HDAC3 is crucial in shear- and VEGF-induced stem cell differentiation toward endothelial cells

Lingfang Zeng,[1] Qingzhong Xiao,[1] Andriana Margariti,[1] Zhongyi Zhang,[1] Anna Zampetaki,[1] Seema Patel,[1] Maurizio C. Capogrossi,[2] Yanhua Hu,[1] and Qingbo Xu[1]

[1]Department of Cardiac and Vascular Sciences, St. George's, University of London, London SW17 0RE, England, UK
[2]Laboratory of Vascular Pathology, Istituto Dermopatico dell'Immacolata, Istituto di Ricovero e Cura a Carattere Scientifico, 104-00167 Rome, Italy

Reendothelialization involves endothelial progenitor cell (EPC) homing, proliferation, and differentiation, which may be influenced by fluid shear stress and local flow pattern. This study aims to elucidate the role of laminar flow on embryonic stem (ES) cell differentiation and the underlying mechanism. We demonstrated that laminar flow enhanced ES cell–derived progenitor cell proliferation and differentiation into endothelial cells (ECs). Laminar flow stabilized and activated histone deacetylase 3 (HDAC3) through the Flk-1–PI3K–Akt pathway, which in turn deacetylated p53, leading to p21 activation. A similar signal pathway was detected in vascular endothelial growth factor–induced EC differentiation. HDAC3 and p21 were detected in blood vessels during embryogenesis. Local transfer of ES cell–derived EPC incorporated into injured femoral artery and reduced neointima formation in a mouse model. These data suggest that shear stress is a key regulator for stem cell differentiation into EC, especially in EPC differentiation, which can be used for vascular repair, and that the Flk-1–PI3K–Akt–HDAC3–p53–p21 pathway is crucial in such a process.

Introduction

Embryonic stem (ES) cells have pluripotent capacity for unlimited growth and self-renewal and the ability to differentiate into mature cells, including vascular cell lineages. Recent studies have revealed the effects of ES cell–derived endothelial cells (ECs) on the formation of new vessels and improvements in cardiac function, implicating the potential of these cells to be used for progenitor cell–associated applications (Pittenger and Martin, 2004). Differentiation of ES cells toward the endothelial lineage is an important step in vasculogenesis, in which signals initiated by vascular endothelial growth factor (VEGF), FGF, and other cytokines have been implicated. Most of our knowledge concerning endothelial differentiation from stem cells is derived from studies using growth factors and hypoxia environments, but less is known about the effect of mechanical forces on endothelial differentiation (Illi et al., 2005; Wang et al., 2005; Yamamoto et al., 2005).

ECs are critical cellular components of blood vessels, functioning as selectively permeable barriers between blood and tissues. The denudation or dysfunction of the intact endothelial monolayer causes lipid accumulation, monocyte adhension, and inflammatory reactions that initiate atherosclerotic lesion development (Ross, 1999; Xu, 2006). Recently, accumulating evidences indicate that endothelial progenitor cells (EPCs), possibly derived from adult stem cells, play an important role in endothelial repair (Werner et al., 2002, 2003; Walter et al., 2002; Sata, 2003; Xu et al., 2003; Rossig et al., 2005). During this process, incorporated EPCs are exposed to shear stress, which is a mechanical force generated by blood flow. Several studies have shown that EPCs can differentiate into endothelial phenotypes when shear stress is applied (Yamamoto et al., 2003; Illi et al., 2005; Wang et al., 2005; Yamamoto et al., 2005). However, the underlying mechanism remains to be elucidated.

The homeostasis between histone acetyltransferases and histone deacetylases (HDACs) regulates the structure and function of chromatin and some transcription factors, leading to gene transcription regulation. The HDAC family consists of 18 members, which are categorized into three classes (Gray and Ekstrom, 2001). HDAC3 belongs to class I HDACs, which contains four members, HDAC1, 2, 3, and 8, and is sensitive to trichostatin A (TSA). HDAC8 is reported to link with smooth muscle cell function (Waltregny et al., 2005). As HDAC1–3 are found expressed in most cell types in vitro, their role in cell differentiation is neglected. The aim of the present study was to investigate how laminar shear-initiated signal pathways were involved in stem

L. Zeng and Q. Xiao contributed equally to this paper.

Correspondence to Qingbo Xu: qxu@sgul.ac.uk

Abbreviations used in this paper: DM, differentiation medium; EC, endothelial cell; eNOS, endothelial nitric oxide synthase; EPC, endothelial progenitor cell; ES, embryonic stem; HDAC, histone deacetylase; HE, hematoxylin and eosin; MOI, multiplicity of infection; Sca, stem cell antigen; TSA, trichostatin A; VEGF, vascular endothelial growth factor.

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cell differentiation into ECs. We demonstrated that shear stress activated HDAC3 through the VEGF receptor 2 (Flk-1)–PI3K–Akt signal pathway, and that HDAC3-mediated p53 deacetylation and p21 activation was crucial for shear stress and VEGF-induced EC differentiation.

Results

Laminar flow promoted ES cell proliferation and differentiation

ES- or stem cell antigen-1–positive (Sca1^{+}) cells (vascular progenitors) were cultured on collagen IV–coated plates in the absence of LIF, and then subjected to 12 dyne/cm^2 laminar shear stress. As shown in Fig. 1 A, laminar flow increased the proliferation by ∼70% for both cells, as compared with static control. RT-PCR analysis revealed that withdrawal of LIF and culture on collagen IV–coated slides for 4 d increased mRNA expression of PECAM-1 (CD31), prominin 1 (CD133), VE-cadherin (CD144), VEGF receptor 1 (Flt-1), and VEGF receptor 2 (Flk-1) in both ES and Sca1^{+} progenitor cells, which was further enhanced by shear stress (unpublished data). Data shown in Fig. 1 B indicate the increase of Flk-1 and endothelial nitric oxide synthase (eNOS) proteins by shear in ES cells. FACS analysis revealed that CD31^{+} or CD133^{+} cells increased slightly, whereas VCAM-1–positive (CD106^{+}) cells increased significantly, in response to shear stress (Fig. 1 C). These results suggest that laminar flow increases the ES cell differentiation toward ECs.

To further explore whether the shear stress–induced, ES cell–derived ECs were functional, in vitro tube formation and in vivo neovascularization assays were performed. As shown in Fig. 1 D, sheared ES cells formed a tubelike structure on Matrigel, and no such structure was observed in the static control. After subcutaneous injection of cells mixed with Matrigel, the sheared cells formed capillary-like structures in all inoculations (6/6) at 1 and 2 wk (Fig. 1 E, shear), whereas only 1/6 of the cells from static group formed capillary-like structures at 2 wk, but 3/6 at 1 wk and 1/6 at 2 wk formed tumorlike structures (Fig. 1 E, static). To distinguish the exogenous ES cells from host cells, the ES cells were infected with the Ad-LacZ virus 12 h before injection. The contribution of shear-induced ECs to the formation of new blood vessels was shown in Fig. 1 E from X-gal staining.

The impact of p53 and p21 in ES cell differentiation

Because the p53–p21 pathway was reported in shear-induced cell cycle arrest in mature ECs (Akimoto et al., 2000; Lin et al., 2000;

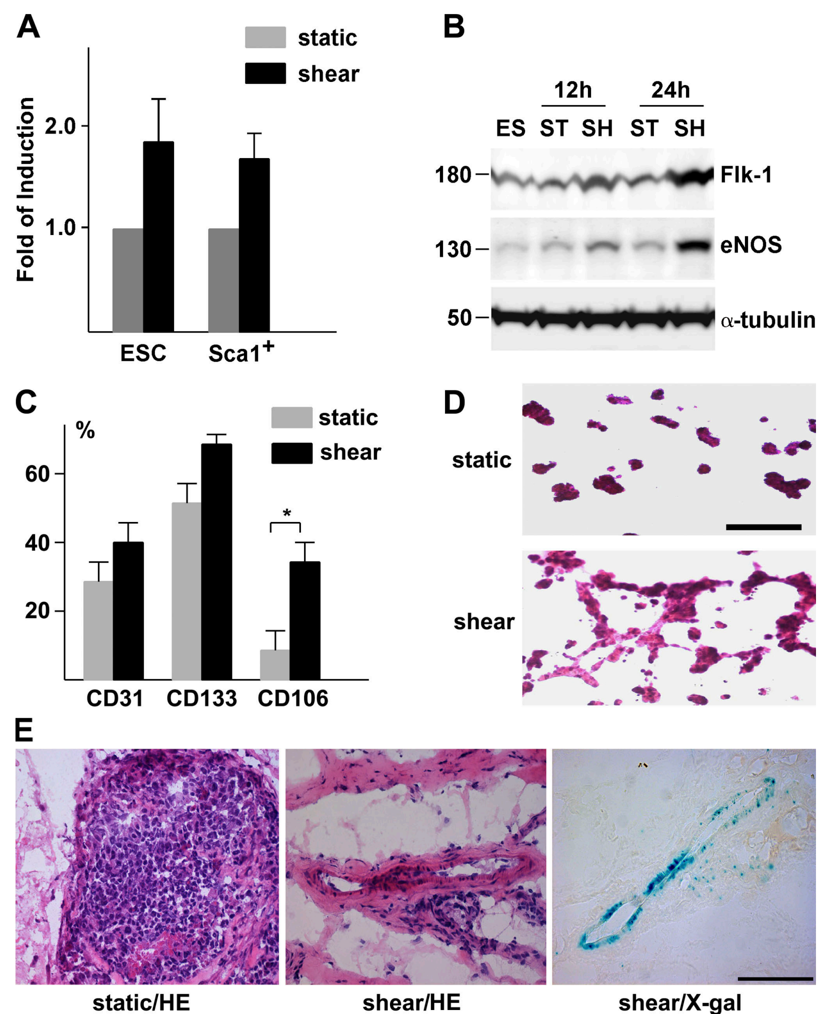

Figure 1. **Laminar flow resulted in ES cell–derived progenitor cell proliferation and differentiation into ECs.** (A) Shear-increased ES and Sca1^{+} cell proliferation. (B) Shear-increased Flk-1 and eNOS protein levels in ES cells. ES, routinely cultured ES cell; ST, static; SH, shear. (C) FACS analysis for EC marker expression, including CD31, CD133, and CD106, in response to shear stress for 24 h. *, $P < 0.05$. (D) In vitro tube-formation assay with static and sheared-ES cells on Matrigel. (E) Hematoxylin and eosin (HE) and X-gal staining for sections from in vivo angiogenesis assay at day 14. The data are representatives or means ± the SD of three independent experiments. Bars, 100 μm.

Mattiussi et al., 2004), we wondered whether such a pathway was involved in shear-induced EC differentiation. When laminar flow was applied, the p53 and p21 proteins increased gradually as shear proceeded (Fig. 2 A). Double immunostaining showed a colocalization of p53 or p21 with EC markers, e.g., CD106 (Fig. 2 B) and CD144 (not depicted). Overexpression of p53 increased CD144- and eNOS-Luc reporter gene expression, but slightly decreased Flk-1–Luc reporter gene expression in ES cells, whereas overexpression of p21 increased expression of all three reporter genes (Fig. 2 C).

To further confirm the impact of p53 and p21 in EC differentiation, we overexpressed p53 in ES cells via adenoviral gene transfer. FACS analysis showed that overexpression of p53 increased $CD31^+$, $CD106^+$, and $CD144^+$ cell numbers, but caused no change in $CD133^+$ cells (Fig. 2 D). Western blot analysis indicated that p53 overexpression led to the increase of CD31, CD144, eNOS, and p21 proteins in a dose-dependent manner (unpublished data). When cells were infected with adenovirus carrying p53 and injected into mice with Matrigel, all inoculates (6/6) showed vascular structures, and only 1/6 of controls displayed such a structure. Immunohistological staining revealed that all vascular structures were positive for p53, p21, and CD144 (Fig. 2 E). These results indicate that p53 up-regulation and the concomitant p21 activation can promote EC differentiation in vitro and in vivo.

To explore whether p53 and p21 were essential for shear-induced EC differentiation, ES cells were transfected with p53 or p21 siRNA for 2 d, followed by 12 dyne/cm^2 laminar flow treatment for 24 h. Western blot analysis showed that p53 siRNA treatment decreased shear-induced p53 and p21 protein production, and that shear-induced eNOS and Flt-1 protein levels were also decreased (Fig. 3 A). Meanwhile, p21 siRNA treatment decreased p21 proteins as expected, and shear-induced eNOS, Flk-1, and Flt-1 induction was ablated (Fig. 3 B). Shear-induced CD144 reporter gene expression was significantly decreased by treatment with either p53 or p21 siRNAs, although both siRNA treatments did not completely block CD144 reporter expression (unpublished data).

p53-mediated p21 activation was through deacetylation by HDAC3

As shown in Fig. 4 A, p53 acetylation at Lys317 and Lys370 sites was decreased, although p53 proteins were increased under shear, suggesting that laminar flow increases p53 deacetylation. To explore the role of HDACs in p53 deacetylation by shear, HDAC activity was detected in cell lysates from static and

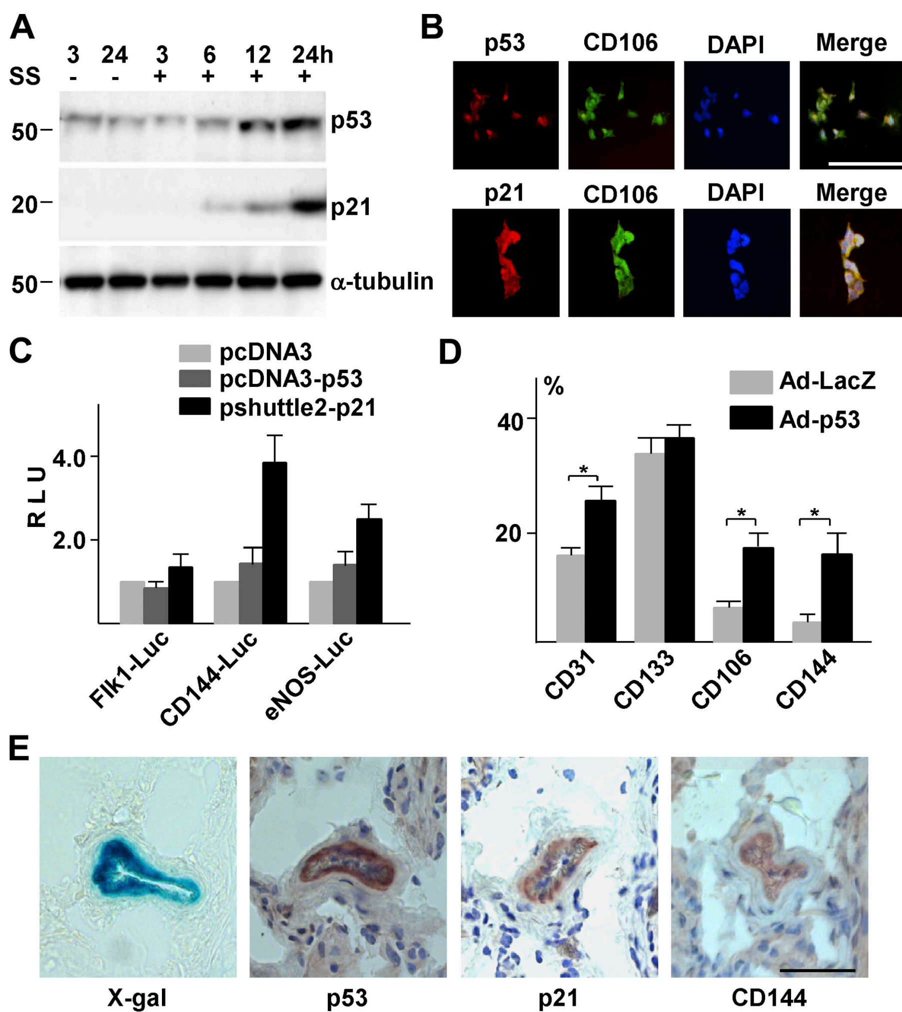

Figure 2. **Up-regulation of p53 and p21 correlated with shear-induced EC differentiation.** (A) Shear up-regulated p53 and p21 proteins in ES cells. SS, shear stress. (B) Double immunofluorescence staining showing the colocalization of p53 or p21 with CD106 in sheared-ES cells. (C) Overexpression of p53 and p21 increased EC marker reporter gene expression in ES cells. (D) FACS analysis showing the effect of overexpression of p53 by Ad-p53 (multiplicity of infection [MOI] = 50) on CD31, CD133, CD106, and CD144 expression. Ad-LacZ virus was included as a control. *, $P < 0.05$. (E) Representative images showing X-gal, anti-p53, p21, and CD144 staining of serial sections of inoculates from Ad-p53–infected ES cells. The data are representatives or means ± the SD from three independent experiments. Bars, 50 µm.

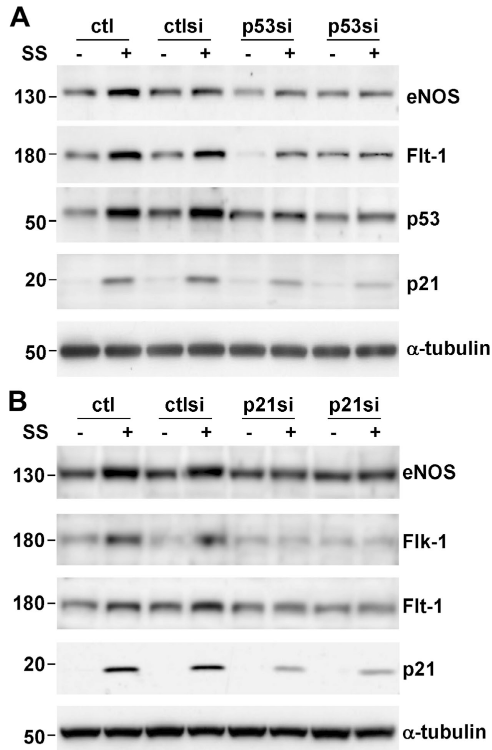

Figure 3. **Knockdown of p53 and p21 ablated shear-induced EC differentiation.** (A) Western blot analysis showing the effect of p53-siRNA on shear-induced p53, p21, eNOS, and Flt-1 proteins. Untransfected cells (ctl) and control siRNA (ctlsi) were included as controls. (B) Western blot analysis showing the effect of p21 siRNA on shear-induced p21 expression, eNOS, Flk-1, and Flt-1 protein production. The data are representative of three independent experiments.

sheared samples. Refreshment of growth medium slightly increased HDAC activity in static cells, which may be caused by growth factor stimulation present in the serum, whereas application of laminar flow on ES cells resulted in a significant elevation in HDAC activity, with a peak at ~6 h (Fig. 4 B). Western blot analysis revealed that HDAC1 and HDAC3 proteins were up-regulated by shear in a pattern similar to HDAC activity (Fig. 4 C), whereas other types of HDACs were undetectable or slightly decreased by shear (not depicted). As further studies revealed that HDAC1 was less relevant in EC differentiation, we focused our studies on HDAC3. Cotransfection assay showed that overexpression of HDAC3 could enhance p53-mediated p21-Luc reporter gene expression (Fig. 4 D).

To further confirm the role of HDAC3 in p53 deacetylation and p21 activation and its potential role in shear-induced EC differentiation, the HDAC inhibitor TSA was included in shear experiments. TSA completely abolished shear-induced HDAC activation (Fig. 5 A). As expected, shear-induced HDAC3 activation and p53 deacetylation were ablated, as revealed by an increase in p53 acetylation. TSA treatment led to an increase in p21 and eNOS proteins in static cells; shear stress could not increase these proteins any more in the presence of TSA, indicating that shear stress does not cooperate with TSA in the enhancement of these gene expressions (Fig. 5 B). When ES cells were transfected with HDAC3 siRNA, shear-induced p53 deacetylation, p21 activation, and Flt-1 expression were significantly reduced, as demonstrated by Western blot analysis (Fig. 5 C). Luciferase reporter assay revealed that HDAC3 siRNA treatment slightly inhibited CD144 reporter gene expression under static conditions, but significantly reduced shear-induced CD144 reporter gene expression (unpublished data). Overexpression of p21 partially rescued HDAC3 siRNA-mediated suppression of Flt-1 reporter gene expression (Fig. 5 D). These results indicate that HDAC3-mediated p53 deacetylation and p21 activation are essential for shear-induced EC differentiation from ES cells.

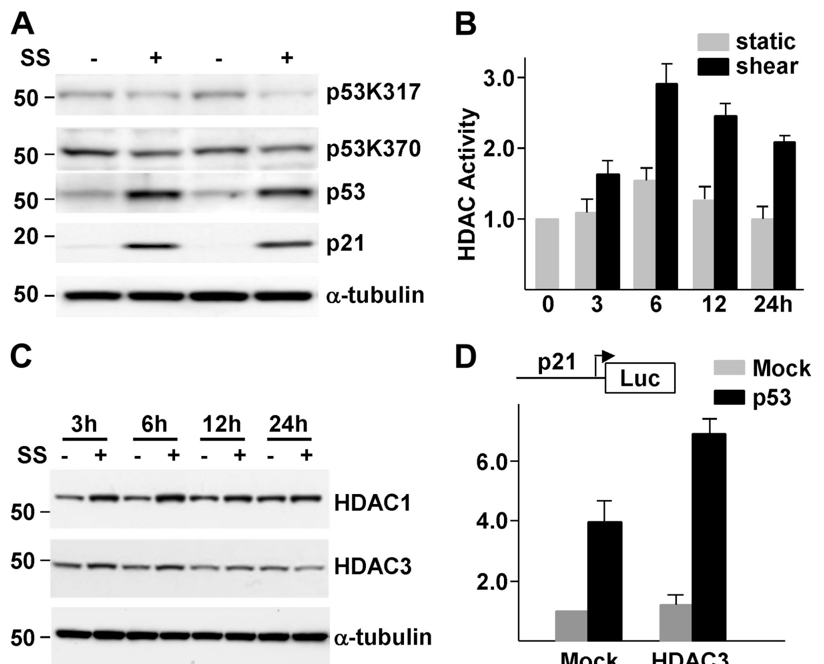

Figure 4. **HDAC3 contributed to shear-induced p53 deacetylation and p21 activation.** (A) Western blot to detect p53 acetylation status at Lys317 and Lys370 and p21 activation in duplicate slides from cells sheared for 24 h. (B) HDAC activity detected in static and shear samples harvested at the times indicated. (C) HDAC1 and HDAC3 proteins were up-regulated by shear treatment. (D) Overexpression of HDAC3 enhanced p53-mediated p21 reporter gene expression. The data are means ± the SD of three independent experiments.

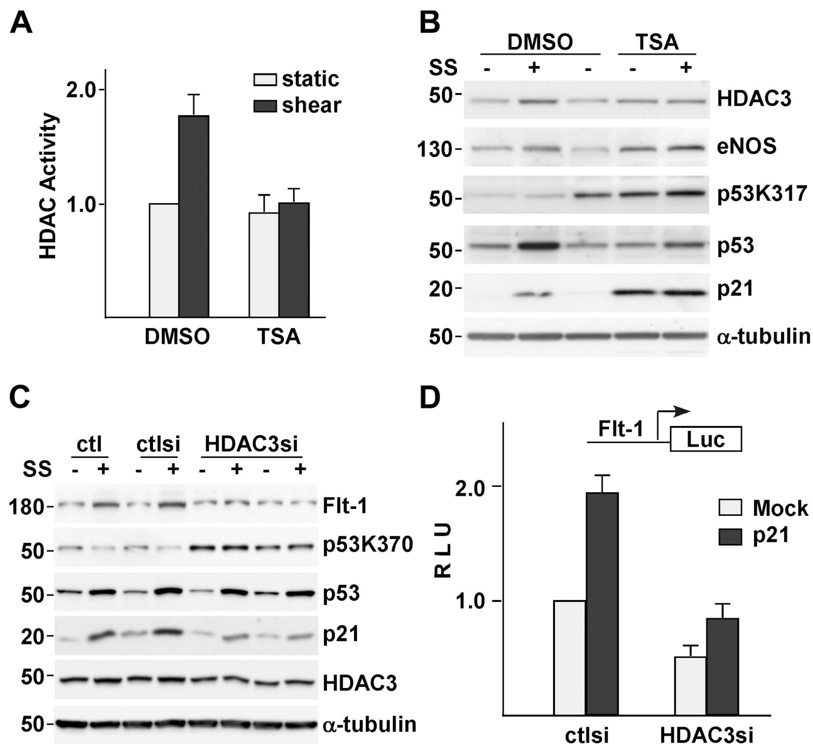

Figure 5. **Inhibition of HDAC3 ablated shear-induced p53 deacetylation, p21 activation, and EC differentiation.** (A) TSA abolished shear-induced HDAC activation. (B) TSA blocked shear-induced HDAC3 activation, p53 deacetylation, p21 activation, and EC differentiation. (C) Western blot data showing the effect of HDAC3 siRNA on shear-induced p53, acetyl-p53Lys370, p21, HDAC3, and Flt-1 proteins. Untransfected cells (ctl) and control siRNA (ctlsi) were included as controls. (D) Overexpression of p21 partially rescued HDAC3 siRNA–mediated suppression of Flt-1 reporter gene expression. The data are representatives or means ± the SD from three independent experiments.

The involvement of Flk-1–PI3K–Akt signal pathways

Western blot analysis demonstrated that both RNA and the protein synthesis inhibitors actinomycin D and cycloheximide decreased HDAC3 protein levels in static cells. When laminar flow was applied, HDAC3 increased by a similar fold (∼1.5-fold) in the absence or presence of the inhibitors (Fig. 6 A), implicating that shear stress up-regulates HDAC3 by post-translation stabilization. To clarify the upstream signal pathways, we studied VEGF and related signal transducers. In the presence of VEGF antagonist SU1498, shear stress could not increase HDAC3 proteins further, and the HDAC activity was abolished (Fig. 6, B and C). Consequently, shear-induced p53 deacetylation, p21 activation, and eNOS expression were ablated (Fig. 6 B). These results indicate that VEGF receptor Flk-1 is crucial for shear-induced HDAC3 stabilization and EC differentiation. It has been reported that shear stress activated Flk-1 in a ligand-independent manner (Jin et al., 2003; Yamamoto et al., 2005). To explore whether ligand-dependent activation of Flk-1 could also contribute to HDAC3 stabilization, the effect of VEGF-165 on HDAC activity and HDAC3 proteins were detected in ES cells. VEGF-165 treatment transiently increased HDAC3 proteins (Fig. 6 D) with concomitant increase of HDAC activity (not depicted). Furthermore, both laminar flow and VEGF-165 treatment resulted in Akt phosphorylation, but different effects were observed on ERK phosphorylation (Fig. 6, E and F). Flk-1 inhibitor SU1498 abolished both shear- and VEGF-induced HDAC3 stabilization and Akt phosphorylation. ERK Kinase (MAKK) inhibitor PD98059 inhibited VEGF-induced ERK phosphorylation, but no effect on shear and VEGF-induced HDAC3 stabilization and Akt phosphorylation, whereas PI3K inhibitor LY294002 inhibited both shear- and VEGF-induced HDAC3 stabilization and Akt phosphorylation.

Similarly, HDAC3 was also demonstrated to be crucial for VEGF-induced EC differentiation, as HDAC3 siRNA ablated VEGF-induced EC marker gene reporter (eNOS-Luc, Flt-1-Luc, and vWF-Luc) expression in ES cells (Fig. 7 A), and high levels of HDAC3, p53, and p21 were detected in VEGF-induced blood vessels (Fig. 7 B).

HDAC3 and p21 was highly expressed in embryonic blood vessel

As described above, the HDAC3–p53–p21 pathway was essential for shear- and VEGF-induced ES cell differentiation toward endothelial lineage in vitro, and might function in the in vivo blood vessel formation derived from these cells. To explore whether such a pathway was also involved in angiogenesis during embryonic development, their expression was checked in blood vessels of 13.5 d *Tie2-LacZ*–transgenic mice embryos. As shown in Fig. 7 C, blood vessels were positively stained for β-gal and CD31 in endothelium, whereas HDAC3 and p21 labeled the whole vessel wall. These results suggest that HDAC3 and p21 may be involved in vasculogenesis during embryo development.

ES cell–derived $Sca1^+$ progenitor cells repair injured vessels

To explore whether the in vitro–produced EPCs could incorporate into the injured vessel and increase the repair in vivo, a femoral artery injury model was conducted in $ApoE^{-/-}$ mice. Considering that in vitro–differentiated ECs directly from ES cells take up only a small portion of the cell population, and that other types of cells might interfere with the experiment, $Sca1^+$

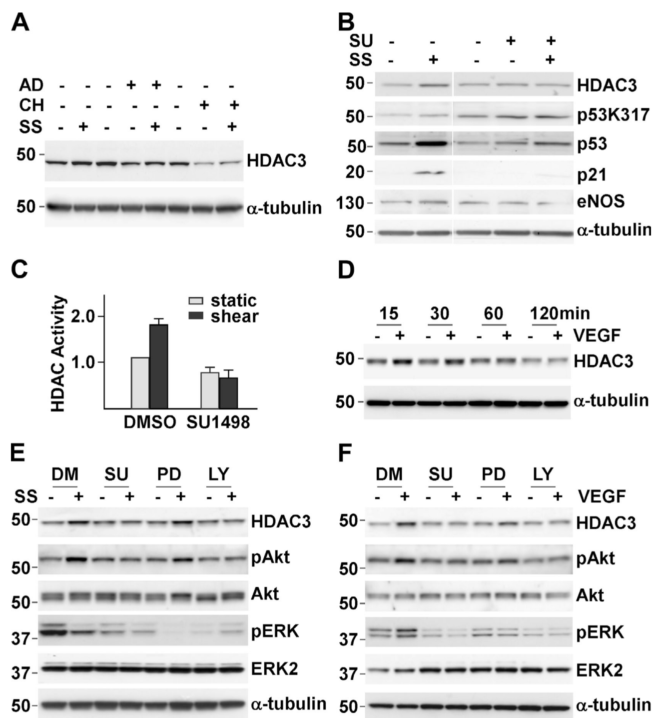

Figure 6. **Shear- and VEGF-stabilized HDAC3 through Flk-1–PI3K–Akt signal pathways.** (A) RNA and protein synthesis inhibitors did not affect shear-increased HDAC3 protein production. (B) Flk-1 inhibitor ablated shear-induced HDAC3 stabilization, p53 deacetylation, p21 activation, and EC differentiation. (C) Flk-1 inhibitor abolished shear-induced HDAC activation. (D) VEGF transiently up-regulated HDAC3 protein induction. (E) Western blot data showed that shear-induced HDAC3 stabilization was related to Flk-1–mediated Akt phosphorylation. (F) Western blot analysis revealed that VEGF-induced HDAC3 stabilization was related to Flk-1–mediated Akt phosphorylation. AD, actinomycin D; CH, cycloheximide; LY, LY294002; PD, PD98059; SS, shear stress; SU, SU1498. The data are representatives or means ± the SD from three independent experiments.

progenitor cells were alternatively used. As shown in Fig. 8 A, β-gal–labeled exogenous VEGF-treated Sca1$^+$ cells incorporated into the injured vessel wall when checked on day 3, which also showed CD31-positive staining. The injured vessel was completely occluded by neointimal lesions 2 wk postoperatively (Fig. 8 B, b). However, local transfer of VEGF-treated Sca1$^+$ cells significantly reduced neointima lesions (Fig. 8 B, c and d). Statistically, the progenitor cell–treated group had neointimal lesions decreased by ∼30–40% (Fig. 8 B, e). To further explore whether shear stress–induced EPCs had similar function, Sca1$^+$ cells were selected from 24-h sheared ES cells. FACS analysis showed that Sca1$^+$ cells took up ∼15% of the population in sheared ES cells before sorting, and >95% after sorting. After expansion, the shear stress–induced Sca1$^+$ cells were used in similar femoral artery injury repair experiments. As expected, shear stress–induced Sca1$^+$ cells could also incorporate into the injured vessel (Fig. 8 C, c) and reduced the neointima formation (Fig. 8 C, b, d, and e) to a lesser extent, as compared with VEGF-treated Sca1$^+$ cells (Fig. 8, B [e] vs. 8 C [e]). These results demonstrate that the in vitro–differentiated EPCs and/or ECs can incorporate into injured artery and reduce neointimal lesions by increased repairing.

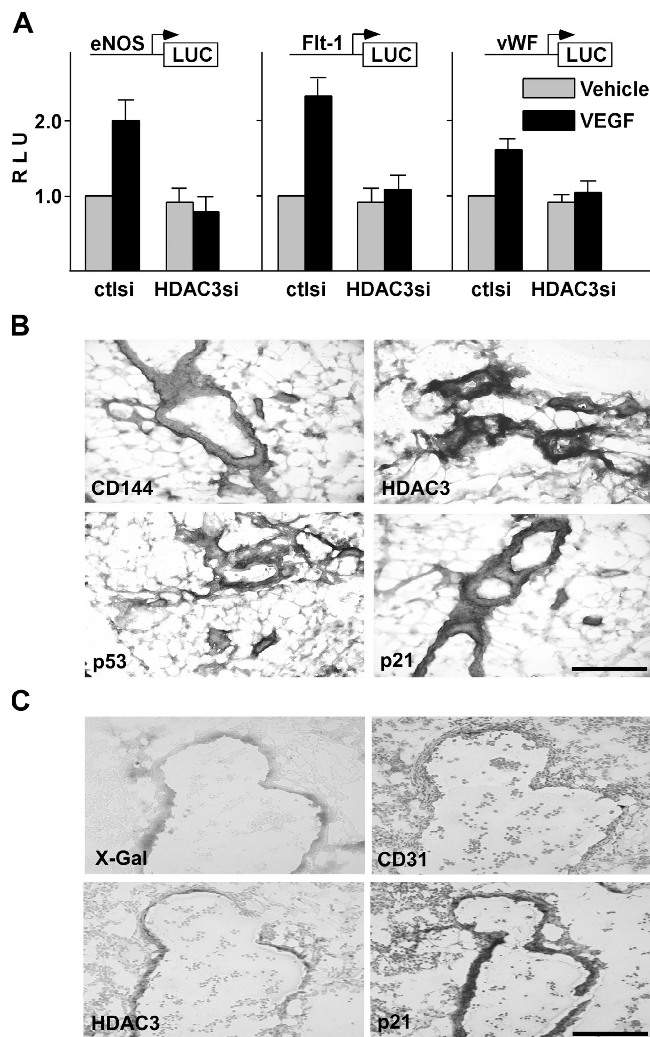

Figure 7. **HDAC3 was involved in VEGF-induced EC differentiation and blood vessel formation in vivo.** (A) HDAC3 siRNA ablated VEGF-induced EC marker expression in ES cells. The data are means ± the SD of three independent experiments. (B) A representative of immunohistological staining for CD144, HDAC3, p53, and p21 in blood vessels formed by VEGF-treated Sca1$^+$ cells. (C) A representative of the X-gal staining and immunohistological staining for CD31, HDAC3, and p21 in blood vessels in D13.5 embryonic sections from *Tie2-LacZ*/ApoE$^{-/-}$ mice. Bars, 200 μm.

Discussion

Under specific stimulation, e.g., growth factors (Schaper and Scholz, 2003), extracellular matrix (Kleinman et al., 2003), mechanical forces (Ingber, 2002; Schaper and Scholz, 2003), and coculture with other cell types (Shen et al., 2004; Wurmser et al., 2004), progenitor cells undergo specific lineage differentiation. In this study, we demonstrate that laminar flow enhances the differentiation of stem cells into functional ECs. We show for the first time that HDAC3 deacetylates p53, leading to p21 activation and resulting in differentiation of stem/progenitor cells into mature ECs. We also provide evidence that shear activates HDAC3 by posttranslation stabilization through Flk-1–PI3K–Akt signal pathways, and that HDAC3 and p21 may be involved in blood vessel formation in vivo. Our findings of mechanistic aspects for shear-induced stem

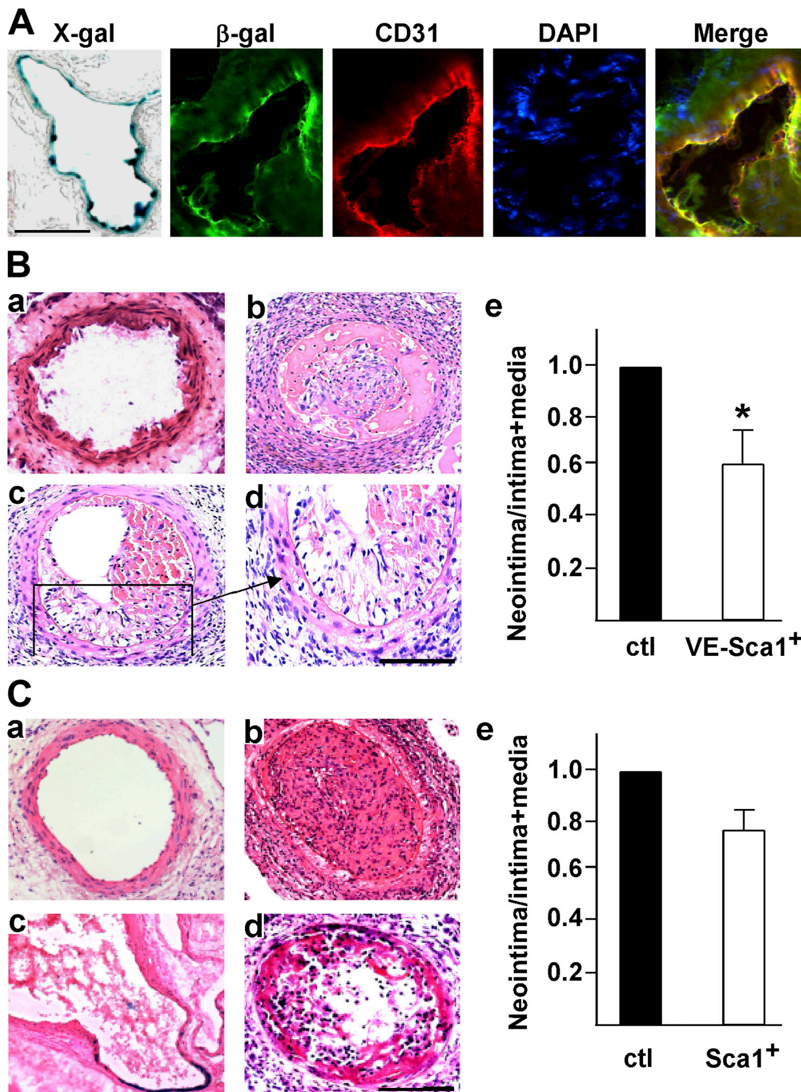

Figure 8. **Sca1$^+$ cells incorporated into injured artery and reduced lesion formation in ApoE$^{-/-}$ mice.** (A) X-gal and double immunofluorescence staining showed the incorporation of VEGF-treated Sca1$^+$ cells into the injured femoral artery. (B) VEGF-treated Sca1$^+$ cell reduced the lesion formation caused by artery injury. a, uninjured artery; b, injured artery without cell treatment; c, injured artery with cell treatment; d, higher magnification of image c; e, statistical analysis of the ratio of neointima to the media. (C) Shear stress–induced Sca1$^+$ cell reduced the lesion formation caused by artery injury. a, uninjured artery; b, injured artery without cell treatment; c, X-gal staining showed the incorporated Sca1$^+$ cell; d, injured artery with Sca1$^+$ cell treatment; e, statistical analysis of the ratio of neointima to the media. Data are means ± the SD from six animals per group. Bars: (A–C) 100 µm; (c) 50 µm.

cell differentiation toward ECs could significantly enhance our knowledge on stem cells involved in vasculogenesis, angiogenesis, and vascular repair.

Cultured on collagen IV–coated plates in the absence of LIF for 3–4 d, ES cells could spontaneously differentiate into EPCs, some even into mature ECs, as identified by the up-regulation of EPC markers, e.g., Flk-1, CD31, CD133, eNOS, and the EC marker CD144. We propose that shear stress significantly enhances both processes, i.e., vascular progenitor differentiation into EPCs, and EPCs into ECs. Shear-produced EPCs could be derived from the differentiation of bipotential vascular progenitor cells. Supporting this notion is the fact that vascular progenitor cells can differentiate into both vascular smooth muscle cells and ECs in response to PDGF and VEGF treatment, respectively (Yamashita et al., 2000). Previous studies showed that shear stress induced EC differentiation, while suppressing smooth muscle differentiation (Wang et al., 2005). In our experiments, we also observed that smooth muscle differentiation was down-regulated by shear stress (unpublished data). It seems that shear-induced EC differentiation is at the cost of reduced smooth muscle differentiation. If this is also true for adult vessels in vivo, a better explanation could be offered as to why high-shear areas in the arterial wall have an integrative endothelium that is resistant to atherosclerosis. Thus, vascular progenitor differentiation toward ECs could be influenced by shear stress in vivo.

VEGF receptor 2 (Flk-1) is a major progenitor cell marker for hematopoietic and endothelial lineage, expressed from hemangioblast to mature ECs (Schatteman and Awad, 2004). Flk-1 mediates not only VEGF-induced EC differentiation but also VEGF-induced mature endothelial function, such as proliferation, migration, permeability, and cell survival (Claesson-Welsh, 2003; Ferrara et al., 2003; Zachary, 2003; Coultas et al., 2005). It was reported that shear stress could activate Flk-1 in a ligand-independent manner (Jin et al., 2003; Yamamoto et al., 2005). We demonstrated that shear stress–induced EC differentiation was mediated through Flk-1, as the Flk-1–specific inhibitor SU1498 abolishes shear-induced eNOS expression. Activation of Flk-1 can trigger several downstream signal pathways, among which the PI3K–Akt pathway is defined to cell survival, whereas the PLC–ERK pathway is defined to proliferation (Fujio and Walsh, 1999; Takahashi et al., 2001; Cross et al., 2003).

In ES-derived progenitor cells, shear stress activated Akt phosphorylation but suppressed ERK phosphorylation, whereas VEGF activated both Akt and ERK phosphorylation. Moreover, we demonstrated for the first time that the PI3K–Akt pathway led to HDAC3 activation. Akt phosphorylation status correlated with HDAC3 protein levels, and its inhibition by SU1498 and LY294002 abolished shear-induced HDAC3 protein increase. Further investigation of how Akt phosphorylation modulates HDAC3 is necessary.

Previous studies reported that inhibition of HDAC activity by TSA blocked tumor angiogenesis, especially hypoxia-induced angiogenesis (Sawa et al., 2002; Liu et al., 2003). It is well known that hypoxia induces tumor angiogenesis and cell survival through the up-regulation of VEGF expression in tumor cells (Harris, 2002; Byrne et al., 2005). These studies provide indirect evidence for the potential role of HDACs in EC differentiation. Although Rossig et al. (2005) reported that shear-activated HDAC1 and HDAC3 were involved in shear-induced adult EPC differentiation through up-regulation of HoxA9, we demonstrated that HDAC3 activation led to p53 deacetylation and, in turn, to p21 activation. In this study, HDAC3 was found to be activated by shear stress and VEGF, whereas inhibition of HDAC activity by TSA or siRNA-mediated knockdown of HDAC3 abolished shear- and VEGF-induced EC marker gene expression. Collectively, these results suggest an essential role of HDAC3 in shear- and VEGF-induced stem cell differentiation in vitro, and a potential role in blood vessel formation in vivo.

Evidence indicates that lack of p21 expression increases EPC sensitivity to apoptosis, with retarded EC maturation (Lai et al., 2003; Bruhl et al., 2004). In this study, we demonstrated that shear stress activated p21 through p53 deacetylation mediated by HDAC3, and that p21 activation was essential for shear-induced EC differentiation. p53 knockdown ablated shear-induced p21 activation, indicating that p21 activation by shear is p53-dependent. Both inhibition of HDAC activity by TSA and HDAC3 siRNA decreased shear-induced p53 deacetylation and p21 activation, implicating that p21 activation is also HDAC3 dependent. Other evidence came from the observations that overexpression of HDAC3 could enhance p53-mediated p21 expression, and high levels of HDAC3 and p21 were detected in blood vessels formed from in vitro–differentiated ECs and during embryogenesis. Other studies showed that p53 acetylation was linked to p21 activation (Di Stefano et al., 2005; Zhao et al., 2006). The discrepancy may be caused by different cell types. In response to different stimuli, native p53 and acetylated p53 recruit different coactivators to form a complex binding to p21 promoter. We show that HDAC3, p53, and p21 are directly linked to EC differentiation in vitro. These findings suggest that p21 is downstream of HDAC3 and plays a central role in shear-induced EC differentiation.

In this study, we also observed that in vitro–differentiated EPCs/ECs could form blood vessels in vivo, and appear to play a crucial role in endothelial restoration and vascular repair. Treatment with these vascular progenitors led to a significant reduction of neointimal lesions in a mouse model of femoral artery injury, through replacement of the denuded endothelium. Newly isolated Sca1$^+$ progenitor cells were less effective than VEGF-treated ones. The discrepancy was derived from the observation that vascular progenitor cells were able to differentiate toward both smooth muscle cell and EC, whereas VEGF induced specific differentiation toward endothelial lineage (Yamashita et al., 2000). Indeed, VEGF-treated cells showed much more EC marker expression. The functionally intact endothelial monolayer suppresses the recruitment of inflammatory cells and, thus, modulates the process of vascular remodeling and down-regulates intima hyperplasia. These observations provide further support to the concept that differentiation of stem cells toward ECs is beneficial to the blood vessel.

In summary, this study demonstrates that shear stress activates Flk-1 and its downstream PI3K–Akt cascade, resulting in the activation of HDAC3, which in turn deacetylates p53 and activates p21. p21 activation results in stem cell differentiation into ECs and an increase in their survival. HDAC3 may also be involved in epigenetic modification of the chromatin to regulate gene transcription, which is essential for modulation of EC marker gene expression, together, leading to stem cell differentiation. Thus, HDAC3 is essential for shear- and VEGF-mediated stem cell differentiation, which can be a new target for therapeutic use.

Materials and methods

Materials

Antibodies against CD31 (rat), Flk-1, and Flt-1 were purchased from Abcam; antibodies against CD106 (rat) and CD144 (rat) were obtained from BD Biosciences; antibody against CD133 (rat) was purchased from eBioscience; antibodies against β-gal, CD31 (goat), CD144 (mouse), HDAC1, p21, p53, p-AktThr308, Akt1/2 (goat), pERK42/44, and ERK2 were obtained from Santa Cruz Biotechnology, Inc.; antibodies against eNOS, HDAC3, and α-tubulin (mouse) were purchased from Sigma-Aldrich; and antibodies against acetylated p53Lys320 and p53Lys373 were obtained from Millipore. All antibodies were raised in rabbit, except those indicated. All secondary antibodies were purchased from DakoCytomation. The inhibitors actinomycin D (1 μg/ml), cycloheximide (30 μg/ml), LY294002 (5 μM), PD98059 (5 μM), SU1498 (1 μM), and TSA (50 nM) were purchased from Sigma-Aldrich, dissolved in DMSO, and used at the indicated concentrations. The cells were pretreated with a specific inhibitor for 1 h before further treatment in the presence of the inhibitor. Other chemicals were also purchased from Sigma-Aldrich.

Cells and cell culture

Mouse ES cells (ES-D3 cell line, CRL-1934; American Type Culture Collection [ATCC]) were maintained as previously described (Vittet et al., 1996). In brief, the ES cells were cultured in gelatin-coated flasks in DME (ATCC) supplemented with 10% FBS (ATCC), 10 ng/ml LIF (CHEMICON International, Inc.), 0.1 mM 2-mercaptoethanol, 100 U/ml penicillin, and 100 μg/ml streptomycin in a humidified incubator supplemented with 5% CO_2, and they were split at a 1:6 ratio every other day. Sca1$^+$ cells were isolated as previously described (Hu et al., 2004), and maintained in the same condition as ES cells. Both ES and Sca1$^+$ cells were used for $<$20 passages in this study. For differentiation, ES or Sca1$^+$ cells were predifferentiated by culture on mouse collagen IV (5 μg/ml)–coated glass slides or flasks or plates in differentiation medium (DM; α-MEM medium [Invitrogen] supplemented with 10% FBS [Invitrogen], 0.05 mM 2-mecaptoethanol, 100 U/ml penicillin, and 100 μg/ml streptomycin) for 3 or 4 d before further treatment. The medium was refreshed every other day.

Shear and VEGF treatment

Shear experiments were conducted as previously described (Zeng et al., 2003). In brief, the predifferentiated cells were exposed to a laminar flow generated by the pressure difference between the upper and lower reservoirs, with the effluent DM medium circulated back to the upper reservoir through a peristaltic pump. The shear stress, which is determined by the

flow rate and the channel dimensions, was 12 dyne/cm^2, which is comparable with the physiological range in human major arteries. The entire flow system was placed in an incubator at 37°C and supplemented with 5% CO_2 to maintain pH. Static controls were cells cultured on slides not exposed to flow. To prevent contamination, 5 µg/ml gentamycin and 0.1 µg/ml doxycycline were included in shear medium.

For VEGF-induced HDAC activation assay, the predifferentiated ES cells were pretreated with serum-free α-MEM supplemented with 0.05 mM 2-mercaptoethanol and antibiotics for 2 h, followed by treatment with 10 ng/ml mouse VEGF-165 (Bender Medsystems) for the time indicated in Fig. 6 D. For HDAC3 siRNA assay, the predifferentiated ES cells were transfected with HDAC3 siRNA or control siRNA for 2 d, followed by 10 ng/ml VEGF-165 treatment in serum-free α-MEM supplemented with 0.05 mM 2-mercaptoethanol, 1% BSA, 10 ng/ml insulin, and antibiotics for 24 h. For VEGF-induced differentiation, Sca1$^+$ cells were cultured in DM medium in the presence of 10 ng/ml VEGF-165 for 1 wk, followed by either an in vivo angiogenesis assay or a femoral injury repair experiment.

Cell proliferation and flow cytometry analysis

A portion of the 24-h sheared or static cells was seeded in 12-well plates in the presence of MTT reagents (Promega) for 1 h, and the cell proliferation was detected according to the protocol provided. The procedure used for flow cytometry was similar to that previously described (Mayr et al., 2000). Rat anti-CD31, CD106, CD133, and CD144 antibodies were used for EC markers.

In vitro tube formation assay

The procedure used was similar to that described previously (Kaufman et al., 2004). Cell suspension containing 4×10^4 static or sheared ES cells was placed on top of the 50 µl/well Matrigel (10 mg/ml; Becton Dickinson) in 8-well chamber slides (Nunc). Rearrangement of cells and the formation of capillary-like structures were observed at 18–24 h. Cells were fixed with 4% paraformaldehyde in PBS at 4°C overnight, and then HE staining was performed. Images were assessed with Axioplan 2 imaging microscope with Plan-NEOFLUAR 10×, NA 0.3, objective lenses, AxioCam camera, and Axiovision software (all Carl Zeiss MicroImaging, Inc.) at room temperature, and were processed with Photoshop software (Adobe).

In vivo angiogenesis assay, embryo section preparation, and femoral artery injury

All cells were labeled with the Ad-LacZ virus (MOI = 20) before in vivo angiogenesis assay to distinguish the in vitro–differentiated cells from the host cells. 10^6 cells were pelleted and mixed with 50 µl Matrigel, and then injected subcutaneously into the back or flank of C57BL/6 mice. Six injections were conducted for each group. The mice were killed on days 7 and 14, and the plaques were harvested and frozen immediately in liquid nitrogen, followed by embedding with OCT and sectioning. 13.5-d embryos were collected from *Tie2-LacZ*/ApoE$^{-/-}$ mice (Hu et al., 2004) and cryosectioned.

For artery injury model, both the left and right femoral arteries of ApoE$^{-/-}$ mice were injured by wire. Ad-LacZ virus–labeled Sca1$^+$ cells (10^6) in 30 µl PBS were injected into one artery, 30 µl PBS was injected into the other side as a control, and both were incubated for 30 min, followed by restoring the blood flow. Arteries were harvested at day 3 for cryosections or day 14 for formalin-fixed sections. The sections were stained with HE or X-gal (Hu et al., 2003), or immunohistologically stained with specific antibodies, and observed under the microscope. Images were assessed and processed as described above except 20×, NA 0.5, and 40×, NA 0.75, objective lenses were used instead. All animal experiments were performed according to protocols approved by the Institutional Committee for Use and Care of Laboratory Animals.

Immunoblotting, immunofluorescence staining, and HDAC activity assay

Cells were harvested and lysed by sonication. 50 µg of whole cell lysate was subjected to standard immunoblotting procedure and probed with primary antibodies as indicated. The bound primary antibody was detected by use of HRP-conjugated secondary antibody and the ECL detection system (GE Healthcare). The procedure used for immunofluorescence staining was similar to that described previously (Xu et al., 2003). Rabbit anti–β-gal, p53, and p21, and rat anti-CD31, CD106, and CD144 antibodies were used. Swine anti–rabbit IgG-FITC and swine anti–rat IgG–TRITC antibodies were used to label the bound primary antibodies, which were illuminated by blue and green light, respectively. Images were assessed and processed as described in the previous paragraph, except that 40×, NA 0.75, objective lenses were used instead. HDAC activity was determined by using the Colorimetric HDAC Activity Assay kit (BioVision) with the protocol provided. The cells were lysed by sonication, and 50 µg of whole cell lysate was used to detect HDAC activity; relative HDAC activity was defined as A405 nm/µg protein, with that of control set as 1.0.

Plasmids and transient transfection and adenoviral infection

The expression vectors for HDAC3, p21, and p53, and the reporter systems for CD144, eNOS, Flk-1, Flt-1, and vWF were cloned with vectors and primers in Table I. For transient transfection, ES cells were cultured in a collagen IV–coated 12-well plate for 3 d, and then transfected with reporter gene (0.33 µg/well) together with expression plasmids (0.33 µg/well) and/or siRNA (0.033 nmol/well) using Fugene 6 reagent (Invitrogen) according to the protocol provided. pShuttle2-LacZ plasmid (0.15 µg/well) was included in all transfection assays as internal control, and pShuttle2 vector and control siRNA were used as a mock control. Luciferase activity assay was performed at 36 or 72 h (siRNA) after transfection. Luciferase and β-galactosidase activities were detected with standard protocol. Relative luciferase unit was defined as the ration of luciferase activity to β-galactosidase activity, with that of controls set as 1.0.

Table I. **Vectors and primer sequences for plasmid construction**

Gene name	Vector name	Primer sequence
HDAC3	pShuttle2-Flag[a]	5′-atg acc ggt acc gtg gcg tat ttc tac gac-3′ 5′-cac agc aag ctt gct gct cta aat ctc cac-3′
p21	pShuttle2-Flag	5′-gag aca ggt acc tcc aat cct ggt gat gtc-3′ 5′-aga gag gaa gct tga ccc aca gca gaa gag-3′
p53	pShuttle2	5′-tgg atg gct agc atg gag gag tca cag tcg-3′ 5′-tgg tga ctt aag cgg gat gca gag gca gtc-3′
CD144 promoter	pGL3-Luc basic	5′-gac aag gag ctc tgg aag agc aac tga tgc-3′ 5′-gtt cag gct cga gct ttg tgg aga gca cag-3′
ENOS promoter	pGL3-Luc basic	5′-acc gac ctc gag aag aac cta gca gtg g-3′ 5′-agt tgc aag ctt cag ctg acc tca act ctg-3′
Flk-1 promoter	pGL3-Luc basic	5′-gac tac ctc gag cca gga agt tca caa cc-3′ 5′-gtc ctg aag ctt ctc agg gca gaa aga gag-3′
Flt-1 promoter	pGL3-Luc basic	5′-gac gtt ctg agc tct tac aag ttg cag gag-3′ 5′-cac tgc gag ctc tca agg acc tcg ctg-3′
VWF promoter	pGL3-Luc basic	5′-tgc cag ctc gag aac ttg taa gac caa ctc-3′ 5′-tgc cag aag ctt ctc tgc ctg caa tag ctc-3′

[a]pShuttle2-Flag was a modified expression vector, which was constructed by replacing the NheI–MCS–AflII fragment with NheI–Flag–MCS–AflII fragment; the latter fragment was amplified from pCMV5-Flag vector with primer set of 5′-tgc ctg gct agc atg gac tac aag gac gac gat-3′ and 5′-tca ctt act taa gct gga tcc tct aga gtc gac-3′

Predifferentiated ES cells were infected with Ad-p53 viruses at a MOI of 50 for 12 h, followed by in vivo angiogenesis assay, or for 36 h, followed by FACS analysis. Ad-tTA and Ad-LacZ viruses were used as controls, respectively.

siRNA knockdown

The siRNAs for p53(sc-29436) and p21(sc-29428) were purchased from Santa Cruz Biotechnology, Inc. The control siRNA and the siRNA for HDAC3 (5′-ccucaucgccuggcauugatt-3′ and 5′-ucaaugccaggcgaugaggtt-3′) were purchased or synthesized from Ambion. For the siRNA knockdown experiments, ES cells were cultured on collagen IV–coated slides (38 × 76 mm) for 3 d, and the medium was refreshed at 24 h and 1 h before transfection. 10 μl of 10 mM siRNA per slide was introduced into the cells with siIMPORTER transfection reagents (Millipore), according to the manufacture's protocol. The untreated and nonrelated siRNA-transfected cells were included as controls. For specific target genes, siRNA transfection was performed in duplicate. The transfected cells were cultured for an additional 48 h and subjected to 12 dyne/cm^2 laminar flow for 24 h, followed by Western blot analysis.

Statistical analysis

Data expressed as the mean ± the SEM were analyzed with a two-tailed *t* test, for two groups, or pair-wise comparisons. A value of $P < 0.05$ was considered to be significant.

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