Histone deacetylase activity is essential for the expression of *HoxA9* and for endothelial commitment of progenitor cells

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The regulation of acetylation is central for the epigenetic control of lineage-specific gene expression and determines cell fate decisions. We provide evidence that the inhibition of histone deacetylases (HDACs) blocks the endothelial differentiation of adult progenitor cells. To define the mechanisms by which HDAC inhibition prevents endothelial differentiation, we determined the expression of homeobox transcription factors and demonstrated that *HoxA9* expression is down-regulated by HDAC inhibitors. The causal involvement of *HoxA9* in the endothelial differentiation of adult progenitor cells is supported by the finding that *HoxA9* overexpression partially rescued the endothelial differentiation blockade induced by HDAC inhibitors. Knockdown and overexpression studies revealed that *HoxA9* acts as a master switch to regulate the expression of prototypical endothelial-committed genes such as endothelial nitric oxide synthase, *VEGF-R*$_2$, and VE-cadherin, and mediates the shear stress–induced maturation of endothelial cells. Consistently, *HoxA9*-deficient mice exhibited lower numbers of endothelial progenitor cells and showed an impaired postnatal neovascularization capacity after the induction of ischemia. Thus, *HoxA9* is regulated by HDACs and is critical for postnatal neovascularization.

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Abbreviations used: BMC, BM cells; butyrate, BuA; EBM, endothelial basal medium; eNOS, endothelial nitric oxide synthase; EPC, endothelial progenitor cell; ES, embryonic stem; HDAC, histone deacetylase; Hox, Homeobox gene; HUVEC, human umbilical vein endothelial cell; MNC, mononuclear cell; mt, mutant; siRNA, small interfering RNA; TSA, Trichostatin A; vWF, von Willebrand factor.

Endothelial progenitor cells (EPCs) can originate from bone marrow–derived progenitor cells, circulate with the blood on mobilization from the bone marrow, and home to sites of active vessel growth, a process termed "adult vasculogenesis" (1, 2). The recruitment of EPCs is involved in tumor vascularization (3, 4) and contributes to ischemia-triggered neovascularization (5–8). Although the exact characterization of the EPCs is not entirely clear, various studies suggest that EPC develop from common endothelial and hematopoietic precursor cells, so-called adult hemangioblasts (1, 2, 9). The molecular mechanisms directing endothelial differentiation from stem or progenitor cells, however, are incompletely understood.

As a vehicle for modulating gene expression, chromatin structure remodeling plays a central role in normal development, the physiological differentiation of cells, and both embryonic and adult stem cell functions (10, 11). Indeed, the acetylation of histones is part of the complex epigenetic regulatory process determining lineage-specific gene expression and cell fate decisions by altering the local structure of chromatin (12). Previous reports suggest that the global deacetylation of histones is necessary for in vitro differentiation of embryonic stem (ES) cells (13) and oligodendrocyte lineage progression (14). The interplay between histone acetyltransferases and histone deacetylases (HDACs) is a key regulator in the dynamics of chromatin structure and function. The family of HDACs comprises at least 17 genes that are classified into three groups. Class I HDACs (*HDAC1*, HDAC2, HDAC3, and HDAC8) and class II HDACs (HDAC4, HDAC5,

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HDAC6, HDAC7, HDAC9, and HDAC10) can deacetylate histone tails and target other cellular proteins (15). Class III HDACs (Sirtuins) were identified on the basis of their similarity with sir2, a yeast transcription repressor requiring NAD^+ as a cofactor (16). Importantly, the inhibition of HDAC was shown not only to block postnatal vessel growth in an animal model of tumor vascularization (17), but also to down-regulate the endothelial nitric oxide synthase (eNOS; reference 18). Thus, we hypothesized that HDAC activity may be required for endothelial differentiation of progenitor cells. Because HDAC inhibitors blocked endothelial differentiation, we further explored the down-stream mechanisms, thereby focusing on the acetylation-dependent regulation of homeobox genes (Hox's).

Hox's encode transcriptional regulatory proteins, which are characterized by a common 60-amino acid DNA-binding motif and regulate differentiation during embryonic development and tissue morphogenesis (19). Members of the Hox family of homeodomain transcription factors play important roles in the embryonic development of the cardiovascular system and also regulate angiogenesis in the adult organism (for review see reference 20). Several Hox transcription factors (e.g., HoxD3, HoxC6, and HoxC8) modulate the expression of integrins, adhesion molecules, and extracellular matrix proteins in mature endothelial cells (21, 22), whereas HoxB5 appears to be involved in the in vitro differentiation of embryonic precursor cells toward endothelial lineage (23). *HoxA9*, which is important for myeloid, erythroid, and lymphoid hematopoiesis (24, 25) and stem cell expansion (26), is also particularly essential for the migration and tube-forming capacity of mature endothelial cells (27) and, thus, could serve as a switch toward endothelial commitment during progenitor cell maturation.

Our present data demonstrate that HDAC inhibition abrogates the endothelial differentiation of progenitor cells and reduces the expression of the homeobox transcription factor *HoxA9*. Knockdown and overexpression studies revealed that *HoxA9* is a critical regulator of postnatal neovascularization and acts as a master switch to direct expression of the endothelial-committed genes.

RESULTS

HDAC inhibitors abrogate endothelial differentiation of progenitor cells

To test the involvement of HDAC activity in endothelial lineage progression, we investigated the effects of HDAC inhibitors on EPCs derived from peripheral blood mononuclear cells (MNCs; references 28, 29). Adherent DiI-Ac-LDL–labeled EPCs express the endothelial marker proteins von Willebrand factor (vWF), CD105, and VE-cadherin and bind lectin (Fig. S1, available at http://www.jem.org/cgi/content/full/jem.20042097/DC1; references 28, 29). Four structurally unrelated HDAC inhibitors—butyrate (BuA), MS-275, Trichostatin A (TSA), and valproate—inhibited the generation of EPCs (Fig. 1, a–c; and Fig. S2).

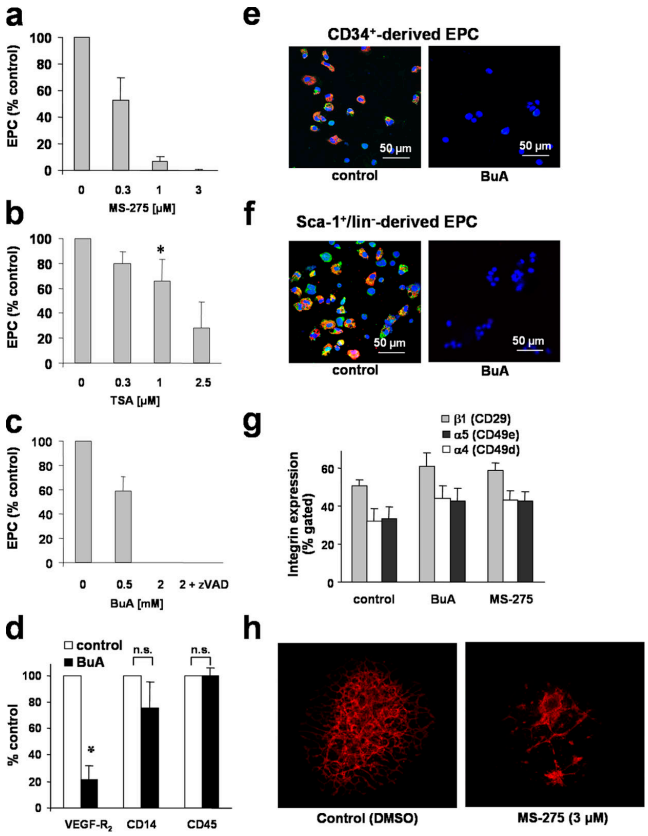

Figure 1. HDAC inhibitors abrogate the ex vivo endothelial maturation of circulating mononuclear precursor cells. (a–c) Dose-dependent effect of MS-275 (a), TSA (b), and BuA (c) on the number of EPCs after 72 h ($n = 3$–7, mean ± SD). (c) The effect of 2 mM BuA on EPC formation under these conditions in the presence or absence of 100 μM of the pan-caspase inhibitor zVAD ($n = 4$). (d) Relative changes in *VEGF-*R_2^+, $CD14^+$, and $CD45^+$ cells in peripheral blood–derived total MNCs after incubation in endothelial growth factor medium for 72 h with or without 2 mM BuA (*, $P < 0.05$ vs. $CD14^+$, $P < 0.005$ vs. $CD45^+$; $n = 6$, mean ± SEM). (e and f) Confocal microscopy of human bone marrow $CD34^+$–derived (e) or murine bone marrow Sca-1^+/lin^-–derived (f) EPCs stained with DiI-Ac-LDL (red fluorescence) and vWF (green fluorescence), and nuclear TO-PRO-3 staining (blue fluorescence) after exposure toward endothelial differentiation conditions for 72 h in the presence or absence of 2 mM BuA. Representative images out of three to six experiments are shown. (g) Flow cytometric analysis of the expression of integrin subunits α4 (CD49d), α5 (CD49e), or β1 integrin (CD29, fibronectin receptor) in peripheral blood MNCs after exposure toward endothelial differentiation conditions for 72 h with or without 2 mM BuA or 3 μM MS-275 ($n = 3$–4). (h) Vascular outgrowth from embryonic allantois explants stained with CD31 antibody.

Moreover, HDAC inhibitors selectively reduced the expression of *VEGF-*R_2 during the culture of MNCs in endothelial differentiation medium, whereas the proportion of $CD45^+$ cells and $CD14^+$ monocytes remained unchanged (Fig. 1 d). Similarly, HDAC inhibitors also prevented the endothelial differentiation of bone marrow–derived human $CD34^+$ hematopoietic progenitor or murine Sca-1^+/lin^-

cells, both of which were exposed to the same endothelial differentiation conditions (Fig. 1, e and f). Control experiments confirmed that the reduction in numbers of EPCs was not related to an antiadhesive or proapoptotic effect of the HDAC inhibitors, because integrin expression (Fig. 1 g) and EPC adhesion and apoptosis rates (Figs. S3 and S4, respectively) were not affected. In addition, caspase inhibitor treatment did not reverse the effect of HDAC inhibitors (Fig. 1 c). In summary, these data suggest that HDAC inhibitors interfere with the ex vivo endothelial lineage progression of circulating peripheral blood- or bone marrow–derived progenitor cells.

To investigate the effect of HDAC inhibitors in a physiological model of vasculogenesis and angiogenesis, we performed an ex vivo analysis of new vessel growth in the allantois assay. The HDAC inhibitors MS-275 (3 μM) and BuA (2 mM) profoundly blocked the formation of a vascular network from embryonic progenitor cells (Fig. 1 h and not depicted), indicating that HDAC activity is required for ex vivo vessel growth.

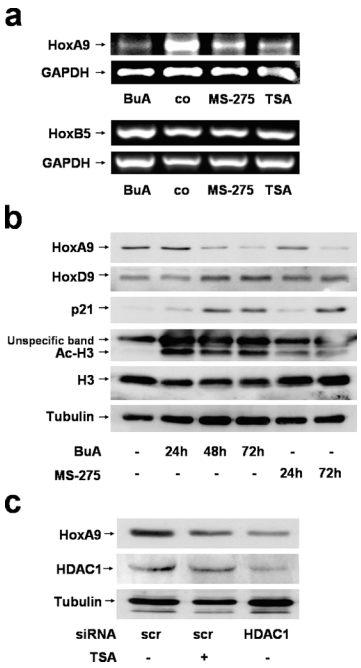

Figure 2. HDAC inhibitors decrease expression levels of *HoxA9* transcription factor. (a) RT-PCR of *HoxA9* (top) and HoxB5 (bottom) after exposure of peripheral blood MNCs to endothelial differentiation conditions for 72 h in the presence or absence of 2 mM BuA, 10 μM MS-275, or 2.5 μM TSA. GAPDH mRNA expression is shown as a control (co; $n = 3$). (b) Western blot analysis of *HoxA9*, HoxD9, p21, histone H3 di-acetylation at lysine residues K9 and K14 (Ac-H3), total histone H3, and tubulin from peripheral blood MNCs after incubating in endothelial medium in the presence or absence of 2 mM BuA or 10 μM MS-275 ($n = 3$–6). (c) Western blot analysis after transfection with siRNA directed against *HDAC1* compared with scrambled (scr) in the presence or absence of 1 μM TSA ($n = 3$).

HDAC inhibition down-regulates *HoxA9* expression and EPC formation

Because homeobox transcription factors play important roles in the embryonic development of the cardiovascular system and in neovascularization in the adult organism (20, 27), we postulated that the expression of Hox proteins might be altered by HDAC inhibition. BuA or MS-275 time- and dose-dependently reduced mRNA expression (Fig. 2 a) and protein levels (17 ± 3% of control; Fig. 2 b) of *HoxA9*. Moreover, the down-regulation of *HDAC1* by small interfering RNA (siRNA) reduced *HoxA9* expression (Fig. 2 c). In contrast, the homeodomain transcription factor HoxD9 was not regulated (Fig. 2 b) and HDAC inhibitors caused only a minor reduction in mRNA levels of HoxB5 (77 ± 11%; Fig. 2 a), indicating that *HoxA9* is rather specifically regulated by HDAC.

HoxA9 is essential for postnatal neovascularization

Next, we determined the role of *HoxA9* in postnatal neovascularization using a previously described hind limb ischemia model and measured the recovery of limb blood flow in WT versus heterozygote $HoxA9^{+/-}$ and homozygote $HoxA9^{-/-}$ mice. As depicted in Fig. 3 a, $HoxA9^{+/-}$ and $HoxA9^{-/-}$

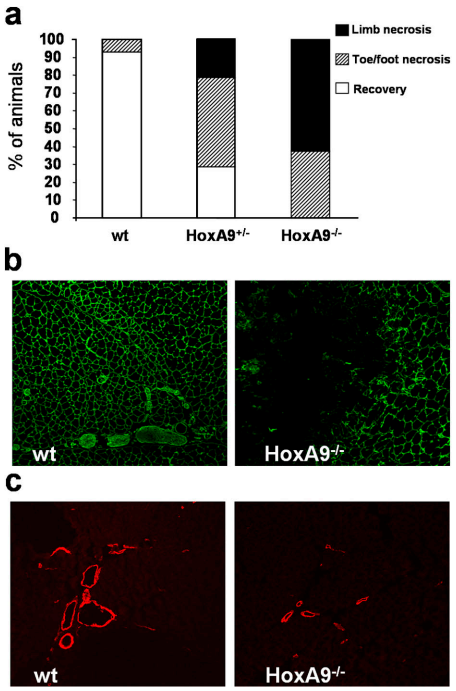

Figure 3. Role of *HoxA9* for postnatal neovascularization after ischemia. (a) Incidence of necrotic limbs in wild-type (WT) versus heterozygote $HoxA9^{+/-}$ and homozygote $HoxA9^{-/-}$ mice after hind limb ischemia by ligation of the femoral artery ($n \geq 6$/group). (b) For morphological analysis, myocytes were identified by staining for laminin (green) in ischemic tissue of WT (left) and homozygote $HoxA9^{-/-}$ mice (right). (c) Conductant vessels were defined by size (>20 μm) and positive staining for α-smooth muscle actin (red).

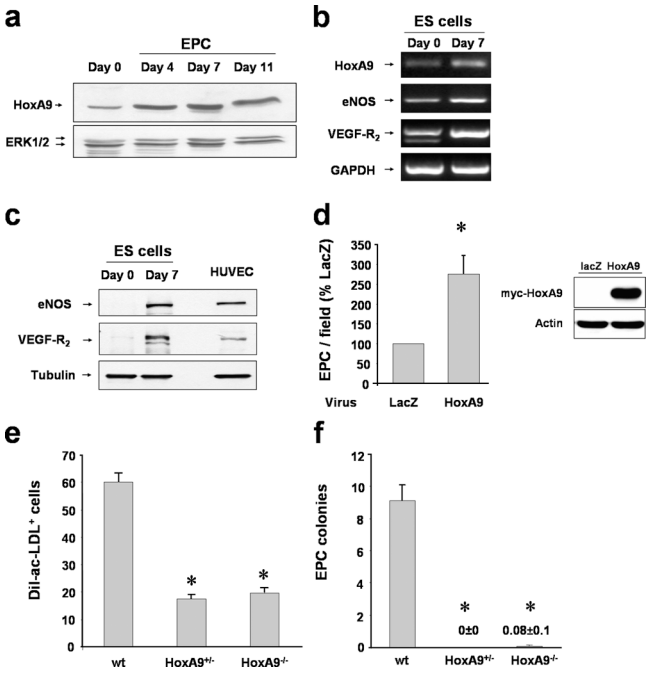

Figure 4. Expression of *HoxA9* during differentiation of progenitor cells. (a) Western blot analysis of *HoxA9* protein expression during ex vivo endothelial differentiation of peripheral blood–derived EPCs for the indicated days. Reprobe with extracellular signal–related kinase (ERK)1/2 indicates equal protein loading ($n = 3$). (b) RT-PCR analysis of *HoxA9*, eNOS, *VEGF-R*$_2$, and GAPDH mRNA expression in ES cells at baseline (day 0) and after exposure toward endothelial differentiation conditions (day 7). (c) Western blot analysis of eNOS and *VEGF-R*$_2$ protein expression in ES cells at baseline (day 0) and after exposure toward endothelial differentiation conditions (day 7). A representative reprobe with tubulin indicates equal protein loading. (d) Numbers of peripheral blood MNC–derived EPCs after transfection with *HoxA9* adenovirus (*, $P < 0.05$ vs. *lacZ* control vector; $n = 10$). Expression control is shown (right). (e and f) Cultivated spleen MNC-derived Dil-ac-LDL$^+$ cells (e) and outgrowing EPC colonies (f) from *HoxA9*$^{-/-}$, *HoxA9*$^{+/-}$, or WT mice (*, $P < 0.05$ vs. WT, $n \geq 6$).

mice showed a severely impaired recovery of blood flow as evidenced by a significantly higher incidence of limb necrosis. Limb perfusion as assessed by laser Doppler 2 wk after the induction of ischemia was significantly reduced in *HoxA9*$^{-/-}$ as compared with WT mice (26.9 ± 14.3% compared with WT; $P < 0.05$). Consistently, *HoxA9*$^{-/-}$ mice exhibited extensive thigh muscle necrosis and a reduced number of arterioles (Fig. 3, b and c), demonstrating that *HoxA9* is essential for postnatal neovascularization and blood flow recovery after hind limb ischemia.

HoxA9 contributes to the endothelial commitment of progenitor cells

To test the hypothesis that *HoxA9* is important for endothelial commitment, we measured *HoxA9* expression during endothelial differentiation of peripheral blood–derived progenitor cells. *HoxA9* expression increased during differentiation of peripheral blood–derived progenitors toward an endothelial phenotype (Fig. 4 a). The increase in *HoxA9* expression during endothelial differentiation and maturation was paralleled by an enhanced expression of the endothelial marker proteins eNOS and *VEGF-R*$_2$ in peripheral blood–derived progenitor cells (reference 30; unpublished data). Additionally, we determined the expression of *HoxA9* during the endothelial differentiation of ES cells as a model of in vitro endothelial differentiation. Similar to the finding in peripheral blood–derived adult EPCs, the endothelial differentiation of ES cells was associated with an increased expression of *HoxA9* that coincided with the increase in endothelial marker genes such as eNOS and *VEGF-R*$_2$ (Fig. 4, b and c). Thus, elevated *HoxA9* gene expression is associated with an increased endothelial commitment, as determined by the expression of endothelial marker genes.

We transduced blood-derived MNCs with a *HoxA9* adenovirus to test whether modulation of *HoxA9* expression is capable of regulating the number of EPCs. *HoxA9* overexpression increased the yield of EPCs significantly (Fig. 4 d). Moreover, we determined the number of EPCs in homozygote and heterozygote *HoxA9*-deficient mice. *HoxA9*$^{-/-}$ and *HoxA9*$^{+/-}$ mice showed a significantly reduced number of Dil-ac-LDL$^+$ adherent cells and outgrowing EPC colonies (Fig. 4, e and f). These data indicate that *HoxA9* plays a critical role in the endothelial commitment of adult progenitor cells.

HoxA9 regulates eNOS, *VEGF-R*$_2$, and VE-cadherin gene expression

To further substantiate that *HoxA9* plays a critical role in the expression of endothelial genes, we determined whether *HoxA9* is required for the maintenance of endothelial marker gene expression in human umbilical vein endothelial cells (HUVECs) as a model for mature endothelial cells. The inhibition of *HoxA9* expression by siRNA reduced the mRNA expression of the endothelial marker genes eNOS, *VEGF-R*$_2$, and VE-cadherin in mature endothelial cells (Fig. 5, a and b). Moreover, *HoxA9* siRNA inhibited eNOS protein expression (Fig. 5 c) and the generation of nitric oxide, as measured by DAF-2DA staining, from 46 ± 6 arbitrary units in scrambled oligonucleotide-treated to 11 ± 3 arbitrary units in siRNA-treated endothelial cells ($n = 4$, $P < 0.001$). *HoxA9*-deficient mice consistently showed a reduced eNOS protein expression in the heart (Fig. 5 d). In contrast, overexpression of *HoxA9* enhanced the expression of eNOS (Fig. 5 e). These data demonstrate that *HoxA9* regulates the expression of endothelial marker genes in vitro and in vivo.

To assess whether *HoxA9* interacts with the eNOS, *VEGF-R*$_2$ and VE-cadherin promoters, we performed chromatin immunoprecipitation assays. After cross-linking, immunoprecipitates of endogenous *HoxA9* were subjected to PCR using primers directed against the eNOS, *VEGF-R*$_2$, and VE-cadherin promoters. Endogenous *HoxA9* bound to the eNOS and the *VEGF-R*$_2$ promoters (Fig. 6 a), whereas

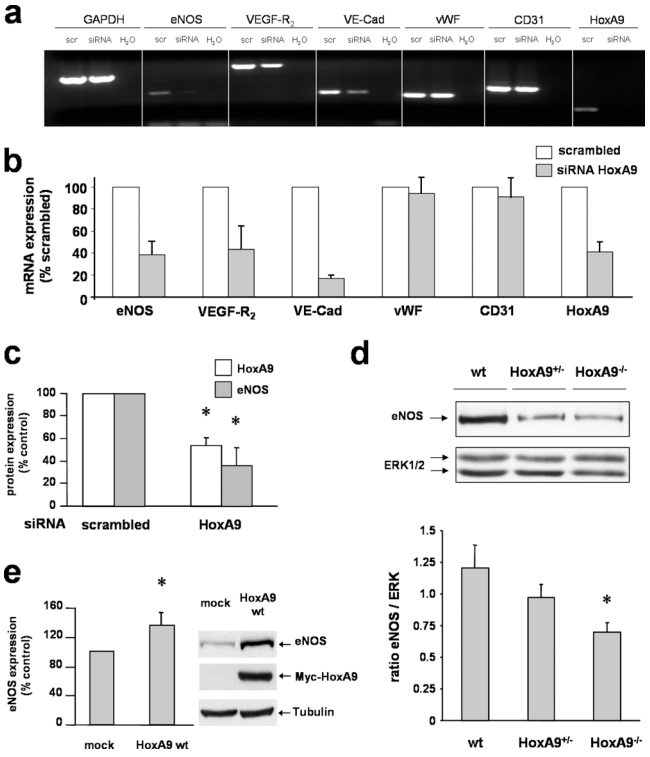

Figure 5. Essential role of *HoxA9* for endothelial lineage marker expression in mature endothelial cells. (a) RT-PCR analysis of mRNA expression of the indicated markers in HUVECs transfected with scrambled siRNA or siRNA against *HoxA9*. A representative agarose gel is shown. (b) Quantitative RT-PCR (Light cycler) of the indicated markers in HUVECs transfected with scrambled siRNA or siRNA against *HoxA9* ($n \geq 4$). (c) eNOS and *HoxA9* protein expression after transfection of HUVECs with scrambled siRNA or siRNA against *HoxA9* (*, $P < 0.05$ vs. scrambled, $n = 4$). (d) eNOS protein expression in hearts of WT, *HoxA9*$^{+/-}$, and *HoxA9*$^{-/-}$ mice (top, representative blot; bottom, quantification; $n = 4$/group;*, $P < 0.05$ vs. WT). (e) eNOS protein expression after transient transfection of a plasmid encoding *HoxA9* in HUVECs (*, $P < 0.05$). (right) Representative Western blot analysis with tubulin as a loading control and staining against c-myc to indicate overexpression of the tagged *HoxA9* insert.

HoxA9 did not interact with the VE-cadherin promoter, at least not within the specific region −1163 to −380 bp upstream of ATG investigated (Fig. 6 a). To test the specificity of the binding of *HoxA9* to the eNOS and *VEGF-R*$_2$ promoters, HUVECs were transiently transfected with WT *HoxA9* or a *HoxA9* mutant (mt) lacking the DNA-binding domain (Δ206-272 [reference 27]; Fig. 6 b). Overexpressed WT *HoxA9* bound to the eNOS and *VEGF-R*$_2$ promoters, whereas the *HoxA9* mt showed no binding (Fig. 6 c), confirming the specificity of the interaction. To determine the promoter activation by *HoxA9*, we used reporter gene constructs driven by the eNOS, *VEGF-R*$_2$, or VE-cadherin promoters. *HoxA9* significantly increased eNOS, *VEGF-R*$_2$, and VE-cadherin promoter activation (Fig. 6, d–f). Thus, *HoxA9* regulates the transcription of prototypic endothelial marker genes in endothelial cells.

Physiological levels of shear stress induce endothelial maturation of progenitor cells in a *HoxA9*-dependent manner

EPCs not only home to sites of ischemia, but also to denuded arteries, thereby promoting the recovery of the endothelial monolayer after a denuding injury. Immediately after attachment, progenitor cells are exposed to laminar blood flow (31–33). Because laminar blood flow increases the expression of various endothelial genes such as eNOS (for review see reference 34) and *VEGF-R*$_2$ (35, 36), we hypothesized that shear stress may enhance the endothelial maturation of progenitor cells by the up-regulation of *HoxA9*. Indeed, shear stress stimulated the expression of *HoxA9* in a time- and dose-dependent manner (Fig. 7 a). Moreover, the exposure of peripheral blood MNC–derived EPCs to shear stress increased the expression of *VEGF-R*$_2$ (Fig. 7 b), eNOS, and the expression of a *VEGF-R*$_2$ promoter–driven reporter gene (unpublished data). Shear stress also enhanced *VEGF-R*$_2$ expression in isolated CD34$^+$ hematopoietic progenitor cells

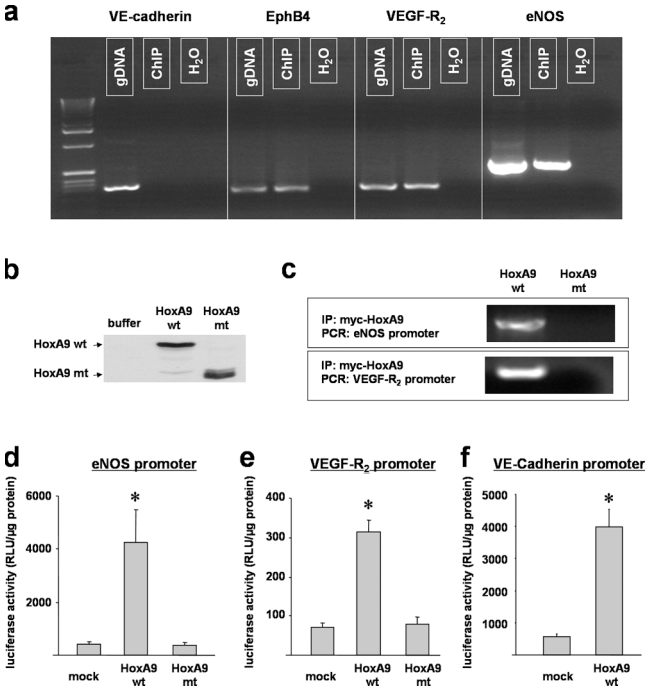

Figure 6. *HoxA9* directly binds to the promoter of eNOS and *VEGF-R*$_2$. (a) Chromatin immunoprecipitation (ChIP) of endogenous *HoxA9* followed by PCR directed against the promoter regions of *VEGF-R*$_2$, VE-cadherin, eNOS, and, as a positive control, EphB4 ($n = 3$). Genomic DNA (gDNA) was used as positive control for PCR. (b) Western blot analysis of immunoprecipitated *HoxA9* WT or *HoxA9* mt in transfected HUVECs. (c) ChIP of DNA-bound myc-tagged *HoxA9* from HUVEC lysates after the transient transfection of *HoxA9* WT or of a truncated *HoxA9* construct deficient in the DNA-binding domain (*HoxA9* mt) followed by PCR analysis with promoter-specific primers against sequences of the eNOS promoter (top) or *VEGF-R*$_2$ promoter (bottom; $n = 3$). (d–f) Transcriptional activation of the luciferase reporter gene under the regulatory control of the eNOS (d), *VEGF-R*$_2$ (e), or VE-cadherin promoter (f) in *HoxA9* WT or *HoxA9* mt cotransfected HUVEC (*, $P < 0.05$; $n = 3$–6 vs. mock; RLU, relative light units).

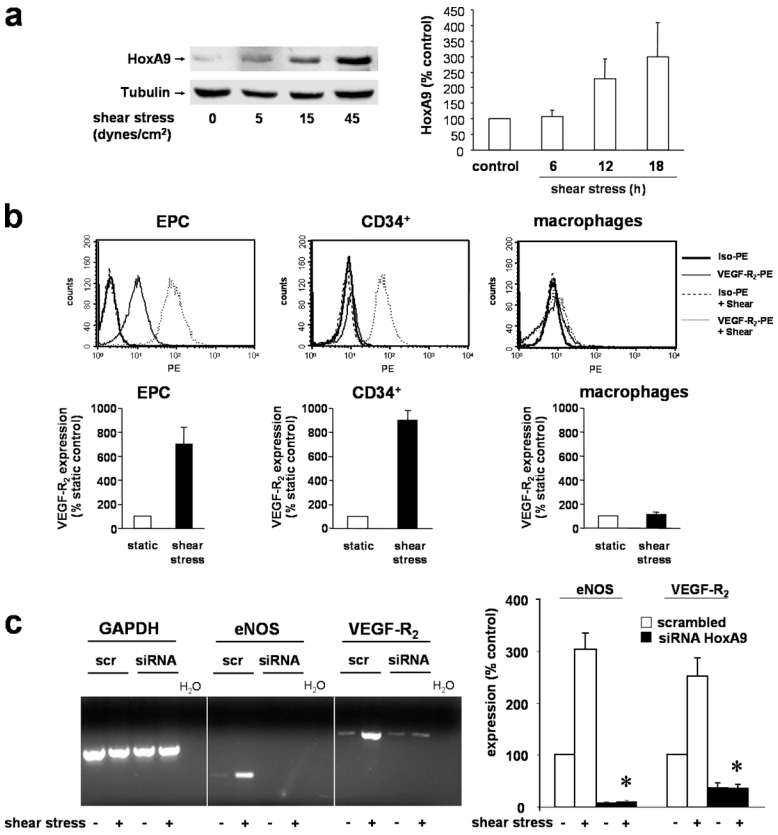

Figure 7. Shear stress induction of *HoxA9*, eNOS, and *VEGF-R_2* is sensitive to HDAC inhibition. (a) Dose- and time-dependent regulation of *HoxA9* expression (Western blot) after exposure of endothelial cells to shear stress. (left) Representative dose-dependency; (right) quantification of time-dependency (15 dynes/cm^2; $n = 3$–6 experiments). (b) FACS analysis of *VEGF-R_2* expression in peripheral blood–derived EPCs (left), human CD34$^+$ cells (middle), and macrophages (right) after exposure of the respective cell type to shear stress (15 dynes/cm^2 for 24 h). (top) Representative traces; (bottom) quantitative analysis. (c) Shear stress (15 dynes/cm^2 for 24 h)–stimulated expression of eNOS and *VEGF-R_2* after *HoxA9* siRNA transfection compared with scrambled oligonucleotides. (left) Representative RT-PCR; right, quantification of $n = 3$ (*, $P < 0.05$ vs. scrambled + shear stress).

(Fig. 7 b). Concomitantly, the expression of the pan-leukocyte marker protein CD45 decreased (unpublished data), which was consistent with a maturation toward the endothelial lineage. In contrast, when macrophages were exposed to shear stress, no increase in *VEGF-R_2* expression was noted (Fig. 7 b), indicating that the capacity of shear stress to induce endothelial lineage marker expression is restricted to progenitor cells and mature endothelial cells. Next, we determined whether the shear stress–induced expression of endothelial-committed genes is indeed mediated via *HoxA9*. A blockade of *HoxA9* gene expression by siRNA abrogated the shear stress–stimulated increase in eNOS and *VEGF-R_2* expression (Fig. 7 c), documenting that shear stress requires *HoxA9* to up-regulate endothelial marker genes.

HoxA9 overexpression partially rescues the HDAC inhibitor–mediated blockade of endothelial differentiation

To finally determine whether the HDAC inhibitor–mediated down-regulation of *HoxA9* is indeed causally involved in the reduction of endothelial commitment and down-regulation of endothelial marker genes by HDAC inhibitor treatment, we overexpressed *HoxA9* by adenoviral transduction. Indeed, *HoxA9* overexpression partially reversed the down-regulation of the number of EPCs by HDAC inhibition (Fig. 8 a). To determine whether the HDAC inhibitor–induced down-regulation of eNOS (18) is mediated by transcriptional repression of *HoxA9*, we overexpressed *HoxA9* before treatment of the cells with HDAC inhibitors. Indeed, the profound down-regulation of eNOS protein expression by HDAC inhibitors was prevented by overexpression of *HoxA9* (Fig. 8 b) suggesting that the transcriptional repression of *HoxA9* by HDAC inhibitors may contribute to eNOS down-regulation and inhibition of endothelial commitment.

DISCUSSION

This study demonstrates that the endothelial lineage commitment of circulating blood- or bone marrow–derived progenitor cells requires HDAC activity. The inhibition of HDACs decreased the expression of the transcription factor *HoxA9* and reduced the number of endothelial cells derived from different progenitor cell sources. *HoxA9* regulates various typical endothelial marker proteins, which are also important for

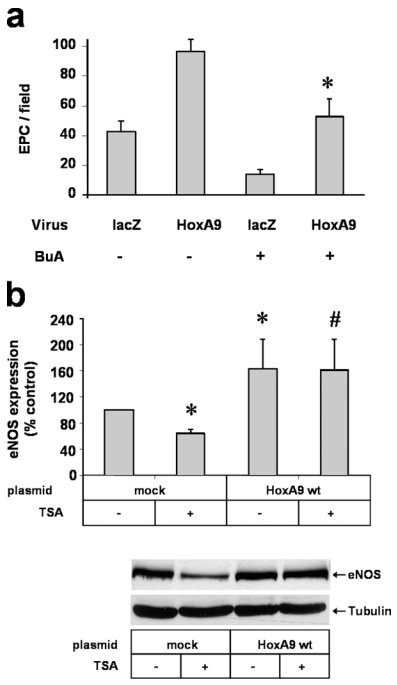

Figure 8. Rescue of HDAC inhibitor–mediated reduction of endothelial progenitors and eNOS expression by *HoxA9* overexpression. (a) Numbers of peripheral blood MNC–derived EPCs after transfection with *HoxA9* adenovirus in the presence or absence of 0.5 mM BuA (*, $P < 0.05$ vs. *lacZ* control vector; $n = 4$). (b) eNOS expression (Western blot analysis) in HUVEC transfected with *HoxA9* WT or pcDNA3.1 control plasmid (mock) after incubation with 2 μM TSA for 24 h. (top) Quantification; (bottom) representative Western blot analysis ($n = 11$; *, $P < 0.05$ vs. mock-transfected cells; #, $P < 0.05$ vs. mock + TSA).

the functional activity of endothelial cells. *HoxA9* deficiency reduces endothelial lineage commitment and results in severe impairment of neovascularization, demonstrating an important role of *HoxA9* in postnatal neovascularization.

HDACs comprise at least 17 genes, of which *HDAC1*, HDAC3, and SIRT1 are expressed in human peripheral blood–derived EPCs (unpublished data). The pharmacological inhibition of class I and II HDACs by structurally different pharmacological HDAC inhibitors abrogated *HoxA9* expression and the endothelial commitment of progenitor cells. Furthermore, a specific down-regulation of *HDAC1* by siRNA reduced the expression of *HoxA9*. In contrast, HoxD9 and HoxB5 were not significantly regulated by HDAC inhibitors, implicating a specific dependency of *HoxA9* transcription on HDAC activity. HDACs are a component of the ALL-1 supercomplex, which binds to the *HoxA9* promoter and is required for *HoxA9* transcription (37). Thus, one may speculate that HDAC activity is necessary for the ALL-1 supercomplex to allow for transcription of *HoxA9* (37).

Our data demonstrate that *HoxA9* is required for postnatal neovascularization after ischemia. Previous studies additionally documented that the inhibition of HDAC blocks tumor angiogenesis (17). However, the importance of HDAC and *HoxA9* for the embryonic development of vascular structures in vivo is unclear. In fact, several lines of evidence indicate that *HDAC1* and *HoxA9* are not essential for embryonic vessel formation. In contrast to *VEGF* gene deficiency, which is lethal because of impaired vessel formation even at the level of haploinsufficiency (38), or *VEGF-R_2* knockout mice, which die between embryonic days 8.5 and 9.5 from the lack of any vascular formation (39), no abnormalities with respect to embryonic vascularization were obvious in *HDAC1*$^{-/-}$ or *HoxA9*$^{-/-}$ mice (40, 41). In our experiments, however, HDAC inhibitors abrogated embryonic angiogenesis and vasculogenesis in the allantois ex vivo assay. This discrepancy might well be rationalized by the broad spectrum inhibitory effect of the pharmacological HDAC inhibitors used in the present study and may indicate that the specific lack of *HDAC1* might be compensated for by the various other members of the HDAC family in vivo. Likewise, the lack of a severe embryonic phenotype of *HoxA9*$^{-/-}$ mice might also be caused by compensation by other members of the Hox family such as HoxB5 during embryonic development in vivo (23). However, our data clearly indicate that *HoxA9* is required for adult vasculogenesis. Indeed, *HoxA9*$^{-/-}$ and *HoxA9*$^{+/-}$ mice showed a severe impairment of endothelial colony formation, blood flow recovery, and functional regeneration after the induction of ischemia. Thus, one may speculate that mechanisms regulating postnatal neovascularization are not necessarily identical to the mechanisms operational during embryonic vascular development.

Why is *HoxA9* important for postnatal neovascularization? In addition to the requirement of *HoxA9* for endothelial progenitor colony formation and adult vasculogenesis, our data indicate that *HoxA9* directly regulates a variety of key endothelial genes that are involved in the functional maturation and activity of endothelial cells. All three *HoxA9* target genes, eNOS (42, 43), *VEGF-R_2* (39), and VE-cadherin (44), are crucial for angiogenesis. Moreover, the reduction of *HoxA9* additionally results in the reduced expression of EphB4 and, thus, inhibits endothelial migration in vitro (27). The reduction of these *HoxA9* regulated genes in vivo (as shown for the eNOS) may contribute to the severe neovascularization defect of *HoxA9*-deficient mice. In addition, *HoxA9* regulates myeloid and lymphoid hematopoiesis (25) and, therefore, may affect inflammation-mediated angiogenesis. However, a reduction of myeloid cells was detected in homozygote, but not in heterozygote, mice (25), whereas in our study heterozygote mice showed a severe impairment of neovascularization and endothelial colony forming activity. Furthermore, the overall peripheral white blood cell counts after the induction of ischemia were not notably different between *HoxA9*-deficient mice and WT littermates (unpublished data). Thus, the impaired neovascularization capacity of *HoxA9*$^{-/-}$ and *HoxA9*$^{+/-}$ mice is most likely not related to a general imbalance of inflammatory cells.

Finally, our data provide novel insights into the prototypic physiological mechanism inducing the maturation of endothelial cells. Shear stress is not only one of the most powerful antiatherosclerotic factors, but is also an absolute prerequisite for a functionally intact endothelial monolayer. We demonstrate that the up-regulation of the endothelial signature gene pattern is dependent on *HoxA9*. Not only did shear stress increase the expression of endothelial marker proteins such as eNOS and *VEGF-R*$_2$, but it also concomitantly down-regulated the pan-leukocyte marker CD45, which is expressed on hematopoietic progenitor cells. These data clearly indicate that shear stress is capable of promoting the commitment of progenitor cells to an endothelial phenotype. The stimulation of *HoxA9* expression and subsequent endothelial maturation by shear stress may considerably contribute to a timely recovery of the endothelial monolayer after injury to prevent atherosclerotic lesion development and restenosis formation, respectively (32, 33, 45, 46).

This study demonstrates that the HDAC-dependent transcription of *HoxA9* is necessary for ex vivo maturation of progenitor cells toward the endothelial lineage. These findings not only contribute to a better understanding of postnatal endothelial maturation, but, given the pivotal role of EPCs for neovascularization of ischemic tissue and reendothelialization, may also provide important therapeutic targets. Of note, the inhibition of adult vasculogenesis by HDAC inhibitors may also contribute to the reported profound antitumor activity of HDAC inhibitors (15).

MATERIALS AND METHODS

Isolation and ex vivo endothelial differentiation of EPCs. Ex vivo EPC differentiation from circulating MNCs was assayed as described previously (28). In brief, MNCs were isolated by density gradient centrifugation with Biocoll separating solution (density 1.077; Biochrom AG) from human peripheral blood buffy coats. Immediately after isolation, 8×10^6 MNC/ml of medium was plated on culture dishes coated with human fibronectin and maintained in endothelial basal medium (EBM; Cambrex) supplemented with EGM SingleQuots (Cambrex) and 20% FCS (GIBCO BRL). After 3 d in culture, nonadherent cells were removed by thorough washing with PBS. Adherent cells were stained with 2.4 μg/ml Dil-Ac-LDL (Harbor Bio-Products) at 37°C for 1 h and fixed with 4% paraformaldehyde for 10 min. Purified human CD34$^+$ BM MNCs (BMCs) were purchased from Cambrex and were differentiated to EPCs under the same conditions as described above.

For the preparation of Sca-1$^+$/lin$^-$ BMC-derived EPCs, murine BM was isolated from the hindlimbs of 6–8-wk-old female C57BL/6 mice. Then, cells were filtrated using a 40-μm pore size cell strainer. After blocking with purified anti–mouse CD16/CD32 (FcγIII/II receptor) antibodies (1:100; BD Biosciences), washed cells were incubated with a biotinylated anti–lineage marker antibody cocktail (Becton Dickinson) and antibiotin microbeads (Miltenyi Biotec), and isolated with an automated magnetic cell sorting device (autoMACS; Miltenyi Biotec). Separated lin$^-$ BMCs were incubated with microbeads directly conjugated to anti–Sca-1 antibodies, and Sca-1$^+$/lin$^-$ BMCs were isolated by a second run through the autoMACS. To assess the capacities of these immature BM stem cells, 0.5–1.0×10^6 pooled Sca-1$^+$/lin$^-$ BMCs were plated on 24-well culture dishes coated with human fibronectin and maintained in EBM supplemented with EGM SingleQuots and 20% FCS. After 3 d in culture, adherent Sca-1$^+$/lin$^-$ BMCs were stained with Dil-Ac-LDL and were fixed. Then, cells were stained for vWF (Acris Antibodies) and the nuclear marker TO-PRO-3 iodide (Molecular Probes), or against 10 μg/ml lectin by incubating with FITC-labeled Ulex europaeus agglutinin I (Sigma-Aldrich) for 1 h. Staining for both vWF or lectin, and Dil-Ac-LDL, was evaluated by confocal microscopy.

For the isolation of spleen-derived EPCs, murine MNCs were isolated from homogenized splenic tissue derived from *HoxA9*$^{-/-}$ (provided by H. Jeffrey Lawrence, University of California, San Francisco, Veteran's Administration Medical Center, San Francisco, CA) or WT littermates by density gradient centrifugation with Biocoll separating solution. 4×10^6 MNCs were plated on fibronectin-coated 24-well plates in 0.5 ml EBM supplemented with EGM SingleQuots and 20% FCS and were stained as described above. Outgrowing colonies were detected after 10 d.

Cell culture. Pooled HUVECs were purchased from CellSystems and cultured as previously described (27). HUVECs were exposed to laminar flow in a cone-and-plate apparatus as previously described (47). For shear stress experiments, human CD34$^+$ hematopoietic progenitor cells were purified from MNCs by positive selection with anti-CD34 microbeads (Miltenyi Biotec; reference 28). For the cultivation of macrophages, CD14$^+$ monocytes were purified from MNCs by positive selection with anti-CD14 microbeads (Miltenyi Biotec) using a magnetic cell sorter (Miltenyi Biotec). Purity assessed by FACS analysis was >95%. CD14$^+$ monocytes were incubated in RPMI 1640 with 10% FCS in the presence of 50 ng/ml M-CSF to induce macrophage differentiation (29).

Allantois assay. Allantoides were isolated as previously described (48). In brief, embryos at 8.5 d postcoitum were dissected in PBS at 4°C, and the allantoides were excised and seeded into four-chambered culture slides (Nalgene Nunc International) containing 0.4 ml DMEM with 10% FCS, 2 mmol/liter L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin (all reagents obtained from Life Technologies). Explants were cultured at 37°C in a 5% CO_2 incubator for 18–20 h in the presence or absence of the HDAC inhibitors. The allantois cultures were fixed for 20 min at 25°C with 4% paraformaldehyde, washed twice in PBS, permeabilized for 15 min at 25°C with 0.02% Triton X-100 in PBS, blocked for 1 h at 25°C with 3% BSA (Sigma-Aldrich) in PBS, and stained with PECAM-1 antibody (MEC13.3; reference 49).

Western blot analysis. Cells were lysed with buffer (20 mmol/liter Tris, pH 7.4, 150 mmol/liter NaCl, 1 mmol/liter EDTA, 1 mmol/liter EGTA, 1% Triton X-100, 2.5 mmol/liter sodium pyrophosphate, 1 mmol/liter β-glycerophosphate, 1 mmol/liter Na_3VO_4, 1 μg/ml leupeptin, 1 mmol/liter PMSF) for 15 min on ice. After centrifuging at 20,000 *g* for 15 min at 4°C, protein content was measured according to the Bradford method. Homogenates (40 μg per lane) were separated on SDS-polyacrylamide gels and transferred to polyvinylidene difluoride membranes, which were then incubated with antibodies to p21 (BD Biosciences), *HDAC1* (Abcam), *HoxA9*, and HoxD9 (Santa Cruz Biotechnology, Inc.), K9-K14-diacetylated histone H3 (Upstate Biotechnology), total histone H3 (Cell Signaling), extracellular signal–related kinase 1/2 (Cell Signaling), eNOS (BD Biosciences), or tubulin (NeoMarkers).

RT-PCR. Total RNA was isolated and either subjected to conventional RT-PCR or quantitative RT-PCR using the oligonucleotide primers summarized in Tables S1 and S2 (available at http://www.jem.org/cgi/content/full/jem.20042097/DC1). Quantification of mRNA was performed in a one-step RT-PCR reaction using the LightCycler (Roche Diagnostics) real-time thermocycler according to the manufacturer's instructions. Amplification was performed with 40 cycles at an annealing temperature of 61°C. Copy numbers were calculated by the instrument software (Roche Diagnostics) from standard curves of an in vitro–transcribed IL-10 cytokine primer mix (Light Cycler control kit RNA; Roche Diagnostics). The specificity of the amplification reaction was determined by a melting curve analysis.

Generation of recombinant adenovirus and adenoviral infection. *HoxA9* (transcript variant 1) was amplified by RT-PCR and cloned into a shuttle pAd Track-CMV vector. This plasmid was linearized by digesting

with restriction endonuclease PmeI and subsequently cotransformed into *Escherichia coli* BJ5183 cells with an adenoviral backbone pAdEasy-1 plasmid (all plasmids and *E. coli* cells were a gift from B. Vogelstein, Howard Hughes Medical Institute, Johns Hopkins Medical Institutions, Baltimore, MD). Recombinants were selected by kanamycin resistance. Finally, recombinants were transfected into HEK293 cells. Recombinant adenoviruses were generated within 7–10 d. The *lacZ* gene codes for the enzyme β-galactosidase. The *lacZ* adenovirus was used as control.

Peripheral blood MNCs (4×10^6 cells/1-cm well) were resuspended in 2.5 ml RPMI 1640 (GIBCO BRL) with 10% FCS and preincubated for 30 min with a mixture of adenovirus, 10 μl Antennapedia peptide (RQIKIWFQNRRMKWKK; 2.5 mM; Biosyntan), and 100 μl Optimem (Life Technologies). After 24 h, 4×10^6 cells were resuspended in 1 ml EBM supplemented with EGM SingleQuots and 20% FCS and plated on fibronectin-coated wells.

Endothelial differentiation of ES cells. CJ7 ES cells, a 129/Sv-derived cell line, were cultivated as previously described (50). To initiate ES cell differentiation and embryonic body formation, ES cells were trypsinized and suspended in IMDM (Life Technologies) with 15% FBS, 10 μg/ml insulin (Sigma-Aldrich), 100 U/ml penicillin, 100 μg/ml streptomycin, 450 μmol/liter monothioglycerol, and endothelial differentiation promoting growth factors including 50 ng/ml recombinant human *VEGF* (PeproTech), 2 U/ml recombinant human erythropoietin (Cilag AG), 100 ng/ml human basic fibroblast growth factor (Genzyme), and 10 ng/ml murine interleukin 6 (Genzyme). After 7 d, ES cell–derived endothelial cells were collected by anti-CD31 immunomagnetic selection (51).

Reporter gene assay. Reporter gene constructs were previously described (1.6-kb human eNOS promoter fragment [reference 52]; 4-kb human *VEGF-R*$_2$ promoter [reference 36]). The VE-cadherin promoter (3,032-kb fragment: −2928/+104) was cloned into KpnI–XhoI restriction sites in pGl3 enhancer plasmid (Promega). 3.5×10^5 HUVECs were transiently transfected with 3 μg plasmid DNA using 18 μl Superfect (QIAGEN) as previously described (47). After incubation, cells were lysed with lysis buffer (Promega), and luciferase activity was measured using the Luciferase System (Promega) with a luminometer (model Luminat LB 9501; Berthold).

Murine ischemic hind limb model. The effect of *HoxA9* on ischemia-induced neovascularization was investigated in a murine model of hind limb ischemia. The present study was performed with the permission of the State of Hesse (Regierungspräsidium Darmstadt), according to section 8 of the German Law for the Protection of Animals, and conforms to the Guide for the Care and Use of Laboratory Animals measurements. In brief, the proximal portion of the femoral artery, including the superficial and the deep branch, as well as the distal portion of the saphenous artery, was ligated and all arterial branches between the ligations were obliterated using an electrical coagulator. The overlying skin was closed using three surgical staples. 2 wk later, we determined the morphology of the limb and measured the ischemic (right) to normal (left) limb blood flow ratio using a laser Doppler blood flow meter (model moorLDI-Mark 2; Moor Instruments). Before initiating scanning, mice were placed on a heating pad at 37°C to minimize variations in temperature. After the recording of complete scan laser Doppler color images, the perfusion of the ischemic and nonischemic limb was calculated on the basis of colored histogram pixels. To minimize variables, including ambient light and temperature, calculated perfusion was expressed as the ratio of ischemic to nonischemic hind limb perfusion.

For morphological analysis, 8-μm frozen sections of the adductor and semimembranous muscles were used. Myocyte membranes were stained using an antibody to laminin (rabbit) followed by anti–rabbit-Alexa 488. Conductance vessels in the adductor and semimembranous muscles were identified by size (>20 μm) and staining using a Cy3 labeled mouse monoclonal antibody for smooth muscle actin (Sigma-Aldrich).

Statistics. Data are expressed as mean ± SEM or as indicated in the figure legends. Two treatment groups were compared with the independent samples *t* test, and three or more groups by one-way analysis of variance followed by post-hoc analysis adjusted with a least significant difference correction for multiple comparisons (SPSS Inc.). Results were considered statistically significant when $P < 0.05$.

Online supplemental material. Fig. S1 shows a representative flow cytometric characterization of endothelial marker expression in adherent human peripheral blood MNC–derived EPCs. Fig. S2 presents a comparison of the effect of structural analoga without HDAC inhibitory capacity (2 mM acetate and 1 mM valpromide) and with HDAC inhibitory capacity (2 mM BuA and 1 mM valproate) on the ex vivo formation of EPCs from human peripheral blood MNCs during 72 h of incubation under endothelial differentiation conditions. Fig. S3 shows cell matrix adhesion. Fig. S4 presents the effect of a 72-h incubation of peripheral blood MNCs with 2 mM BuA, 10 μM MS-275, or 1 mM valproate (VPA) under endothelial differentiation conditions on apoptosis. Table S1 summarizes the sequences of the oligonucleotide primer pairs (forward and reverse) used for RT-PCR. Table S2 summarizes the sequences of the oligonucleotide primer pairs (forward and reverse) used for quantitative RT-PCR (Light Cycler). Online supplemental material is available at http://www.jem.org/cgi/content/full/jem.20042097/DC1 or from the authors by request.

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