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Endothelial cells of different organs exhibit heterogeneity in von Willebrand factor expression in response to hypoxia

Anahita Mojiri[a,1], Parnian Alavi[a], Maria Areli Lorenzana Carrillo[a], Maryam Nakhaei-Nejad[a], Consolato M. Sergi[b], Bernard Thebaud[c], William C. Aird[d], Nadia Jahroudi[a,*]

[a] Department of Medicine, University of Alberta, Edmonton, Canada
[b] Department of Laboratory Medicine and Pathology, University of Alberta, Edmonton, Alberta, Canada
[c] Ottawa Hospital Research Institute & CHEO Research Institute, Pediatrics, Ottawa, Ontario, Canada
[d] Center for Vascular Biology Research and Division of Molecular and Vascular Medicine, Beth Israel Deaconess Medical Center, Harvard Medical School, Boston, MA, USA

HIGHLIGHTS

- von Willebrand factor (VWF) upregulation in response to hypoxia exhibits organ specificity.
- Hypoxia-induced upregulation of VWF correlates with platelets aggregate formation.
- Distinct molecular mechanisms regulate hypoxia-induced expression of VWF in heart compared to lung endothelial cells.

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ABSTRACT

Background and aims: We have previously demonstrated that in response to hypoxia, von Willebrand factor (VWF) expression is upregulated in lung and heart endothelial cells both *in vitro* and *in vivo*, but not in kidney endothelial cells. The aim of our current study was to determine whether endothelial cells of different organs employ distinct molecular mechanisms to mediate VWF response to hypoxia.
Methods: We used cultured human primary lung, heart and kidney endothelial cells to determine the activation of endogenous VWF as well as exogenously expressed VWF promoter in response to hypoxia. Chromatin immunoprecipitation and siRNA knockdown analyses were used to determine the roles of VWF promoter associated transacting factors in mediating its hypoxia response. Platelet aggregates formations in vascular beds of mice were used as a marker for potential functional consequences of hypoxia-induced VWF upregulation *in vivo*.
Results: Our analyses demonstrated that while Yin Yang 1 (YY1) and specificity protein 1 (Sp1) participate in the hypoxia-induced upregulation of VWF specifically in lung endothelial cells, GATA6 mediates this process specifically in heart endothelial cells. In both cell types, the response to hypoxia involves the decreased association of the NFIB repressor with the VWF promoter, and the increased acetylation of the promoter-associated histone H4. In mice exposed to hypoxia, the upregulation of VWF expression was concomitant with the presence of thrombi in heart and lung, but not kidney vascular beds.
Conclusions: Heart and lung endothelial cells demonstrated VWF upregulation in response to hypoxia, using distinct mechanisms, while this response was lacking in kidney endothelial cells.

1. Introduction

Von Willebrand factor (VWF) is a molecule, strictly specific to endothelial cells and megakaryocytes, which mediates the adhesion of platelets to endothelial and subendothelial surfaces, thus playing a major role in the initiation of thrombus formation [1]. VWF expression is commonly used as a marker of endothelial cell phenotype, but endothelial cells of distinct vascular beds display heterogeneity in their

Abbreviations: ChIP, chromatin immunoprecipitation; HFF1, human foreskin fibroblasts cell line; HPRT, hypoxanthine phosphoribosyltransferase; HIF-1α, hypoxia inducible factor 1α; IHC, immunohistochemistry; I51HSS, intron 51 hypersensitive sequence; MVEC, microvascular endothelial cells; MOI, multiplicity of infection; NFI, nuclear factor I; OCT, optimal cutting temperature compound; Sp1, specificity protein 1; VWF, von Willebrand factor; YY1, Yin Yang 1

* Corresponding author. Department of Medicine, University of Alberta, Edmonton, Alberta, T6G 2S2, Canada.
E-mail address: nadia.jahroudi@ualberta.ca (N. Jahroudi).
[1] Present address: Department of cardiovascular Sciences, Houston Methodist Research Institute, Houston, TX, USA.

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levels and patterns of VWF expression *in vivo* [2–4]. Previously, we have demonstrated that VWF gene expression is upregulated in response to hypoxia, although the response to hypoxia is not uniform in the endothelial cells of all vasculature [5]. For example, VWF expression is primarily found in the large vessels of the lung in control, untreated mice, whereas the VWF expression pattern is altered in the hypoxic lung so that it is detected in both micro- and macro-vessels [5]. VWF upregulation in response to hypoxia was observed in the vasculature of other major organs including heart, brain and liver, but not kidney vasculature [5]. To determine the mechanism of hypoxia-induced transcriptional upregulation of the VWF gene in the lung, we took advantage of previous findings regarding VWF transcriptional regulatory elements and their function *in vitro* and *in vivo*.

The characterization of the promoter and regulatory regions of the VWF gene in transgenic mice had revealed that distinct regions and regulatory elements are required for its transcriptional activation in the endothelial cells of different organs [6–11]. The VWF proximal region (sequences −487 to +247) functioned as an endothelial-specific promoter with activity that was exclusive to brain vasculature, whereas a hypersensitive region in intron 51 of the VWF gene (referred to as I51HSS), which interacts with YY1 transacting factor, was shown to confer lung endothelial cell-specific activity to the promoter [7,8,12]. Additionally, a mutation in the VWF proximal promoter that interfered with the binding of the NFI family of transacting factors (a repressor of the VWF promoter) [13] was shown to result in proximal promoter activation in the endothelial cells of heart, lung, and brain [9]. The targeted NFI in lung and heart endothelial cells was shown to be primarily the isoform NFIB [9].

Previously, to gain insight into the mechanism of the induction of the *VWF* gene by hypoxia, we had exposed transgenic mice carrying the transgene VWF-LacZ-HSS to hypoxia. In this transgene, the *LacZ* gene was fused to the *VWF* proximal promoter (sequences −487 to +247) and the intron 51 lung-specific enhancer sequences (I51HSS) [8]. The analyses of LacZ transgene expression in mice demonstrated that these combined VWF sequences (−487 to +247 and the I51HSS) contain elements that are required to mediate the hypoxia-response of the *VWF* gene in lung endothelial cells [5]. Analysis in cultured human lung endothelial cells demonstrated that YY1 interaction with the I51HSS was required for basal *VWF* gene activation, as well as the hypoxia induction of the *VWF* promoter in these cells. We also demonstrated that the interaction of the NFIB repressor with the *VWF* promoter in lung endothelial cells was significantly reduced in response to hypoxia.

Our analyses of VWF-LacZ-HSS transgenic mice demonstrated that in the absence of hypoxia, transgene expression was confined to lung and brain endothelial cells, whereas in response to hypoxia, it was detected in the vascular endothelial cells of the heart [5]. VWF-LacZ-HSS transgene activation was not observed in endothelial cells of any other organ analyzed. This observation suggested that hypoxia induced an alteration in the transcriptional machinery of heart endothelial cells *in vivo*, leading to the activation of the transgene VWF regulatory sequences that were otherwise silent in these cells. These results also suggested that the elements mediating the hypoxia-response of the *VWF* gene in heart endothelial cells are likely to be located within the *VWF* promoter proximal sequences (−487 to +247) and/or the intron 51 lung specific enhancer sequences (I51HSS).

In this study, we report that the hypoxia-induced upregulation of the *VWF* gene in heart vascular endothelial cells involves a mechanism that shares certain elements, but is distinct from that of lung endothelial cells. We also demonstrate that the functional consequences of hypoxia-induced VWF upregulation are correlated with thrombi generation in the vascular beds of specific organs. Collectively, our results demonstrate heterogeneity in the levels and mechanisms of VWF response to hypoxia in endothelial cells of different organs.

2. Material and methods

2.1. Plasmids used for the generation of adenoviral vectors

Plasmids LacZK and LacZKHSS (previously referred to as VWF-LacZ-HSS) were generated as described previously [7–9]. HPRT-targeted transgenic mice harboring LacZKHSS were generated in C57BL/6 strain mice as described previously [11]. Adult transgenic mice were euthanized and harvested organs were frozen in OCT for cryostat sectioning. Animals were maintained and euthanized at Harvard Medical School in compliance with the animal use and care committee of Harvard Medical School. Adenoviral vectors containing transgenes LacZK and LacZKHSS, referred to as AdLacZK, and AdLacZKHSS were generated using corresponding plasmids by Amy Barr at the Cardiovascular Research Core Facility Center at the University of Alberta. Adenovirus AdCMVLacZ was a gift of Dr. Jason Dyke at University of Alberta. Adenoviral injections into mice were performed, and mice were maintained and euthanized in compliance with the animal care and use committee at the University of Alberta.

2.2. In vivo delivery of adenoviral vectors

Adenoviruses (MOI 5×10^9) were administered into mice via tail vein injections. After 48 h, mice were euthanized and all major organs were harvested, embedded in OCT and frozen for cryostat sectioning.

2.3. Organ harvest of control and hypoxia-exposed mice

Hypoxia-exposed mice were generated and analyzed as described previously [5]. Briefly, mice were euthanized 35 days after hypoxia exposure, and organs that were harvested from control and hypoxic mice were maintained frozen in OCT or paraffin-embedded [5]. The other animals that were used were mice injected with adenoviral vectors (Supplementary Fig. 1). Hypoxia exposure, adenoviral delivery, maintenance and euthanasia of mice were performed in compliance with the animal care and use committee at the University of Alberta.

2.4. Immunofluorescent and immunohistochemistry staining

Immunofluorescent (IF) staining and confocal imaging were performed as described previously [5]. OCT-embedded organs of adenovirus-injected mice (Supplementary Fig. 1) were sectioned 4 μm thick, fixed in acetone, blocked and probed for VWF (sheep FITC-preconjugated anti-VWF, Abcam-ab8822) and LacZ (goat anti-β-galactosidase, Abcam-ab12081) [5]. Paraffin-embedded tissues were sectioned, deparaffinized and blocked for 1 h at room temperature, followed by overnight incubation with antibodies for detection of either VWF and CD41 (platelet marker, Cemfret analytics, Germany) or VWF, CD41 and fibrinogen (Sigma-Aldrich). The secondary antibodies used were donkey anti-goat Alexa 594 (A-11058) and donkey anti-rabbit Alexa 488 A-21206) from Invitrogen, and AlexaFluor 647 affinipure Donkey anti-rabbit (711-605-152, Jackson Immunoresearch). Tricom Immunohistochemical (IHC) staining was performed on paraffin-embedded heart sections using standard staining techniques.

2.5. Cell cultures, hypoxia treatment, adenovirus transfections and siRNA knockdown

Human endothelial cells (EC) isolated from microvessels of the heart (cardiac MVEC) and lung (lung MVEC) were purchased from Lonza. Human glomeruli endothelial cells (glom MVEC) were a gift from Dr. Ballermann. ECs were cultured on gelatin-coated dishes in endothelial medium (EGM supplemented with microvascular growth supplements, EGM-2, Lonza), and grown in 5% CO_2 at 37 °C. Human foreskin fibroblasts cell lines (HFF1) were cultured in DMEM supplemented with 10% FBS (Invitrogen, ON). For hypoxia treatment, endothelial cells at

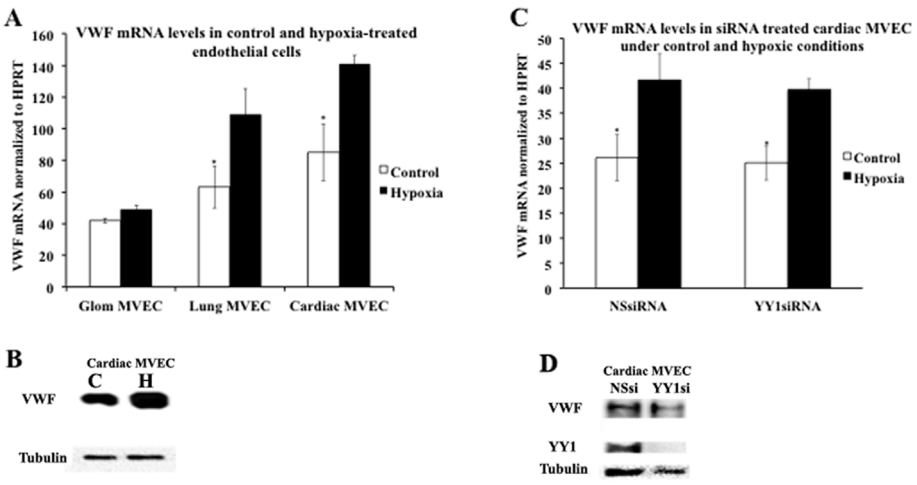

Fig. 1. Analysis of VWF expression in control and hypoxia-treated human cardiac, lung and glomeruli endothelial cells.
(A) Glomeruli (Glom), lung and cardiac microvascular endothelial cells (MVEC) were either maintained as controls (untreated) or exposed to hypoxia (1% oxygen for 1 h). Cells were then maintained in normoxic conditions for 48 h, after which RNAs were collected and subject to quantitative RT-PCR to detect endogenous *VWF* and *HPRT* mRNAs. (B) Western blot analyses to detect VWF protein levels in human cardiac MVEC that were maintained as control and those that were exposed to hypoxia as described for (A). (C) Human cardiac MVEC were transfected with non-specific siRNA (NSsiRNA) or YY1-specific siRNA (YY1siRNA) prior to hypoxia treatment and *VWF* mRNA detection was done as described for (A). The graphs represent the relative levels of target gene mRNAs on the Y-axis as values normalized to *HPRT* using ΔCT, and are the average of three to four independent experiments ($*p < 0.05$). (D) Western blot analyses of siRNA treated human cardiac MVEC demonstrating protein levels of VWF, YY1 and tubulin.

approximately 80–85% confluence were placed in a hypoxia chamber (Ruskinn Invivo2 Hypoxia-Gas Mixer Q) containing 1% oxygen for 1 h, and then maintained at normoxic (normal oxygen levels) conditions for 48 h. After 48 h, cells were harvested for RNA isolation. MVECs at 70% confluence were transduced with AdLacZK, AdLacZKHSS, or AdCMVLacZ (125 MOI), the adenoviruses were removed after 48 h and the cells were exposed to hypoxia as described for non-transduced ECs, or maintained as controls. For transient knockdown of target transcription factors, cells were transfected twice within a 48-hour period with non-silencing or specific silencing siRNAs (10 nmol/L). The sources and sequences of siRNAs are presented in Supplementary Table 1. Transfections were performed using Lipofectin 2000 (Thermofisher) according to the manufacturer's protocol. All siRNA transfections of cells were performed prior to hypoxia exposure, and cells were analyzed for RNA and protein expression 48 h post-hypoxia treatment, as described previously [5].

2.6. RNA analyses

RNA was prepared from cultured cells (hypoxia-treated and controls) and subject to quantitative reverse transcription-polymerase chain reaction (RT-PCR) to detect mouse endogenous VWF and HPRT, as well as transgene LacZ mRNA expression as described previously [5,9]. The primers that were used are presented in Supplementary Table 1.

2.7. Chromatin immunoprecipitation assay

Chromatin immunoprecipitation (ChIP) assays were performed as previously described [5] using antibodies to specifically determine the interactions of NFIB, YY1, GATA6, SP1, HIF-1α, acetylated histones H3 and H4 (all purchased from Abcam Cambridge, MA, USA) with the *VWF* promoter. Anti-IgG antibody was used as negative control and the binding capacity of each transcription factor was normalized to IgG. All antibodies were purchased from Abcam Inc. (Abcam, Cambridge, MA, USA). Briefly, following the reversal of cross-linking of immunoprecipitated chromatins, purified DNA were subjected to quantitative real-time PCR using primers (Supplementary Table 1) that specifically amplified VWF proximal promoter sequences or intron 51 (I51HSS region), as described previously [5].

2.8. DNA methylation analysis

The two potential methylation sites (cytosines in a CpG dinucleotide) located in the *VWF* promoter (−422 and + 119) were analyzed to determine their methylation status using OneStep qMethyl kit (Zymo Research, Irvine, CA), according to the manufacturer's protocol. Briefly, DNA extracted from cells (1 μg) was either digested or not treated with the methylation sensitive restriction enzyme Hpall. Digested and undigested products were used as templates for quantitative real-time PCR. Two sets of primers were used to amplify DNA sequences centered on the target CpG dinucleotide sequences. If the target sites in the promoter DNA sequences are methylated, they were protected from digestion and amplified, otherwise no or little amplification products were detected.

2.9. Protein analysis

Cell lysates (30 μg protein) were prepared for Western blot analyses as described previously [5]. The antibodies used were human-specific anti-VWF antibody (Dako Omnis, Denmark), or tubulin, GATA6, SP1 and HIF1α antibodies (all purchased from Abcam, Cambridge, MA, USA).

2.10. Statistics

All experiments were performed a minimum of three times and the data represent the mean ± standard error of the mean. Student *t* tests were used to analysis results and $p < 0.05$ was considered statistically significant.

3. Results

3.1. Endothelial cells of the lung, heart and kidney exhibit heterogeneity in VWF expression in response to hypoxia

To determine whether cultured endothelial cells of various organs maintain the VWF expression of their *in vivo* counterparts, we explored the VWF response to hypoxia in cultured human cardiac, kidney glomerular, and lung microvascular endothelial cells (MVEC). These endothelial cells were exposed to hypoxia (1%) for 1 h followed by 48 h of normoxia, as described previously [5]. In response to hypoxia, a significant upregulation of VWF mRNA was observed in cultured human

lung and cardiac MVEC, but not kidney glomerular MVEC (Fig. 1A). These results were consistent with previous *in vivo* analyses that had demonstrated the hypoxia-induced upregulation of VWF in lungs and hearts, but not kidneys, of mice. We had previously reported that in lung MVEC, *VWF* mRNA upregulation was accompanied with increased levels of VWF protein; and that YY1 mediated this process [5]. We had also demonstrated that YY1 knock down significantly decreased the basal level of VWF in lung MVEC [5]. Thus, we proceeded to determine whether in cardiac MVEC increased VWF protein accompanies increased *VWF* mRNA and whether YY1 was also necessary for hypoxia-induced VWF upregulation. Western blot analyses demonstrated that VWF protein levels were upregulated in hypoxia-exposed cardiac MVEC compared to control (Fig. 1B). Next, YY1 knockdown assay was performed using YY1 specific siRNA (YY1siRNA) as described previously for lung MVEC [5]. The results demonstrated that YY1 knockdown did not alter the hypoxia response of the VWF gene in cardiac endothelial cells (Fig. 1C). Moreover, in contrast to lung endothelial cells, YY1 knockdown did not significantly alter basal levels of VWF expression in cardiac endothelial cells (Fig. 1C–D). Collectively, these results demonstrated that: (i) when cultured *in vitro*, endothelial cells of various organs maintain their organ-specific VWF response to hypoxia, and (ii) unlike lung MVEC, cardiac MVEC do not require YY1 to regulate basal expression or hypoxic induction of the *VWF* gene.

3.2. Characterization of the hypoxia-responsive regulatory regions of the VWF gene in cardiac endothelial cells

Since the YY1 transcription factor associates with lung-specific enhancer sequences in the intron 51 (I51HSS) region of the *VWF* gene, we hypothesized that I51HSS sequences may be necessary for VWF activation in response to hypoxia in lung, but not heart, endothelial cells. To test this hypothesis, we proceeded to determine the hypoxia response of the proximal *VWF* promoter sequences in the absence and presence of I51HSS sequences in cultured cardiac and lung endothelial cells. To obtain efficient transgene delivery in cultured primary endothelial cells, we proceeded to generate adenoviral vectors containing either the *VWF* proximal promoter sequences - 487 to + 247 (AdLacZK), or the proximal promoter plus I51HSS sequences (AdLacZKHSS), fused to the *LacZ* gene (Fig. 2A). The VWF sequences used were similar to those described previously for the generation of LacZK and VWF-LacZ-HSS transgenic mice respectively [7,8].

Since in transgenic mice these sequences were shown to exhibit strict organ-specific regulatory characteristics, we first explored and compared their basal expression patterns in cultured lung, heart and kidney MVEC *in vitro*, and in organs of transduced mice *in vivo*. Analyses of the basal expression patterns in cultured endothelial cells did not demonstrate a pattern of expression that was reflective of the highly strict, organ-specific activities of the *VWF* promoters (Fig. 2, comparing controls in various cell lines). For instance, while *in vivo* proximal promoter sequences alone (sequences −487 to +247) are not active in lung or heart endothelial cells, these sequences exhibited significant activity in cultured human lung and heart endothelial cells. However, after the *in vivo* delivery of these adenoviral vectors into mice, they demonstrated a pattern of expression that closely mimicked the expression pattern of the corresponding transgenes in transgenic mice. LacZ expression from AdLacZK was restricted to the brain, whereas with AdLacZKHSS, LacZ was detected in brain and lung endothelial cells (Supplementary Fig. 1). These results demonstrated that the *VWF* promoter sequences in adenoviral vectors do not exhibit organ-specific activity when transduced into cells *in vitro* (which is consistent with the results of previous analyses of the plasmids containing various VWF promoter sequences that were transfected in cultured cells), but maintain their organ-restricted and endothelial cell-specific activity when transduced *in vivo*.

Next, we proceeded to determine the hypoxia responses of the *VWF* regulatory sequences in cultured endothelial cells that were transduced with AdLacZK and AdLacZKHSS. Transduced cultured cells (cardiac, lung, and kidney glomerular MVEC) were maintained as controls or exposed to hypoxia as described above for non-transduced cells. The RNAs prepared from control and hypoxia-exposed cells were subjected to RT-PCR analyses to detect the *LacZ* transgene mRNA levels. The results demonstrated that cardiac MVEC, which were transduced with AdLacZK exhibited a significantly increased level of *LacZ* mRNA in response to hypoxia (Fig. 2B), whereas human lung MVEC that were

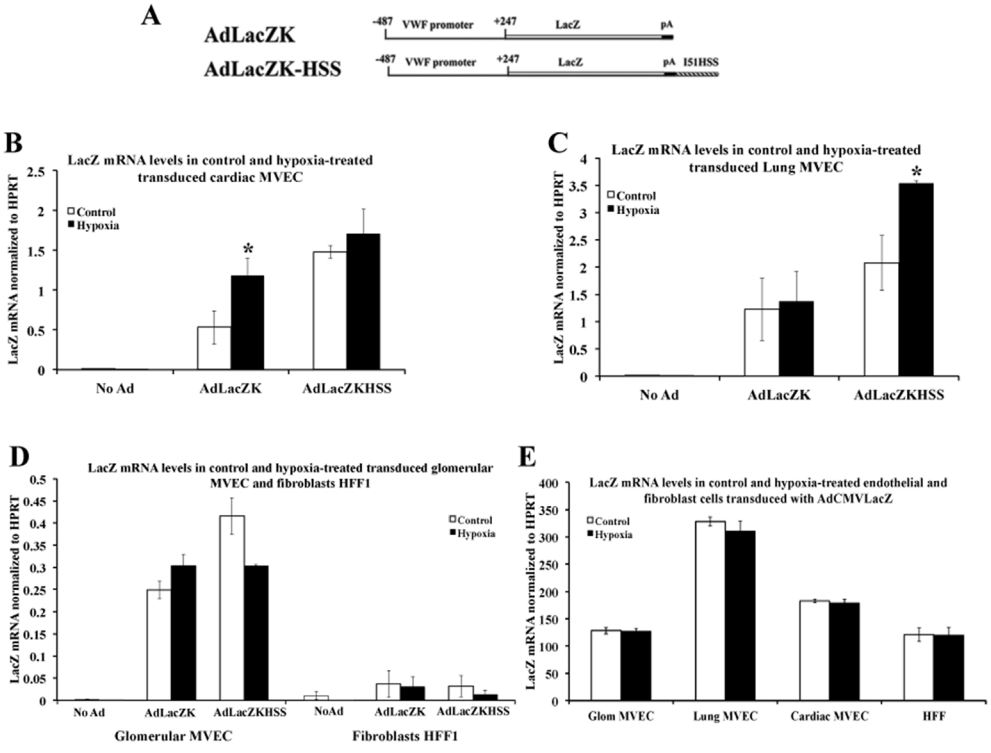

Fig. 2. Analyses of LacZ expression in various transfected endothelial cells and fibroblast in control and hypoxia-treated cells.
(A) Schematic representations of transgenes in which the *VWF* promoter and regulatory sequences as well as *LacZ* are incorporated into adenoviruses to generate adenoviral vectors AdLacZK and AdLacZKHSS. Human cardiac, lung and glomeruli MVEC and human fibroblasts (HFF1) were transduced with either AdLacZK, AdLacZKHSS, or AdCMVLacZ (an adenovirus vector containing LacZ under the regulation of the CMV promoter) as described in Materials and methods, and 48 h after transduction they were maintained as controls or exposed to hypoxia as described for Fig. 1. (B–E) Graphs represent the levels of *LacZ* mRNA detected by RT-PCR, normalized to *HPRT*. Results represent the averages of three independent experiments for each cell type (*$p < 0.05$).

transduced with AdLacZKHSS, but not AdLacZK, exhibited a similar response (Fig. 2C). These results demonstrate that although I51HSS enhancer sequences are required for the hypoxia response of the *VWF* promoter in the lung, they are not required for this response in cardiac endothelial cells in culture. Moreover, the results demonstrated that the elements necessary to mediate the hypoxic induction of the VWF promoter in cardiac endothelial cells are located within the −487 to +247 sequence. In kidney glomeruli endothelial cells neither AdLacZK nor AdLacZKHSS were responsive to hypoxia (Fig. 2D), which was consistent with the lack of a hypoxic response of the endogenous *VWF* gene in cultured human glomerular endothelial cells (Fig. 1A), and in mouse kidney *in vivo* as previously reported [5].

For controls, a human non-endothelial cell type, namely HFF1, was transduced with these adenoviruses and then exposed to hypoxia; also, all of the cell types studied were transduced with AdCMVLacZ (an adenovirus containing the *LacZ* gene under the regulation of the ubiquitous cytomegalovirus promoter) and then examined for response to hypoxia. The results demonstrated that there was no significant expression of LacZ in HFF1 transduced with AdLacZK or AdLacZKHSS, either before or after hypoxia treatment (Fig. 2D). The residual LacZ levels detected in HFF1 were not significantly higher than controls (no adenovirus) and may represent background expression from the adenovirus vectors independent of the promoter. Also, the level of LacZ expression did not change in various endothelial cells transduced with AdCMVLacZ after hypoxia (Fig. 2E), demonstrating that the observed hypoxic response was due to the VWF regulatory sequences.

3.3. Characterization of the transacting factors that participate in hypoxia-induced VWF promoter upregulation in heart and lung MVEC

Based on the results of deletion analyses, we hypothesized that factors interacting with sequences within −487 to +247 region mediate the hypoxic induction of the VWF gene in cardiac MVEC. To test this hypothesis, we first analyzed and compared the binding of a number of transacting factors to the *VWF* promoter in control and hypoxia-exposed cardiac and lung MVEC. These factors had been previously shown to participate in *VWF* promoter regulation or have a potential binding site on the *VWF* promoter. HIF-1α, a well-known mediator of hypoxic response [14], was included in this analysis although its binding site was not detected on the *VWF* proximal sequences. The VWF regulatory factors compared included NFI family members (functioning as repressors of the VWF promoter) [9,13], GATA family members 2, 3 and 6 (functioning as activators of the VWF promoter) [15–17], SP1 (which has a potential binding site on the VWF promoter) [15] and YY1 [5,8].

Chromatin immunoprecipitation analyses were performed using specific antibodies against the target transacting factors, and primers for PCR that amplified *VWF* proximal regulatory sequences, or I51HSS sequences specific for YY1. For the NFI and GATA families, we chose to target NFIB and GATA6, since we have previously demonstrated that NFIB preferentially interacts with the *VWF* promoter in cultured human lung microvascular endothelial cells, and is present preferentially in the heart and lung endothelial cells of mice [9]; also, GATA6 was shown to preferentially interact with the *VWF* chromatin in human umbilical vein endothelial cells (HUVEC) [16,17]. The results demonstrated that in response to hypoxia, the association of the repressor NFIB with the *VWF* promoter proximal sequences was significantly reduced in both cardiac and lung MVEC (Fig. 3A–C). However, a distinctly different pattern of association of transacting factors that function as activators (or may potentially interact with the *VWF* promoter) was observed between the two cell types in response to hypoxia. Hypoxia resulted in increased association of YY1 (consistent with previously reported results) and SP1 specifically in lung endothelial cells, whereas an increased association of GATA6 and HIF-1α were observed specifically in heart endothelial cells (Fig. 3B and C). Notably, there were no detectable levels of SP1 association with the *VWF* promoter in control lung or heart endothelial cells (values normalized to IgG, and those at less than 1 indicate bindings similar to IgG, and thus non-specific), suggesting that SP1 does not participate in basal regulation of VWF in endothelial cells. This is consistent with our previous report that mutation of the SP1 binding site did not alter the activation pattern of the *VWF* promoter transgene in transiently transfected endothelial cells [15]. Also, in control lung and heart endothelial cells, there were no significant associations of GATA6 with the *VWF* promoter, although we have previously demonstrated the association of GATA6 with the *VWF* promoter in human umbilical vein endothelial cells (HUVEC). Since GATA2 and 3 were also shown to interact with the GATA site, this observation may indicate that GATA family members other than GATA6 are predominantly interacting with the *VWF* promoter in lung and heart endothelial cells under normoxic conditions. The lack of significant HIF-1α association with the *VWF* promoter is consistent with the expectation that under normoxic conditions, this transacting factor is not recruited to the nucleus.

Our previous analyses of the VWF regulation in hypoxia-induced lung endothelial cells had demonstrated that there were no significant alterations in the total protein levels of either YY1 or NFIB in lung endothelial cells in response to hypoxia, although a modest but significant increase in the mRNA levels of both factors were detected. The role of YY1 was demonstrated to be through its increased translocation into the nucleus. RT-PCR analyses of the control and hypoxia-treated cardiac endothelial cells demonstrated no significant alterations in the levels of *GATA6, NFIB* or *YY1* mRNAs in response to hypoxia (Supplementary Fig. 2). Thus, to directly determine whether target factors participate in hypoxia induction of the *VWF* gene, we used specific siRNA to knockdown target transacting factors that demonstrated hypoxia-induced altered binding in lung or cardiac endothelial cells, and then determined the hypoxia response of *VWF* gene in the cells. Western blot analyses were used to determine protein levels of VWF and targeted transacting factors. In both cardiac and lung MVEC, NFIB knockdown (using NFIBsiRNA) abolished the hypoxia-induced upregulation of VWF (Fig. 3D and E). These results are consistent with the hypothesis that hypoxia somehow “lifts” the repression imposed by NFIB due to decreasing its association with the *VWF* promoter. Thus, if the NFIB repression is alleviated as a result of its knockdown, hypoxia exposure is expected to have no further effects. We also determined the effects of GATA6 and HIF-1α knockdown in cardiac MVEC, as well as the knockdown of SP1 in lung MVEC. We had previously reported in detail that YY1 knockdown inhibited VWF induction by hypoxia in lung MVEC [5] and in Fig. 1C and D here, we demonstrated that YY1 knockdown does not alter this response in cardiac endothelial cells. The results of GATA6, HIF-1α and SP1 knockdown demonstrated that the knockdown of either GATA6 or HIF-1α lead to the inhibition of hypoxia-induced VWF upregulation in cardiac MVEC (Fig. 3D), while knockdown of SP1 inhibited the hypoxic induction of VWF in lung MVEC (Fig. 3E). Collectively, these results demonstrated that alterations in the associations of NFIB, GATA6, HIF-1α and SP1, as well as YY1, with the *VWF* promoter in cardiac and lung endothelial cells are functionally relevant in regulating the hypoxia response of the VWF gene in these two cell types. Although the decreased association of repressor NFIB is common to both cell types, cardiac and lung endothelial cells exhibit distinct patterns of activator associations. Cardiac MVEC exhibit increased GATA6 and HIF-1α associations, while lung MVEC exhibit increased SP1 and YY1 associations with the *VWF* gene regulatory sequences.

To determine whether knock down of specific transacting factors alters the hypoxia-induction of other potential target genes in endothelial cells, we explored the expression of genes that were reported to be hypoxia-inducible in endothelial cells as well as being targets of the transacting factors that participated in hypoxia-induction of VWF. *TGFβ2* gene is hypoxia-responsive, while it is also reported to be a target of negative regulation by GATA6 in endothelial cells [18,19]. SP1 is reported to positively regulate the expression of eNOS, which is a

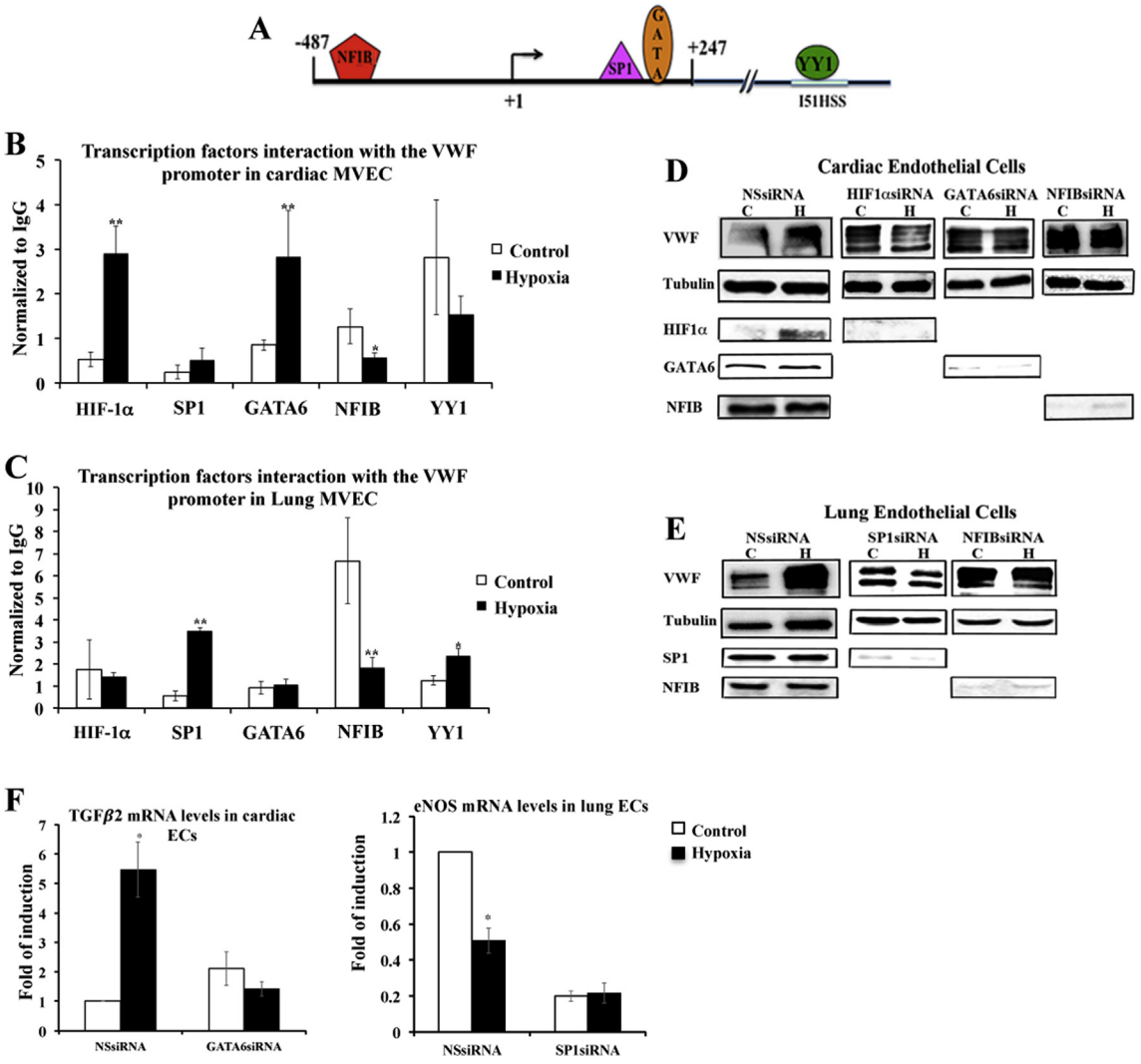

Fig. 3. Determination of the chromatin binding and participation of specific transacting factors in the hypoxia response of VWF in lung and heart MVEC.
(A) Schematic representation of *VWF* regulatory sequences and corresponding transacting factors. (B–C) Chromatin immunoprecipitations (ChIP) were performed to determine the associations of HIF-1α, SP1, GATA6, NFIB and YY1 with the *VWF* regulatory sequences in control and hypoxia-treated (B) cardiac and (C) lung MVEC. Graphs represent the averages of 3–5 independent experiments for each factor (*$p < 0.05$). (D–E) Human cardiac (D) and lung (E) MVEC were transfected with either non-specific siRNA (NSsiRNA) or siRNAs that specifically targeted NFIB (NFIBsiRNA), GATA6 (GATA6siRNA) and HIF-1α (HIF-1αsiRNA) or SP1 (SPsiRNA) prior to hypoxia exposure. Western blot analyses were performed on control and hypoxia-treated cells to determine the levels of VWF protein and the protein levels of the specific siRNA-targeted transacting factors. The results are representative of 3 independent experiments for each siRNA transfection. (F) RNA prepared from GATA6siRNA treated cardiac MVEC and SP1siRNA treated lung MVEC, as well as corresponding NSsiRNA treated cells maintained as control and exposed to hypoxia were used to detect TGFβ2 in cardiac MVEC, or eNOS in lung MVEC as well as *HPRT* mRNA in all cells. Graphs represent fold induction of target transacting factors mRNA levels (normalized to *HPRT*) in hypoxia compared to control. Results represent the averages of three independent experiments for each cell type (*$p < 0.05$).

hypoxia-responsive and an endothelial specific molecule [20,21]. However, while hypoxia upregulates *TGFβ2* mRNA expression it was reported to down regulate *eNOS* mRNA levels in endothelial cells [18,20]. Thus, we explored the expression of TGFβ2 in control and hypoxia exposed cardiac MVEC that were treated with either NSsiRNA or GATA6siRNA; and we determined the expression of eNOS in control and hypoxia-exposed lung MVEC that were treated with NSsiRNA or SP1siRNA. The results demonstrated that in cardiac MVEC that are treated with NSsiRNA, hypoxia treatment significantly upregulated *TGFβ2* mRNA levels while GATA6siRNA treatment abolished this hypoxia response. Consistent with the negative role of GATA6 on the basal levels of TGFβ2 expression, GATA6siRNA treatment lead to increased levels of TGFβ2 in control cells in the absence of hypoxia. In lung MVEC, knock down of SP1 abolished hypoxia-induced down regulation of eNOS, and reduced basal level of eNOS (consistent with the positive role of SP1 on regulation of basal level of eNOS expression) (Fig. 3F).

3.4. Epigenetic modification of the VWF promoter in response to hypoxia

We have previously reported that endothelial specific regulation of VWF transcription, as well as its induction in response to irradiation, are correlated with the increased acetylation of histones, specifically that of histone H4 [17,22]. To determine whether hypoxic induction also alters the acetylation pattern of VWF-associated histones, we performed ChIP analyses using antibodies specific to acetylated histones H3 and H4. There was a significant increase in the association of acetylated histone H4 with the *VWF* promoter in both lung and cardiac MVEC in response to hypoxia (Fig. 4A and B). These results demonstrated that the hypoxia-induced upregulation of VWF, regardless of the transcriptional machinery that is invoked in different endothelial cells, leads to increased acetylation of promoter-associated histone H4 as a pathway towards increased transcription.

Epigenetic modification can also occur through DNA methylation, which functions as a regulatory mechanism for modulating gene

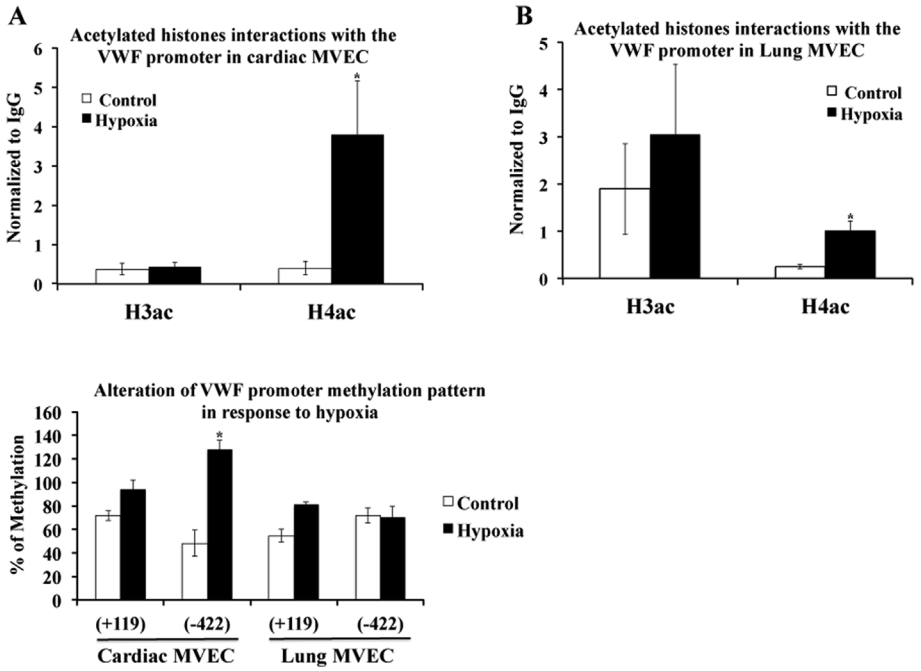

Fig. 4. Histone acetylation and DNA methylation analyses of the *VWF* promoter in control and hypoxia-treated cardiac and lung MVEC.
(A and B) Control and hypoxia-treated lung and cardiac MVEC were subject to ChIP analyses to determine the association of acetylated histones H3 and H4 with the *VWF* promoter. (C) Isolated DNA from cardiac and lung MVEC was digested with methylation-sensitive restriction enzymes. Then, the digested DNA was subject to quantitative-PCR analyses using *VWF* promoter-specific primers to determine the relative methylation status of the CpG dinucleotides at positions −422 and + 119, as described in Materials and methods. The results for ChIP and methylation analyses are presented as averages of 4 independent experiments for each cell type (*$p < 0.05$).

expression [23]. Recent data has revealed that DNA methylation of the *VWF* gene is another factor that participates in regulating its cell type specificity, as well as mosaic pattern of expression [24,25]. The *VWF* promoter contains 8 CpG sites that are targets for methylation and these sites are hypermethylated in non-endothelial cells. In endothelial cells, however, there are differential patterns of methylation that correspond to low *vs.* high levels of VWF [24,25]. Thus, we proceeded to determine whether hypoxia-induced VWF upregulation in lung and cardiac MVEC is associated with alterations in the methylation patterns of the CpG dinucleotides in the *VWF* promoter. We determined the methylation status of two specific CpG elements located at −422 and + 119 on the *VWF* promoter that were recently reported to be non-methylated specifically where VWF is expressed [24]. For these analyses, we used methylation sensitive restriction enzymes and PCR analyses as described in Materials and methods. Our results demonstrated that in cardiac MVEC, site −422 specifically exhibits significantly increased methylation in response to hypoxia, whereas there were no significant changes in the methylation pattern of either of the two sites in human lung MVEC in response to hypoxia (Fig. 4C). Since the dinucleotide CpG in site −422 is in proximity to the NFIB binding site (−442 to −471), we hypothesize that the methylation of this dinucleotide may interfere with NFIB repressor association with the *VWF* promoter, thereby contributing to increased transcription.

3.5. Detection of occluded blood vessel lumens in the heart and lung, but not kidney, vasculature of mice exposed to hypoxia

Increased levels of high molecular weight VWF have been associated with increased thrombosis [26,27]. Consequently, we explored and compared the vasculatures of hearts, lungs and kidneys of hypoxic and control mice for platelet aggregate formation using antibody to the platelet-specific marker CD41 in immunofluorescent (IF) staining analysis. The analyses were performed on organs of mice that were used in our previously reported study [5]. Significant platelet aggregates were detected specifically in the hypoxic heart and lung vessels, but not in kidney vessels (Fig. 5A). Quantification of occluded vessels (50–80% occlusion) in the heart demonstrated that hypoxia exposure results in a significantly higher number of vessels with platelet aggregates leading to partial or full lumen occlusion (Fig. 5B). Double IF staining using VWF and CD41-specific antibodies demonstrated the presence of VWF in the platelet aggregates (Fig. 5C–F). Since fibrinogen deposits have been reported to be associated with thrombus formation [28], we also explored the presence of fibrinogen in the aggregates by triple staining to detect fibrinogen, as well as platelets and VWF (Supplementary Fig. 3A). To obtain a clear histological representation of any morphological changes that may occur in the heart vasculature of hypoxic mice, we also performed immunohistochemistry (IHC) assays using Tricom staining. IHC demonstrated that in vessels of the hypoxic heart, endothelial cells were swollen and intima-media thickness was increased. Smooth muscle cell nuclear hypertrophy (nucleomegaly) and perivascular edema were also observed. The levels of microthrombosis and aggregation of erythrocytes in lumina were significant (Supplementary Fig. 3B). Together, these results suggest that hypoxia-induced VWF upregulation specifically in the heart and the lung of mice is associated with thrombus formation.

4. Discussion

Previous analyses of the mechanism of hypoxia-induced VWF upregulation in lung endothelial cells demonstrated that transcription factor YY1 participates in this process, in addition to mediating the basal level expression of VWF [5]. Here, we have demonstrated that in heart endothelial cells, GATA6 and HIF-1α, but not YY1, participated in the hypoxic induction of VWF. Since we did not identify a HIF-1α binding sequence (HRE) on the *VWF* promoter, we hypothesize that specifically in cardiac MVEC, HIF-Iα may be recruited to the *VWF* promoter through its interaction with partners that directly bind to the *VWF* promoter sequences. In lung MVEC exposed to hypoxia, we did not observe an increased association of GATA6 or HIF-1α with the *VWF* promoter. However, we did observe an increased association of SP1 with the *VWF* promoter (and YY1, as previously reported), and demonstrated that the inhibition of SP1 by siRNA also abrogated the hypoxic induction of VWF. While these results demonstrate contribution of distinct transacting factors and specific regions of the *VWF* promoter that participate in its hypoxia-induction they do not exclude the possibility that other factors may also contribute to this process. A significant region of the chromatin that contains *VWF* gene and its extended 5' sequences (chr12:6,150,727-6,343,962 approximately 100 Kb upstream of transcription start site to exon 18) has been identified as a super enhancer region, with potential binding sites for many

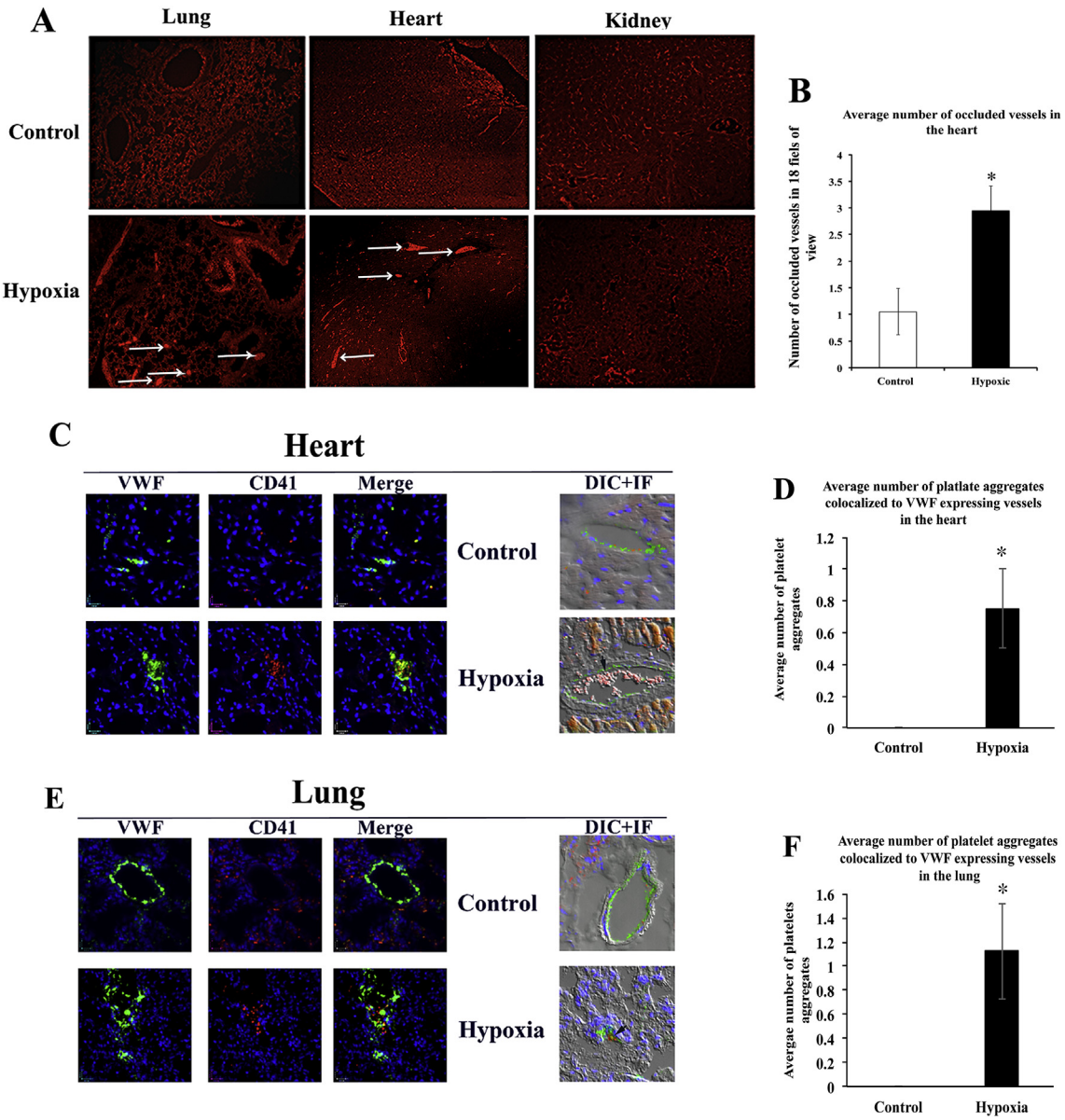

Fig. 5. Determining the presence of platelet aggregates in the organs of control and hypoxic mice.
(A) Frozen sections (5 μm) of lungs, hearts and kidneys from control and hypoxia-exposed mice were subjected to immunofluorescence (IF) analyses using CD41 (platelets marker) specific antibody (red) as described in Materials and methods. Arrows show representative aggregates. (B) Graph shows the quantification of 50–80% occluded vessels in the heart of control and hypoxia-exposed mice. Quantification was performed based on analyzing 18 fields of view of heart sections from 6 control and 6 hypoxia treated mice (*$p < 0.05$). (C and E) IF and confocal microscopic analyses of heart and lung were performed using double staining for CD41 (red) and VWF (green), as described in Material and methods. Platelet aggregates (red) were detected in vascular areas showing VWF expression in hypoxic hearts and lungs (merged figure and DIC). Colocalization (yellow) is also representative of VWF expression by platelets, which is observed in non-aggregated platelets in the heart and lungs of control mice. Right panels in (C) and (E) represent differential interference contrast (DIC) images superimposed with IF staining for VWF (green) and platelets (red) of heart and lung vessels. Nuclei are detected by DAPI staining (blue). The results are representative of 2 independent stainings from 3 different controls or hypoxia-treated mice. Panels D and F represent the quantification of > 50% occluded vessels in the lung and heart of control and hypoxia-exposed mice. Quantification was performed based on analyzing 8 fields of view of heart sections of the 3 control and the 3 hypoxia treated mice.(*$p < 0.05$).

transacting factors, some of which may also contribute to hypoxia-response of *VWF* gene [29]. Nevertheless, our analyses demonstrate that the participations of above-mentioned transacting factors are necessary for hypoxia-induced VWF upregulation in an organ-specific manner. The participation of SP1 in the hypoxic induction of VWF in lung endothelial cells is consistent with a recent report demonstrating the upregulation of VWF in the lungs of mice that were exposed to acute hypoxia [30]. Hypoxia-induced VWF upregulation was mediated through a pathway that involved high mobility group box-1 (HMGB1) activation of toll-like receptor 2 (TLR2) and associated MYD88 signaling protein leading to SP1 phosphorylation and association with the *VWF* promoter. A pharmacological inhibitor of SP1 activity was shown to inhibit hypoxia-induced upregulation of VWF in the lungs of mice [30]. However, our deletion analyses demonstrated that in the absence of intron 51 sequences that bind YY1, the VWF proximal promoter sequences that contain the SP1 binding site were not sufficient to support the hypoxic induction of VWF in cultured lung endothelial cells. These results strongly suggest that while both SP1 and YY1 are necessary, individually they are not solely sufficient for hypoxic induction of the *VWF* promoter in lung endothelial cells, and the cooperative action of both YY1 and SP1 is required for this process.

In contrast to the activators, NFIB, which is a repressor of the *VWF*

promoter [9,13], participated in mediating the hypoxia response in both cardiac and lung MVEC. In addition, in both cell types, hypoxia-induced activation was concomitant with the increased acetylation of histone H4. Specifically in cardiac MVEC however, the hypoxia response was also associated with increased methylation of a CpG site in proximity to the NFIB binding site. Based on these observations, we propose the following hypothesis to describe hypoxic induction of VWF in cardiac and lung endothelial cells.

We propose that the hypoxia response of endothelial cells (both lung and cardiac) regarding VWF expression is centered on "lifting" the NFIB repression of the *VWF* promoter, through its decreased association with the promoter. We also propose that lifting the repression is associated with increased acetylation of histone H4, and consequently, increased transcription. We further hypothesize that lung and cardiac endothelial cells arrive at this central process through two distinct pathways. In lung MVEC, increased binding of YY1 to the VWF I51HSS sequences (which, as previously shown, is brought in close proximity to the NFIB binding site through chromatin looping) may mediate this process, and association of SP1 may cooperatively or independently enhance transcription. However, in cardiac MVEC, increased association of GATA6 with the VWF promoter may be the mediator of this process. A role for GATA6 does not exclude the participation of other potential activators/coactivators, as demonstrated by the recruitment of HIF-1α to the *VWF* promoter specifically in cardiac MVEC. Recruitment of factors/cofactors may also result in epigenetic modifications, which in turn, could influence the association of various factors. This hypothesis is consistent with the increased methylation at the CpG site located in close proximity to the NFIB binding site, which may contribute to the process by interfering with NFIB binding. Collectively, these analyses demonstrate that the organ-specific pattern of VWF expression in response to hypoxia is maintained in the cultured MVEC and reflective of their corresponding intact organs. Moreover, despite certain common elements (i.e. reduced NFIB association with the promoter), lung and cardiac MVEC employ distinct molecular pathways for this process. Analyses of two other hypoxia-responsive genes, TGFβ2 and eNOS, that are also targets of GATA6 and SP1 regulation, demonstrated that these transacting factors participate in hypoxia-induction of at least two other genes that were explored in endothelial cells as well. Thus, a vascular bed-specific modulation of these transacting factors function in response to hypoxia may contribute to an additional level of regulation (potentially to a subset of target molecules) that confers organ-specificity to hypoxia-response of endothelial cells.

The heterogeneity of endothelial cells in structure, function and response to the environment is coordinated with the requirements of the organs/tissues that are served by their specific vascular beds [4,31–33]. While some organs/tissues, such as the lung, may be exposed to high levels of oxygen, others, such as kidney glomeruli, are exposed to a significantly less oxygenated environment. Normally, microvascular endothelial cells of these organs are adapted to varying degrees of oxygenation and may have evolved distinct mechanisms to differentially regulate their response to hypoxia. This process has been previously reported [34] and shown here in the development of distinct mechanisms to regulate *VWF* gene expression in response to hypoxia. For instance, a lack of upregulation of VWF in response to hypoxia in kidney glomeruli endothelial cells maybe reflective of these cells adaptation to their normally less oxygenated environment.

The differential hypoxia response by distinct vascular beds regarding VWF expression raised the question of whether this process has functional consequences *in vivo*. Increased plasma sodium concentration was recently shown to be a stimulus for increased VWF production, leading to the increased generation of microthrombi [35]. While hypoxia-response of endothelial cells has been known to result in degranulation of Weibel-Palade bodies (VWF storage organelles) and the release of stored VWF (in addition to VWF transcriptional upregulation) both *in vitro* and *in vivo* (thus suggesting a potential prothrombotic consequence); there has been reports that hypoxia alters platelets function in a manner that impairs their aggregation [36–39]. However, this effect was observed in isolated platelets and not in plasma rich platelets [37]. Additionally, in a recent study a delay in clot formation was observed in healthy volunteers that were acclimatized to high altitude, but also an increased in clot strength were reported in the same study [40]. These results point to a complex and as yet unclear role for various components of platelets aggregate/clot formation in response to hypoxia *in vivo*, although an overall prothrombotic consequence for hypoxia has emerged [26,27,40].

The analyses of various organs of control and hypoxia-exposed mice demonstrated the presence of significant platelet aggregates, as well as fibrin containing thrombi, in vasculatures of hearts and lungs, but not kidneys of hypoxic mice. Although these results are consistent with an increased VWF expression correlated with prothrombotic consequences, they do not directly demonstrate that increased VWF levels are responsible for increased platelet aggregate formation. The effect of hypoxia on endothelial cells is not restricted to the expression of VWF. For example, increased levels of other adhesion molecules, including E-selectin and P-selectin, as well as downregulation of anti-thrombotic factors such as thrombomodulin, have also been demonstrated [41,42]. Additionally, platelet aggregates/thrombus formation is also regulated by thrombolytic molecules (including VWF cleaving enzymes), and the levels of these molecules may also be altered in response to hypoxia. Decreased levels of thrombolytic molecules may have a net effect of increased platelet aggregate/thrombus formation. We hypothesize that the heterogeneity of the response of endothelial cells to hypoxia may also extend to other pro and/or anti-thrombotic molecules, which could also contribute to differential thrombus formation in the vasculature of different organs.

The observed heterogeneity in the response of distinct vascular beds to external stimuli underscores the tools that may allow organ-restricted targeting of specific vasculature for desired manipulations. We have generated HPRT-targeted transgenic mice harboring LacZK51HSS and demonstrated specific brain and lung endothelial cell expression of LacZ (Supplementary Fig. 1). This, in addition to other previously reported HPRT-targeted *VWF* promoter-*LacZ* transgenes [11,43], confirms that transgene copy numbers and integration sites are not responsible for the observed organ-specific activation pattern of the VWF regulatory sequences. However, from transgenic mice analyses, it is not clear whether the organ-restricted and endothelial cell specificity of the VWF promoter fragments are maintained when transgenes are delivered somatically *in vivo*, and/or remain episomal (two characteristics that may be desirable for development of therapeutic *in vivo* targeting tools). Here, we demonstrated that when delivered somatically *in vivo,* VWF regulatory sequences in adenoviral vectors maintained their endothelial-specific and organ-restricted activities (Supplementary Fig. 1), suggesting that they are attractive tools for somatic targeting of desired molecules to distinct vascular beds.

Conflicts of interest

The authors declared they do not have anything to disclose regarding conflict of interest with respect to this manuscript.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.atherosclerosis.2019.01.002.

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