Epigenetic Regulation of Angiogenesis by JARID1B-Induced Repression of HOXA5


Objective—Altering endothelial biology through epigenetic modifiers is an attractive novel concept, which is, however, just in its beginnings. We therefore set out to identify chromatin modifiers important for endothelial gene expression and contributing to angiogenesis.

Approach and Results—To identify chromatin modifying enzymes in endothelial cells, histone demethylases were screened by microarray and polymerase chain reaction. The histone 3 lysine 4 demethylase JARID1B was identified as a highly expressed enzyme at the mRNA and protein levels. Knockdown of JARID1B by shRNA in human umbilical vein endothelial cells attenuated cell migration, angiogenic sprouting, and tube formation. Similarly, pharmacological inhibition and overexpression of a catalytic inactive JARID1B mutant reduced the angiogenic capacity of human umbilical vein endothelial cells. To identify the in vivo relevance of JARID1B in the vascular system, Jarid1b knockout mice were studied. As global knockout results in increased mortality and developmental defects, tamoxifen-inducible and endothelial-specific knockout mice were generated. Acute knockout of Jarid1b attenuated retinal angiogenesis and endothelial sprout outgrowth from aortic segments. To identify the underlying mechanism, a microarray experiment was performed, which led to the identification of the antiangiogenic transcription factor HOXA5 to be suppressed by JARID1B. Importantly, downregulation or inhibition of JARID1B, but not of JARID1A and JARID1C, induced HOXA5 expression in human umbilical vein endothelial cells. Consistently, chromatin immunoprecipitation revealed that JARID1B occupies and reduces the histone 3 lysine 4 methylation levels at the HOXA5 promoter, demonstrating a direct function of JARID1B in endothelial HOXA5 gene regulation.

Conclusions—JARID1B, by suppressing HOXA5, maintains the endothelial angiogenic capacity in a demethylase-dependent manner. (Arterioscler Thromb Vasc Biol. 2015;35:1645-1652. DOI: 10.1161/ATVBAHA.115.305561.)

Key Words: angiogenesis; epigenetics; growth and development; Kdm5b protein, mouse; promoter regions, genetic

A healthy vascular endothelium is antiatherosclerotic, promotes vasodilatation, provides an anticoagulatory surface, and is able to perform angiogenesis. During endothelial dysfunction, these properties are lost and endothelial cells (ECs) promote vascular disease development.1,2 Understanding the fundamental mechanisms governing endothelial phenotype switching offers novel approaches to prevent and alter the course of vascular disease. Epigenetic mechanisms conserve the cellular identity and give rise to developmental processes. Changing epigenetic marks therefore is an innovative approach to promote changes of the cellular phenotype. The epigenetic control of EC biology, however, is incompletely understood.3,4

Histone methylation is an important mechanism of epigenetic regulation. Trimethylation at histone 3 lysine 4 (H3K4me3), located in promoter regions, is associated with gene expression.5,6 Modifiers of H3K4me3, histone deacetylases and demethylases, have fundamental roles in biological processes, such as embryonic development, stem cell biology,6 and cancer development.7

Multiple enzymes are involved in the demethylation of H3K4, among them enzymes of the JARID1 family (JARID1A to JARID1D). Up to now, the mechanisms rendering gene-specific H3K4-demethylation are not understood and the function of several of the H3K4 demethylases in the cardiovascular system has not been studied in detail. The demethylase LSD1 has...
been shown to maintain endothelium-dependent relaxation. For JARID2, an important role of endocardial-driven cardiac development has been reported. The function of the other JARID enzymes in the cardiovascular system has not been studied. In this study, we found the enzyme JARID1B, which has been implicated in cell fate decision, cancer progression, and stem cell self-renewal, to be highly expressed in ECs. By its JmjC domain, JARID1B catalyzes the demethylation of H3K4me2 and H3K4me3. Through this function, JARID1B is involved in the repression of transcription during embryonic development and in embryonic and hematopoietic stem cells. Therefore, genetic deletion of JARID1B impairs mouse and porcine embryonic development. Considering its effect on gene regulation, we hypothesized that JARID1B affects the expression of endothelial genes and examined the consequences on angiogenesis in human umbilical vein ECs (HUVECs), tamoxifen-inducible conditional, and in the endothelial-specific Jarid1b knockout mouse.

Materials and Methods
Materials and Methods are available in the online-only Data Supplement.

Results
JARID1B Is Highly Expressed in HUVECs
To study whether JARID1B has a role in the endothelium, we used HUVECs. Microarray expression analysis of several histone-modifying enzymes particularly demethylases revealed that JARID1B was of the studied genes the second most highly expressed, after LSD1, histone-modifying enzyme in ECs (Figure 1A). Quantitative reverse transcription polymerase chain reaction validation of the array data, however, indicated that JARID1B was expressed at higher levels than all other histone demethylase, including LSD1 (Figure 1B). Western blot analysis confirmed the expression of JARID1B protein in HUVECs, whereas expression in smooth muscle cells and blood-derived angiogenic myeloid cells were much lower (Figure 1C). The expression level of JARID1B in angiogenic myeloid cells was also lower than that in HUVECs (Figure 1D).

Catalytic Activity of JARID1B Is Essential for Endothelial Angiogenic Capacity in Culture
To identify a functional role for JARID1B in ECs, the expression of the enzyme was decreased using a lentiviral JARID1B shRNA approach (Figure I in the online-only Data Supplement). Depletion of JARID1B had no effect on endothelial proliferation or apoptosis (data not shown). The downregulation of JARID1B, however, attenuated EC sprouting and tube forming capacity (Figure 2A and 2B). To determine whether this feature of JARID1B requires its enzymatic activity, experiments were repeated with a catalytically dead mutant (H499A/E501A) with an inactive JmjC domain. Compared with overexpression of the wild-type JARID1B plasmid, overexpression of the mutant JARID1B reduced EC sprouting (Figure 2C). In line with this, 2–4(4-methylphenyl)-1,2-benzisothiazol-3-(2H)-one, a pharmacological inhibitor of the JARID family, diminished the angiogenic potential of ECs (Figure 2D and 2E). Vessel outgrowth also depends on cell migration. Consistently, the knockdown of JARID1B, as well as the inhibition of the JARID family, attenuated the migration of HUVECs in the scratch wound as well as in the

Figure 1. JARID1B is highly expressed in human umbilical vein endothelial cells (HUVECs). A and B, Expression of the indicated histone-modifying enzymes in HUVECs as determined by gene array (A) and quantitative reverse transcription polymerase chain reaction (qRT-PCR; B). C, Representative Western blot for JARID1B, endothelial nitric oxide synthase (eNOS), and β-actin in THP-1, aortic smooth muscle cells (ASMC), HEK293, HUVEC, angiogenic myeloid cells (AMC), and fibroblast (Fibro). D, qRT-PCR for JARID1B in HUVECs and AMCs.

*P<0.05; n>3.
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Boyden chamber assay (Figure 2F–2I). Collectively, these findings suggest that JARID1B and its enzymatic activity are required for the angiogenic capacity of HUVECs in culture.

JARID1B Contributes to Angiogenesis In Vivo

To corroborate these findings in a physiological system, we generated tamoxifen-inducible global knockout mice, in which depletion of exon 6 results in a frameshift and a premature stop codon. As global Jarid1b knockout mice are born at sub-Mendelian ratio and frequently die around birth with complex developmental problems, a tamoxifen-induced knockout mouse was breed. Activation of Cre-recombinase by tamoxifen resulted in a significant loss of Jarid1b protein, whereas the expression of an unspecific, closely located cross-reacting band was unchanged. As determined by quantitative reverse transcription polymerase chain reaction, Cre-recombinase activation reduced Jarid1b level by ≈80% (Figure 3A and 3B).11,19 Ten-week old mice with induced conditional deletion of JARID1B were vital and showed no obvious phenotype. However, EC sprouting, detected by the number of CD31-positive cells, was abnormal in explanted aortic rings: both the sprout number and the total sprout length were markedly reduced in aortic rings from knockout mice (Figure 3C). Also EC migration in vivo was reduced after deletion of Jarid1b as observed in the carotid artery electro injury model (Figure 3D). Importantly, JARID1B also contributed to physiological angiogenesis as its knockdown delayed the rate of retinal vascularization, as well as the density of the developed vascular plexus (Figure 3E).

Figure 2. Catalytic activity of JARID1B is essential for endothelial angiogenic capacity. Spheroid outgrowth assay (A, C, and D), tube formation assay (B and E), Boyden chamber assay (F and G), and scratch wound assay for migration (H and I) of human umbilical vein endothelial cells (HUVECs) transduced with 2 different control shRNAs shScrambled (shCtl-1) and shGFP (shCtl-2) or Jarid1B shRNAs (shJ1B; A, B, F, and H) or after overexpression of plasmids coding for JARID1B wild-type (WT) or the H449A/E501A mutant (C) or in the presence (JARID1 inhib.) or absence (Solvent) of the JARID1 inhibitor 2 to 4(4-methylphenyl)-1,2-benzisothiazol-3-(2H)-one (D, E, G, and I). If not indicated otherwise, HUVECs were pretreated for 12 h with 10 μmol/L of JARID1 inhibitor in 1% FCS with EBM medium. Spheroid outgrowth was assessed in the presence of vascular endothelial growth factor A 10 ng/mL. *P<0.05; n≥3.

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Interpreting the results of studies performed after the global downregulation of JARID1B is complicated by the fact that the enzyme is a reportedly important regulator of hematopoietic stem cell activity.\(^8,12\) Given the contribution of bone marrow–derived cells in angiogenesis, studies were repeated using a tamoxifen-inducible endothelial-specific JARID1B knockout mouse. In these animals, tamoxifen significantly attenuated EC JARID1B levels (Figure 3F and 3G) and reproduced the delayed retina vascular growth and the decreased vascular plexus density (Figure 3H).

**Figure 3.** Endothelial-specific knockout of JARID1B decreases retina angiogenesis in mice. Western blot for JARID1B and tubulin (A and F), quantitative reverse transcription polymerase chain reaction for JARID1B (B and G; n=3) from mouse lung tissue and isolated lung endothelial cells, aortic outgrowth assay (C; n=5), carotid artery injury assay (D; n=6), and retina vascularization (E and H; n=6) of JARID1BFlox/Flox (WT) and JARID1BFlox/Flox-Cre-ERT2\(^{+/0}\) (KO; A–E) and endothelial specific JARID1BFlox/Flox-CDH5-Cre-ERT2\(^{+/0}\) mice (F–H). Tamoxifen was applied to all animals. Adult mice were injected (IP) for 3 days with tamoxifen followed by 3 weeks of washout (A–H), pubs received tamoxifen by intragastric injections from P1–P5. *P<0.05. TSS indicates transcription start site.

JARID1B Represses HOXA5 in ECs

To determine how JARID1B contributes to angiogenesis, microarray experiments were carried out in HUVECs to identify genes regulated by this enzyme. In keeping with the proposed inhibitory effect of JARID1B on the promoter activity, downregulation of the histone demethylase increased the expressions of a larger number of genes (256 genes, >20%; \(P<0.05\)) than it downregulated expression (26 genes, <20%; \(P<0.05\); Figure II in the online-only Data Supplement). These findings confirm that JARID1B acts as a transcriptional repressor.\(^{16,22}\) Gene ontology analyses of the arrays revealed that JARID1B regulates genes involved in embryogenesis, vision/eye, liver/biliary systems, respiratory system, and hematopoietic system (Table I in the online-only Data Supplement). One of the genes derepressed by the downregulation of JARID1B in HUVECs was the homeobox gene HOXA5 (Figure II in the online-only Data Supplement). A comparison of the JARID1B microarray with HOXA5 Chip-Seq revealed that 20% of the induced genes consistent with the peaks of HOXA5 Chip-Seq (Figure III and Table II in the online-only Data Supplement). We validated several genes (AP1S3, BTF3L4, CCNE2, and LGR4) for being induced by HOXA5 overexpression and by depletion of JARID1B (Figure IV in the online-only Data Supplement).
HOXA5 is important for tumorigenesis but has also been attributed antiangiogenic effects. In agreement with the microarray data, both HOXA5 protein and mRNA were upregulated in lung endothelial cells isolated from Jarid1b knockout mice when compared with wild-type mice (Figure 4A and 4B). Importantly, HOXA5 was specifically suppressed by JARID1B as silencing of JARID1A and JARID1C had no effect on HOXA5 expression (Figure 4C). In line with this, overexpression of JARID1B reduced HOXA5 transcription (Figure 4D). Similar to the effects of JARID1B on cell function, the suppression of HOXA5 was dependent on demethylase activity, as the overexpression of the catalytically inactive mutant or the addition of the JARID inhibitor both increased HOXA5 expression in HUVECs (Figure 4E and 4F).

If HOXA5 was epigenetically regulated by JARID1B in ECs, the activating H3K4me3 modification of the HOXA5 promoter should be increased in cells lacking JARID1B. Indeed, chromatin immunoprecipitation assays revealed that the depletion of JARID1B resulted in an increase in H3K4me3 marks at 900 bp upstream of the HOXA5 transcription start site and this was accompanied by a reduced binding of JARID1B. The effect was specific for the promoter of HOXA5 where JARID1B binding was observed, whereas the H3K4 histone modifications around the transcription start site of HOXA5 or in the promoter of GAPDH did not show enrichment for JARID1B (Figure 4G).

Thus, JARID1B suppresses the HOXA5 gene expression in HUVECs through a mechanism requiring enzymatic activity of this demethylase.

**HOXA5 Inhibits Endothelial Angiogenic Capacity**

The link between JARID1B and HOXA5 implied that HOXA5 could affect the angiogenic capacity of ECs. Indeed, when HOXA5 was overexpressed in HUVECs (Figure 5A), their tube forming capacity and sprouting from EC spheroids were significantly attenuated (Figure 5B and 5C). To determine whether induction of HOXA5 mediated the inhibitory effect of JARID1B shRNA on angiogenic capacity, HOXA5 was downregulated. Silencing of HOXA5 expression in JARID1B-depleted cells increased the sprouting and tube formation (Figure 5D and 5E).

Thus, the role of JARID1B in angiogenesis can be attributed in part to the repression of HOXA5 gene transcription.

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**Figure 4.** JARID1B represses HOXA5 in endothelial cells. Representative Western blot and statistics of densitometry for JARID1B, HOXA5, and GAPDH (A; n=4) and quantitative reverse transcription polymerase chain reaction (qRT-PCR; B; n=5) for HOXA5 in lung endothelial cells cultured from Jarid1b<sup>fl/−/−</sup> (WT) and Jarid1b<sup>fl/−/−-Cre-ERT2+/−</sup> (KO) mice after tamoxifen treatment. C–F, HOXA5 expression as determined by qRT-PCR in human umbilical vein endothelial cells (HUVECs) transduced with shRNA against JARID1B (shJ1B), JARID1A (shJ1A), JARID1C (shJ1C), or control shRNA (shCtl). C, shRNA validation shows the expression of the respective JARID mRNA. Overexpression of a plasmid coding for WT JARID1B (J1B WT) or GFP (D) or H449A/E501A mutant (J1B Mut; E) and pretreatment with the JARID1 inhibitor 2 to 4(4-methylphenyl)-1,2-benzisothiazol-3-(2H)-one (12 h, 10 μmol/L) or solvent (F). n≥3; *P<0.05. G, Chromatin immunoprecipitation of HUVECs transduced with control shRNA (shCtl) or JARID1B shRNA (shJ1B) with the antibodies indicated followed by PCR for GAPDH or HOXA5 using primers binding at the transcription start site (TSS) or 900 and 650 base pairs, respectively, upstream of the TSS. n=3; *P<0.05.
In this study, we identified JARID1B as the most highly expressed histone demethylase in cultured HUVECs. Knockout or inhibition of JARID1B attenuated the angiogenic capacity of human ECs in vitro and exerted similar effects on murine ECs in vivo. We propose that this is at least partially because of an inhibitory effect of JARID1B on the expression of HOXA5, an antiangiogenic factor. JARID1B was found to occupy the promoter region of HOXA5 and to reduce the H3K4me3 levels there. The effect was specific to JARID1B because knockdown of the isoforms JARID1A or JARID1C did not lead to upregulation of HOXA5.

There is growing evidence from embryonic and hematopoietic stem cells that JARID1B is a central regulator of key developmental genes. It regulates the differentiation of stem cells and embryonic development. During neural differentiation, JARID1B binds predominantly at transcription start sites of genes and regulates H3K4 methylation level and subsequently represses lineage-inappropriate genes. It seems that JARID1B has a similar function in the mature endothelium as our microarray analysis revealed that depletion of JARID1B induced the expression of numerous genes and suppressed the expression of only a few genes.

Of the regulated genes, HOXA5 was the focus of further investigation as the HOX genes are indispensable for embryogenesis, and during this process, they also regulate vascularization and angiogenesis. Moreover, HOXA5 drives ECs into a resting state and thus inhibits basal angiogenesis, as well as responsiveness to angiogenic stimuli, such as vascular endothelial growth factor. Our finding that HOXA5 is repressed by JARID1B could account for the downregulation of the gene under quiescent conditions. Moreover, chromatin immunoprecipitation analysis established a direct link between the epigenetic activity of JARID1B and HOXA5 suppression. It is, however, unclear how chromatin-modifying enzymes are recruited to a specific site. DNA binding of JARID1B depends on the ARID site, whereas the demethylase activity is located in the JmjC-domain, a structure similar to the hypoxia-inducible factor hydroxylation prolyl hydroxylases. In fact, demethylation by JARID1B is a 2-step mechanism: the nitrogen is first hydroxylated and subsequently, the methyl group cleaved off. The present data using an inactive mutant and a JARID1B inhibitor show that the data presented here depend on this enzymatic activity.
As mentioned above, many genes were repressed by JARID1B, and it was interesting to note that several of them were occupied by HOXA5. Our decision to focus the further analysis on HOXA5 was based on its known role in developmental function. This rational, obviously, is somewhat arbitrary, and in fact, HOXA5 was not the most strongly repressed gene by JARID1B—this was tetraspanin-8. Gene ontology term analysis, however, indicated that JARID1B acts on numerous cellular functions, and for most of these genes, a link to angiogenesis still needs to be established. This aspect, however, is beyond the scope of this study.

Consistent with our in vitro data, inducible JARID1B knockout mice exhibit an impaired endothelial sprout outgrowth from aortic rings. Recently, it was shown that JARID1B regulates hematopoietic stem cell activity and acute but not constitutive genetic deletion of JARID1B increased peripheral blood T cells.8,12 To exclude that bone marrow–derived cells mediate the effects on angiogenesis, endothelial–specific knockout mice were generated. These clearly demonstrate the importance of JARID1B in ECs for vessel growth.

In summary, we established JARID1B as an important mediator of endothelial angiogenesis. Loss or inhibition of JARID1B attenuates tube formation, spheroid outgrowth cell migration, vascular repair, and vessel growth. JARID1B maintains angiogenesis presumably by repression of antiangiogenic transcriptional regulators, such as HOXA5.

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Disclosures
None.

References


**Significance**

Here, we demonstrate that the histone demethylase JARID1B is essential for angiogenesis. Downregulation or inactivation of JARID1B attenuated endothelial migration and angiogenesis in a demethylase-dependent manner. This could be attributed to the induction of the angiogenic factor HOXA5. In endothelial cells, JARID1B binds to the HOXA5 promoter and removes histone 3 lysine 4 methylation associated with active transcription. Thus, JARID1B represses HOXA5 expression and thereby maintains the angiogenic capacity of endothelial cells. Inhibition of JARID1B could be a novel antiangiogenic strategy.
Epigenetic Regulation of Angiogenesis by JARID1B-Induced Repression of HOXA5


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Supplemental Material

Epigenetic regulation of angiogenesis by JARID1B-induced repression of HOXA5

Christian Fork¹, Landa Gu¹, Juliane Hitzel¹, Ivana Josipovic¹,⁵, Jiong Hu²,³, Michael Szeka Wong¹, Yuliya Ponomareva³, Mareike Albert³, Sandra U. Schmitz², Shizuka Uchida³, Ingrid Fleming²,³, Kristian Helin⁴, Dieter Steinhilber⁵, Matthias S. Leisegang⁶, ⁷, and Ralf P. Brandes¹,⁵, ⁷

Materials and Methods

Materials

Anti-JARID1B antibodies were from Sigma (#HPA027179) and Bethyl (#A301-813A), anti-HOXA5 from Santa Cruz (#sc-28599), anti-eNOS from Invitrogen (#334600), anti-beta-actin (#A1978), anti-GAPDH from sigma (#G8795), anti-tubulin beta (#sc-9104) was acquired from Santa Cruz. The JARID1 inhibitor 2-(4-(4-(methylphenyl)-1,2-benzisothiazol-3-2H)-one (PB1T) described in ¹ was from Sigma (#PH009215).

Experimental animals and animal procedures

All experimental procedures were approved by the local governmental authorities (approval numbers: FU28/40) and were performed in accordance with the animal protection guidelines. Only male mice, age 8 to 12 weeks were used. JARID1B flox/flox mice were provided by one of the co-authors. Targeted ES cell lines were initially generated by the European Gene Trap Consortium (see www.mousephenotype.org). Cdh5-CreERT² mice were kindly provided by Ralf Adams, MPI Münster, CMV-CreERT² mice were from Harald von Melchner, Goethe-University, Frankfurt. Activation of CreERT2 was achieved by intraperitoneal injection (i.p.) of tamoxifen in sunflower oil on 3 consecutive days followed by a "wash-out" phase of 3 weeks. For retina angiogenesis, tamoxifen was administered by intragastric injection as reported previously by others. In all experiments Cre positive (denoted as Cre+0) as well as the Cre negative (denoted as Cre0/0) control animals received tamoxifen to exclude direct effects of this anti-estrogen. Breeding of the Cre-lines was carried out by crossing Cre +0 and Cre 0/0 animals so that Cre +/0 and Cre 0/0 littersmates could always be compared side by side. Mice were housed in a specified pathogen-free facility with 12/12 hours day/night cycle and free access to chow and water.

Carotid artery injury model

Electric-injury was performed under general anesthesia by ketamin/rompun as described. Briefly, the carotid artery was exposed to electric injury using a hemostatic electric coagulation forceps (ERBOTOM ICC 50 HF, ERBE, Tübingen, Germany) twice juxtapose for 3 seconds to induce a 3 mm wide denudation. Wounds were closed by staples and animals were allowed to recover for 3 days followed by Evans blue staining for determination of the denuded area. Evans blue solution (2%, 200 μL) was injected via the tail vein and allowed to circulate for 10 minutes. Subsequently, animals were perfused transcardially with NaCl 0.9% to remove the excessive dye. Carotid arteries were isolated and imaged by an infrared-based laser fluorescence scanner (Odyssey, Licer, Bad Homburg, Germany) or photographed on a macro stage.

Retina angiogenesis

For whole-mount immunohistochemistry, retinas were fixed in 4% PFA for 2 h at room temperature, or overnight at 4°C. After fixation, retinas were blocked and permeabilized in 1% BSA and 0.5% Triton X-100 overnight at 4°C. Then retinas were washed three times in Pblec buffer (0.5% Triton X-100, 1 mM CaCl₂, 1 mM MgCl₂, and 1 mM MnCl₂ in PBS, pH 6.8) and incubated overnight in Pblec containing FITC labeled Isolectin B4 (1:100; Sigma-Aldrich). Subsequently, the retina were flat-mounted in mounting medium (Dako). All quantifications were done with high-resolution images taken using a laser scanning confocal microscope (LSM-510; Carl Zeiss).

Aortic ring assay, tube formation and spheroid sprouting assay

These assays were performed as described in ⁷.

Cell Migration

Scratch wound-healing assays were performed in 24-well plates. Cells were cultured in endothelial basal medium (EBM) containing FCS (1%). Endothelial cell migration was monitored by live cell imaging (Zeiss TIRF System LASOS77). The distance migrated was calculated using ImageJ software.

Endothelial cell migration in Boyden chamber assays were investigated in a modified transwell chamber system. 2x10⁵ cells were seeded on membrane inserts (Fluoroblok, 3 μm pore size, BD Bioscience, Heidelberg, Germany) in the presence of EBM. The lower chamber contained EBM supplemented with 2% FCS. After 20 hours, the cells on the upper surface of the filter were removed mechanically. Then cells that had migrated into the lower compartment were fixed (4% paraformaldehyde in PBS), stained with DAPI and counted (8 images per well, x200 magnification).

Cell Culture

Human umbilical vein endothelial cells (HUVECs) were purchased from Lonza (#CC-2519, Lot No.186864; 191772; 192485; 76524; 76921, 7F3111, Walkersville, MD, USA) and PELOBiotech (#PB-CH-190-8013, Lot No. QC-18P13F11, Planegg, Germany). Cells were cultured on fibronectin-coated (#356009, Corning Incorporated, Tewksbury, MA, USA).
USA) dishes in endothelial growth medium (EGM), consisting of endothelial basal medium (EBM) supplemented with human recombinant epidermal growth factor (EGF), EndoCGS-Heparin, (PELOBiotech, Planegg, Germany), 8% fetal calf serum (FCS) (#S0113, Biochrom, Berlin, Germany), penicillin (50 U/ml) and streptomycin (50 µg/ml) (#15140-122, Gibco (lifeTechnologies, Carlsbad, CA, USA) in a humidified atmosphere of 5% CO₂ at 37 °C. For each experiment at least three different batches of HUVEC from passage 3 were used.

Human embryonic kidney (HEK) 293T/17 cells (#CRL-11268) were purchased from ATCC (Manassas, VA, USA). Cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM), high glucose, GluMax from Gibco, lifeTechnologies (Carlsbad, CA, USA), supplemented with 8% fetal calf serum (FCS), penicillin (50 U/ml), and streptomycin (50 µg/ml) in a humidified atmosphere of 5% CO₂ at 37 °C.

Human aortic smooth muscle cells (HAoSMC)(#354-05a) were purchased from PELOBiotech (Planegg, Germany). Cells were cultured in Smooth Muscle Cell Medium (#PB-MH-200-2190) supplemented with 8% fetal calf serum (FCS), penicillin (50 U/ml), and streptomycin (50 µg/ml) in a humidified atmosphere of 5% CO₂ at 37 °C.

Human foreskin fibroblasts were cultured in DMEM/F12 (#11039-021) from Gibco (lifeTechnologies, Carlsbad, CA, USA) supplemented with 8% fetal calf serum (FCS), penicillin (50 U/ml), and streptomycin (50 µg/ml) in a humidified atmosphere of 5% CO₂ at 37 °C.

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Human angiogenic myeloid cells (AMC) were isolated according to 8 and murine lung endothelial cells were isolated and cultured as described in 8.

**shRNA, siRNA and plasmid transfection**

For shRNA treatment, endothelial cells were infected with lentiviral particles according to Addgene “pKIO.1 Protocol” (http://www.addgene.org/tools/protocols/pkio/). Cells were selected with puromycin (0.5 µg/ml). The shJARID1B-1 target sequence was: 5'-CCTGAGGAAGAGGAGTTCTT-3', for shJARID1B-1 5'- CGAGATGGAAATACAGTCTT-3'. Control shRNA against green fluorescent protein (shGFPl) and shScrambled (shScr) were purchased from Addgene. For siRNA treatment, endothelial cells (80–90% confluent) were transfected with GeneTrans II according to the instructions provided by MoBiTec (Göttingen, Germany). All siRNAs (Stealth RNAi) were from Invitrogen (#HSS173871 JARID1B, HSS179327 HOXA5). siCtrl was used as a negative control: scrambled Stealth RNAi™ Med GC (#12935-300) from lifeTechnologies (Carlsbad, CA, USA). Plasmid overexpression was achieved with the Neon electroporation system (Invitrogen). The plasmids JARID1B-WT (vector pCMV-HA) and JARID1B-Mut (H499A, E501A, vector pCMV-HA) were provided by one of the co-authors (K.R.), pCMV6-HOXA5 and pCMV6-DDK were purchased from Origene (#RC502156, # PS100001).

**Quantitative RT-PCR**

Total RNA was extracted with the RNA Mini Kit (Bio&Sell). cDNA was prepared with SuperScript III reverse transcriptase (Invitrogen) and random hexamer together with oligo(dT) primers (Sigma #O4387). Quantitative real-time PCR was performed with Eva Green Master Mix and ROX as reference dye (Bio&Sell #76.580.5000) in a Mx3005 cycler (Stratagene). Relative expression of target genes were normalized to (RNA)II (DNA-directed) polypeptide A (POLR2A) or β-Actin and analyzed by the delta-delta Ct method with the MxPro software (Agilent Technologies, Santa Clara, CA, USA).

**Protein isolation and western blot analysis**

Cells were lysed with Triton X-100 lysis buffer (20 mM TRIS/Cl pH 7.5, 150 mM NaCl, 10 mM NaPP, 20 mM NaF, 1% Triton, 2 mM orthovanadate (OV), 10 mM okadaic Acid, protein-inhibitor mix (PIM), 40 µg/ml phenylmethylsulfonylfluorid). Cells were centrifuged for 10 min at 16,000 g. Supernatant and pellet were used and after determination of protein concentration by the Bradford assay, equal amounts of proteins were boiled in Laemmli buffer and separated by SDS-PAGE gel electrophoresis. Infrared-fluorescent-dye-conjugated secondary antibodies were purchased from Lincor (Bad Homburg, Germany) and detected with an infrared-based laser scanning detection system (Odyssey Classic, Lincor, Bad Homburg, Germany).

**Chromatin immuno-precipitation (ChIP)**

Cell preparation, crosslinking and nuclei isolation were performed with the truCHIP™ Chromatin Shearing Kit (Covaris, Woburn, USA) according to the manufacturers protocol. Afterwards, the lysates were sonicated with the Bioruptur Plus (9 cycles, 30 seconds on, 90 seconds off; Diagenode, Seraing, Belgium) for 3 hours at 4°C, subsequently washed twice for 5 minutes with each of the wash buffers 1-3.
Supplemental Material

(Wash Buffer 1: 20 mmol/L Tris/HCl pH 7.4, 150 mmol/L NaCl, 0.1% SDS, 2 mmol/L EDTA, 1% Triton X-100; Wash Buffer 2: 20 mmol/L Tris/HCl pH 7.4, 500 mmol/L NaCl, 2 mmol/L EDTA, 1% Triton X-100; Wash Buffer 3: 10 mmol/L Tris/HCl pH 7.4, 250 mmol/L lithium chloride, 1% Nonidet, 1% sodium deoxycholate, 1 mmol/L EDTA) and finally washed with TE-buffer pH 8.0. Elution of the beads was done with elution buffer (0.1 M NaHCO3, 1% SDS) containing 1x proteinase K (Diagenode, Seraing, Belgium) and shaking at 600 rpm for 1 hour at 55°C, 1 hour at 62°C and 10 minutes at 95°C. After removal of the beads, the eluate was purified with the QiaQuick PCR purification kit (Qiagen, Hilden, Germany) and subjected to qPCR analysis.

Microarray
HUCECs were transfected with control or JARID1B siRNA. RNA was isolated 48 hours after transfection with the miRNeasy Mini Kit (QIAGEN). GeneChip Human Exon 1.0 ST array (Affymetrix) was used and data were analyzed by using the noncoder web interface10 and the RMA normalization method GO terms were determined by using GO-Elite pathway analysis (version 1.2).

HOXA5 ChIP-Seq
HOXA5 ChIP-Seq data was obtained from Gene Expression Omnibus: http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSM1239461

Statistics
Unless otherwise indicated, data are given as means ± standard error of mean (SEM). Calculations were performed with Prims 5.0 or BiAS.12.12. The latter was also used to test for normal distribution and similarity of variance. In case of multiple testing, Bonferroni correction was applied. For multiple group comparisons ANOVA followed by post hoc testing was performed. Individual statistics of unpaired samples was performed by T-test and if not normally distributed by Mann-Whitney test. P values of <0.05 was considered as significant. Unless otherwise indicated, N indicates the number of individual experiments.

Reference List
### Primer

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### Primers for ChIP-PCR

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Supplemental Table I

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Supplemental Figure II: Impact of JARID1B down-regulation on gene expression

Heatmap (A) of microarrays (Affymetrix GeneChip Human Exon 1.0 ST) of 3 different HUVEC batches treated with JARID1B siRNA or control siRNA (siCtl). Only genes are indicated, whose expression were at least 20% changed by knockdown of JARID1B and with a p-value <0.05. Gene ontology analysis (Tab. S1) calculated by GO-Elite pathway analysis.

Supplemental Figure I: Effect of shRNA against JARID1B on protein abundance. HUVECs were transduced with lentivirus coding for two different JARID1B shRNAs (shJ1B-1 & shJ1B-2) or two different controls (shCtl-1 (shScr) and shCtl-2 (shGFP)). Cells were kept in selection medium (0.5 µg/ml puromycin) and JARID1B and β-Actin protein expression were determined by Western blot 5 days later. (p<0.05, n>3)
Supplemental Figure III: Venn diagram for genes recovered by HOXA5 Chip-Seq and genes induced by Jarid1B shRNA determined by microarray in HUVEC. HOXA5 ChIP data were obtained from Gene Expression Omnibus.

Supplemental Table II: Matched genes between HOXA5 ChIP-seq and JARID1B microarray.

Gene Symbol: AP1S3, ARSJ, AXL, BTF3L4, CALML4, CCND1, CCNE2, CDC42SE2, CISD1, CLIC4, CSNK1G1, DDX21, DERL1, DLG1, EXT1, GLS, GNG2, GOLIM4, HDCC2, HMGA2, IARS, INSIG1, LGR4, MAML2, MAN1A2, MICAL2, NCEH1, NTM, PHF19, PHF2, PICALM, PIGW, PLXNA2, Pomgnt1, PTP4A2, RAB11FIP2, RBMS3, RPRD1A, SH3YL1, SLC12A2, SMC5, SMCHD1, TMEM123, TMEM62, TRPC1, TSPAN8, UAP1, UGCG, ZNF254, ZNF92

Supplemental Figure IV: Gene validation of matched genes between HOXA5 ChIP-seq and JARID1B microarray. HUVECs were transfected with plasmids coding for HOXA5 (pCMV6-HOXA5) or a control plasmid (pCMV6-DDK) or transduced with shRNA against JARID1B (shJ1b) or control shRNA (shCtl). AP1S3 (adaptor-related protein complex 1, sigma 3 subunit), BTF3L4 (basic transcription factor 3-like 4), CCNE2 (cyclin E2), LGR4 (leucine-rich repeat containing G protein-coupled receptor 4). n=4. *p<0.05.