Epigenetic Regulation of Endothelial Lineage Committed Genes in Pro-Angiogenic Hematopoietic and Endothelial Progenitor Cells

Kisho Ohtani, Georgios J. Vlachojannis, Masamichi Koyanagi, Jes-Niels Boeckel, Carmen Urbich, Ruxandra Farclas, Halvard Bonig, Victor E. Marquez, Andreas M. Zeiher, Stefanie Dimmeler

Rationale: Proangiogenic hematopoietic and endothelial progenitor cells (EPCs) contribute to postnatal neovascularization, but the mechanisms regulating differentiation to the endothelial lineage are unclear.

Objective: To elucidate the epigenetic control of endothelial gene expression in proangiogenic cells and EPCs.

Methods and Results: Here we demonstrate that the endothelial nitric oxide synthase (eNOS) promoter is epigenetically silenced in proangiogenic cells (early EPCs), CD34⁺ cells, and mesoangioblasts by DNA methylation and prominent repressive histone H3K27me3 marks. In order to reverse epigenetic silencing to facilitate endothelial commitment, we used 3-deazaneplanocin A, which inhibits the histone methyltransferase enhancer of zest homolog 2 and, thereby, reduces H3K27me3. 3-Deazaneplanocin A was not sufficient to increase eNOS expression, but the combination of 3-deazaneplanocin A and the histone deacetylase inhibitor Trichostatin A augmented eNOS expression, indicating that the concomitant inhibition of silencing histone modification and enhancement of activating histone modification facilitates eNOS expression. In ischemic tissue, hypoxia plays a role in recruiting progenitor cells. Therefore, we examined the effect of hypoxia on epigenetic modifications. Hypoxia modulated the balance of repressive to active histone marks and increased eNOS mRNA expression. The reduction of repressive H3K27me3 was associated with an increase of the histone demethylase Jmjd3. Silencing of Jmjd3 induced apoptosis and senescence in proangiogenic cells and inhibited hypoxia-mediated up-regulation of eNOS expression in mesoangioblasts.

Conclusions: These findings provide evidence that histone modifications epigenetically control the eNOS promoter in proangiogenic cells. (Circ Res. 2011;109:1219-1229.)

Key Words: Jmjd3 ■ eNOS ■ hypoxia ■ proangiogenic cells

Endothelial or hematopoietic progenitor cells contribute to vascular repair and postnatal neovascularization. Administration of progenitor cells augmented neovascularization and functional recovery after critical ischemia studies. However, the mechanisms underlying the beneficial effect of progenitor cells are unclear, and it is still intensely debated whether endothelial and hematopoietic progenitor cells can undergo differentiation toward the endothelial lineage and incorporate into newly formed blood vessels. The basal mechanisms for endothelial specification and endothelium-specific gene expression are poorly understood and an endothelial-specific master regulator has yet to be identified. Obviously, many transcription factors such as members of Ets, GATA, and forkhead families play a key role in endothelial gene activation and unique combinational regulation of multiple transcription factors is required for endothelial specification and differentiation. However, many of these transcription factors implicated in vasculogenesis are also expressed in nonendothelial cells like hematopoietic stem cells but are not sufficient to activate endothelial genes. Likewise, expression of the prototypical gene endothelial NO synthase (eNOS) is controlled by a variety of transcription factors such as Sp-1, Ets-1, and YY1, but these transcription factors are also expressed in nonendothelial cells. These observations have led to the hypothesis that additional mechanisms including chromatin modifications might regulate the accessibility of transcription factors and thereby control endothelial lineage commitment.
Gene expression is regulated by chromatin-based epigenetic pathways, which include DNA methylation and covalent modification of histones. Methylation of DNA occurs at cytosine residues in CpG dinucleotides (CpGs) and is catalyzed by DNA methyltransferases. DNA methylation is essential in mammalian development for the repression of imprinted genes and for X-chromosome inactivation. The N-terminal tails of histones are subject to various post-translational modifications such as acetylation, methylation, phosphorylation, ubiquitination, and sumoylation. In general, acetylation of histone H3 and H4 is associated with increased gene expression and was shown to regulate the angiogenic function of endothelial cells. The effect of histone methylation on transcription depends on the specific lysine (K) residues and degree of methylation (mono-, di-, or trimethylation), attached to the specific sites. Histone H3 modification at K4 and K36 is associated with activation of gene expression, whereas the effect of K9 methylation depends on the location of the mark. Methylation of the lysine residue 27 (K27) is involved in epigenetic silencing of gene expression and is tightly controlled by the Polycomb group proteins such as the enhancer of zeste homolog 2 (Ezh2), a histone methyltransferase that can trimethylate H3K27. The presence of H3K27me3 at transcription start sites is correlated with repression of gene expression. H3K27me3 has initially been considered as an irreversible silencing mark; however, several recent studies suggested reversibility by the Jmjd3 domain-containing proteins Jmjd3 and UTX as genuine H3K27 demethylases. The reversion of H3K27me3 silencing by Jmjd3 and partially histone deacetylase inhibitors augmented the expression of eNOS.

We and others have demonstrated that administration of bone marrow mononuclear cells or cultured early endothelial progenitor cells (EPCs), which express the myeloid markers CD14 and CD45, improved the functional recovery after ischemia in experimental models of hind limb and myocardial ischemia and modestly augmented the ejection fraction in patients with acute myocardial infarction. Mechanically, we have shown that eNOS is essential for progenitor cells mobilization and function and that increasing eNOS expression by eNOS enhancers improved functional activity of patient-derived cells. Recent data demonstrated that particularly eNOS expressing cells contribute to the maintenance of functional recovery after ischemia when using lineage-committed suicide vectors to deplete bone marrow mononuclear cells 2 weeks after injection in a myocardial infarction model. Together, these data suggest that eNOS expression is important for the functional effects achieved by administration of progenitor cells for the treatment of ischemia.

Here, we aim to elucidate the epigenetic control of endothelial gene expression in cultured EPCs (early EPCs, now termed proangiogenic cells [PACs] because of the coexpression of hematopoietic markers), isolated CD34+ cells, and mesoangioblasts. Based on preliminary findings that hypoxia and inflammation enhance the expression of epigenetic regulators of gene expression, we hypothesize that, under conditions of severe stress, the epigenetic profile of cells is modulated, thereby facilitating endothelial commitment. Using the eNOS as a prototypic endothelial gene, we demonstrate that the eNOS promoter is characterized by a profound DNA methylation and repressive trimethylation of histone H3 at lysine 27 in CD34+ cells and cultured PACs. Pharmacological inhibition of silencing histone H3K27me3 marks in combination with induction of histone acetylation by histone deacetylase inhibitors augmented the expression of eNOS. Most importantly, we demonstrate that hypoxia increased the expression of the histone demethylase Jmjd3 and partially reversed the repression of the endothelial genes.

### Methods

Experimental protocols are described in the Supplemental Methods section, available online at http://circres.ahajournals.org.

### Results

#### DNA Methylation Status of the 5’-Flanking Region of the Human eNOS Gene

DNA methylation controls the expression of eNOS gene. To determine the epigenetic state of endothelial commitment at the level of DNA methylation, we analyzed DNA methylation of the eNOS promoter region 800 bases upstream of the transcription start site, which spans the proximal promoter that controls basal eNOS expression by bisulfite genomic DNA sequencing (Figure 1A). Whereas the proximal eNOS promoter was either devoid of or very lightly methylated in differentiated human endothelial cell types including human umbilical vein endothelial cell (HUVEC) and microvascular endothelial cell, the promoter was heavily methylated in all examined proangiogenic cells such as peripheral blood-derived PACs (early EPCs), bone marrow-derived CD34+ hematopoietic progenitor cells, and mesoangioblasts, which are isolated from peripheral blood and express mesenchymal and endothelial markers (Figure 1A). Because cultured PACs and CD34+ cells comprise a heterogeneous cell population and contaminations might have affected our results, we additionally isolated the subpopulation of CD34+ cells that coexpressed the endothelial marker KDR (Figure 1B). Although KDR is also expressed in stem cells, the CD34+KDR+ population was assumed to be enriched in

### Non-standard Abbreviations and Acronyms

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tr>
<td>ChIP</td>
<td>chromatin immunoprecipitation</td>
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<tr>
<td>DZNep</td>
<td>3-deazaneplanocin A</td>
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<tr>
<td>eNOS</td>
<td>endothelial nitric oxide synthase</td>
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<tr>
<td>EPC</td>
<td>endothelial progenitor cells</td>
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<tr>
<td>Ezh2</td>
<td>enhancer of zeste homolog 2</td>
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<tr>
<td>HUVEC</td>
<td>Human umbilical vein endothelial cell</td>
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<td>K</td>
<td>lysine</td>
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<tr>
<td>PAC</td>
<td>pro-angiogenic cell</td>
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<td>TSA</td>
<td>Trichostatin A</td>
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<tr>
<td>VE-cadherin</td>
<td>vascular endothelial-cadherin</td>
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EPCs. However, isolated CD34+/KDR+ cells also did show a hypermethylated status of the eNOS promoter (Figure 1B). Collectively, these results revealed that the eNOS promoter is highly methylated irrespective of the progenitor cell subpopulation studied, suggesting a limited commitment of the cells toward the endothelial lineage under steady state conditions.

Figure 1. DNA methylation status of endothelial nitric oxide synthase (eNOS) 5′-flanking region in vasculogenic progenitor cells. A, Top, Schematic illustration of the human eNOS promoter and transcription factor binding sites which are involved in regulating eNOS expression. Middle, Schematic diagram of the CpG sites of eNOS gene in vasculogenic progenitor cells and endothelial cells. The degree of methylation on each CpG dinucleotide was obtained from 3 individual clones in the indicated cells. The horizontal numbers indicate positions of the CpG sites relative to the transcription start site. B, Schematic diagram of the CpG sites of eNOS gene in sorted subpopulation of bone marrow derived cells. The 3 representative clones are shown for each cell type. Open squares represent unmethylated cytosines, black-filled squares represent methylated cytosines, and gray squares indicate failure of sequencing. HUVEC indicates human umbilical vein endothelial cell; MVEC, microvascular endothelial cell; PAC, pro-angiogenic cell.

Histone Methylation Status of the Human eNOS Promoter

Besides gene repression by DNA methylation, post-translational modifications of histone N-terminal tails are dynamic and essential components in the epigenetic regulation of genes. Therefore, using a quantitative chromatin immunoprecipitation (ChIP) technique, we examined post-
translational histone modifications at the eNOS promoter in cultured PACs, CD34+ cells, and HUVEC. Histone modifications were specifically detected in regions of the eNOS promoter, which were shown to control eNOS gene expression such as positive regulatory domains I (−104/−95) and II (−144/−115), the redox-sensitive-AP-1 binding site (−662/−656), TGF-β activating Smad2 binding site (−1000/−720), and the hypoxia-responsive element site (−5375/−5366) that are thought to be important for hypoxia-induced transcription of eNOS (Figure 2A, Supplemental Material). We observed that the repressive H3K27me3 mark was highly enriched at all sites studied in PACs, whereas another repressive histone modification, H3K9me3, was rather low (Figure 2A). Some progenitor cells are reported to display “bivalent” domains, which are characterized by H3K4me3 and H3K27me3 marks. However, almost no enrichment of active H3K4me3 marks was observed at the eNOS promoter in PACs, and acetylation of histone H3 was only modest (Figure 2A). Similar results were obtained in CD34+ cells (Figure 2A). Again, the repressive H3K27me3 mark was enriched at the eNOS promoter, whereas the active H3K4me3 was absent and histone H3 acetylation was only slightly elevated. In contrast, in endothelial cells (HUVEC), only active (histone H3 acetylation and H3K4me3) marks were detected (Figure 2A). However, at the 5′ region of the eNOS promoter (−9730/−9670), which lacks endothelial transcription factor binding sites, we observed that high levels of repressive histone modifications (H3K27me3 and H3K9me3) were dominant also in HUVEC demonstrating that the lack of repressive marks in the regulatory regions of the eNOS promoter in HUVEC is not due to a failure of the ChIP experiment (Supplemental Figure I). These results indicate that the eNOS promoter is epigenetically silenced by H3K27me3 marks in PACs and CD34+ cells compared to HUVEC. The prevalence of DNA methylation and repressive histone marks at the eNOS promoter in PACs and CD34+ cells correlate closely with a low eNOS mRNA expression levels in these cells compared to HUVEC (Figure 2B).
Histone Methylation Status of the Promoters of Other Endothelial Genes

To determine whether the repression of endothelial genes in proangiogenic cell subtypes is specific for eNOS or extends to other endothelial committed genes, we examined the histone modification status of the endothelial marker genes KDR, von Willebrand factor (vWF), and vascular endothelial-cadherin (VE-cadherin) promoter regions. We observed that H3K27me3 was highly enriched at all 3 promoters studied in both PACs and CD34+ cells (Figure 3A). The repressive H3K9me3 mark was also detected at the KDR promoter in PACs and CD34+ cells, although at a lower level. The levels of histone H3 acetylation marks were higher at the VE-cadherin promoter in PACs and the vWF and VE-cadherin promoter in CD34+ cells (Figure 3A). The active H3K4me3 mark was overall rather low. In contrast, in differentiated endothelial cells, active marks are dominant (Figure 3A).

Despite active histone H3 acetylation marks at some promoters (Figure 3A), the mRNA expression levels of KDR, vWF, and VE-cadherin are very low in PACs and CD34+ cells compared to HUVEC (Figure 3B) indicating that silencing H3K27me3 plays a dominant role in repressing gene expression of these endothelial genes.

Increased Expression of eNOS in Proangiogenic Cells After DZNep and Trichostatin A Treatment

Because the promoters of all endothelial committed genes are characterized by high repressive H3K27me3 in PACs and CD34+ cells, we determined whether inhibition of this repressive histone modification might facilitate endothelial lineage commitment. Therefore, we treated PACs with 3-deazaneplanocin A (DZNep), an S-adenosylhomocysteine hydrolase inhibitor, which inhibits Ezh2 and acts as an inhibitor of H3K27 trimethylation.16 DZNep dose-dependently reduced overall H3K27me3 levels as shown by Western blot and inhibited H3K27me3 at the eNOS promoter (Figure 4A and 4C). However, eNOS mRNA expression remained low and was not influenced by DZNep treatment despite the efficient reduction of the silencing marks (Supplemental Figure IIA and Figure 4C). Because eNOS expression also requires active histone acetylation,17 we next combined DZNep with the histone deacetylase inhibitor Trichostatin A (TSA). Indeed, the combination of DZNep and TSA modestly increased histone H3 acetylation and signifi-
cantly increased eNOS mRNA expression in PACs (Figure 4B and 4D). The activation of the eNOS promoter was further confirmed by ChIP experiments showing that DZNep/TSA treatment increased the binding of the eNOS activating transcription factor Sp1 to the regulatory region of the eNOS promoter (Figure 4E). Interestingly, DZNep treatment alone significantly increased eNOS expression in CD34<sup>+/H11001</sup> cells and the combination of DZNep and TSA induced a further augmentation of eNOS that exceeds the response of PACs (PACs: 1.4-fold increase; CD34<sup>+/H11001</sup> cells: 9.2-fold increase). In contrast, DZNep and TSA did not increase but even reduced eNOS expression in differentiated cells such as HUVEC or smooth muscle cells (SMCs) (Supplemental Figure IIB) suggesting a cell type-specific response to these pharmaco-

tological modulators. Together, our data suggest that the reduction of repressive H3K27me3 marks in combination with pharmacological augmentation of histone H3 acetylation can derepress the eNOS promoter in proangiogenic cells.

**Hypoxia Increases the Balance of Active Versus Repressive Histone Marks at Endothelial Promoters**

Among the many environmental signals in ischemic tissue, hypoxia is one of the strongest stimuli to enhance recruitment of progenitor cells and induce angiogenesis. Therefore, we investigated whether hypoxia modulates epigenetic control mechanisms to sensitize PACs for endothelial differentiation. To address this question, we exposed PACs to a hypoxic environment (1% O<sub>2</sub>) and analyzed the mRNA level of eNOS and KDR. Both eNOS and KDR mRNA expression were time-dependently upregulated by hypoxia (Figure 5A). In line with the increase mRNA expression, eNOS protein was upregulated in PACs after hypoxic stimulation (Supplemental Figure IIIA and IIIB). In parallel, hypoxia significantly (P<0.01) reduced the overall levels of repressive histone H3K27me3 marks (Figure 5B and 5C). Furthermore, H3K27me3 marks are significantly reduced at the eNOS promoter, and the ratio of active H3K4me3 to repressive H3K27me3 marks were increased at all sites of the eNOS promoter studied after exposure of the cells to hypoxia (Figure 5D). Similarly, hypoxia increased the ratio of active H3K4me3 versus repressive H3K27me3 marks at the KDR (393±103%), vWF (659±301%), and VE-cadherin (298±81%) promoters. These data suggest that hypoxia changes the epigenetic signatures toward an active state and, thereby, facilitates endothelial gene expression in PACs.

**Hypoxia Upregulates the Expression of Demethylases**

Because previous studies suggest that hypoxia can stimulate the expression of proteins that belong to the JmjC-domain
family, we specifically determined whether Jmjd3 and UTX, which both were shown to reverse H3K27me3 marks, are regulated. Indeed, Jmjd3 and UTX mRNA expression were time-dependently upregulated by hypoxia in vitro (Figure 6A) and were increased in muscle tissue after induction of hind limb ischemia in vivo (9.1-fold in UTX and 10.2-fold in Jmjd3 at day 4). Consistent with the increase of mRNA expression, Jmjd3 protein and, to a minor extent, UTX protein levels were up-regulated after hypoxic stimulation of PACs (Figure 6B). To address whether Jmjd3 is recruited to the eNOS promoter, we further performed ChIP experiments. These experiments demonstrate that Jmjd3 is recruited to some sites at eNOS promoter (Figure 6C). Taken together, these data demonstrate that hypoxia up-regulated endo-thelial nitric oxide synthase (eNOS) gene expression and down-regulated H3K27me3 at the eNOS promoter. A. Quantitative eNOS, KDR mRNA levels in proangiogenic cells (PACs) exposed to hypoxia (1% O2). The expression level of eNOS and KDR gene under normoxic condition was set as 1 and fold induction is shown. Data represents means±SEM, n=5 to 6. B. Western blot analysis of histone proteins in PAC exposed to hypoxia (1% O2). Histone H3 is used as a loading control. C. Immunofluorescence image of H3K27me3 (pink) in PAC exposed to normoxia or hypoxia (1% O2). Nuclei are stained in blue. Bars represent 20 μm. D. Quantitative chromatin immunoprecipitation (ChIP) analysis of H3K27me3 (top), the ratio of active vs repressive chromatin marks (H3K4me3/H3K27me3, bottom) at the eNOS promoter locus in PAC exposed to normoxia or hypoxia for 36 hours. Data were expressed as % input as means±SEM. The enrichment level of H3K27me3 and the level of H3K4me3/H3K27me3 under normoxic condition was set as 1 and fold induction is shown. n=6 to 12. *P<0.05.

Silencing of Jmjd3 Induces Cell Death and Senescence

To investigate whether the increase of Jmjd3 expression indeed causally contributes to the hypoxia-induced expression of eNOS, we generated lentiviral vectors expressing short hairpin RNAs (shRNA) against Jmjd3. However, despite testing 5 different sequences, we only could reduce the level of Jmjd3 by about 35% in PACs and silencing of Jmjd3 was associated with an induction of apoptosis and an increased expression of senescence markers (p14 ARF, p16 INK, p21 Cip, and p53, Figure 7A through 7C). Moreover, the combination of shJmjd3 and hypoxia resulted in the death of more than 30% of PACs indicating that Jmjd3 expression is essential for PAC survival. Therefore, we were unable to address the question whether Jmjd3 regulates eNOS expression in PACs. However, because previous studies demonstrated that in other cell types Jmjd3 might have a different effect on senescence, we additionally evaluated the function of Jmjd3 in mesoangioblasts. In these cells, silencing of Jmjd3 did not affect survival and did not affect the expression of senescence marker genes (data not shown) allowing us to test the requirement of Jmjd3 for the hypoxia-induced regulation of eNOS. Indeed, silencing of Jmjd3 significantly reduced the hypoxia-induced increase in eNOS expression (Figure 7D).

Discussion

The data of the present study demonstrate that endothelial committed genes are epigenetically silenced in different subtypes of human EPCs including cultured PACs and isolated bone marrow-derived CD34+ cells. Silencing of promoters of endothelial committed genes occurred at the level of DNA methylation and histone modifications. Partic-
ularly, H3K27me3 was prominent at all promoters studied (eNOS, KDR, vWF, and VE-cadherin) suggesting that the regulation of H3K27 methylation may play a crucial role in regulating endothelial commitment of adult progenitor cells. In addition, most of the promoters of endothelial committed genes lacked active histone marks that are typically required for active gene expression in PACs and CD34+/H11001 cells. These data indicate that the cells studied are not (yet) determined to the endothelial lineage and that the induction of endothelial commitment may require epigenetic remodeling at the promoters of the endothelial lineage committed genes.

In order to reverse epigenetic silencing to facilitate commitment to an endothelial cell fate, we used a pharmacological approach to inhibit the methylation of H3K27, the silencing mark that was highest at the promoters of the EPCs. The pharmacological inhibitor DZNep thereby blocks the enzyme Ezh2, which catalyzes the trimethylation of H3K27. Although DZNep reduced the levels of overall H3K27me3 and the prevalence of H3K27me3 at the eNOS promoter, DZNep alone was not sufficient to augment eNOS expression in PACs. Because active H3 marks are necessary for eNOS expression in embryonic stem cells and DZNep exhibits some inhibitory effects on active histone marks (see Figure 4A), we speculated that activating histone modifications may be required to support eNOS expression. Therefore, we combined DZNep with low concentrations of the histone deacetylase inhibitor TSA and indeed showed that the combination of the substances facilitates eNOS expression. Of note, these effects were dose-dependent and higher concentration of TSA and DZNep were exhibiting toxic effects and even suppressed eNOS expression. This finding is consistent with previous data showing that the complete blockade of histone deacetylases reduces eNOS levels in EPCs.

Interestingly, the derepression of eNOS was cell-type specific. In CD34+ cells, DZNep alone significantly induced eNOS expression and the combination of DZNep and TSA further increased to levels much higher compared to PACs, whereas in HUVEC and SMC no increase in eNOS expression was seen. These data indicate that CD34+ cells (and PACs) are sensitive to the derepression of the eNOS promoter by pharmacological inhibition of the methyltransferase Ezh2. Consistently, the expression of Ezh2 was higher in CD34+ cells and PACs compared to HUVEC and SMC (Supplemental Figure IIC). However, the increases in eNOS expression—although significant—were only modest and the levels achieved under optimal dosing of both inhibitors in PACs and CD34+ cells were not comparable to that of HUVEC. These findings are consistent with a remaining DNA methylation of the eNOS promoter observed in PACs after incubation with DZNep and TSA (Supplemental Figure V). Therefore, the removal of silencing histone marks is not sufficient to induce a DNA demethylation of the eNOS promoter within a few days. Because it is known that DNA methylation, particularly of promoters with low CpG island content (as the eNOS promoter), does not fully block transcriptional activation, the removal of silencing histone modification and increase of activating histone marks may be sufficient to partially activate the eNOS promoter.

In the second part of the article, we evaluated the influence of physiological factors that may modify the epigenetic signatures of PACs. Because hypoxia plays a critical role in recruiting proangiogenic cells, we specifically determined the effect of hypoxia. Hypoxia indeed increased the expression of eNOS and KDR and altered histone modifications. Thereby, the silencing H3K27me3 mark was significantly reduced by hypoxia as shown by Western blotting and as confirmed by immunohistochemistry and ChIP of the eNOS promoter. In addition, hypoxia increased the levels of active histone marks. These findings are consistent with data from others showing that hypoxia improves EPC functions and activates...
eNOS expression in endothelial cells. However, a recent study reported that hypoxia reduces histone H3 acetylation and represses eNOS in endothelial cells via sustained hypoacetylation at the eNOS promoter leading to decreased nucleosome accessibility. Consistent with this study, in HUVEC histone acetylation (histone H3) at the proximal eNOS promoter marginally decreased on hypoxia, whereas in PACs histone acetylation at the eNOS promoter did not decrease on hypoxia (data not shown). Beyond that, hypoxia did not increase Jmjd3 expression and did not decrease H3K27me3 marks in HUVEC (Supplemental Figure IV). The reason for this discrepant reaction and the precise mechanisms regulating Jmjd3 recruitment is unclear but may be due to the different cell types used in the studies (mature endothelial cells versus progenitor cell populations). Indeed, EPCs and HUVEC are reported to display different responses to hypoxia. Whereas hypoxia induced an increased proliferation in EPCs, a proliferation arrest was induced in HUVEC.

The mechanisms by which hypoxia modulates histone modifications are not entirely solved but may include the upregulation of histone demethylases of the JmjC-domain containing family. Indeed, Jmjd3 mRNA and protein was significantly increased by hypoxia and recruited to the eNOS promoter. However, when we attempted to study the causal involvement of Jmjd3 in hypoxia-mediated up-regulation of eNOS, we observed that Jmjd3 silencing lead to induction of apoptosis and senescence in PACs, which precluded testing the original hypothesis. The induction of apoptosis and senescence in PACs by Jmjd3 silencing was surprising because it has been reported that Jmjd3 can induce oncogene-mediated senescence in cancer cells. However, Jmjd3 was recently shown to have an essential function in myeloid cells. Because PACs resemble monocytic cells, our observation may be related to a crucial role of Jmjd3 in this cell type. To address the principal role of hypoxia-mediated up-regulation of Jmjd3, we therefore used an alternative circulating cell, mesoangioblast, which does not express myeloid or hematopoietic markers. Although mesoangioblasts also showed a high DNA methylation pattern of the eNOS promoter, eNOS expression was upregulated by hypoxia and this effect was prevented in the absence of Jmjd3. These results provide evidence that, in some cell types, Jmjd3 expression contributes to the regulation of the eNOS promoter on induction of hypoxia.

Together, the present study is the first to evaluate the epigenetic signature of promoters of endothelial committed
genes in different subsets of cells that have been used to augment neovascularization. Our findings demonstrating an epigenetic repression of endothelial genes indicate that the cells studied are not predetermined to the endothelial lineage. This is consistent with previous reports showing that endothelial marker gene expression detected in PACs might be a consequence of transferred mRNA by contaminating cells, such as platelets, as well as by the lack of direct differentiation of isolated hematopoietic stem cells to the endothelial lineage. However, several other groups have shown that hematopoietic cells can acquire an endothelial cell fate. Therefore, we cannot exclude that the different culture protocols might modify (or reprogram) the cells. Indeed, a combination of pharmacological substances as well as hypoxia changed the epigenetic signature indicating that modulation in the cell environment might potentially result in a change in cell fate.

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Disclosures

None.

References

Novelty and Significance

What is Known?

● Several subsets of endothelial progenitor cells (EPCs) and proangiogenic hematopoietic cells contribute to postnatal neovascularization.

● The mechanism by which these cells contribute to neovascularization is controversially discussed and includes paracrine effects and differentiation to endothelial cells.

What New Information Does This Article Contribute?

● We demonstrate that the endothelial genes are epigenetically silenced in several subsets of endothelial progenitor cells and proangiogenic hematopoietic cells including CD34+ cells and early EPCs.

● Epigenetic silencing of endothelial promoters is mediated by repressive histone methylation at lysine 27 (H3K27me3).

● Pharmacological inhibition of histone methylation and histone deacetylation or physiological stimulation with hypoxia in part reversed epigenetic silencing and enhanced expression of endothelial marker genes.

Although various subsets of EPCs were shown to therapeutically augment angiogenesis and recovery after ischemia, it is unclear whether these cells are committed to the endothelial lineage. Here, we demonstrate that endothelial promoters are epigenetically silenced by repressive histone methylation marks in different populations of EPCs demonstrating that these cells are not committed to the endothelial lineage. Treatment with pharmacological inhibitors of the histone methyltransferase Ezh2 and histone deacetylases reversed the silencing and induced expression of endothelial marker genes such as the endothelial nitric oxide synthase. Likewise, hypoxia modulates the epigenetic signature in part by inducing the histone demethylase Jmjd3. These data demonstrate that pharmacological inhibitors or physiological stress can modulate cell fate decisions in EPCs.
Epigenetic Regulation of Endothelial Lineage Committed Genes in Pro-Angiogenic Hematopoietic and Endothelial Progenitor Cells

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SUPPLEMENTAL MATERIAL

Detailed Methods

Isolation and cell culture of PACs, CD34⁺, HUVEC, and Mesoangioblasts

Approval for these studies was obtained from the ethics review board of J. W. Goethe University, and the study was conducted with the Declaration of Helsinki. Informed consent was obtained from each patient. Mononuclear cells (MNCs) were isolated by density gradient centrifugation with Biocoll (Biochrom) from human peripheral blood buffy coats as previously described.¹ Immediately after isolation, 8 × 10⁶ MNCs/ml were plated on culture dishes coated with human fibronectin (Sigma) and maintained in endothelial basal medium (EBM; Lonza) supplemented with hydrocortisone, bovine brain extract, gentamicin, amphotericin B, epidermal growth factor and 20% fetal calf serum. After 3 days in culture, nonadherent cells were discarded by thorough washing with PBS and adherent cells were incubated in fresh medium for 24 h before starting the experiments. Mesoangioblasts were isolated as previously reported.² Briefly, MNCs were isolated from blood which were extracted during open heart surgery and plated on a fibronectin-coated dish. On 14 day after plating, growing cells are collected and re-plated on another dish. To eliminate the contamination of other cells, re-plating was repeated at least 3 times before use. Surface marker profile of these outgrowing cells is distinct from hematopoietic and endothelial progenitor cells (absence of CD45, CD34, CD133, CD31, and CD14) and represents a phenotype of embryonic dorsal aorta-derived mesoangioblasts (the expression of mesenchymal markers such as CD13 and CD73, and endothelial marker such as KDR). Human umbilical vein endothelial cells (HUVECs) were purchased from Cambrex and cultured until the third passage. CD34⁺ cells for ChIP experiment (Figure 2 and 3) were purchased from Lonza and cultured in HPGM medium (Lonza) without cytokines for 4 hours before using in experiments to keep primitive state. Immunomagnetically selected CD34⁺ hematopoietic progenitor cells for DNA methylation analysis were obtained from cryopreserved samples.³ CD34⁺ hematopoietic progenitor cells for drug treatment and hypoxia experiments were isolated from bone marrow and purified by immunomagnetic beads conjugated with anti-CD34 antibody and cultured in medium containing SCF 25ng/mL, TPO 50ng/mL, and FL 50ng/mL. For drug treatment, PACs were treated with 0.5 to 5 µM DZNep (kindly gifted by Dr. Marquez VE, NCI), 32nM TSA (Sigma), and 1 to 10 µM 5-Azacytidine (5-aza, sigma) for the indicated times.

Cell sorting

Immunomagnetically selected CD34⁺ hematopoietic progenitor cells (a purity of more than 97% was determined by flow cytometry) were labeled with allophycocyanin (APC)-anti CD34 (555824, BD
pharmingen) and phycoerythrin (PE)-anti KDR (FAB357P, R & D) and subsequent separation of CD34+/KDR+/- subpopulations was performed by a FACSARia (Becton Dickinson, San Jose, CA).

**Hypoxic treatment**
In order to induce hypoxia, PACs were cultured in hypoxic chamber (BINDER, Germany) with a humidified atmosphere of 1% oxygen for the indicated duration.

**RNAi knockdown**
PACs or mesoangioblasts were transduced with pLKO.1-lentivirus carrying shRNA encoding human Jmjd3 (CCGCGCGCTGACCATTACAAA) or non-target shRNA control (both from Sigma).

**Sodium bisulfite sequencing**
Sodium bisulfite treatment of genomic DNA was carried out according to manufacturer’s protocol (EZ DNA methylation kit, ZYMO). Amplified bisulfite PCR products were subcloned into TOPO TA vector (Invitrogen) and sequenced to determine the presence of methylated cytosines. The primers used in this assay are available upon request.

**Quantitative PCR**
Total RNAs were isolated using TRIZol (Invitrogen) or RNeasy PlusMini Kit (Qiagen). RNA was reverse transcribed to cDNA and subjected to quantitative PCR on StepOnePlusTM Real Time PCR System (Applied Biosystems). 28srRNA was used in hypoxia experiments and GAPDH was used in other experiments to normalize gene expression data. Primer sequences are available upon request.

**Western blot**
PACs were lysed in cell lysis buffer (Cell Signaling) and briefly sonicated. Equal amounts of proteins were separated in SDS-polyacrylamide gel and then transferred to a polyvinylidene difluoride (PVDF) membrane. Immunoblotting was performed with the antibody against H3K4me3 (07-473 Millipore), H3K9me3 (07-422 Millipore), H3K27me1 (07-448 Millipore), H3K27me2 (07-322 Millipore), H3K27me3 (07-449 Millipore), Acetylated H3 (06-599 Millipore), Histone H3 (9715 Cell Signaling), Jmjd3 (AP1022a ABGENT), UTX (ab36938 abcam), and β-actin (Sigma).

**Immunostaining**
Cells were fixed with 4% paraformaldehyde and permeabilized with 0.2% saponin (Sigma). Cells were incubated with the antibody against H3K27me3 (07-449 Millipore) and eNOS (610296 BD Transduction
Laboratories). Nuclei were counterstained with DAPI according to the manufacturer’s instructions. The images were recorded by confocal microscope (LSM510-META, Carl Zeiss, Germany) and analyzed by LSM 510 microscope software.

**Chromatin immunoprecipitation (ChIP)**

ChIP assays were performed according to the manufacture’s protocols (PACs; ChIP-IT Express, Active Motif, CD34+ cells and HUVEC; Magna-ChIP, Millipore). Briefly, after fixation by 1% formaldehyde, PACs chromatin was enzymatically digested by enzymatic shearing cocktail according to the protocol of the manufacturer (Active Motif), whereas in CD34+ cells and HUVEC chromatin was sheared by sonication using the Branson 450 Sonifier. Chromatin shearing was optimized for the different cell types and the efficiency (chromatin DNA fragments ranging from 200 to 1000 bp) was confirmed for all protocols. Sheared chromatin was immunoprecipitated with antibodies against H3K4me3 (17-614 Millipore), H3K9me3 (07-442 Millipore), H3K27me3 (07-449 Millipore), AcetylH3 (06-599 Millipore), IgG (12-370 Millipore), Jmjd3 (AP1022a ABGENT, ARP40102 AVIVA), and Sp1 (17-601 Millipore). The purified chromatin was used in quantitative real-time PCR (Applied Biosystems). ChIP-quantitative PCR data were normalized as percent input. For that purpose, Ct values obtained from the ChIP were divided by the Ct value from the input control. The primer sequences used to amplify the target regions were designed according to previous publications characterizing the promoter regions that are involved in endothelial gene expression and by rVISTA software. Primer sequences are available as requested.

**Annexin V staining**

Apoptotic cells were stained with Annexian-V (BD Pharmingen) and analyzed by a flow cytometer, BD FACS Calibur cell sorter (BD Bioscience).

**Statistical analysis**

Data are represented as mean ± SEM. Unnormalized data were analyzed by using Unpaired Student’s t-test, Mann Whitney test, and Paired t-test when comparing two groups, or a one-way ANOVA with Bonferroni’s comparison test when comparing multiple groups. Normalized data are analyzed by using Paired t-test, One sample t-test, and Wilcoxon Signed Rank test when comparing two groups, or Repeated Measures ANOVA with Bonferroni’s comparison test when comparing multiple groups. The data obtained after Probability values of < 0.05 were considered statistically significant and tests were performed two-sided.
References


Online Figure I. Histone modifications of eNOS and KDR upstream promoters in vasculogenic cells. Quantitative ChIP analysis of histone H3 acetylation (Ace H3), trimethylation of histon H3 at lysine 4, 27, and 9 (H3K4me3, H3K27me3, and H3K9me3) of eNOS (-9730/-9670) and KDR (-5902/-5837) promoter of the indicated cell types. IgG was used as a negative control. Data was expressed as % input as means ± SEM. Green and red color indicate active and repressive histone modification, respectively. N=3-6.
Online Figure II. Cell context dependent effects of DZNep and TSA on eNOS expression (A) Quantitative eNOS mRNA levels after DZNep (0.5-5 µM) treatment for 3 days. The expression level of untreated control cells was assigned the arbitrary value of 1. Data represents means ± SEM, n=3 (B) Quantitative eNOS mRNA levels were evaluated in indicated cells treated with DZNep (1 µM) and/or TSA (32 nM) for 3 days. (C) Quantitative Ezh2 mRNA levels in indicated cells.
Online Figure III. Hypoxia up-regulated eNOS expression.  
(A) Immunofluorescence image of eNOS (green) in PACs exposed to normoxia or hypoxia (1% O₂) for 36h. Nuclei are stained in blue. Bars represent 5 μm. (B) Quantitative analysis of eNOS positive PACs versus whole nuclei. Data represents means ± SEM, n=3.
Online Figure IV. Hypoxia down-regulated eNOS gene and reduced the ratio of active H3K4me3 versus repressive H3K27me3 at the proximal eNOS promoter in HUVEC.

(A) Quantitative eNOS mRNA levels in HUVEC exposed to hypoxia (1% O_2) for 36h. The expression level of eNOS gene under normoxic condition was set as 1 and fold induction is shown. Data represents means ± SEM, n=5. *P<.05. (B) Quantitative Chip analysis of H3K27me3 (top), H3K4me3 (middle), and the ratio of active versus repressive chromatin marks (H3K4me3/H3K27me3, bottom) at the eNOS promoter locus in HUVEC exposed to normoxia or hypoxia (1% O_2) for 36h. Data was expressed as % input. The enrichment level of H3K27me3 and H3K4me3 under normoxic condition was set as 1 and fold induction is shown compared to the normoxic cells.
Online Figure V. DNA methylation status of eNOS 5’-flanking region after treatment
(Upper) Schematic illustration of the human eNOS promoter and transcription factor binding sites which are involved in regulating eNOS expression. (Lower) Schematic diagram of the CpG sites of eNOS gene in PACs treated in hypoxic condition for 36hr or with DZNep (1 uM) and TSA (32 nM) for 3 days. The degree of methylation on each CpG dinucleotide was obtained from three individual clones. The horizontal numbers indicate positions of the CpG sites relative to the transcription start site. Open squares represent unmethylated cytosines, black filled squares represent methylated cytosines, and gray squares represent unavailable data.