed by the expression of mRNAs encoding alkaline phosphatase, osteopontin, osteocalcin, osterix, and Runx2, and induced calcium deposition [Lee et al., 2011]. Hisahara et al. [2008] reported that SIRT1 modulated neuronal differentiation by its nuclear translocation from cytoplasm of embryonic and adult neural precursor cells.

However, the effect of SIRT1 on differentiation in muscle and fat cells is different from that described above. SIRT1 overexpression actually inhibits their differentiation [Fulco et al., 2003; Picard et al., 2004]. Similarly, glucose restriction-mediated activation of AMPK prevents differentiation of skeletal muscle cells, which is followed by an increased SIRT1 activity [Fulco et al., 2008]. Homma's study inferred that SIRT1 was not involved in mesoblastic cells differentiation [Homma et al., 2010], because SIRT1 expression was not significantly different before and after VEGF-simulation. And inhibition of SIRT1 by the addition of 25 or 50 mM sirtinol during the induction period did not alter endothelial cell differentiation efficiency.

The reason for the opposite effects may be the differences of in vitro induction protocols, and SIRT1 may regulate different signaling pathways in different cells. It is reported that loss function of SIRT1 led to a significant decrease in the levels of all three dishevelled proteins, which serves as key messengers for as many as 19 Wnt ligands [Holloway et al., 2010]. This may explain the diverse physiological responses observed in different cellular contexts.

As a deacetylase localized in nuclear and cytoplasm, SIRT1 responses to shear stress may be mediated by upstream signal pathway. The PI3k/Akt pathway mediates stem/progenitor cells differentiation induced by shear stress or other stimuli through different downstream signal molecules [Zeng et al., 2006; Ye et al., 2008]. As referred before, Flk-PI3k/Akt-HDAC3-p53-p21 signal pathway promoted endothelial differentiation of mouse embryonic stem cells [Zeng et al., 2006]. Akt-FOXO3 signaling affects human EPC differentiation [Mogi et al., 2008]. Here, we demonstrated that Akt phosphorylation was associated with up-regulation of SIRT1. Referring to the downstream of SIRT1, histone H3 was studied. Wang et al. [2008] found that Sirt1−/− embryos die at middle gestation stages, increased acetylation of H3K9 and H4K16, reduced chromosome condensation, impaired heterochromatin formation, and abnormal mitosis. In this study, we found endothelial differentiation of EPCs under shear stress was companied by the decreased expression of Ac-H3K9, which may be partly responsible for SIRT1 mediated EPC differentiation. It was reported that oxidation results in a stronger association of SIRT1 to the Mash1 promoter, leading to a targeted and local deacetylation of H3K9 and subsequently to Mash1 inhibition in the process of redox regulated neural progenitors differentiation [Prozorovski et al., 2008]. Whether Mash1 or other SIRT1 target promoters are involved in the process of EPC differentiation needs to be determined in further research.

The current study initially demonstrated the effect of shear stress on SIRT1 expression in EPCs and elucidated the possible molecular mechanism of SIRT1 on the shear stress-induced EPC differentiation. Although further investigations are required in vivo, the present findings could significantly enhance our knowledge on the role of EPCs in vasculogenesis and vascular repair, and provide new insight into the development of vascular regenerative cell therapy for ischemic diseases. In summary, this study demonstrated that the shear stress activated a PI3k/Akt cascade, resulting in the up-regulation of SIRT1, which in turn deacetylated histone H3 and led to human blood cord derived-EPC differentiation toward vascular endothelial lineages while suppressed that of SMCs.

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