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-R2, 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.

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 hematopoietic stem cells (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,
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 Di-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 matura- tion 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). (d) The effect of 2 mM BuA on HDAC formation under these conditions in the presence or absence of 100 μM of the pan-caspase inhibitor zVAD (n = 4). (e) Relative changes in VEGF-R2⁺, 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). (f and g) Confocal microscopy of human–bone marrow CD34⁺–derived (e) or murine bone marrow Sca-1⁻/lin⁻–derived (f) EPCs stained with Dil-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-R2 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.

**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−/−

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**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).

**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 = 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).
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-R2 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-R2 (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-R2, 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-R2, 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-R2, 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-R2, and VE-cadherin promoters. Endogenous HoxA9 bound to the eNOS and the VEGF-R2 promoters (Fig. 6 a), whereas
HoxA9 did not interact with the VE-cadherin promoter, at least not within the specific region \( /H11002_{1163} \) to \( /H11002_{380} \) bp upstream of ATG investigated (Fig. 6 a). To test the specificity of the binding of HoxA9 to the eNOS and VEGF-R2 promoters, HUVECs were transiently transfected with WT HoxA9 or a HoxA9 mutant (mt) lacking the DNA-binding domain (\( /H9004_{206-272} \); reference 27; Fig. 6 b). Overexpressed WT HoxA9 bound to the eNOS and VEGF-R2 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-R2, or VE-cadherin promoters. HoxA9 significantly increased eNOS, VEGF-R2, 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-R2 (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-R2 (Fig. 7 b), eNOS, and the expression of a VEGF-R2 promoter–driven reporter gene (unpublished data). Shear stress also enhanced VEGF-R2 expression in isolated CD34+ hematopoietic progenitor cells.
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...
transfected cells; #, P

representative Western blot analysis ( vs. HoxA9 (a) Numbers of peripheral blood MNC–derived EPCs after transfection with HoxA9 (n

tal neovascularization after ischemia. Previous studies addi-

tional HDAC and HoxA9 for the embryonic development of vascular structures in vivo is unclear. In fact, several lines of evi-
cence indicate that HDAC1 and HoxA9 are not essential for embryonic vessel formation. In contrast to VEGF gene
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obvious in HoxA9+/− mice (40, 41). In our experiments, however, HDAC inhibitors abrogated embry-
onic 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
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Likewise, the lack of a severe embryonic phenotype of
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clearly indicate that HoxA9 is required for adult vasculogen-
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vere impairment of endothelial colony formation, blood
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tion of ischemia. Thus, one may speculate that mechanisms
regulating postnatal neovascularization are not necessarily
identical to the mechanisms operational during embryonic
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Why is HoxA9 important for postnatal neovasculariza-
tion? In addition to the requirement of HoxA9 for endo-
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data indicate that HoxA9 directly regulates a variety of key
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tion and activity of endothelial cells. All three HoxA9 target
genes, eNOS (42, 43), VEGF-R2 (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 neovas-
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sis. However, a reduction of myeloid cells was detected in
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our study heterozygote mice showed a severe impairment of
neovascularization and endothelial colony forming activity.
Furthermore, the overall peripheral white blood cell counts
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the functional activity of endothelial cells. HoxA9 deficiency
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tant 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 differ-
cent pharmacological HDAC inhibitors abrogated HoxA9 expres-
sion 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 com-
ponent 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 neces-
sary for the ALL-1 supercomplex to allow for transcription
of HoxA9 (37).

Our data demonstrate that HoxA9 is required for postna-
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![Figure 8](image_url)
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-R2, 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 (BMNCs) 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- to 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 (FcγRII/II receptor) antibodies (1:100; BD Biosciences), washed cells were incubated with a biotinylated anti-lineage marker antibody cocktail (Becton Dickinson) and biotin-labeled microbeads (Miltenyi Biotec), and isolated with an automated magnetic cell sorting device (autoMACS; Miltenyi Biotec). Separated lineage-negative 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 europeaus agglutinin I (Sigma-Aldrich) for 1 h. Staining for both vWF and 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 Boccoll 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 con-endothelial progenitor cells were puriﬁed 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. Allantois were isolated as previously described (48). In brief, embryos at 8.5 d postcoitum were dissected in PBS at 4°C, and the allantois were excised and seeded into four-chambered culture slides (Nalge Nunc International) containing 0.4 ml DMEM with 10% FCS, 2 mmol/l-glutamine, 100 U/ml penicillin, and 100 µg/ml streptomycin (all reagents obtained from Life Technologies). Embryos were cultured at 37°C in a 5% CO2 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 PECA1-1 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 Na3VO4, 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 poly(vinylidene difluoride) membranes, which were then incubated with antibodies to p21 (BD Biosciences), HDAC7 (Abcam), Hox9, and HoxD9 (Santa Cruz Biotechnology, Inc.), K9-K14-diacetylated histone H3 (Upstate Biotechnology, Inc.), or tubulin (Neomarkers) by the use of the Western blotting systems 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 Pmel and subsequently cotransformed into Escherichia coli BJS183 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 × 10⁶ 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 × 10⁶ 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-Alrich), 100 U/ml penicillin, 100 μg/ml streptomycin, 450 μmol/l 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 (Glycylne), and 10 ng/ml murine interleukin 6 (Glycynne). 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-R2 promoter [reference 36]). The VE-cadherin promoter (3,032-kb fragment: −2928/+104) was cloned into KpnI–Xhol restriction sites in pGEm enhancer plasmid (Promega). 3.5 × 10⁶ HUVECses 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 morphological and physiological parameters. After 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 semimembranosus muscles were used. Myocyte membranes were stained using an antibody to laminin (rabbit) followed by an anti–rabbit-Alexa 488. Conductance vessels in the adductor and semimembranosus muscles were identified by size (>20 μm) and staining using a Cy3 labeled mouse monoclonal antibody for smooth muscle actin (Sigma–Alrich).

Statistics. Data are expressed as mean ± SEM or as indicated in the figure legends. Two treatment groups were compared with the independent sam-
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