Transcriptional regulation of von Willebrand Factor gene in response to hypoxia and in cancer cells

By

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A thesis submitted in partial fulfillment of the requirements for the degree of

Doctor of Philosophy
in
EXPERIMENTAL MEDICINE

Department of MEDICINE

University of Alberta

2016

Abstract

Von Willebrand Factor (VWF) is a pro-coagulant, glycosylated protein that is exclusively expressed in endothelial cells and megakaryocytes. An acute phase protein, *VWF* is upregulated or released from activated or injured endothelial cells. In addition, it is a carrier for factor VIII that mediates the adhesion of platelets to the sub-endothelium at the injury sites of blood vessels. Both low and high levels of VWF protein may contribute to diseases associated with abnormal thrombosis. Low levels of VWF protein are the cause of the most prevalent inherited bleeding disorder, known as von Willebrand disease, while high levels of VWF protein, when dysregulated, are an independent risk factor for cardiovascular disease. Although VWF is a marker of endothelial cells, it exhibits a heterogeneic expression pattern throughout the vasculature. However, neither the molecular bases of *VWF* transcription, nor its heterogeneic expression pattern, nor the mechanisms of its response to external stimuli are completely known. Investigation of the *VWF* transcription process has led to the identification of distinct regions, as well as specific regulatory cis- and trans-acting factors, which contribute to this process. To gain more insight into the mechanisms that regulate *VWF* gene activity, we explored *VWF* expression in response to hypoxia *in vivo* and *in vitro*. We also explored *de novo* expression of *VWF* in cancer cells of non-endothelial origin.

We investigated the *VWF* expression at protein and RNA levels in a mouse model of pulmonary hypertension that occurs as a result of hypoxia exposure. We demonstrated increased expression of *VWF* in all of the major organs of hypoxic compared to control mice. Furthermore, we specifically investigated the mechanisms underlying *VWF* upregulation in the hypoxic lung and heart. In the hypoxic lung, *de novo* expressions of *VWF* were detected in small microvessels, while in control mice, *VWF* expression was predominantly limited to large vessels of the lung. Hypoxia-induced upregulation of *VWF* in the lung endothelial cells was associated with increased binding of the YY1 transacting factor to its cognate binding site (intron 51 region), which was concomitant with increased translocation of YY1 to the nuclei. *VWF* upregulation in the lung was also associated with decreased binding of NFIB repressor to the promoter.

We demonstrated that organ-specific mechanisms are involved in the hypoxia-induced regulation of *VWF* transcription. While hypoxia resulted in *VWF* upregulation in the heart and lungs, this was not observed in kidney endothelial cells either *in vivo* or *in vitro*. Furthermore, distinct mechanisms participated in the hypoxia-response of endothelial cells in the lung compared to those in the heart. This included a reduction in the binding of the repressor NFIB to the *VWF* promoter in both cell types. However, in lung endothelial cells, YY1 participated in the hypoxia-induction of *VWF*, while in cardiac endothelial cells, GATA6 and HIF participated in this process. Analysis of the methylation pattern demonstrated that a CpG dinucleotide located in the proximity of the repressor NFIB binding site was hypermethylated in response to hypoxia, specifically in cardiac endothelial cells. Increased *VWF* expression in the heart and lung of hypoxic mice was associated with platelet accumulation and aggregation in blood vessels, and occluded vessels were observed in the heart and lung.

Increased plasma levels of VWF and thrombogenesis are commonly observed in cancer patients. It has been proposed that the source of these increased levels of VWF is either endothelial cells or platelets. However, we have demonstrated that a series of malignant glioma as well as osteosarcoma cell lines express *VWF*, which in turn, mediate increased cancer cell adhesion, transmigration and extravasation from endothelial cells. Analyses of the mechanisms of transcriptional activation of the *VWF* gene in cancer cell lines demonstrated a binding pattern of transacting factors and epigenetic modifications that was generally consistent with that observed in endothelial cells.

(Preface)

The information in this thesis is the original work performed by Anahita Mojiri. Chapter Two is a published manuscript and Chapter Three and Four are submitted for publication. The projects described in Chapter Two and Four have been completed in collaboration with Dr. Michelakis and Dr. Lewis from University of Alberta, respectively. Dr. Michelakis lab generated pulmonary hypertension (PH) in mice and performed PAAT test to confirm PH in our transgenic mice. DR. Lewis lab performed CAM assay as well as experimental mice model of metastasis using our cancer cells. Their contributions have been specifically with regard to animal studies and exovo, which were performed in accordance with their animal ethics approvals from University of Alberta ASUC. Experimental design, performance and analysis of data, as well as manuscripts composition were completed by Anahita Mojiri and her supervisor Dr. Nadia Jahroudi.

“The true sign of intelligence is not knowledge but imagination”
Albert Einstein

I dedicate this work to my family, whom without their generous support and love, none of this would have been achievable.

Acknowledgments

I would like to express my deepest thanks to my supervisor, Dr. Nadia Jahroudi, for giving me the opportunity to work with her. I appreciate her support, patience and inspiration during the years of my work in her lab. She has been a great mentor and has shown by example how to be a skilled, dedicated and conscientious scientist.

Special thanks go to my supervisory committee members, Dr. Murray and Dr. Pasdar, for their invaluable input and help throughout my project.

I would like to thank our recent students in the lab, Radya Yousef Abdualla and Maria Areli Lorenzana Carrillo, who made my time working in the lab much more fun.

I would like to thank all my friends and collaborators, Dr. Ballermann, Dr. Laiji Li, Dr. Maria Obeidat and Dr. Mahtab Tavasoli, for sharing their space and knowledge with me. I would also like to thank other members of the nephrology group, Dr. Maikel Farhan, Dr. Huang and Abul Azad, for their friendship and help.

I wish to thank my good friend, Dr. Zulika Zak, for kindly giving an office to me to study and write my thesis. Special thanks go to Dr. Nicolas Touret for his continuous support and guidance with microscope imaging.

I wish to thank my best friend, Dr. Maryam Nakhaie-Nejad, for her friendship and everyday help and support. Thank you for making lunchtime more fun and energetic. I would also like to thank my friend, Dr. Furugh Sanaee, for her support.

I would like to express my heartfelt thanks to my family, my dear parents, my sister Soheila and my brothers Maziyar and Arash for your love and encouragement. Without your support I wouldn't have made it.

I must thank my husband Babak. Your support made me feel stronger in challenges I have had through my studies. Thank you for being so patient and understanding in my journey. I am here today because of you.

Ahoura, you are the best precious gift any mom could ask for. Your smiles, kisses and hugs were the only things that could relax me everyday after work. Thank you.

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List of abbreviations

3'UTR	Three prime Untranslated region
5'UTR	Five prime Untranslated region
ADAMTS13	A Disintegrin And Metalloproteinase with a Thrombospondin Type 1 motif, member 13
ALI	Acute Lung Injury
CAM	Chick Chorioallantoic Membrane
ChIP	Chromatin Immunoprecipitation
CMEC	Cardiac microvascular endothelial cells
DAPI	4',6-diamidino-2-phenylindole
DNase-1	Deoxyribonuclease I
EC	Endothelial Cells
ECM	Extracellular Matrix
EGM	Endothelial Growth Medium
eNOS	Endothelial nitric oxidesynthase
ETS	E-Twenty-six
FACS	Fluorescence-Activated Cell Sorting
FITC	Fluorescein Isothiocyanate
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GATA	GATA-binding factor
GFP	Green Fluorescent Protein
GS-IB4	Griffonia simplicifolia
H3	Histone H3
H4	Histone H4
HAT	Histone acetyltransferases
HCC	Hepatocellular Carcinoma
HDAC	Histone deacetylases
HFF1	Human foreskin fibroblasts
HIF-1	Hypoxia Inducible Factor-1
HLP	Histone-Like Protein
HPRT	Hypoxanthine-guanine phosphoribosyl transferase
HRE	HIF Responding Element
HRP	Horseradish peroxidase
HSS51	Hypersensitive Intron 51
HUVEC	Human Umbilical Vein Endothelial Cells
I51HSS	Intron 51 Hypersensitive
IF	Immunofluorescence
IHC	Immunohistochemistry
IL-11	Interleukin- 11
IL-6	Interleukin -6
LMEC	Lung Microvascular endothelial cells

LPS	Lipopolysaccharides
miR-24	microRNA-24
MMP2	Matrix Metalloproteinase-2
MMP9	Matrix Metalloproteinase-9
MOI	Multiplicity Of Infection
MVEC	Micro Vascular Endothelial Cells
NET	Neutrophil Extracellular Traps
NFAT	Nuclear Factor of Activated T-cells
NFAT5	Nuclear factor of activated T-cells 5
NFI	Nuclear Factor I
NFY	Nuclear transcription Factor Y
OCT	Octamer-binding Transcriptionfactor
OCT	Optimum cutting temperature
PAAT	Pulmonary Artery Acceleration Time
PCAF	P300/CBP-associated factor
PH	Pulmonary Hypertension
PRMT1	Protein arginine N-methyltransferase 1
PTEC	Proximal Tubule Epithelial cells
RT-PCR	Reverse transcription polymerase chain reaction
SiRNA	Small interfering RNA
SP1	Specificity protein 1
STAT3	Signal transducer and activator of transcription 3
Tie2	Tyrosine kinase with immunoglobulin-like and EGF-like domains
TNFα	Tumournecrosis factor alpha
TTP	Thrombotic Thrombocytopenic Purpura
ULVWF	Ultra Large VWF
VEGF	Vascular endothelial growth factor
VHL	Von Hippel-Lindau
VWD	Von Willebrand Disease
VWF	Von Willebrand Factor
WPBs	Weibel–Palade Bodies
YY1	Yin Yang 1

CHAPTER 1

VON WILLEBRAND FACTOR GENE REGULATION

INTRODUCTION

The vascular tree is comprised of endothelial cell units that form arteries, veins and capillaries. The blood circulating through the body is enclosed in the vasculature, and endothelial cells are the first barrier between the blood and other cells and tissues [1]. Endothelial cells play a critical role in regulating hemostasis and the homeostatic balance of the body, and perform many other physiological functions [2, 3]. The most remarkable aspect of endothelial cells is their heterogeneity, which corresponds to their vascular bed needs and structure.

Endothelial cell heterogeneity was defined for the first time when cells were studied using electron microscopy [4]. Endothelial cells display heterogeneity in their structure, function and gene expression in order to deal with the diverse functions of these organs [1-3]. For example, in the blood-brain barrier, endothelial cells have minimum permeability due to the presence of tight junctions, ensuring the maximum protection of neurons and brain parenchyma from blood-based fluctuations and effects, such as sudden increased lipids, toxic material or changes in blood pressure [2, 5]. In contrast, the discontinuous, highly fenestrated endothelial cells in the liver are maximally permeable, allowing increased transportation of material from the blood to

the hepatocytes. Distinct phenotypes of endothelial cells in other organs and also in different segments of the vascular loop of one organ have been reported [2, 6].

Von Willebrand Factor (VWF) is a multimeric glycoprotein that is exclusively expressed in endothelial cells and megakaryocytes (platelets precursors) [7]. As Aird et al. (2007a, b) note, VWF protein distributions through vasculature display a very heterogenic pattern, which is reflective of the heterogenic transcriptional activity of the *VWF* gene. *VWF* expression, as they observe, changes upon culturing endothelial cells *in vitro*. While *VWF* expression is almost absent in glomeruli endothelial cells *in vivo* [8] , these cells start to express *VWF* when cultured *in vitro* [9, 10]. The following sections summarize the current knowledge of VWF protein and gene regulation in physiological and pathophysiological *VWF*-related events.

VON WILLEBRAND FACTOR (VWF)

VWF protein

VWF is a heavily glycosylated protein which, in endothelial cells, is stored in Weibel-Palade bodies (WPBs) and in platelets, is stored in the alpha organelles. WPBs consist of tubular, multimeric forms of VWF that are arranged in parallel and surrounded by a lipid bilayer [11]. Glycosylated VWF displays a variety of sizes due to assembly of the monomers [12-14]. While dimer forms of VWF protein exist in the cytoplasm and are released constitutively from endothelial cells, extra-large multimers of VWF protein are exocytosed from WPBs upon endothelial cell activation or released from injured endothelial cells [15]. *VWF* mRNA is approximately 8 kb, and the

translated protein is comprised of 2,813 amino acids, with a signal peptide of 22 aa and a propeptide of 741 aa. The mature 2,050 aa VWF monomer is extensively glycosylated [7].

VWF protein contains multiple domains (A1, A2, A3, B1, B2, B3, C1, C2, CK, D', D1, D2, D3, D4), each of which contributes to one or more functions of VWF through its capacity to bind with different proteins. For example, D' binds to factor VIII, A1 adheres to platelets, and both A1 and A3 bind to ECM proteins (collagen, fibronectin) in the endothelial basement membrane and connective tissue [16, 17]. Mutational changes in these domains induce functionally deficient forms of the VWF protein, which have been identified in various types of von Willebrand disease [18, 19].

The glycosylated VWF monomers are assembled as dimers or multimers through a specific post-translational maturation process. Disulfide bridge formation at two sites in the C- and N-termini leads to dimeric and multimeric forms of VWF. During endoproteolytic processing in the Golgi apparatus by the furin enzyme [20], the VWF-propeptide is cleaved and the multimeric, mature VWF, which is the active form of the protein with high density (ULVWF) (roughly 1.3 $\mu g/cm^3$) [21], is packed and stored in the WPBs [22].

VWF expression pattern is representative of endothelial heterogeneity

The distribution pattern of the VWF protein throughout the vascular tree is a remarkable example of endothelial cell heterogeneity [23, 24]. While VWF tubules are organized similarly in WPBs, the sizes, shapes and number of WPBs appear to vary, corresponding to the cytoplasmic space of endothelial cells (the smaller the space in

cytoplasm, the smaller the size and number of WPBs) [11]. Immunohistochemistry experiments on endothelial cells of the lung, heart, brain, liver and kidney have demonstrated strong *VWF* expression in veins as well as in larger vessels, with moderate expression in arteries and smaller vessels, and expression in patches in the capillaries of these major organs [23-25].

The heterogeneic expression pattern of *VWF* has also been illustrated in various organs upon stimulation [8]. Many organ-specific characteristics of endothelial cells are lost when these cells are removed from their native environment and grown in culture [9, 26]; this underscores the significance of *in vivo* vs. *in vitro* studies of gene regulation and raises questions of how applicable and predictive our *in vitro* findings are to *in vivo* conditions. Transient ON/OFF switch of the *VWF* promoter activity has been shown during the development of capillaries in the heart, lung and brain; this effect contributes to the heterogeneic pattern of *VWF* expression [27]. This stochastic switching may simply induce a "moment" of signaling, which could potentially control random cell fate decisions. The heterogeneic pattern of *VWF* expression was shown to occur even in the population of cells that arose from a single cell, suggesting that the extra/intracellular signal variations lead to transitional phenotypes from one endothelial cell to another [27]. These observations have motivated researchers to investigate and identify the molecular bases of *VWF* heterogeneity in the vasculature.

VWF Function

VWF is a central component of the hemostasis system, which balances normal thrombosis. VWF is not an enzyme, but performs fundamental roles in the hemostatic

balance of the body [7]. Two major functions of VWF protein regarding hemostasis are: (i) mediating the adhesion of platelets to injured endothelial cells or sub-endothelium extracellular proteins at the site of injury to stop bleeding, and (ii) functioning as a carrier and stabilizer for factor VIII. In addition, recent studies have demonstrated novel roles for VWF in angiogenesis [28] and immune response [29]. It was shown that inhibition of *VWF* expression induces angiogenesis, which is mediated by increased vascular endothelial growth factor receptor-2 [28]. In the adaptive immune response, leukocytes transmigrate through endothelial cells; this migration is mediated by various adhesion molecules on the endothelial cells [30]. Direct binding of VWF with released DNA from stimulated leukocytes under shear stress also have been shown to potentially facilitate leukocyte adhesion to endothelial cells and their extravasation [29].

Under normal physiological conditions, the ADAMTS13 protease digests and removes the excess released VWF, and maintains the level of mature VWF in the blood at about 10 μg/ml (1U/ml) [31], thereby avoiding unnecessary thrombus formation [32]. Qualitative or quantitative (below 0.5-0.3 U/ml) variations in the VWF protein can lead to Von Willebrand disease (VWD), which is an inherited bleeding disorder [33-35]. Low levels of VWF, which can be moderate or severe, may cause type 1 or 3 VWD, respectively, and VWF protein that is inefficient in its capacity to bind to platelet or factor VIII leads to type 2 VWD phenotypes [36].

VWF GENE AND PROMOTER

The *VWF* gene is located on chromosome 12 at 12p13.2 and is comprised of 52 exons [37]. The sequence of the *VWF* 5' untranscribed region includes the standard TATA box and downstream CCAAT box, followed by the transcription start site [38].

In vitro experiments, in which constructs of various *VWF* gene sequences fused to reporter genes were transfected into cultured endothelial and non-endothelial cells, have demonstrated that *VWF* gene sequences comprised of nucleotides -487 to +246 function as an endothelial specific promoter [39]. This *VWF* promoter consists of a core promoter (sequences spanning nucleotides -90 to +22, relative to the transcription start site), which is active in both endothelial and non-endothelial cells. It also contains a negative regulatory region (spanning nucleotides -500 to -300) that, when combined with the core promoter alone, inhibits the core promoter activity in both endothelial and non-endothelial cells [39, 40]. However, a positive regulatory region (located in the first exon, spanning nucleotides +155 to +247) could not only overcome the inhibitory effect of the negative regulatory region, but also trigger the activation of the *VWF* promoter only in endothelial cells, thereby imposing the cell-type specific transcriptional activity to the promoter *in vitro* (Fig. 1-1) [39].

Specific cis-acting elements and corresponding transacting factors that function as either activators {GATA, ETS, NFAT, NFY (at -18 site) and HLP} or repressors {NFI, OCT, E4BP4, NFY (at +226 site)} of the *VWF* promoter activity have been identified (Fig. 1-1) [39, 41-48].

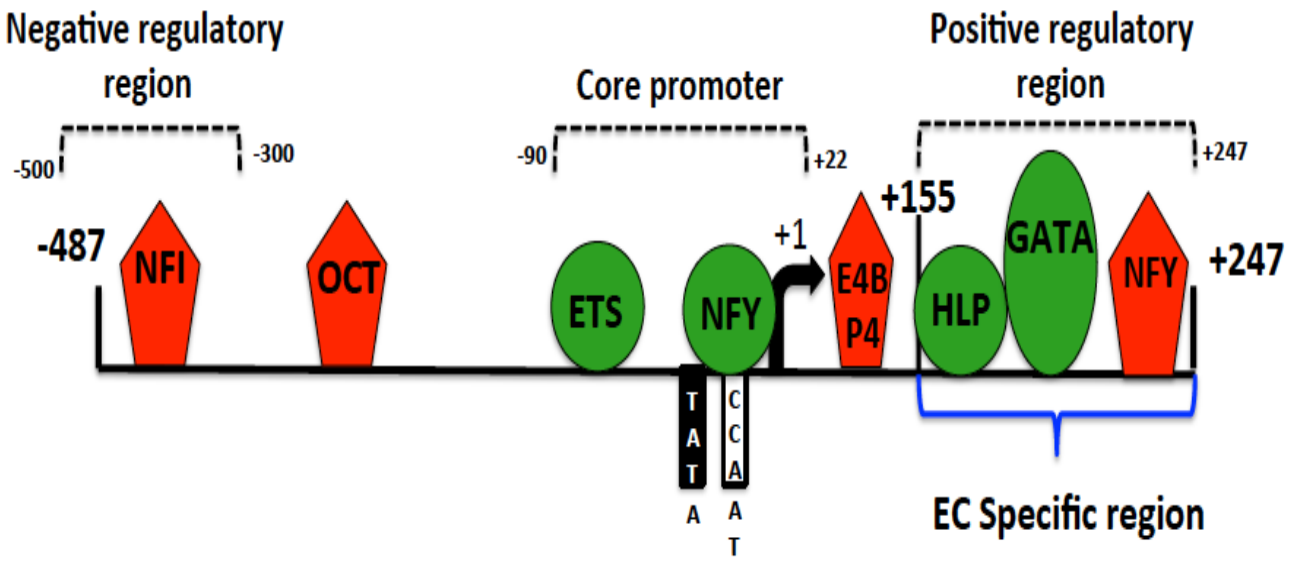


Figure 1-1 Von Willebrand Factor promoter sequences -487 to +247 and several known transcription factors bind to the cis-sequences. Transcription factors that function as repressors (shown in red) include Nuclear Factor I (NFI), octamer-binding transcription factor (OCT), nuclear transcription factor Y (NFY) and E4BP4. Transcription factors that function as activators (shows in green) include E-Twenty-six (ETS), nuclear transcription factor Y (NFY), histone-like protein (HLP), and GATA-binding factor (GATA).

VWF PROMOTER ACTIVITY *IN VITRO*

Based on site mutation experiments, it has been shown that the binding of GATA family members (2,3 and 6) to the positive regulatory region (+220 to +6) is essential for the basal expression levels of promoter sequences (-487 to +247), or an extended 3kb (-2,812 to the end of first intron) promoter activity in endothelial cells. Mutation of this GATA binding site abolished the activities of both promoter sequences in cultured endothelial cells [39, 49]. Furthermore, Liu et al. (2011) demonstrated that ETS (a member of ERG family) binding at the -56 site is necessary for 3kb *VWF* promoter activation in HUVEC, and this activation was significantly reduced by site mutation. Certain repressors are important in modulating the levels of *VWF* gene activity. Mutation of the Oct-1 binding site (located in the negative regulatory elements) increases *VWF* gene activation in endothelial cells [47]. It has also been demonstrated that E4BP4, which binds to the +96 to +105 position, represses the promoter activity in both non-endothelial and endothelial cells, leading to the lack of expression and the reduced expression of activated *VWF* in these cells, respectively [48]. The E4BP4 repression is due to its direct interaction with the cis-element of *VWF* promoter in non-endothelial cells. In endothelial cells, however, this repression appears to be indirect, and potentially occurs through the activity of a complex of proteins, which have yet to be identified [48].

Nuclear factor I family of transcription factors include four separate genes coding for isoforms NFIA, NFIB, NFIC and NFIX proteins [50, 51]. NFI proteins bind to DNA sequences TTGGC(N5)GCCAA on the promoter to impose their effect either as

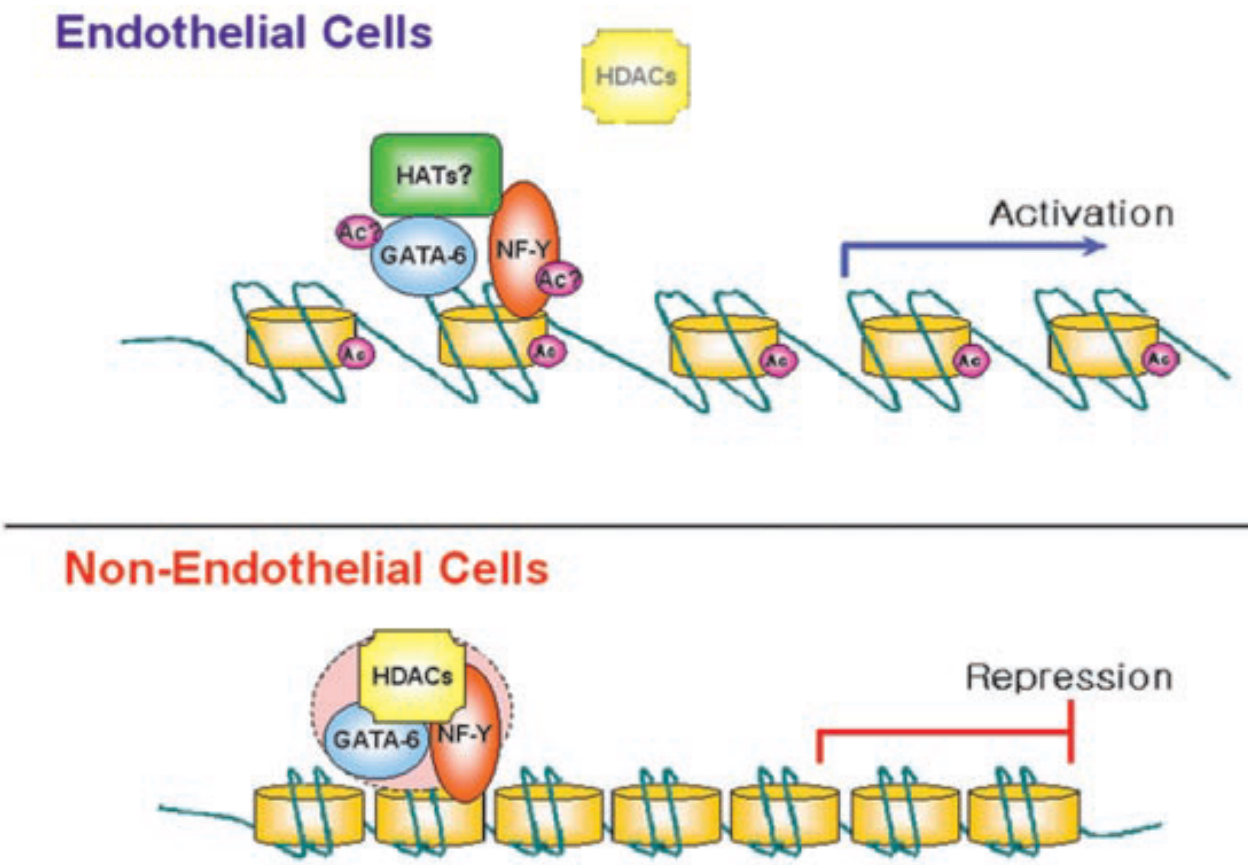
repressor or activators of the gene [52] NFI isoforms are important to regulate gene expression and developments of brain and lung tissues [53, 54]. NFIB role in mesenchymal cell maturation of lung tissue was shown to be essential for lung development [54]. The pattern of NFI isoforms expression in endothelial cells of various organs also displays organ specificity [55]. NFI has binding site on VWF promoter and was shown to be a major repressor in regulation of VWF gene in vitro and in vivo [41, 45, 55]

NFY (transcription factor Y) was shown to act as both an activator and a repressor of the *VWF* promoter, depending on its binding site [42]. NFY bound to the consensus sequence at -18 (CCAAT), functions as an activator in endothelial cells, while NFY bound to the sequence +226 to +234 functions as a repressor in non-endothelial cells. It has been shown that NFY can mediate nucleosome assembly and change chromatin structure by recruiting histone modifiers {PCAF or HDAC} to its binding site [56]. In non-endothelial cells, the repressive function of NFY was shown to be mediated specifically through the recruitment of histone deacetylase HDAC1 and 2 to the *VWF* promoter [57]. Recruitment of HDAC to the *VWF* promoter in non-endothelial cells occurred concomitant with the presence of deacetylated histone H4, potentially conferring a silent chromatin structure, which is consistent with the observed lack of *VWF* promoter activity in non-endothelial cells. In endothelial cells, by contrast, HDACs had significantly reduced association with the NFY. They were thus not recruited to the *VWF* promoter and, consistent with promoter activity in these cells, promoter associated histone H4 proteins were acetylated (Fig. 1-2) [57].

In addition, specifically in non-endothelial cells, the NFY-HDAC complex was also associated with GATA6, which led to the hypothesis that the NFY-HDAC-GATA6 complex in non-endothelial cells may render GATA6 ineffective, thus contributing to the inhibition of promoter activity [57]. In endothelial cells, however, GATA6 that is not sequestered by a NFY-HDAC complex may be free to function as activator, and may be potentially associated with histone acetylases, thus recruiting them to the promoter, leading to observed acetylation of promoter-associated histone H4, and consequently active chromatin structure which favors transcriptional activation. This hypothesis has been supported by the demonstration of GATA6 association with PCAF specifically in endothelial cells (Jahroudi, unpublished results).

It was demonstrated that mutation of both NFY and NFI (both repressors) sites in the 748 bp promoter significantly induced *VWF* promoter activity in non-endothelial cells [42, 45].

Together, then, as noted above, several transacting factors are involved in the regulation of *VWF* expression in endothelial and non-endothelial cells *in vitro*. However, these transcription factors are distributed in all organs and none are identified as endothelial-specific factors, thus we hypothesize that a pathway of vascular bed signaling may mediate their specific function in regulating *VWF* expression in a cell type-specific or an organ-specific manner.



[57] (Figure appears precisely in this form in Peng and Jahroudi (2003), p. 83892.)

Figure 1-2. " Model describing the activation and repression function of NFY-binding site in the *VWF* promoter. The model represents the *VWF* region corresponding to sequences -155 to -247. The solid cylinders represent potential nucleosomes, and circles with Ac represent acetylation. In endothelial cells a complex composed of NFY, GATA6, and potentially an endothelial specific HAT (unknown thus represented as HATs?) may be formed. The putative HAT may acetylate the GATA6 and NFY as well as histone H4 in the nucleosomes, and these modifications of trans-acting factors and nucleosomes could facilitate promoter activation. In non-endothelial cells, the absence of endothelial specific HATs could result in the increased pool of NFY associated with HDACs, and a complex consisting of NFY-HDAC-GATA6 may be

recruited to the *VWF* promoters which deacetylate the histone H4 in the nucleosomes and potentially maintain a deacetylated form of GATA6 and NFY. These may contribute to inhibition of the promoter activity by deacetylation of histone H4 and potentially inhibiting the activating function of GATA6 and NFY, thus turning the entire complex to a repressor."

(Caption appears precisely in this form in Peng and Jahroudi (2003), p. 83892.)

VWF PROMOTER ACTIVITY *IN VIVO*

The *VWF* gene is activated in the mouse embryo at an early stage in the development of vascular structure, in which a heterogeneic pattern of its expression is also observed [58]. Researchers have detected early *VWF* expression ot E8 (embryonic day 8) in just "dorsal aortae", while endothelial cells forming vessels were displayed in paraxial and lateral plate mesoderm at E7 [58]. Furthermore, heterogeneic patterns of *VWF* expression were demonstrated at E12, when many vessels become VWF positive, but *VWF* expression was absent in the majority of endothelial cells in capillaries [58]. To investigate whether the endothelial cell-type specific activity of the *VWF* promoter sequence -487 to +247 is maintained *in vivo*, Aird et al. (1995) generated chimeric transgenic mice harboring a *LacZ* transgene (under the control of *VWF*-derived promoters) as a marker. Transgenic mice carrying 748 bp of the *VWF* promoter showed a nonhomogeneous pattern of *LacZ* transgene expression in the yolk sac. However, in adult transgenic mice, *LacZ* expression was exclusive to a population of brain endothelial cells and no expression was observed in other organs [59]. The complete absence of *LacZ* in the vessels of other organs (containing endothelial cells with high levels of endogenous VWF) suggested the potential importance of other *VWF* gene sequences that were not included in the transgenic, and also highlighted the significance of microenvironmental signals [59].

ORGAN SPECIFIC ACTIVITY OF THE *VWF* PROMOTER

The findings of the above study suggest that more sequences of the *VWF* gene are involved in the promoter activity. The observation also led to the hypothesis that

repressors might participate in silencing the *VWF* promoter in the endothelial cells of organs other than brain. To test these hypotheses, we and other researchers performed analyses to determine the role of additional gene sequences, as well as identifying repressor-binding cis-acting elements of the *VWF* gene, using transgene mice models carrying various elements of the *VWF* promoter.

The role of 5' and intron 1 in *VWF* promoter activation in the endothelial cells of heart and skeletal muscle

To determine the elements that contribute to the activity of the *VWF* promoter in other organs, sequences of the *VWF* gene immediately upstream and downstream of the 748 bp were added to the original promoter and fused to the *LacZ* transgene. Analysis of transgenic mice carrying the *LacZ* transgene under the regulation of the *VWF* promoter sequence extending from -2645 to the end of the first intron (3 kb) showed expression of the *LacZ* transgene in the endothelial cells of heart and skeletal muscle, as well as the brain [25, 60-62]. The pattern of expression occurred mainly in the veins and venules in both heart and skeletal muscle, with random low expression observed in arteries of the diaphragm [62]. Further analysis showed that although the coronary arteries, penetrating arteries, and endocardium of heart expressed endogenous *VWF*, they did not show the activation of exogenous promoter [62].

Using the various constructs and mutational analysis of the 5' upstream region of the *VWF* promoter, Liu *et al.* (2011) further defined the effect of the specific regions of the *VWF* gene in regulating promoter activity. They found that specific sequences in region from corresponding -843 to -620 activated transgene expression in

the capillaries of heart and skeletal muscle, while region -620 to +1475 was shown to contain information necessary for *VWF* expression in veins/venules and partially/patchy expression in arterioles of heart and skeletal muscle [62]. These results demonstrate that the sequences of -843 to -620 and -620 to 1475 have site-specific information. Specifically, however, *in vitro*, HUVEC transfected with an expression construct of the 3 kb *VWF* promoter with or without the region -843 to -620 showed similar levels of expression, indicating that distinct sequences of *VWF* gene are important for activation in various vascular beds within an organ [62].

The significance of vascular bed signaling on *VWF* expression was most clearly demonstrated by Aird *et al.* (1997), who implanted a neonatal heart in the ear of a transgenic mouse that showed *LacZ* expression in the endothelial cells of the brain, heart and skeletal muscle. When the ear's microvasculature engulfed the implanted heart and received biophysical signals provided by cardiomyocytes of the implanted heart, the ear endothelial cells surrounding ventricular myocytes started to express the *LacZ* transgene [25]. The induction effects of cardiomyocytes on transgene activation were also shown to be reproducible and significant in several coculture experiments involving wild-type myocytes with cardiac microvascular endothelial cells (CMEC) from transgenic mice [25]. A key finding was that the CMEC lost *LacZ* expression after growing 4 days in culture, and then regained *LacZ* expression upon coculture with myocytes to a level that was 2.6-fold higher than that detected in freshly isolated CMEC [25]. These observations suggested that organ-specific signaling cascades, which participate in *VWF* transcription, are invoked. Indeed, the

recapitulation of the organ-specific *VWF* expression phenotypes by endothelial cells is determined and mediated partly by the surrounding extracellular milieu.

The role of intron 51 in *VWF* promoter activation in the endothelial cells of the lung

The requirement for additional sequences of the *VWF* gene to achieve a pattern of expression that corresponds to that of endogenous *VWF* prompted the search for other organ-specific elements in the *VWF* gene. Basing their work on a screening study using the DNase-I-hypersensitive technique, Kleinschmidt *et al*. (2008) identified sequences within intron 51 of the *VWF* gene in which chromatin had potentially lost its condensed structure and exposed the DNA to transcription factors or transcriptional protein complexes. The intron 51 DNase1 hypersensitive region (HSS51) was shown to contain a binding site for transcription factor YY1. *In vitro* analyses demonstrated that the HSS51 sequences, when fused to a heterologous promoter, functioned as an enhancer specifically in endothelial cells, and this enhancer activity was dependent on an intact YY1 binding site [63].

To investigate the effect of intron 51 *in vivo,* the authors generated and analyzed transgenic mice harboring *LacZ* reporter and *VWF* 734 bp promoter sequences with I51HSS positioned either upstream of the *VWF* promoter (HSS-VWF-LacZ) or downstream of *LacZ* gene (VWF-LacZ-HSS). Results demonstrated that *LacZ* expression was only detected in subpopulations of the lung as well as brain endothelial cells [63]. *LacZ* expression displayed a heterogeneous pattern and was mainly restricted to the endothelial cells of the parenchyma near the pleura. The authors

proposed that YY1 binding to intron 51 induces chromatin looping, which brings the distal element into proximity of the transcription initiation site on the promoter and affects transcription. It was shown that the YY1 transcription factor bind to its cognate sequence in intron 51 and maintain the activity of the *VWF* promoter in endothelial cells of the lung [63].

Similar constructs, while maintaining endothelial cell specificity, did not show significant differences in *LacZ* expression between HUVEC, human and bovine lung endothelial cells *in vitro* [63]. The brain- and lung-specific pattern of *LacZ* expression in transgenic mice indicated that the contributions of other *VWF* gene sequences are required for *VWF* transcription in other organs.

The role of repressors NFI and NFY in organ-specific regulation of *VWF* promoter

The regulation of the *VWF* gene involves complex mechanisms and indicates that although transcription activators are important to enhance *VWF* activity in endothelial cells, the contribution of repressors is significant in maintaining endothelial and organ specificity of the *VWF* promoter. Nassiri *et al.* (2010) found that transgenic mice carrying the *VWF* promoter (747 bp) with mutation of the NFI binding site which inhibited NFI (repressor) binding, demonstrated expression of *LacZ* in lung and heart endothelial cells, as well as brain endothelium. In addition, mutations in the NFY binding site induced significant *VWF* transcription in brain and kidney endothelial cells. The inhibition of the binding of both NFI and NFY repressors by mutations in their binding sites resulted in *LacZ* expression in the endothelial cells of

all major organs in transgenic mice [55]. These data indicate that inhibitors (NFI, NFY) are contributing significantly to the organ-specificity of *VWF* expression throughout the vascular tree [55].

It was demonstrated that NFI isoforms have organ-specific distribution patterns throughout the vasculature, with significant expression of NFIC and NFIX predominantly in the brain. NFIB and NFIA were most detectable in heart. NFIX expression was significantly detected in kidney, and NFIB was most detectable in the lung [55]. An important role of NFIB in lung development has also been shown [64]. Significant activation of the *VWF* promoter with mutation in NFI binding site in the lung, in addition to preferential detection of NFIB in the lung endothelial cells, suggests that NFIB plays a role in regulating lung specific activity of the *VWF* promoter. Also, as discussed above, intron-51 lung-enhancer sequences that interact with YY1 participate in lung-specific activation of the *VWF* promoter. These two contributing elements (NFIB repressor and YY1-activator) were shown to be brought in close proximity to each other through chromatin looping [55, 63].

Based on these observations, it was hypothesized that repressive function of NFIB may be bypassed by activating function of YY1 in lung endothelial cells. However, the exact mechanisms by which YY1 influences NFI are not yet clear. It is also possible that the functions of these two factors are regulated independently of each other. It has been shown that the NFI transcription factor loses its DNA binding capacity through some modification, such as phosphorylation [65]. It is possible that cell signaling in the lung could induce NFI phosphorylation and inhibit its binding to

the *VWF* promoter. The combination of these mechanisms could also remove the repression effect of NFIB, leading to the activation of the *VWF* promoter in the lung.

These and other examples of vascular bed-specific promoters of *VWF* activity could provide a great opportunity to construct a targeting vector to investigate endothelial cells in specific organs.

The role of activators in *VWF* promoter activity *in vivo*

While repressors control the organ-specific activation pattern of the *VWF* promoter, mutational analyses have shown that the activator GATA (+220-5) is potentially involved in regulating *VWF* promoter activity [39, 49]. The mutation of the GATA binding site in the 3 kb promoter (-2182 +first exon +first intron) led to lower levels of transgene activity, but preserved the expression pattern in vessels of the brain, heart and skeletal muscle of mice compared to the wild type promoter [49]. Furthermore, Liu *et al*. (2011) demonstrated that mutation of the ETS binding site (at -56), in the context of the 3 kb *VWF* promoter fused to *lacZ* gene, abolished *LacZ* expression in the heart and skeletal muscles of transgenic mice, with a trace of activity in the hindbrain vessels [62]. The roles of transacting factors that function as activators of the *VWF* promoter were also studied in regard to response to stimuli, which will be discussed in more detail in other sections.

THE ROLE OF DNA METHYLATION AND RNA SPLICING IN REGULATION OF *VWF* PROMOTER ACTIVITY

DNA methylation of the *VWF* promoter

Recent data have revealed that DNA methylation of the *VWF* gene is another key factor in the cell type specificity and mosaic pattern of *VWF* expression [66]. Epigenetic modification through DNA methylation has been shown as a regulatory mechanism in activating or silencing genes [66]. The *VWF* promoter contains 8 CpG sites that are targets for methylation, and these sites (for the most part) are hypermethylated in non-endothelial cells and hypomethylated in endothelial cells. Changes towards increased methylation levels could potentially change the *VWF* promoter activity. A study by Yuan *et al*. has shown that DNA methylation of the *VWF* gene at the CpGs not only regulates promoter silencing in non-endothelial cells, but also regulates the transient activation of the VWF gene [27]. This study showed that methylation patterns are not stable, and levels are inversely associated with *VWF* expression in endothelial cells *in vitro.* Also, transitional methylation was found to be potentially necessary to provide signals for a normal healthy environment *in vivo* [27]. DNA methylation is functionally important for most of the endothelial specific genes. It has been shown that the level of methylation in the proximal promoter of *CD31, eNOS* and *VWF* is significantly higher in smooth muscle cells than in HUVEC [67] [27].

For endothelial-specific genes such as *eNOS*, it is important that the promoters are integrated into the genome in order to be methylated at certain sites to exhibit an

activation pattern/level similar to that of endogenous promoters [68]. Indeed, chromatin integration has been shown to be essential for cell-type specific of *eNOS* expression in transgenic mice. Analyses of *VWF* promoter methylation and its correlation with expression also emphasize a role for this epigenetic modification in silencing promoter activity in non-endothelial cells [67].

The role of intron splicing in regulation of the *VWF* promoter activity

Emerging research on *VWF* gene regulation underscores the complexity of the mechanisms involved in the heterogeneic expression of this gene. It has been shown that intron splicing can modulate *VWF* expression, and that in cell culture this expression is dependent on the location and direction of the intron [69]. Whereas the expanded *VWF* promoter {from -2182 to +246 (no intron included)} was activated only in the brain, adding the first intron of *VWF* or even the second intron from the human beta-globin gene could activate the promoter in heart and skeletal muscle, in addition to brain endothelial cells [62]. Since the intron undergoes splicing, these data demonstrated that splicing is involved in the activity of the promoter, and that intron sequences (even from other genes) are required for organ specificity.

It is worth noting that the presence or absence of this intron does not change the *VWF* promoter activity in megakaryocytes. Further analysis of the *LacZ* knock-in mice (at the *VWF* locus) with *LacZ* located immediately after first intron showed that the reporter gene expression was maintained at a level equivalent to that of the endogenous *VWF* [69]. However, changing the location of the *LacZ* transgene in the genome of the knock-in mouse to after the first exon (no intron) almost abolished *LacZ* expression in

the whole vasculature [69]. Further, Yuan *et al*. (2013) found that the intron-splicing effect is observed on post-translational modification, since the mRNA level analyses from both *VWF* promoter constructs (with or without the intron) showed equal amounts of mRNA both *in vitro* and in transgenic mice. Intron splicing did not influence the level of mRNA, mRNA trafficking or mRNA polyadenylation. It was also demonstrated that in transgenic mice, a heterogeneous intron from other species could rescue the expression (at various levels) of the *LacZ* transgene when it was located after the first exon [69]. Clearly, intron splicing has an effect on *VWF* expression (even though potential mechanisms are not known).

CIRCADIAN EFFECTS ON *VWF* PROMOTER ACTIVITY

Researchers have found that a unique regulation of *VWF* expression occurs by a specific circadian factor, BmalI, which modulates the optimal fluctuation and periodicity pattern of *VWF* expression within 24 hours in the plasma [70]. The 5'-UTR of the *VWF* promoter contains E-box sequences which are capable of binding to BmalI. BmalI is a component of a circadian transcriptional complex, and BmalI-deficient mice were shown to have increased thrombogenecity in their arteries due to *VWF* upregulation, increased subendothelial VWF deposits and increased release of VWF multimers into the plasma [70]. This study suggested that BmalI binds to the *VWF* promoter and maintains an optimal fluctuation of *VWF* expression within 24 hours, as seen in normal mice. The lack of BmalI, however, removes the repressive effect and activates the *VWF* promoter, resulting in the growth of thrombi, especially in high shear flow locations such as arteries [70].

VWF EXPRESSION IN DISEASES

Under normal physiological conditions, endothelial cells express and release an inventory of pro- and anti-coagulating factors, which regulate the tone and permeability of blood vessels [71]. VWF is a key initiator of thrombosis and is an acute phase protein which links the role of VWF with all of the diseases associated with thrombogenesis. While low levels of VWF are associated with VWD, excessive plasma levels of VWF are an independent risk factor for cardiovascular mortality and are observed in many pathophysiological conditions including atherosclerosis [72], myocardial infarction injury [73] [74], thrombotic thrombocytopenic purpura (TTP) [75, 76], diabetes [77-79], inflammation diseases [80, 81], sepsis [82], malaria [83] and cancers [84, 85]. Increased plasma levels of VWF could be due to either the exocytosis/release of WPBs from activated/injured endothelial cells, or transcriptional upregulation of the gene. Both or either of these processes can occur in response to micro-environmental signals [86].

VWF REGULATION IN RESPONSE TO STIMULATION

Although VWF levels in circulation were long believed to be regulated post-translationaly, primarily through the release of VWF from storage organelles by specific secretagogues (including TNFα, IL-6, and DDVAP, among many others) [87-89], it has been found that external stimuli can also mediate transcriptional upregulation of the *VWF* gene. Specifically, LPS [24] [49], irradiation [90], hypoxia [86], IL-11 [91], osmotic pressure (high sodium) [92], and glucose [79] were shown to upregulate *VWF* transcription.

VWF regulation in response to Lipopolysaccharide (LPS)

Endothelial gene activation in response to various stimulations have been shown previously [93]. Differential expression of the *VWF* mRNA isolated from endothelial cells of various organs suggests that the regulation of *VWF* promoter activity may differ in endothelial cells of different organs [94]. To understand whether the VWF synthesis level is fixed or changes according to signaling within vascular beds, mice responses to intraperitoneal administration of LPS in regard to *VWF* were analyzed [24, 49]. Results indicated that *VWF* mRNA levels were reduced by endotoxemia in all major organs [24]. Since activators are generally responsible for inducing gene activity, Liu *et al.* (2009) investigated the potential involvement of the GATA transcription factor in this scenario. The role of GATA was analyzed in transgenic mice carrying the 3 kb *VWF* promoter, which show endothelial expression of *LacZ* in brain, heart and skeletal muscles. The authors observed that in LPS-injected transgenic mice, although *LacZ* expression was significantly reduced in brain, heart and skeletal muscles endothelial cells, the level of reduction was similar whether transgenic mice were harboring a transgene with a wild type or mutated GATA binding site. This suggests that the reduction in *VWF* level as a result of LPS treatment is not mediated through GATA [49]. The roles of other activators such as NFY or ETS, however, need to be determined in response to LPS treatment.

The pathophysiological changes in *VWF* expression in response to stimuli have suggested a possible treatment for VWD (deficiency of the VWF). When heterozygous VWD dogs (showing VWF:Ag levels less than 60%) were treated with recombinant human Interleukin 11 (rhIL-11), *VWF* mRNA levels gradually increased

in their heart, aorta and spleen [91]. Consecutive rhIL-ll treatments also increased VWF activity and factor VIII levels in the plasma close to the normal range, which was sustained for 24 hours [91]. These findings indicate the importance of understanding the molecular bases of *VWF* heterogeneity in relation to the vascular bed, which could lead to potential therapeutic targets in various pathophysiological conditions.

The mechanisms of *VWF* upregulation in response to irradiation and hypoxia have been analyzed in detail, whereas *VWF* upregulation in response to high sodium and glucose has been studied in the context of its relevance to the pathophysiological consequences of this upregulation. In the following sections, the underlying mechanisms of *VWF* gene regulation in response to specific conditions are described.

VWF regulation in response to irradiation

Irradiation is a major element of cancer treatment, but damage to healthy tissues, specifically the vasculature of tissues surrounding the tumor, is an undesired side effect of this type of therapy [95, 96]. The increase in thrombus formation in patients undergoing irradiation therapy has led to the hypothesis that increased *VWF* expression may be a factor contributing to increased thrombosis [97]. In mouse kidney glomeruli, *VWF* expression and accumulation in the sub-endothelium were significantly increased by irradiation and were associated with a large population of leukocytes recruited to the cortex, which consequently had the potential to impair renal function due to inflammation and vascular damage [98, 99]. Analyses by Sporn *et al*. (1984) have demonstrated that irradiation of human umbilical vein endothelial

cells led to VWF release from WPBs. However, increased *VWF* mRNA in response to irradiation has also been demonstrated [90].

A detailed analysis by Bertagna *et al*. (2001) demonstrated the mechanism of increased *VWF* mRNA levels in response to irradiation in which irradiation directly upregulates VWF transcription, and this process is mediated through the NFY transcription factor that interacts with the CCAAT element. NFY was shown to be associated with two opposing chromatin modifying cofactors, histone acetylase PCAF and histone deacetylase HDAC [57]. Irradiation resulted in increased NFY-PCAF association and decreased NFY-HDAC association. This led to increased recruitment of PCAF to the *VWF* promoter, leading to increased acetylation of promoter-associated histone H4 and consequently, increased transcription [100]. Together, these analyses provided the first evidence of a molecular mechanism controlling the direct transcriptional upregulation of the *VWF* promoter in response to external stimuli in cultured endothelial cells. Additionally, the results clearly demonstrated the significant contribution of changes in epigenetic modifications of the *VWF* chromatin towards transient modulation of *VWF* transcription in response to stimuli. [57, 100, 101].

VWF regulation in response to hypernatremia

Dehydration from insufficient intake of water, prolonged vomiting or/and high fever has been shown to increase the plasma level of VWF, and thus, the risk of thrombosis [89, 102]. Some of these stimuli increase VWF plasma as they cause the exocytosis of WPBs and the release of preexisting stored VWF. However, transcriptional upregulation of various genes in response to certain cellular

physiological conditions can lead to increased *VWF* gene activity. In the kidney of normal mice, it has been shown that blood perfusion in the medulla contains higher NaCl levels compared with those of the cortex; at the same time, *VWF* expression is significantly higher in medulla than in the cortex [89]. This observation also links high salt with the high density of VWF protein in the kidney. However, significant levels of ADAMTS13 expressed by podocytes and deposited in the basement membrane of glomeruli in the kidney [103, 104] could be a protective mechanism from unnecessary thrombus generation [105]. *In vitro* investigations have shown that the level of hydration and salt intake by endothelial cells generated adaptive cellular responses to compensate for dehydration leading to hypernatremia [92].

The cellular response to dehydration is to induce NFAT5 activation, which is a master regulator of hypertonicity. Recent data have indicated a correlation between increased *VWF* and *NFAT5* mRNA levels and increased plasma sodium both *in vitro* and *in vivo* [92]. The *VWF* promoter sequence includes an NFAT5 binding site upstream of the transcription start site suggesting that *VWF* upregulation is mediated by NFAT5 in hypernatremia. Indeed, *in vitro* analyses of HUVEC grown in high salt media have shown that NFAT5 binding to its target site on the *VWF* promoter was significantly increased, triggering *VWF* transcription, production and secretion [92]. *In vivo*, significantly increased *VWF* mRNA levels in mice with mild dehydration were detected in lung and liver, and to a lesser extent in other major organs. In addition, NFAT5 upregulation in the spleen, thymus and kidney was detected. In dehydrated mice, there was microthrombus formation in the capillaries of liver as well as

increased D-dimer in the blood as a marker for disseminated intravascular coagulation [92].

These data implied that the role of increased VWF protein in inducing microthrombosis was a result of the dehydration of various organs and one of the potential consequences of cardiovascular complications. Dehydration has been associated with age [106], diabetes and kidney diseases [89], all of which have been linked with the increased plasma VWF and coagulability [92].

VWF regulation in response to hyperglycemia

Data from a long-term study of patients with type-2 diabetes mellitus have shown that high levels of VWF were significantly correlated with an increased risk of cardiovascular disease in this population [107]. Endothelial cells grown in media with a high glucose concentration were shown to have larger and a higher number of WPBs than in untreated cells [108]. Endothelial dysfunction and vascular abnormality have been observed in hyperglycemia, which is associated with diabetes mellitus [109].

Increased thrombotic complication in diabetes mellitus is a major risk factor for cardiovascular disease and mortality. Analysis of plasma VWF has shown various sizes of uncleaved VWF (dimer and small multimers) in a mouse model of diabetes mellitus (types 1&2)[107]. This observation was associated with low levels of miR-24 in both patients with diabetes and mouse models of diabetes [79]. miR-24 is a non-coding microRNA containing nucleotide sequences that are complementary to the 3'UTR of the *VWF* mRNA (90-106 bp). Recently, Xiang *et al.* (2015) have shown that miR-24 directly binds to and inhibits the translation of the *VWF* mRNA, thereby

negatively controlling the translation of the messenger RNA of target genes. Indeed, increased glucose diets in the diabetic mice model resulted in decreased miR-24 expression, which in turn relieved its repression of the *VWF* mRNA. *In vitro*, it has been shown that miR-24 down-regulation by high glucose leads to both excess translation of *VWF* mRNA and more than 1.5-fold secretion of stored ultralarge VWF from WPBs [79]. Furthermore, in mice fed a high glucose diet, increased VWF was associated with platelet aggregation in arteries, which was revealed by platelet-activated CD41 staining [79]. VWF protein is an acute phase protein that is released from activated endothelial cells in response to blood fluctuations, as in the diseases described above. These data suggest that *VWF* regulation not only occurs at the level of the promoter activity, but may also be regulated at the post-transcriptional level of VWF, which depends on the signaling responses from the microenvironments within different organs.

VWF regulation in pulmonary hypertension and in response to hypoxia

Pulmonary hypertension is associated with increased *VWF* expression in lung endothelial cells and their consequent injury [110]. Acute lung injuries, in turn, cause endothelial dysfunction, vascular remodeling and the release of VWF along with other pro-thrombic factors [111]. Pulmonary hypertension also can arise from hypoxia which potentially occurs in people affected by exposure to high altitude [112]. We have studied *VWF* expression in mice model that have developed pulmonary hypertension as a result of long-term hypoxia exposure. We observed that *VWF* expression in response to hypoxia is significantly and differentially increased in all major organs, with the exception of the kidney vasculature. Increased *VWF* expression

specifically in the lung is associated with acute lung injury, which is characterized by pulmonary hypertension [110, 113]. Furthermore, it has been shown that the pattern of VWF protein distribution in pulmonary hypertension induced by hypoxia was changed in the lung, moving primarily from larger vessels to microvessels. We have shown previously that transgenic mouse carrying the *VWF* promoter (747 bp linked to intron 51 and *LacZ* marker sequences) specific to brain and lung endothelial cells display *LacZ* expression colocalized with endogenous VWF primarily in the large vessels [63]. Using these transgenic mice to induce pulmonary hypertension, we demonstrated that exogenous *VWF* promoter was upregulated in heart and lung, consistent with endogenous *VWF* upregulation in response to hypoxia. In the hypoxic lung, however, increased *VWF* promoter activity was accompanied by *de novo* expression of *VWF* and *LacZ* in some microvessels as well as larger vessels, resulting in a redistribution pattern of *VWF* and *LacZ* expression.

We have demonstrated the molecular bases of hypoxia induced-*VWF* in the lung endothelial cells, and results are presented in detail in Chapter Two.

VWF expression in cancer cells

Aberrant platelet activation and aggregation have been found in many cancers and are thought to be early steps in the metastatic process [85]. The importance of VWF in platelet adhesion and thrombus formation suggests that VWF and VWF/platelet adhesion may play a role in cancer growth and metastasis [114]. An abnormal increase in VWF protein levels has been observed in the plasma of patients with different cancers, such as ovarian [115], bladder [116] and colon cancer [117]. It

has been shown that VWF fibers released from the activated endothelial cells of microvessels of tumors could induce thrombogenesis and facilitate cancer cells extravasation [118]. Since *VWF* expression has long been believed to be an exclusive property of endothelial cells and megakaryocytes, it has been assumed that the source of the increased VWF is the tumor vasculature or injured endothelial cells surrounding the tumor. Surprisingly, however, a small amount of published work has indicated that several cancer cell lines of non-endothelial origin can acquire the ability to express *VWF*.

Osteosarcoma is an aggressive tumor, which rapidly metastasizes. The analysis of multiple paired primary and metastatic tumor samples from osteosarcoma patients has indicated an increased *VWF* expression in progressive metastatic tumor samples compared to that in primary tumors. SAOS2, an osteosarcoma cell line, has been shown to express VWF protein, which could have a potential role in metastatic spreading [119]. It has also been observed that human colorectal SW480 cancer cells express *VWF*, which mediate the adhesion of these cells to collagen type III, thereby increasing their migratory ability. Antibodies against VWF have been shown to abolish this phenomenon [120]. In addition, *VWF* expression has been detected in HepG2 and BEL7402 hepatocellular carcinoma cells, which may facilitate hepatitis B virus replication and favor hepatocarcinoma metastasis [121]. It was suggested that *VWF* expression may cause STAT3 pathway activation, leading to the induction of MMP2 and MMP9, which modifies the extracellular matrix and favors tumor growth and metastasis in hepatocarcinoma [121].

The mechanisms underlying *VWF* gene activation in these cancer cells of non-endothelial origin are unknown. Furthermore, it is unclear how cancer cells take advantage of *VWF* expression to increase their growth or metastasis.

CONCLUSION

Among endothelial genes, *VWF* is a well-known representative of heterogeneity, which shows differential patterns of expression throughout the vasculature. A complex mechanism is invoked in regulation of *VWF* transcription in endothelial cells, in response to external stimuli, and in specific circumstances in cancer cells of non-endothelial origin. The mechanism employs a cohort of transacting factors that, although generally not endothelial-specific, exhibit both cell-type and vascular bed-specific activity. These factors include repressors as well as activators, and their cell-/and or vascular bed-specific function may stem from their association with cofactors, other transacting factors, and/or post-translational modifications. These regulators also participate in the regulation of *VWF* in response to external stimuli and thus may contribute to pathological consequences of unregulated *VWF* expression, leading to thrombogenic consequences.

CHAPTER 2

HYPOXIA RESULTS IN UPREGULATION AND *DE NOVO* ACTIVATION OF VON WILLEBRAND FACTOR EXPRESSION IN LUNG ENDOTHELIAL CELLS[1]

INTRODUCTION

Von Willebrand Factor (VWF) is a glycosylated protein that is expressed exclusively in endothelial cells and megakaryocytes. This highly restricted expression pattern is used to distinguish endothelial cells from other cell types [122]. However, VWF is not uniformly, expressed by endothelial cells of all vascular beds [2, 3]. In lung, VWF is highly abundant and uniformly expressed in vessels of diameters 40 μm or above; however, expression in capillaries and microvessels is low and displays a mosaic pattern [8, 123-125]. Both low and high levels of VWF lead to diseases [87, 126-130].

Qualitative and quantitative deficiencies of VWF lead to the most common inherited bleeding disorder: von Willebrand Disease (VWD) [130]. Conversely, excessive circulating levels of VWF are an independent risk factor for cardiovascular mortality [131]**.** Increased *VWF* expression specifically in lung is associated with acute lung injury (ALI) which has a degree of pulmonary hypertension (PH) among its characteristics [88, 111, 113, 132-135]. Pathophysiological changes in pulmonary vasculature that are associated with ALI include endothelial dysfunction as well as vascular remodeling and increased vascular tone, which consequently alters the production of endothelial derived molecules leading to an imbalance of factors that

[1] Arterioscler Thromb Vasc Biol. 2013;33:1329-1338

regulate vascular tone as well as initiation of inflammation, coagulation and thrombogenecity [111]. A prothrombotic state is favoured with increased production of procoagulant molecules such as VWF. Despite well-established association of increased VWF with PH, its mechanism has not been determined. Increased VWF in circulating plasma may result from the release of VWF protein from storage organelles (Weible-Palade bodies) [136], and/or regulation of *VWF* gene expression at various levels including posttranscriptional or transcriptional activity.

In vitro and *in vivo* analyses of the mechanism of transcriptional regulation of *VWF* gene expression has led to identification of distinct regions that are necessary for activation in vascular endothelial cells of specific organs, as well as identification of a number of transactivators that participate in regulation of *VWF* promoter activity [25, 39, 41, 42, 44, 46, 47, 49, 55, 57, 59-63]. A proximal *VWF* promoter region spanning sequences -487 to +247 is necessary for endothelial specific activation but targets expression only to brain vascular endothelial cells *in vivo*, while mutation of an NFI binding site that functions as a repressor in this promoter fragment results in the promoter activation in lung and heart endothelial cells as well as brain [39, 55, 59]. Additionally we had previously reported identification of a DNase 1 hypersensitive region in intron 51of the *VWF* gene that was shown to be necessary for transcriptional activation in endothelial cells of lung in transgenic mice [63]. Our characterization of the region that regulates the *VWF* transcription specifically in lung and generation of transgenic mice that express *LacZ* gene under the regulation of the *VWF* proximal promoter and the intron 51 sequences (HSS-VWF-LacZ and VWF-LacZ-HSS) in brain and lung endothelial cells [63] provided the opportunity to explore whether

pulmonary hypertension generating conditions such as hypoxia results in modulation of the *VWF* transcriptional activity in lung endothelial cells and exploration of the underlying molecular mechanism.

MATERIALS AND METHODS

RNA preparation and analyses

RNA were prepared from cultured cells (hypoxia treated and controls) and harvested organs (lung, brain, heart, liver and kidney) of control and hypoxia exposed transgenic mice and subjected to quantitative reverse transcription-polymerase chain reaction (RT-PCR) to detect mouse endogenous *VWF, GAPDH, Tie-2,* and *LacZ* transgene as previously described [55]. Primers list is presented in table 2-1.

VWF, NFIB, YY1, LacZ and isolectin binding detection using immunofluorescence and confocal microscopy

Analyses of mice organs: Harvested organs (lung, brain, heart, liver and kidney) from hypoxia exposed and control transgenic mice were either used to generate formalin embedded blocks for immunohistochemical staining with hematoxylin and eosin or frozen in OCT for immunofluorescence analysis as previously described [55, 63]. Immunofluorescence analyses were generally performed as previously described [55] with minor modifications are described below, antibodies used were as follows: anti goat polyclonal β-galactosidase (Abcam Cat#ab-12081-100), two antibodies for detection of VWF [a polyclonal rabbit anti-VWF antibody (SIGMA-ALDRICH Cat# F3520) and a sheep FITC preconjugated anti-VWF antibody (Abcam-ab8822), rabbit anti YY1

antibody (SANTA CRUZ, SC-1703). The secondary antibodies were donkey anti-goat Alexa 594 (cat#A11058) and donkey anti rabbit Alexa 488 (cat# A-21206) (all from Invitrogen). For colocalization experiments, anti-β galactosidase antibody (goat anti-β galactosidase, Abcam, Cat#ab-12081-100) and the FITC preconjugated sheep anti VWF antibody were used, thus requiring only the use of one secondary antibody namely donkey anti goat Alexa 594 (Cat#A11055). Primary antibodies were used at dilutions of 1:50 to 1:250 and secondary antibodies were used at 1:1000 to 1:2000 dilutions.

For detection of isolectin binding, isolectin GS-IB4 from Griffonia simplicifolia conjugated with Alexa Fluor® 568 (Invitrogen) diluted (1:200) in 5% fish gelatin was used. Sections were fixed with acetone (10 min. at 4^{o}C) and blocked with 5% fish gelatin (1 hour at room temperature) and then incubated with diluted isolectin at 4^{o}C overnight. Sections were washed with high salt PBS and incubated with sheep FTCI preconjugated anti-VWF antibody and subjected to confocal analyses.

Analyses of cultured cells: Cells were fixed with 4% paraformaldehyde, permeabilized with 2% Triton X100, and blocked with 5% donkey sera plus 2.5% fish gelatin in PBS for one hour at room temperature. Double staining were performed using antibodies to detect NFIB (rabbit anti-NFIB, Cat#ARP32716-T100, AVIVA & systems Biology), sheep FITC preconjugated anti-VWF antibody (Abcam-ab8822), and rabbit anti YY1 antibody (SANTA CRUZ, SC-1703). The secondary antibody was donkey anti rabbit Alexa 594 (INVITROGEN cat# A21207). Primary antibodies were used at a 1:250 dilution and secondary antibody at a 1:1000 dilutions. Confocal microscopy analyses and imaging were performed as previously described [55].

Platelet activity under shear stress

Laminar flow adhesion assays were done using 35 mm tissue-culture plates as the lower surface of a parallel-plate laminar flow chamber (127μm gap; Glycotech, Rockville, MD). The chamber was mounted on the stage of an inverted phase-contrast microscope (Leica DM IRB, Leica Microsystems, Richmond Hill, ON), and platelet/endothelial cell interactions were observed through a 20x objective and captured using a CCD camera (Pixelink, Vitana Corporation, Ottawa, ON) at 6 frames/sec for 4-10 minutes periods. Endothelial cells were grown on a 35 mm glass coverslip. Endothelial cells were exposed to shear stress for about 30 seconds and then platelets were perfused over the endothelial cells monolayer while the flow rate was gradually increased to 1 dyne/cm2 and was kept constant throughout the assay by perfusion of medium containing platelets. Analysis of platelet activity was done by counting the platelet chains in 5 different fields of view between 9-10 minutes of shear stress. To confirm platelet activity and binding to endothelial monolayer, after 10 minutes of shear stress the flow chamber was carefully removed and the endothelial cells -platelet cocultre was fixed with 2% paraformaldehyde solution and stained for mouse anti human cd42b conjugated to PE (BD Biosciences- Canada), or sheep FITC preconjugated anti-VWF antibody plus cd42b conjugated to PE . Endothelial cells were located by nucleus staining using DAPI in the mounting medium. The images were acquired using a LSM 510 confocal microscope (Zeiss, Toronto, ON).

Chromatin Immunoprecipitation and western blot analyses:

Chromatin immunoprecipitation using anti NFIB and YY1 antibody and control IgG were performed as previously described [55]. For Western blot analyses protein were

prepared from nuclei or from total cell lysate and subjected to Western blot analyses using standard techniques as described [101]. Antibodies used for detection of NFIB (Rabbit anti NFIB, Cat# ARP32716-T100, AVIVA system biology) and YY1 were used for immunofluorescent analyses as described above. The following antibodies were used to detect VWF protein, tubulin (mouse anti-β1 tubulin, Cat#05-661, Upstate) and lamin (rabbit anti-laminB1, Cat# SC-20682, SANTA CRUZ). The secondary antibodies to detect tubulin and lamin were goat anti-mouse-HRP, (Cat#1858413, PIERCE) and goat anti-rabbit-HRP, (Cat#12-348, Millipore) respectively.

Transfection with siRNA : Monolayer of LMEC were transfected using TransIT® -2020 transfection reagent (Mirus Bio LLC, Madison, WI) as suggested by manufacturer. The si RNAs used were as follows:

Hs _YY1_3 Target Sequence 5'-ATGCCTCTCCTTTGTATATTA-3' (QIAGEN Cat. no.: SI00051926)

Hs_VWF_4 Target Sequence 5'-AACATGGAAGTCAACGTTTAT-3' (QIAGEN Cat. no.: SI00011830)

ALLStars Neg. Control siRNA used as non silencing siRNA (QIAGEN Cat. no.: I027281)

Table 2-1

Primers for qRT-PCR	Company & Cat#
VWF	**QIAGEN-QT00051975**
NF-IB	**QIAGEN-QT00048139**
VEGF	**QIAGEN-QT01036861**
GAPDH	**QIAGEN-QT01192646**
YY1	**QIAGEN-QT00052738**
Beta-Galactosidase	**QIAGEN-QT01976079**
Tie-2	**QIAGEN-QT02050895**

Primers for CHIP assay	sequences
Human VWF intron 51-YY1 binding region	
Forward	**CGCAGGGAAGAGAAGGGAAATAAAC TGG**
Reverse	**GACTGATCTTCAAGAATTGTGGCCC**
Human VWF NF1-binding region	
Forward	**TAGGCTTGTGGCCAAGACCTTCAT**
Reverse	**CGGTCCTGGCCCTGACACAAA**

Generation and hypoxia exposure of transgenic mice and cultured cells

Transgenic mice expressing *LacZ* gene under the regulation of the *VWF* promoter (sequences -487 to +247) and DNase 1 hypersensitive region of the intron 51 (referred to as HSS) positioned either upstream (HSS-VWF-LacZ) or downstream (VWF-LacZ-HSS) of the *VWF* promoter were generated and analyzed for *LacZ* expression pattern as previously described [63]. Transgenic mice were exposed to hypoxia by placing them in a hypoxic chamber (BioSpherix) to establish PH as previously described [137] with minor modifications. Mice were maintained in the hypoxic chamber for 35 days and the oxygen level was reduced from 18 to 10% gradually for the first two weeks and maintained at 10% for 3 weeks. Establishment of PH in selected mice was determined by echocardiogram as previously described [137-139]. The Health Sciences Animal Policy

and Welfare Committee at the University of Alberta approved all animal housing and experimentation. Human Lung Microvascular Endothelial Cells (LMEC) were obtained from Lonza (CC-2527 HMVEC-L) and were grown as described by manufacturer. For hypoxia treatment LMEC at about 80% confluence were placed in hypoxia chamber (Ruskinn Invivo$_2$ Hypoxia- Gas Mixer Q), exposed to 1% oxygen for 1 hour and then maintained at normoxic condition for periods of 1-72 hours.

Statistical analyses

Data are given as mean (SD) and statistical analyses used the paired *t* test.

RESULTS

Hypoxia results in upregulation of *VWF* transcription and its redistribution from primarily large vessels to microvasculature in lung

To determine whether the increased levels of VWF associated with pulmonary hypertension is regulated at the transcriptional level, we chose to take advantage of our previously generated transgenic mice (HSS-VWF-LacZ and VWF-LacZ-HSS) that were used to demonstrate the lung-specific enhancer activity of the *VWF* intron 51 sequences (referred to as HSS) [63]. In these transgenic mice *LacZ* expression was regulated through the activities of the proximal *VWF* promoter in combination with the HSS region positioned either upstream (HSS-VWF-LacZ) or downstream (VWF-LacZ-HSS) of the proximal promoter. The presence of HSS, irrespective of its position, was shown to target the expression of *LacZ* gene to endothelial cells of lung as well as brain, while the proximal *VWF* promoter alone targeted *LacZ* expression only to brain endothelial cells

[63]. Lung specific *LacZ* expression was not uniform in all endothelial cells of the vasculature and this was consistent with previous reports that endogenous *VWF* expression is not uniformly detected in all endothelial cells of the lung vasculatures [63] and is primarily detected in larger vessels [140].

We aimed to determine whether exposure of these mice to hypoxia as a model of PH generating condition, is correlated to quantitative and/or qualitative changes in the activity of the *VWF* transcriptional regulatory sequences, reflected in the levels and pattern of *LacZ* expression in these mice. Towards this goal, we chose a well-established model of hypoxia-induced PH, which results from prolonged (3-5 weeks) exposure of mice to progressively reduced oxygen levels [137]. Transgenic mice (6 mice of HSS-VWF-LacZ and 3 mice of VWF-LacZ-HSS, all aged approximately 3 months) were exposed to hypoxia for 35 days, with equal numbers of aged matched littermates for each line maintained as control. The establishment of PH in hypoxia treated mice was determined by Doppler Echocardiography that was used to calculate pulmonary artery acceleration time (PAAT) as previously described [138, 139, 141] and by medial hypertrophy of vessels demonstrated in hematoxylin and eosin staining of lung sections (Fig. 2-1). PAAT is a standard clinically used parameter that correlates inversely to mean pulmonary artery pressure (i.e. the lower the PAAT the higher the mean PA pressure). We showed a significant ($P<0.05$) decrease in PAAT in the hypoxic mice compared to age matched normoxic controls. In addition we showed histological evidence of pulmonary vascular remodeling (medial hypertrophy) in the hypoxic mice. Taken together these data confirm that our hypoxic exposure was effective in terms of inducing pulmonary hypertension.

Lungs and other organs (brain, heart, liver and kidney) were harvested from hypoxia treated and control mice and processed for immunofluorescence analyses to detect endogenous VWF, CD31 (as a marker of micro and macrovascular endothelial cells) and LacZ protein expression, as well as binding to isolectin GS-IB4 from Griffonia simplicifolia (as a marker of microvascular endothelial cells [142]). Harvested organs were also processed for RNA analyses (quantitative RT-PCR) to detect endogenous *VWF, GAPDH, Tie2* and transgene *LacZ* transcripts. Immunofluorescence analyses to detect endogenous VWF and CD31 protein levels demonstrated VWF expression in primarily large vessels of lung in control mice. CD31 expression marked both micro and macrovascular endothelial cells. In lungs of control mice VWF and CD31 colocalization was detected in endothelial cells in large vessels, but not those of small vessels (Fig. 2A). In contrast, colocalization of VWF and CD31 was demonstrated in a significant number of small vessels as well as large vessels in lungs of mice exposed to hypoxia (Fig. 2-2A). Isolectin GS-IB4 staining, which preferentially marks small vessels, colocalized with VWF in significantly higher number of microvessels in lungs of hypoxic mice compared to control mice (Fig. 2-2B and C). These results demonstrated a significant change in distribution pattern of VWF with increasing number of lung microvessels exhibiting VWF expression in response to hypoxia.

Analyses of *LacZ* transgene expression in these mice similarly demonstrated a redistribution of *LacZ* transgene expression in lungs from primarily larger vessels in control to microvessels in hypoxia treated mice (Fig. 2-2F). Co-localization immunofluorescence analyses demonstrated that *LacZ* transgene expression colocalized to endothelial cells expressing endogenous *VWF* in both control and hypoxia treated mice

(Fig. 2-2G). Endogenous *VWF* and transgene *LacZ* RNA analyses demonstrated that redistribution patterns of expression were accompanied by an approximately 2 and 1.6 fold increase in the levels of *VWF* and *LacZ* mRNA, respectively, in lungs of hypoxia-treated mice compared to control (Fig. 2-2D and E). *VWF* mRNA levels were normalized to *Tie-2* as well as *GAPDH* which demonstrated that this increase was independent of potential increase in vascular density that may result from hypoxia/PH [2, 143-145].

Together these results demonstrate that hypoxia leads to activation of *VWF* transcription (endogenous and exogenously expressed *VWF* promoter fragment) in a subset of microvascular endothelial cells of the lung that do not normally express this gene.

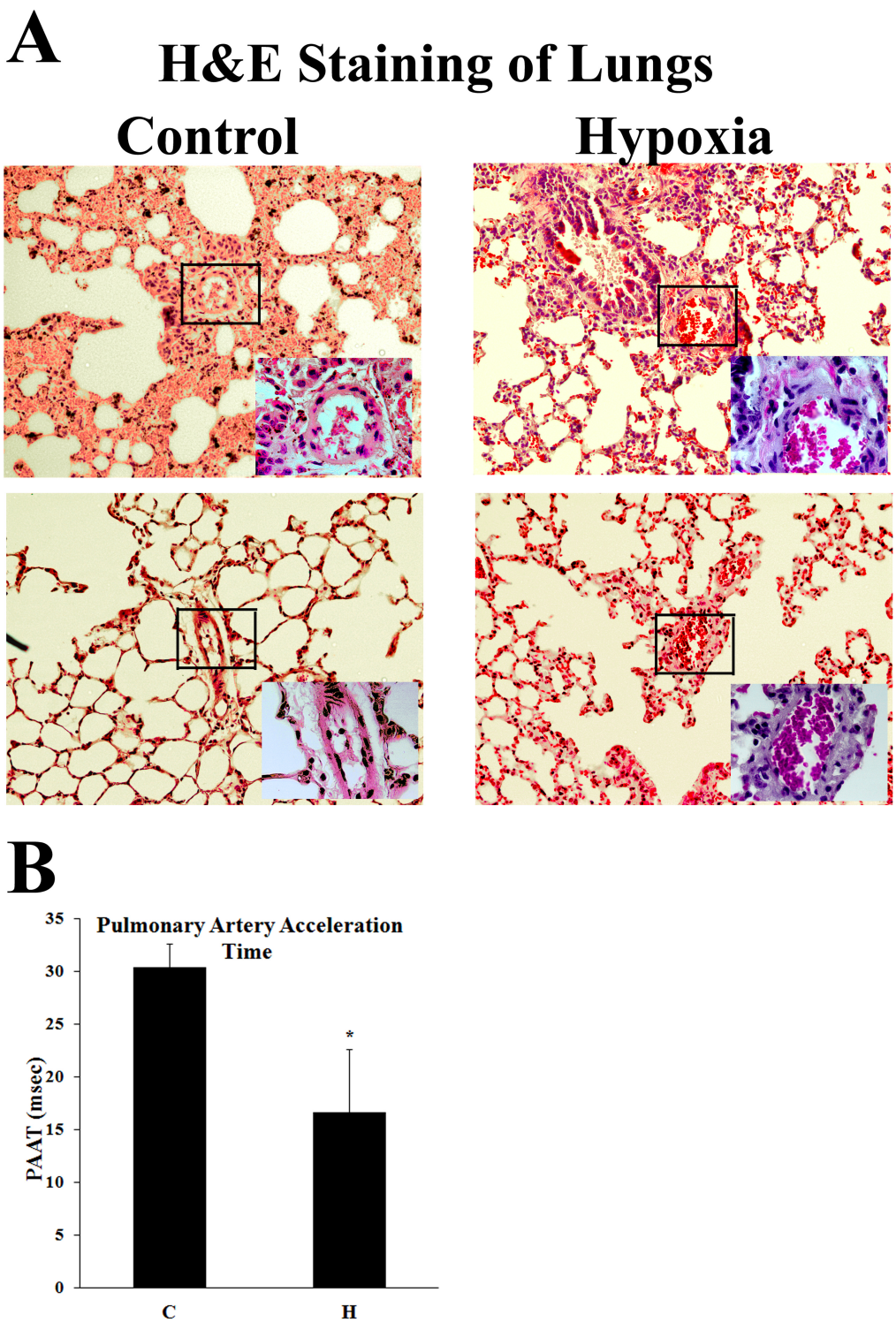


Figure 2-1- Chronic hypoxia exposure results in pulmonary vascular remodeling and reduced pulmonary artery acceleration time (PAAT) in mice. (A) Hematoxylin and eosin staining of 5 μm sections of lung from control (c) and hypoxia exposed mice

shown at magnifications 200X and inset (1000X). The inset is the higher magnification (1000X) of the area selected in rectangle representing pulmonary arteries and demonstrating medial hypertrophy in vessels of hypoxic mice compared to control. Images are representative of two independent mice from control and hypoxia treated groups. **(B)** Doppler Echocardiography was used to calculate Pulmonary Artery Acceleration Time (PAAT) represented in the bar graph as milliseconds (msec) in 6 control and 9 hypoxia treated mice. A significant (*$P<0.05$) decrease in PAAT in the hypoxic mice compared to age matched controls was observed.

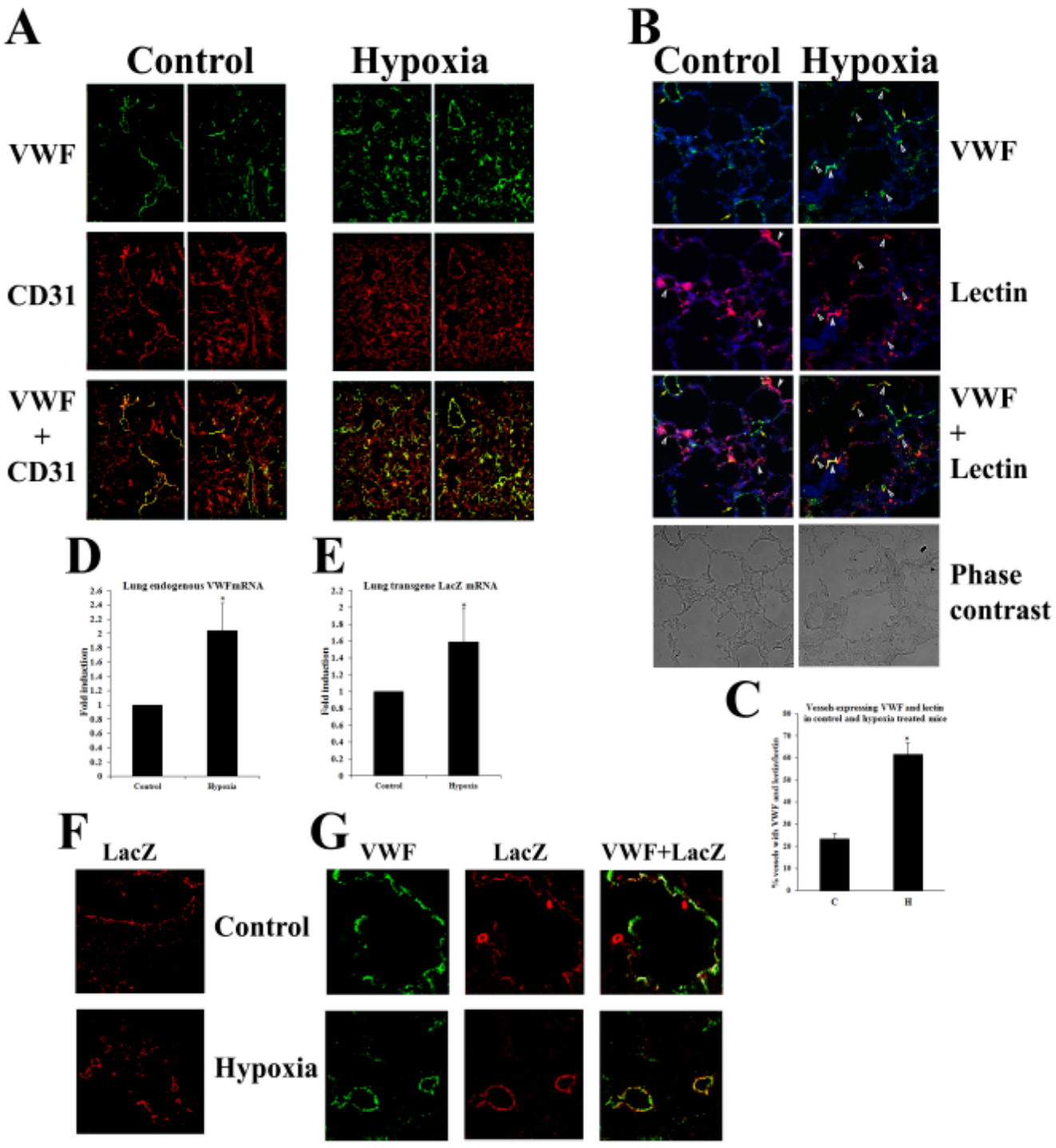


Figure 2-2. Analyses of *VWF* and *LacZ* expression in lungs of control and hypoxia-exposed transgenic mice. Immunofluorescence and confocal microscopy analyses of CD31 and LacZ were performed on 5 μm OCT frozen sections of lungs from control and hypoxia exposed VWF-LacZ-HSS transgenic mice as previously described [55, 63].

Staining for isolectin GS-IB4 binding was performed similarly with minor modifications as described in methods. **(A)** Sections were treated with preconjugated sheep anti-VWF antibody (1:250 dilution), rat anti-CD31 antibody (1:200 dilution) and donkey anti-rat Alexa 594 (1:1000 dilution) followed by confocal microscopy analyses as previously described [55]. Tile scanning was performed to provide a composite image of a region covering approximately 0.9 mm^2 of lung sections. VWF expression was detected by green , CD31 by red , and colocalization by yellow fluorescent signal. Magnification 200X. **(B)** Sections were treated with Alexa Fluor® 568 conjugated isolectin GS-IB4 (1/200 dilution) and anti-VWF antibody and subjected to confocal microscopy analysis as described for panel A. Panel showing isolectin GS-IB4 binding (detected as red fluorescent signal) is labelled as Lectin. Colocalization of VWF and lectin is detected as yellow fluorescence in panel Lectin + VWF. Yellow arrows indicate larger vessels that express VWF (green) but do not demonstrate isolectin GS-IB4 binding and white arrows indicate smaller vessels that exhibit lectin binding. The bottom panel represents phase contrast representation of the sections. Magnification 200X. **(C)** Quantification of vessels that express both VWF and lectin binding as percentage of total vessels that exhibit lectin binding in 10 fields of view (*$P < 0.05$). **(D and E)** RNAs (1 μg) prepared from lungs of control and hypoxia treated transgenic mice [VWF-LacZ-HSS(3 mice) and HSS-VWF-LacZ (6 mice)] were subjected to reverse transcription and real-time PCR analyses to detect endogenous *VWF* and *LacZ* transgene mRNAs, as well as *GAPDH* and *Tie2* mRNAs. Graphs represent fold induction of the levels of *VWF* and *LacZ* normalized to *Tie2* and *GAPDH* in hypoxia treated compared to aged matched control mice (*$P < 0.05$). **(F)** Sections were treated with anti-β-galactosidase antibody (goat polyclonal 1:50 dilution) and donkey anti goat Alexa 594 (1:1000) to detect LacZ (red) in the left panel (Magnification 200X). **(G)** Immunofluorescence and confocal microscopy analyses to detect VWF and LacZ colocalization were performed as described for panel A**,** except that goat anti-β-galactosidase antibody was used (as described for panel F) in conjunction with anti-VWF antibody. Red and green fluorescent signals represents LacZ and VWF respectively and yellow represents colocalization. Magnification 400X. All immunofluorescence results are representative of 2-4 independent experiments from 2 different mice for each control and hypoxia treatment.

Endogenous *VWF* mRNA is upreguleted in brain, heart and liver but not kidney of mice exposed to hypoxia

To determine whether increased *VWF* mRNA is a general response of all major organs to hypoxia (and/or developed pulmonary hypertension), we performed similar RT-PCR analyses as described above for lungs, in brains, kidneys, livers and hearts of hypoxic and control mice. The results demonstrated that endogenous *VWF* mRNA in brains, hearts and livers but not kidneys of hypoxic mice were significantly increased compared to control (Fig. 2-3A). Since in these transgenic mice *VWF* promoter directed the *LacZ* expression to the brain as well as lung, we performed RT-PCR analyses of the *LacZ* transgene in brains of hypoxic mice. The results (Fig. 2-3B) demonstrated that although there appeared to be some increase in *LacZ* transgene expression in the brain, results were highly variable among the number of hypoxic animals that were analyzed and not statistically significant. Next, to determine whether hypoxia resulted in *de novo* activation of the transgene VWF-LacZ-HSS, we analyzed *LacZ* expression in hearts, livers and kidneys of control and hypoxia treated mice. As expected, RT-PCR analyses on hearts, livers and kidneys demonstrated that there was no *LacZ* transgene expression in any of these organs in control mice, and neither was there detectable *LacZ* expression in livers and kidneys of hypoxic mice. However, in heart, detectable levels of *LacZ* mRNA expression were observed in response to hypoxia exposure in both lines, (although most consistently in VWF-LacZ-HSS) of transgenic mice (Fig. 2-3C).

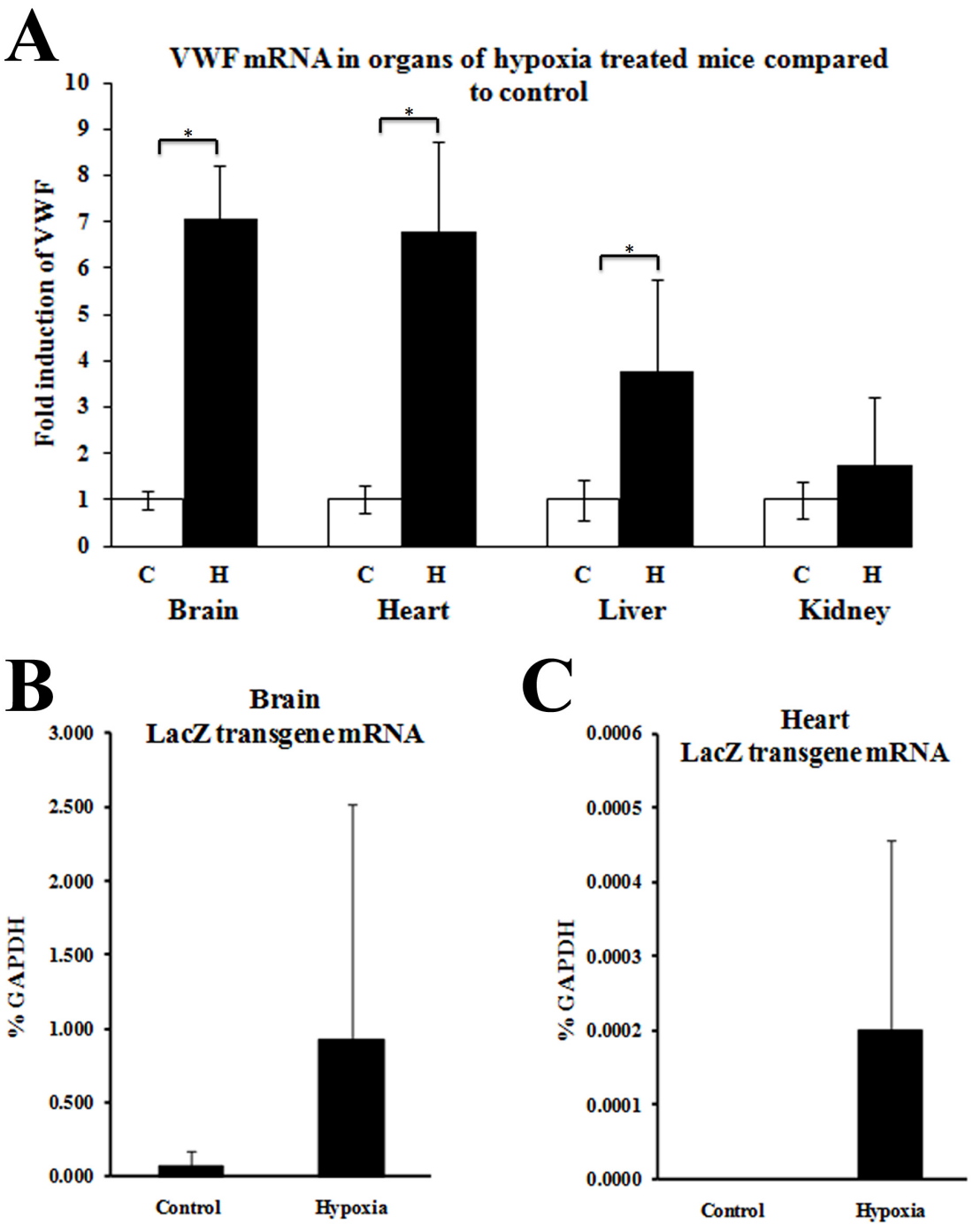


Figure 2-3. Real-time PCR analyses of endogenous *VWF* mRNA and *LacZ* transgene in organs of control and hypoxia treated transgenic mice. RNAs (1 μg) prepared from various organs (brain, heart, kidney and liver) of transgenic mice were subjected to reverse transcription and real-time PCR analyses to detect endogenous VWF and

GAPDH mRNA, as well as *LacZ* transgene. *VWF* or *LacZ* levels were normalized to *GAPDH*. There were no detectable levels of *LacZ* expression in livers and kidneys of either control or hypoxia treated mice, thus no graphs are presented for these analyses. *LacZ* expression was detected in the brain and heart of hypoxia treated mice as shown. **(A)** For endogenous *VWF* expression, results represent the averages of RNA from organs of 9 control and hypoxia treated mice [HSS-VWF-LacZ (6 mice) and VWF-LacZ-HSS (3 mice) (*P<0.05). **(B and C)** For *LacZ* transgene mRNA, results from three mice of VWF-LacZ-HSS line are shown. Induction in the brain was not statistically significant and statistical analysis for the heart was not performed since there were no detectable levels in control to perform paired *t* test.

LacZ transgene expression is activated in the heart of transgenic mice exposed to hypoxia

Next we determined whether observed *LacZ* mRNA in hearts of hypoxia treated mice correlate with LacZ protein expression and endothelial localization of this protein. Additionally, we determined whether the observed increase in endogenous *VWF* mRNA is accompanied by vascular redistribution of the VWF protein (as observed for lung). For these analyses we performed immunofluorescence confocal microscopy to detect endogenous VWF and LacZ is brains, hearts, livers and kidneys of control and hypoxia treated mice as described above for lung. The results demonstrated that there were no significant changes in cellular pattern of endogenous VWF and LacZ transgene expression in brain (Fig. 2-4A). Similarly there were no significant changes in the pattern of VWF expression in livers and kidneys, and consistent with RNA analyses there was no detectable *LacZ* expression in these organs in either control or hypoxia treated mice (Fig. 2-4C and D). In heart however, consistent with the results of RNA analyses, *LacZ* expression, which was not detected in control mice, was clearly detectable in the heart vessels and co-localized to *VWF* expressing endothelial cells (Fig. 2-4B). These results demonstrate that hypoxia treatment induces activation of the VWF-HSS promoter fragment in heart endothelial cells, which in control condition do not support the activity of this promoter fragment. The results demonstrate that hypoxia neither significantly alters distribution pattern of endogenous VWF in brain, heart, liver and kidney, nor that of LacZ transgene in brain, liver and kidney, while in heart it leads to *de novo* activation of VWF-LacZ-HSS transgene expression in endothelial cells.

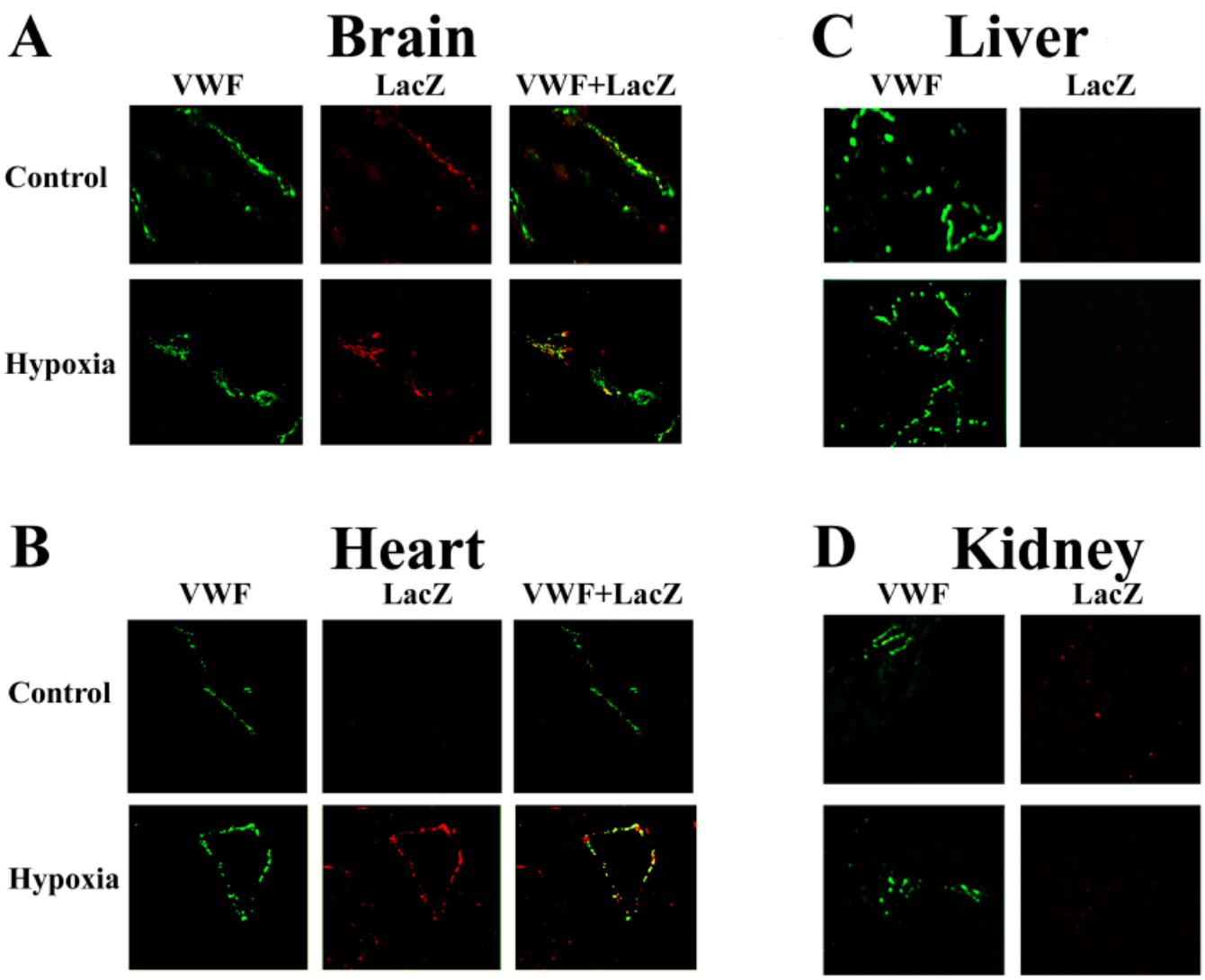


Figure 2-4. Immunofluorescence analyses and colocalization of VWF and LacZ expression in organs of control and hypoxia treated mice. OCT frozen sections of brains, hearts, livers and kidneys from control and hypoxia exposed VWF-LacZ-HSS mice were analyzed as described in Figure 2E for lung. Results are representative of 2 independent experiments from 2 different mice for each control and hypoxia treatment (magnification 400X).

VWF gene is upregulated in cultured lung microvascular endothelial cells in response to hypoxia

Significant increase in *VWF* expression and its redistribution specifically in lung vasculature demonstrated that endothelial cells of this organ have a distinct response to hypoxia and/or conditions generated as a result of hypoxia (i.e. PH). To determine whether hypoxia directly targets *VWF* gene regulation, and if so to explore the mechanism of its action, we used human lung microvascular endothelial cells (LMEC) in culture and performed analyses of *VWF* expression in these cells in response to hypoxia. Cells were maintained as control or exposed to hypoxic conditions (1% oxygen compared to normal 21%). Following 1 hour of hypoxia treatment, cells were transferred to normoxic conditions and RNA was collected at various time points (1 - 72 hours). RT-PCR analyses of *VWF* transcripts demonstrated that within 1 hour (1 hour of normoxia after hypoxia treatment) an increase in *VWF* mRNA levels was detected; however, this increase reached statistical significant ($P<0.05$) and was maximal at 48 hours (Fig. 2-5A). As a control we also determined the levels of *VEGF* transcript (a previously well-established target of hypoxia [146]) and demonstrated that at 48 hours post hypoxia treatment, *VEGF* mRNA levels were also significantly increased and then declined by 72 hours (Fig. 2-5B). Next we performed Western blot analysis of the VWF protein from cellular lysates as well as those secreted in media and demonstrated that the increase in *VWF* mRNA at 48 hours was concomitant with a significant increase in intracellular and conditioned media released VWF protein levels (Fig. 2-5C, D and E). These results demonstrated that hypoxia directly targets regulation of *VWF* and results in its increased production both at protein and RNA levels.

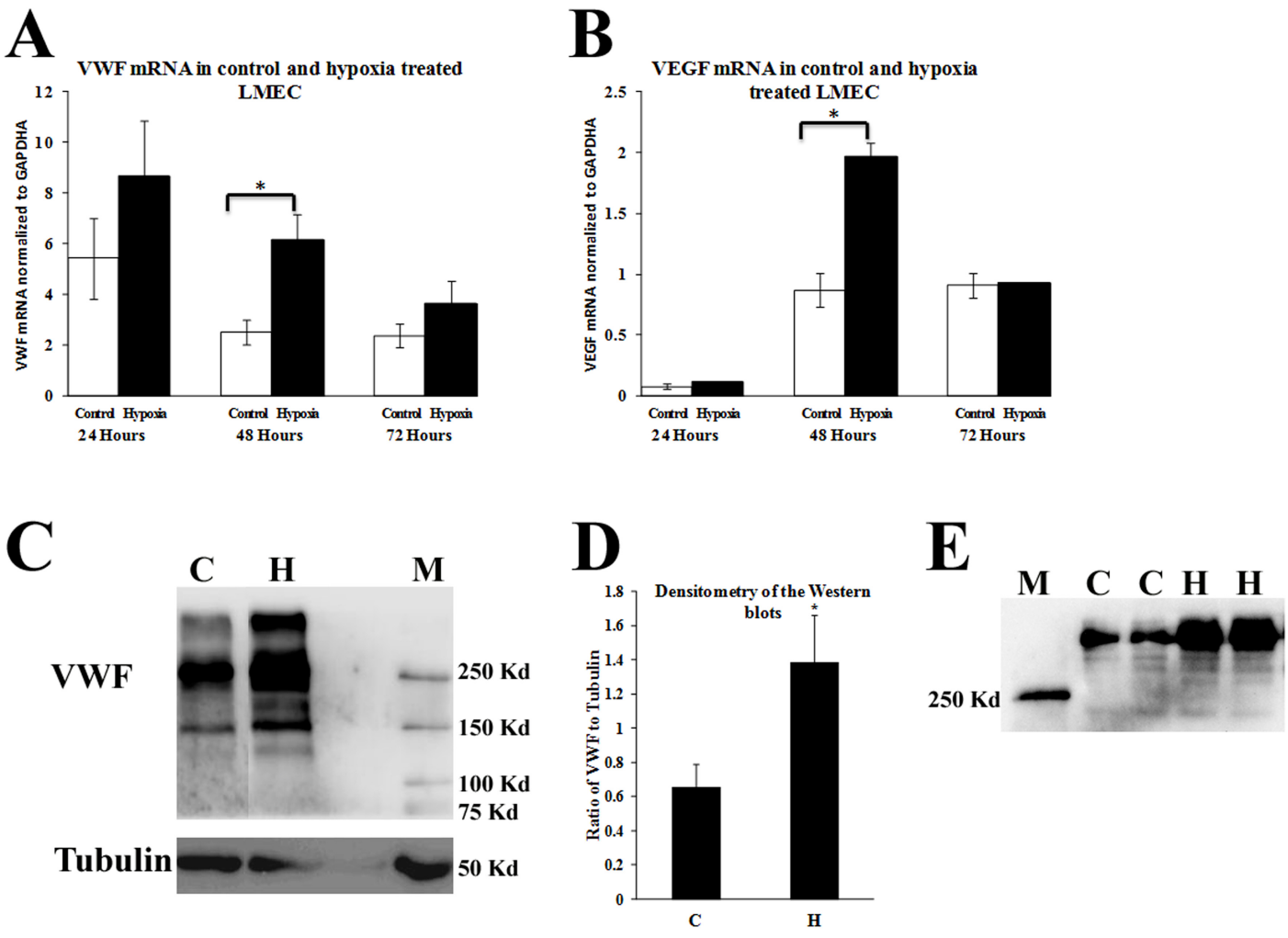


Figure 2-5. Analyses of *VWF* expression in control and hypoxia treated LMEC in culture. LMEC were maintained as control or exposed to hypoxia (1% oxygen for 1 hour), followed by 24, 48 and 72 hours of normoxia. **(A and B)** RNAs (150 ng) were collected and subjected to Real-time PCR analyses to detected *GAPDH* and **(A)** endogenous *VWF* mRNA as well as **(B)** *VEGF* as described in methods. *VWF* and *VEGF* levels were normalized to *GAPDH* and demonstrated significant (*P<0.05) increase at 48 hours post hypoxia induction. **(C)** Total protein (50 μg) collected from cells at 48 hour time point (1 hour hypoxia followed by 48 hours normoxia) and corresponding control were exposed to Western blot analyses to detect VWF and tubulin. **(D)** Densitometry tracing of the VWF signal compared to tubulin from three independent blots indicated significant (*P<0.05) increase in VWF signal intensity in hypoxia compared to control. **(E)** Conditioned media (30 μl) collected from

control and hypoxia treated cells at 48 hour time point (1 hour hypoxia followed by 48 hours normoxia) were directly used in Western blot analyses to detect secreted VWF as described for panel C. VWF signal detected at above 250 kDa compared to Western blot analyses of lysate may be indicative of multimerized VWF that is secreted from the cells into the cultured media.

Transcription factors NFI and YY1 participate in upregulation of *VWF* gene in response to hypoxia

HIF-1 transcription factors are well known regulators of gene expression in response to hypoxia and although we cannot rule out their participation in regulation of the *VWF* gene in response to hypoxia, our initial analyses of regulatory DNA sequences of the *VWF* gene did not reveal a hypoxia responsive element. However, analyses of *VWF* gene expression *in vitro* and *in vivo* previously demonstrated that several transacting factors positively and negatively participate in its transcriptional regulation. Specifically, we demonstrated YY1 association with the *VWF* intron 51 sequences that target lung-specific activity of the *VWF* promoter [63]. Also recently we have demonstrated that NFIB transcription factor functions as repressor of *VWF* promoter in lung and heart endothelial cells *in vivo* [55].

Our observation that hypoxia exposure resulted in *VWF* transgene (VWF-LacZ-HSS) *de novo* activation in heart vascular endothelial cells (Fig. 2-4B) as well as lung microvascular endothelial cells, raised the possibility that NFIB levels and/or activity may be targets of hypoxia. Additionally, specific association of YY1 with lung-specific enhancer sequences (HSS) of the *VWF* gene suggested that possible modification of YY1 activity may also contribute to *VWF* transcription regulation in response to hypoxia specifically in lung. To test the above hypothesis we first determined whether levels of *NFIB* and *YY1* are modified in LMEC in response to hypoxia. RNA prepared from control and hypoxia exposed LMEC at 24, 48 and 72 hours were analysed to detect *NFIB* and *YY1* mRNA by RT-PCR as described above for *VWF* mRNA. The results demonstrated that both *NFIB* and *YY1* mRNA levels were increased in response to

hypoxia maximally at 48 hours, however the level of increase in *YY1* was more extensive and it was maintained for longer period of time (Fig. 2-6A and B). Next, we determined whether this increase in mRNA correlates to an increase in NFIB and YY1 proteins by Western blot analyses of total, as well as nuclear protein preparation of control and hypoxia treated LMEC. The results demonstrated that NFIB protein levels were not significantly altered in either total lysate or nuclear fractions in response to hypoxia, while a significant increase in nuclear fraction, but not total lysate levels of YY1 protein was detected (Fig. 2-6C).

To determine whether functional activities of NFIB and YY1 were altered in response to hypoxia, we next determined the levels of associations of these transacting factors with their cognate binding sites on the *VWF* gene regulatory elements. Chromatin immunoprecipitation assays were performed for these analyses and the results demonstrated that association of NFIB with the *VWF* promoter was significantly reduced, while association of YY1 with its cognate binding site on intron 51 HSS sequences was significantly increased (Fig. 2-6D).

To directly determine the role of YY1 in hypoxia induction of *VWF*, we transfected LMEC with siRNA directed against *YY1* transcript and determined the effect of *YY1* knockdown on *VWF* expression in control and hypoxia treated cells. For these analyses LMEC were transfected with YY1 specific or a non-silencing siRNA as described in methods and 48 hours post-transfection, cells were maintained as control or exposed to hypoxia as described above. Whole lysate proteins were prepared 48 hours after hypoxia treatment and subjected to Western blot analyses to determine the levels of YY1 and VWF. The results demonstrated that YY1 specific siRNA treatment significantly reduced

the levels of *YY1* and this was accompanied by a significant decrease in the levels of *VWF* in both control and hypoxia treated LMEC (Fig. 2-6E). Chromatin immunoprecipitation were also carried out and demonstrated that YY1 specific siRNA treatment abolished hypoxia-induced increase in YY1 association with its target binding site on *VWF* chromatin (Fig. 2-6F). These results demonstrated that YY1 participates in basal level as well as hypoxia-induced transcription of *VWF* in LMEC.

Since analyses of *YY1* levels demonstrated that there was a significant increase in nuclear YY1 protein levels in response to hypoxia we next determined whether this increase is also observed *in vivo* in the lung endothelial cells of hypoxia treated mice as well as hypoxia exposed cultured LMEC. For these analyses we chose to perform confocal immunofluorescence analyses of YY1 on lung sections of control and hypoxia treated mice. To correlate this with *VWF* expression, we used VWF staining for colocalization. We first performed these analyses on cultured cells and demonstrated that consistent with the results of Western blot analyses, there was a significant increase in nuclear YY1 concomitant with increased levels of VWF detectable by immunofluorescence analyses (Fig. 2-7A). Next, similar analyses were performed on OCT frozen lung section of hypoxia exposed and control mice and the results demonstrated that there was a significant increase of YY1 in the nucleus of VWF expressing endothelial cells in lung of hypoxia treated mice compared to control (Fig. 2-7B). Together these results demonstrate that increased YY1 in the nucleus is concomitant with increased binding of YY1 with the *VWF* promoter, and this process combined with decreased association of NFIB repressor with the *VWF* promoter correlates with increased *VWF* transcriptional activity.

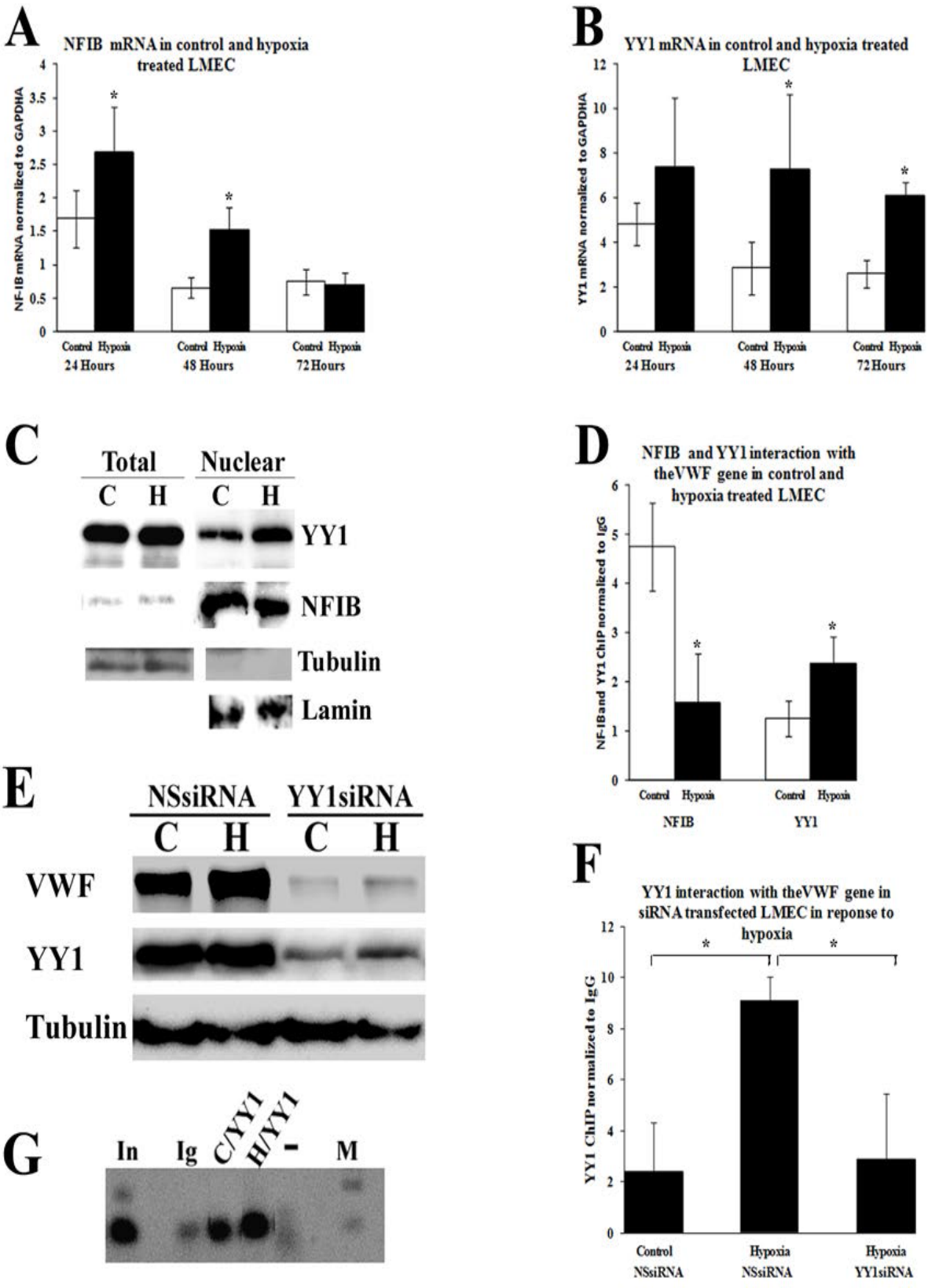

A
NFIB mRNA in control and hypoxia treated LMEC
NF-IB mRNA normalized to GAPDHA
Control Hypoxia
24 Hours
48 Hours
72 Hours
B
YY1 mRNA in control and hypoxia treated LMEC
YY1 mRNA normalized to GAPDHA
C
Total
Nuclear
C H C H
YY1
NFIB
Tubulin
Lamin
D
NFIB and YY1 interaction with theVWF gene in control and hypoxia treated LMEC
NF-IB and YY1 ChIP normalized to IgG
NFIB
YY1
E
NSsiRNA
YY1siRNA
VWF
YY1
Tubulin
F
YY1 interaction with theVWF gene in siRNA transfected LMEC in reponse to hypoxia
YY1 ChIP normalized to IgG
Control NSsiRNA
Hypoxia NSsiRNA
Hypoxia YY1siRNA
G
In
Ig
C/YY1
H/YY1
-
M

Figure 2-6. Analyses of *VWF, NFIB* and *YY1* expression and interaction with *VWF* chromatin in control and hypoxia treated LMEC in absence and presence of YY1 specific siRNA. LMEC were maintained as control or exposed to hypoxia as described for Fig. 5. (**A and B**) RT-PCR analyses on collected RNA were performed using specific primers (Table 1. supplementary material) to detect *NFIB* and *YY1* mRNA and *GAPDH* at the specified time points. Time points indicating significant (*P<0.05) increase are marked (*). **(C)** Nuclear and total lysates (50 μg for total and 100 μg for nuclear component) collected from cells at 48 hour time point (1 hour hypoxia followed by 48hours normoxia) and corresponding control were exposed to Western blot analyses to detect NFIB, YY1, tubulin and lamin. Lack of tubulin detection in the nuclear extract is indicative of lack of cytoplasmic contamination of nuclear extract preparation. Lamin was used as loading control for nuclear extracts. **(D)** Chromatin immunoprecipitations were performed on control and hypoxia treated cells (48 hour time point) using antibodies specific to NFIB, YY1 or IgG (as negative control). Isolated DNA from chromatin was subjected to quantitative real time PCR analyses to detect amplified *VWF* DNA sequences that corresponded to NFI binding sites or YY1 binding sequences in intron 51 as previously described[55]. The primer sequences are presented in (Table 2-1) in supplementary data. Results represent fold increase in amplified DNA detected by NFIB or YY1 immunoprecipitation compared to IgG from 4 independent experiments (*P<0.05). **(E and F)** LMEC were transfected with either siRNA specific for YY1 or non-silencing siRNA (NS), as described in methods. Cells were then exposed to hypoxia or normoxia for 1hour and after 48 hours cells were harvested for protein analyses and chromatin immunoprecipitation. **(E)** A representative blot of 6 independent experiments, indicating efficient YY1 knockdown by siRNA transfection of cells and also reduction of *VWF* expression in YY1 knock down cells. **(F)** Chromatin IP of cells using antibodies specific to YY1 or IgG. Purified chromatin was amplified with real time-PCR using specific primers to detect sequences corresponding to YY1 binding site on the *VWF* gene, as described in methods. Results represent fold change in amplified DNA detected by YY1 immunoprecipitation compared to IgG from 3 independent experiments (*P<0.05). **(G)** A representative gel analysis of real-time PCR product of chromatin immunoprecipitation assay demonstrating lack of PCR products in negative control

(water only, labelled as "-") and significantly lower levels of detected PCR products in chromatin immunoprecipitated with non-specific IgG antibody, while specific PCR products were detected from input chromatin (positive control labelled as "In") and those immunoprecipitated with YY1 antibody from control (C/YY1) and hypoxia (H/YY1) treated cells.

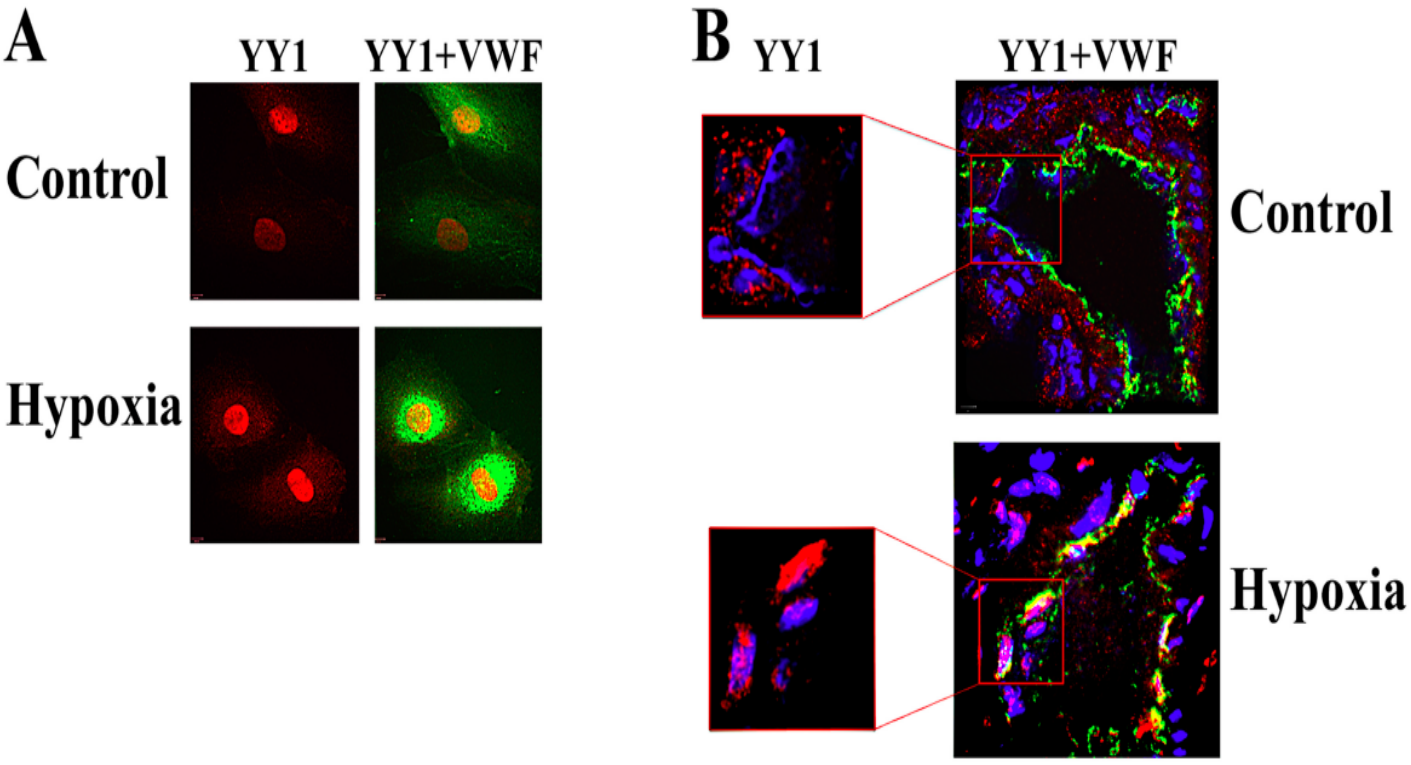


Figure 2-7. Immunofluorescence analyses of YY1 expression in human LMEC *in vitro* and *in vivo* in response to hypoxia. (A) Cultured LMEC maintained as control or exposed to hypoxia (1 hour hypoxia and 48hours normoxia), and **(B)** OCT frozen sections of lung from control and hypoxia treated mice were subjected to immunofluorescence analyses and confocal microscopy to detect YY1 (red) and VWF (green) as described in methods and Figure 1E. Nuclei were stained with DAPI (blue). Results are representative of 2 independent experiments for cultured LMEC and 2 mice for each control and hypoxia treatment (magnification 600X).

Hypoxia results in increased platelet adhesion to monolayer of cultured lung microvascular endothelial cells

To determine the functional effect of increased *VWF* expression in response to hypoxia, we performed a platelet binding assay (laminar flow adhesion assay) that would demonstrate whether increased levels of VWF protein correlate with increased platelet binding activities. For this assay, control and hypoxia treated LMEC monolayer grown on cover slips were exposed to shear stress while isolated human platelets were perfused over the monolayer as described in methods. Platelet chains were observed by an inverted phase-contrast microscope (Fig. 2-8A) and the number of platelet chains forming after 4 minutes of exposure to shear stress were determined in 5 different fields of view (Fig. 2-8D). Cover slips containing LMEC-platelet coculture were then gently removed from shear stress, fixed with 4% paraformaldehyde and subjected to immunofluorescence analyses using cd42b antibody conjugated to PE to detect platelets (Fig. 8B), or subjected to confocal immunofluorescence analyses using cd42b and VWF specific antibodies to detect colocalization of platelets and VWF (Fig. 2-8E). The results of phase-contrast microscopy analyses demonstrated that there was a significant increase in the number of platelet chains formed over the monolayer of hypoxia treated LMEC compared to control {Fig. 2-8D compare NT (no treatment) in control and hypoxia}. We also observed that upon removal of shear stress the platelet chains dissociated rapidly but the dissociation occurred at a slower rate in hypoxia-treated compared to control. This observation was supported by immunofluorescence analyses of LMEC-platelet coculture demonstrating a significant number of platelet chains on hypoxia treated LMEC while the number of platelet chains on control LMEC were few or non-detectable (Fig. 2-8B). These results

demonstrate that increased *VWF* expression in response to hypoxia leads to increased platelet adhesion and formation of platelet chains with increased stability. To determine whether this increased platelet chain formation was dependent on VWF and also to determine whether YY1 activity contributed to this process, we performed similar experiments with cells that were transfected with siRNA that specifically targets *VWF* or *YY1*. As previously shown in (Fig. 2-6E) we demonstrated that siRNA targeting of *YY1* significantly reduced VWF as well as YY1 protein levels in the cells. Efficacy of the siRNA targeting the *VWF* was demonstrated by Western blot analyses of LMEC cells that were transfected with VWF siRNA (SiVWF) compared to a non-specific (NS) siRNA (Fig. 2-8C). The results of platelet binding assays demonstrated that both VWF and YY1 specific siRNAs (SiVWF and SiYY1) compared to non-specific siRNA (NS), significantly reduced the number of platelet chains binding to LMEC in hypoxia treated cells (Fig. 2-8D). Non-specific siRNA transfection compared to no transfection (NT) also appeared to modestly but significantly reduce the platelet chain formation in hypoxia treated cells, and more significantly in control cells. This may be due to the process of transfection that may induce the release of VWF, thus reducing the overall levels of VWF in cells prior to hypoxia treatment and binding assays. Nevertheless in comparing control and hypoxia treated cells, both NT (no treatment) and non-specific siRNA (NS) treatment demonstrated significantly higher platelet chain formation on hypoxia treated cells, while SiVWF and SiYY1 treatment abolished this increased platelet binding to similar levels observed with control cells (Fig. 2-8D). Confocal immunofluorescence analyses also demonstrated formation and colocalization of platelet chains (labeled by red fluorescence of cd42b conjugated antibody and shown as yellow when colocalizing with VWF) on

extended VWF molecules (shown by green fluorescence) in hypoxia-exposed cells both in NT and NS transfected cells. The extended VWF molecule and colocalizing platelets were not detected in SiVWF and SiYY1 transfected cells in either hypoxia or control cells (Fig. 2-8E).

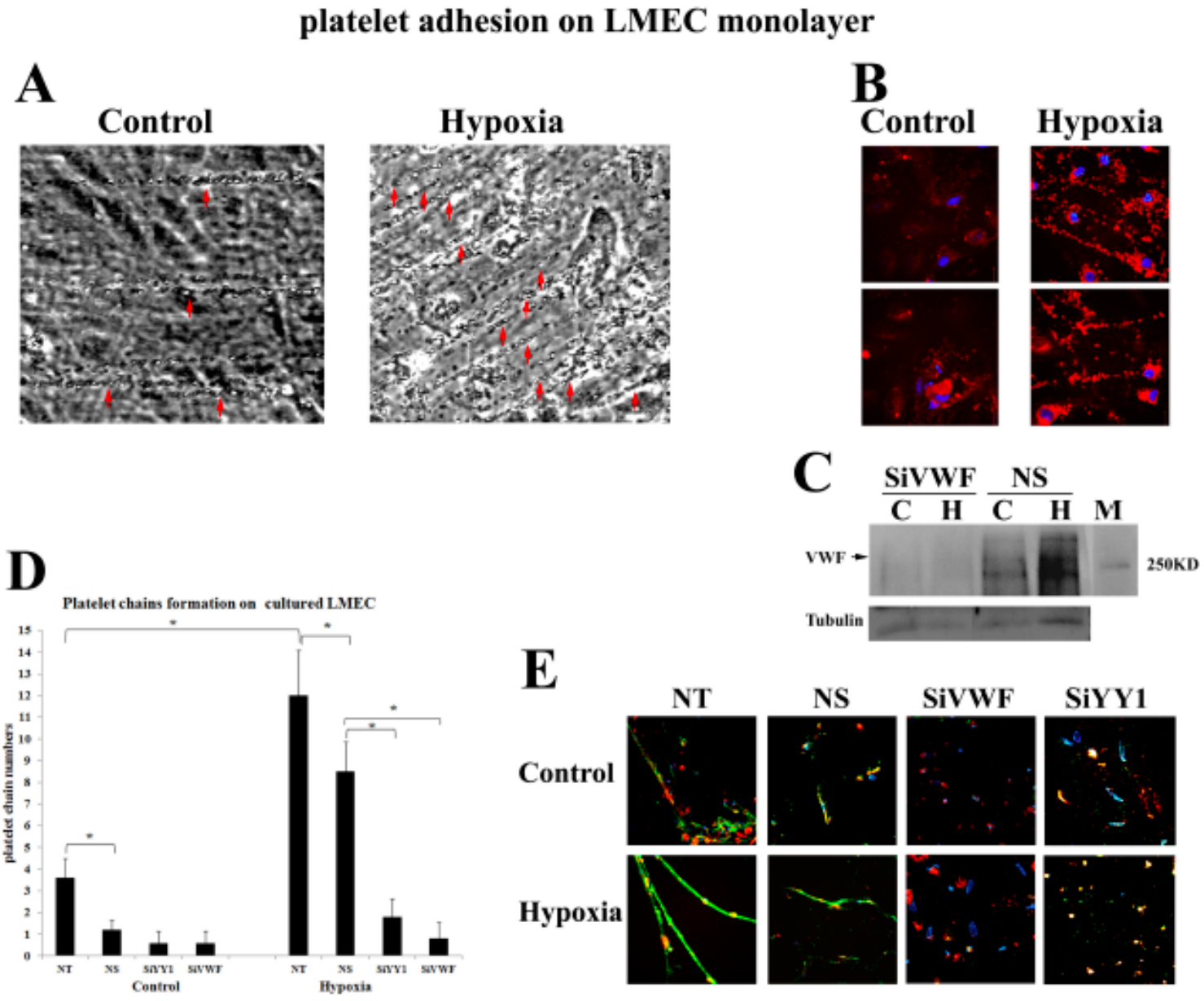


Figure 2-8. Endothelial exposure to hypoxia increases platelet binding to endothelial monolayer under shear stress. (A and B) LMEC were incubated in hypoxic conditions as described in Fig. 5. The endothelial monolayer was exposed to 1 dyne/cm^2 shear stress for about 30 seconds and then freshly isolated human platelets were perfused over the monolayer for 10 minutes. **(A)** a bright-field zoomed area of the movie acquired at 10 minutes in both control and hypoxia-induced monolayer. **(B)** Immunofluorescence staining of the endothelial cells (nucleus stained with DAPI: blue) and platelets (cd42b antibody: red) interactions as described in Methods. **(C-D)** LMEC were non- transfected

(NT) or transfected with non-silencing siRNA (NS), siRNA specific for YY1 (SiYY1) or siRNA specific for VWF (SiVWF) as described in methods. Cells were then exposed to hypoxia or normoxia for 1 hour and after 48 hours cells were used in platelet binding assay as described above and used in Western blot analyses to demonstrate efficacy of VWF siRNA treatment. **(C)** A representative blot of 2 independent experiments, indicating efficient *VWF* knockdown by SiVWF transfection of cells. **(D)** Quantification of number of platelet chains observed in 5 different fields of view after 4 minutes. **(E)** Immunofluorescence staining and confocal microscopy analyses of the VWF (green) and platelets (cd42b antibody: red) interactions as described in Methods. Magnifications for all Immunostainings are 400X.

DISCUSSION

Transgenic mice expressing the LacZ transgene under the regulation of brain and lung specific transcriptional regulatory elements of the *VWF* gene were used to explore the mechanism(s) of *VWF* upregulation in a mouse model of pulmonary hypertension that involves hypoxia exposure [137]. Immunofluorescence analyses of endogenous *VWF* and *LacZ* transgene expression in hypoxia treated mice compared to control demonstrated that while in control mice expression was mainly restricted to larger vessels, in hypoxia treated mice expression was redistributed to include significant regions of the microvasculature. RNA analyses demonstrated a concomitant increase in the levels of endogenous *VWF* and LacZ transgene transcripts in lungs of hypoxia-exposed compared to control mice. These results demonstrated that hypoxia alters transcriptional activity of the *VWF* promoter in lung endothelial cells in a manner that exhibits upregulation, as well as *de novo* activation in endothelial cells that normally do not express VWF protein. Our immunofluorescence analyses of the endogenous VWF and LacZ transgene expression in the brain of control and hypoxia treated mice did not demonstrate a significant alteration in the pattern of expression. However, RNA analyses demonstrated a significant increase in the levels of endogenous *VWF* mRNA. Similarly a significant increase in the levels of endogenous *VWF* mRNA in heart and liver but not kidneys of hypoxia treated mice compared to control was demonstrated. These observations suggest that while in lung *de novo* activation of the *VWF* transcription in microvascular endothelial cells is detected, increased transcriptional activity of *VWF* in endothelial cells that support the basal expression of *VWF* in lung, brain, heart and liver is also observed. This suggests that either a common mechanism or two independent processes may be

invoked by hypoxia treatment/PH leading to increased and *de novo* activation of *VWF* transcription. The observed increase in *VWF* mRNA in multiple organs but not in kidney may be a reflection of differential sensitivities of endothelial cells of these organs to hypoxia. Analyses of *VWF* transcriptional regulation in cultured LMEC demonstrated that hypoxia treatment resulted in an approximately three fold increase in the levels of *VWF* mRNA, accompanied by increased levels of VWF protein, leading to increased chains of platelets that are formed in association with the LMEC monolayer. This increased levels of platelet chain formation was abolished by treatment of cells with siRNA that specifically targeted the *VWF* expression. Thus our results demonstrate that the increased levels of VWF protein functionally contribute to increased platelet adhesion to lung microvascular endothelial cells and as such may contribute to thrombus formation *in vivo*.

An intriguing observation in the analyses of *LacZ* transgene expression was detection of transgene in the heart endothelial cells of hypoxia treated mice. Lack of *LacZ* transgene expression in all organs tested, except lung and brain, in control mice was consistent with previous analyses of these transgenic mice and consistent with lung and brain endothelial activation pattern of these *VWF* regulatory elements (-487 to +247 combined with intron 51 sequences). However, the activation of the transgene in response to hypoxia in heart endothelial cells suggested that the molecular mechanism(s) leading to *VWF* activation in response to hypoxia may include alterations in functions of transacting factors that normally participate as repressors of the *VWF* promoter activity. We have recently reported that two repressors, NFI and NFY, participate in organ-specific activity of the *VWF* promoter [55]. Specifically mutation of NFI binding site was

shown to activate *VWF* promoter sequences (-487 to +247) in lung and heart endothelial cells, in addition to brain; while the wild type promoter was activated only in brain endothelial cells [55]. Additionally we demonstrated that among four NFI isoforms (A, B, C and X), NFIB was predominantly detected in lung and heart endothelial cell nuclei [55]. Based on these results we hypothesized that NFIB may be a target of hypoxia and alteration in its levels and/or function may contribute to transgene activation in endothelial cells of the heart as well as lung microvascular endothelial cells. However, lung-specific activity of the *VWF* promoter sequences were also shown to be regulated through sequences in intron 51 region of the *VWF* gene. We have shown that YY1 interacts with these sequences and contributes to its regulatory activities. Additionally, through a process involving chromatin looping YY1-binding intron 51 sequences are brought in close proximity to the proximal NFIB binding *VWF* region, potentially allowing for YY1 to influence NFIB function and alleviate its repressive effect [55]. Thus, we also hypothesized that YY1 activity may be a target of hypoxia, and alteration of its levels or function may also contribute to hypoxia-induced upregulation of *VWF* specifically in lung endothelial cells. Thus, we explored the roles of these two transacting factors, NFIB and YY1, in hypoxia induction of *VWF*.

Our analyses demonstrated that hypoxia-induced *VWF* upregulation was not accompanied by any changes in the levels or distribution of NFIB; however, association of NFIB with the *VWF* promoter sequences was significantly decreased. This result is consistent with the hypothesis that alleviation of NFIB repressive function on the *VWF* promoter may contribute to *VWF* transcriptional upregulation in response to hypoxia. RNA and protein analyses of *YY1* demonstrated a significant increase in the levels of *YY1*

mRNA, concomitant with a significant increase in the nuclear levels of YY1 protein in hypoxia treated cells compared to control. Immunofluorescence analyses also demonstrated that both *in vitro* and *in vivo*, levels of *YY1* are significantly upregulated in the nuclei of hypoxia treated cultured LMEC, as well as in lung microvascular endothelial cells of hypoxia treated mice. In addition, chromatin immunoprecipitation analyses demonstrated that YY1 interaction with its cognate binding site on the intron 51 HSS sequences of *VWF* gene were significantly enhanced in response to hypoxia. Furthermore, we demonstrated that knock down of *YY1* by siRNA treatment significantly reduced the basal and hypoxia-induced levels of *VWF* and eliminated increased platelet chain formation on the surface of cultured LMEC. These results demonstrate that YY1 regulation is a target of hypoxia and positively contributes to upregulation of *VWF*. Additionally, the significant decrease in basal levels of *VWF* activity in YY1-specific siRNA transfected LMEC is consistent with our previous results demonstrating the role of YY1-interacting intron 51 sequences in lung-specific activation of the *VWF* promoter.

NFIB and YY1 may independently contribute to *VWF* upregulation and may contribute differentially to transcriptional upregulation (observed in most organs) vs. *de novo* activation (observed in lung microvascular endothelial cells). Alternatively, the close proximity of these two negative and positive regulators of the *VWF* promoter through chromatin looping may influence binding activity of one by another. Specifically, enhanced binding of YY1 in response to hypoxia may lead to decreased binding of NFIB, resulting in increased and/or *de novo* activation of *VWF* transcription in lung microvascular endothelial cells. This is also consistent with previous reports that demonstrate YY1 can influence activities of other transacting factors including p53 and

HIF-α [147, 148]. YY1 is accumulated in cytoplasm, but phosphorylated YY1 is activated and moves to the nucleus, where it can interact with various molecules, modify them, and ultimately alter their functions [149].

In conclusion our data here is consistent with a hypothesis that pulmonary hypertension and/or conditions leading to this process, including hypoxia are associated with a "phenotypic shift" of endothelial cells from anticoagulant to procoagulant activity. We additionally propose that this process of "phenotypic shift" includes modification of specific transacting factors including NFIB and YY1 that target regulation of *VWF* and potentially other endothelial-specific genes.

CHAPTER 3

ENDOTHELIAL CELLS OF DISTINCT ORGANS EXHIBIT HETEROGENEITY IN RESPONSE TO HYPOXIA WITH REGARD TO VON WILLEBRAND FACTOR TRANSCRIPTIONAL REGULATION

INTRODUCTION

Von Willebrand factor (VWF) is a prothrombotic molecule that is expressed exclusively in endothelial cells and megakaryocyte. It mediates adhesion of platelets to the sub endothelium matrix and functions as carrier and stabilizer of factor VIII in the circulation [150]. Due to its highly endothelial-specific expression pattern VWF is commonly used as a marker to distinguish endothelial cells from other cell types. Although all cultured endothelial cells express *VWF*, levels and pattern of VWF expression is highly dependent on the size and location of the vascular bed *in vivo*, and thus reflective of endothelial cell heterogeneity [8, 24, 151]. Characterization of *VWF* promoter and regulatory regions in transgenic mice demonstrated that distinct regions and regulatory elements of the *VWF* gene are required for its transcriptional activation in endothelial cells of distinct organs [25, 55, 59, 63]. A region of the *VWF* gene spanning sequences -487 to +247 functions as an endothelial specific promoter, but *in vivo*, this region is sufficient for transcriptional activity exclusively in a subset of brain endothelial cells. A hypersensitive region in intron 51 of the *VWF* gene, which interacts with YY1 transacting factor, was shown to confer lung

endothelial cell specific activity to the promoter [63]. Additionally a mutation in the *VWF* proximal promoter that interfered with binding of NFI transacting factor (a repressor of the *VWF* promoter) was shown to result in proximal promoter activation in endothelial cells of heart and lung, as well as brain. The target NFI in lung and heart endothelial cells was shown to be primarily the isoform NFIB [55].

Hypoxia-induced release of stored VWF from Weibel Palade bodies were previously reported [152], but we have recently demonstrated that *VWF* gene expression is also upregulated in response to hypoxia, and that hypoxia-response is not uniform in endothelial cells of all vasculature [86]. Specifically in lung, the pattern of *VWF* expression was altered from primarily large vessels in control to that of micro- as well as macro- vessels in hypoxic lung. *VWF* upregulation in response to hypoxia was also observed in the vasculature of heart, brain and liver in addition to lung, albeit at differing levels; however, there was no up regulation in kidney vasculature.

To gain insight into the mechanism of hypoxia induction of the *VWF* gene, we exposed to hypoxia transgenic mice harboring *LacZ* gene fused to the *VWF* proximal promoter sequences (-487 to +247) and the intron 51 lung-specific enhancer (I51HSS) referred to as VWF-LacZ-HSS [63]. Analyses of *LacZ* transgene expression demonstrated that these *VWF* sequences (-487 to +247 and the HSS) contain necessary elements to mediate hypoxia-response of the *VWF* gene in lung endothelial cells [86]. We explored the mechanism of hypoxia-induced upregulation of the *VWF* promoter in lung endothelial cells and demonstrated that interaction of YY1 transacting factor with the lung-specific enhancer in intron 51 was required for

this process. We also demonstrated that interaction of NFIB repressor with the *VWF* promoter was significantly reduced in response to hypoxia.

We also analyzed VWF-LacZ-HSS expression in vascular beds of other organs, in addition to lung, in hypoxia treated mice. Our analyses demonstrated that, although in the absence of hypoxia, the activities of these *VWF* regulatory sequences were confined to lung and brain endothelial cells, we observed that in response to hypoxia exposure these sequences lead to *LacZ* expression in cardiac vascular endothelial cells [86]. This activation was not observed in endothelial cells of any other organ analyzed including liver or kidney. These results suggested that the elements mediating hypoxia-response of the *VWF* gene in cardiac endothelial cells are likely to be located within the *VWF* promoter proximal (sequences -487 to +247), and/or intron 51 lung specific enhancer sequences (I51HSS). Additionally, the data suggested that hypoxia resulted in an alteration in transcriptional machinery of cardiac endothelial cells *in vivo*, which lead to activation of the *VWF* regulatory sequences that are otherwise silent in these cells.

Here we report that hypoxia-induced upregulation of *VWF* 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 functional consequences of hypoxia-induced *VWF* upregulation are correlated with thrombi generation in vascular beds of specific organs. Collectively, our results demonstrate heterogeneity in levels and mechanism of *VWF* gene response to hypoxia in endothelial cells of different organs. These observations suggest that environmental and genetic/epigenetic factors contribute to the regulation of *VWF* gene expression

and a complex mechanism has evolved to tightly regulate the sites, levels, and stimuli-responses of its expression.

MATERIALS AND METHODS

Plasmids used for generation of adenoviral vectors

Adenoviral vectors containing transgenes *LacZ*K and LacZKHSS referred to as AdLacZK and AdLacZKHSS were generated using corresponding plasmids [55, 59, 63] by Amy Barr at Cardiovascular Research Core Facility Center at University of Alberta. Adenovirus CMVLacZ was a gift of Dr. Jason Dyke at University of Alberta.

In vivo delivery of adenoviral vectors

Adenoviruses ($5x10^9$) were administered into mice via tail vein injections. After 48 hours mice were euthanized according to animal care and use committee at the University of Alberta. All major organs were harvested and preserved in OCT embedding medium and frozen for cryostat sectioning.

Analyses of organs of control and hypoxia exposed mice

Hypoxia exposed mice were previously generated and described [86]. Mice were euthanized 35 days after hypoxia exposure, and organs harvested from control and hypoxic mice were maintained as frozen OCT or paraffin embedded. Hypoxia exposure, maintenance and euthanasia of mice were performed according to animal care and use committee at the University of Alberta.

Immunofluorescent (IF) and immunohistochemistry (IHC) staining

Immunofluorescence staining and confocal imaging were performed as previously described [86]. OCT embedded hearts were sectioned 4μm thick, fixed in acetone, blocked and immunestained for VWF (sheep FITC preconjugated anti-VWF, Abcam-ab8822) and LacZ (goat β-galactosidase, Abcam Cat#ab-12081-100) [86]. Paraffin embedded tissues were cut, deparaffinized and blocked for 1 hour at room temperature, followed by over-night incubation with antibodies for detection of VWF, platelets [CD41specific antibody (Cemfret analytics- Germany)] and fibrinogen (anti fibrinogen antibody from Sigma-Aldrich). The secondary antibodies (Invitrogen, Burlington, ON) used were donkey anti goat Alexa 594 (cat#A11058) and donkey anti rabbit Alexa 488(cat# A-21206).

Cell cultures, hypoxic conditions and adenovirus transfections

Endothelial cells isolated from microvessels of heart and lung were purchased from Lonza. Glomeruli endothelial cells were a gift from Dr. Barbara Ballermann (University of Alberta). Human MVECs were cultured on gelatin coated dishes in endothelial medium (EGM supplemented with microvascular growth supplements, EGM-2, Lonza) and grown in 37 °C, 5% CO_2. Human foreskin fibroblasts (HFF1) were cultured in DMEM supplemented with 10% FBS (Invitrogen, ON). For hypoxia treatment, endothelial cells at approximately 80-85% confluence were placed in an hypoxia chamber (Ruskinn Invivo2 Hypoxia- Gas Mixer Q) at 1% oxygen for 1 hour and then maintained at normoxic condition for 48 hours, after which cells were harvested for RNA isolation. For hypoxia treatment of transduced cells, MVECs at

70% confluence were first transduced by adenoviral vectors (125 MOI), and after 48 h media were replaced and cells were exposed to hypoxia treatment and RNA collection as described for non-transduced endothelial cells.

RNA analysis

RNA was extracted (Qiagen kit) from cultured cells and subjected to quantitative reverse transcription-polymerase chain reaction (qRT-PCR) analyses using syber green master mix (Invitrogen) to detect human endogenous *VWF, HPRT* and *LacZ* as previously described [55, 86]. Primers used to perform qRT-PCR for *VWF, HPRT* and b-Galactosidase (*LacZ*) were purchased from Qiagen and catalog numbers are provided in (Table 3-1). Relative levels of target gene mRNAs were presented on Y axis as values normalized to the HPRT using ΔCT.

Chromatin immunoprecipitation assay

Chromatin immunoprecipitations (ChIP) were performed using antibodies to specifically determine interactions of HIF1a, SP1, GATA6, NFIB, YY1, 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 binding capacity of each transcription factor was normalized to IgG. All antibodies were purchased from Abcam Inc. (Abcam, Cambridge, MA, USA). ChIP assays were performed, using primers (Table 3-1) for PCR that specifically amplify *VWF* proximal promoter sequences (Human VWF NF1-binding region), or intron 51 (I51HSS region) as previously described [2].

DNA methylation analysis

The two potential methylation sites (cytosines in 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 manufacturer's protocol. Briefly, DNA extracted from cells (1 μg) was either digested or not with methylation sensitive restriction enzyme Hpall. Digested and undigested products were used as templates for quantitative 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 will be protected from digestion and amplified, otherwise no or little amplification products are detected. Primers sequences are shown in (Table 3-1).

Generation of transcription factors knocked-down endothelial cells

For transient knock down, cells were transfected twice within 48 hours with non-silencing or specific silencing siRNAs (10 nmol/L). siRNAs source and sequence are presented in (Table 3-1) . Transfections were performed using Lipofectin 2000 (Thermo Fisher) according to the manufacturer's protocol. All siRNA transfections were performed prior to hypoxia exposure and cells were analyzed for RNA and protein expression 48 hours post hypoxia treatment as previously described [86].

Protein analysis

Cell lysates (30 μg protein) were prepared for Western blot analyses as previously described [86]. Antibodies used were human specific anti-VWF antibody

(Dako Omnis, Denmark), or tubulin, GATA6, SP1 and HIF1α

(all purchased from Abcam, Cambridge, MA, USA).

Statistics

All experiments are performed with a minimum of triplicate and data represent the mean ± standard error of the mean. Student *t* test was used to analysis results and $P < .05$ was considered statistically significant.

Table 3-1

Primers for q-RT-PCR	
VWF	QIAGEN - QuantiTect Primer Assay Cat. no.: QT00116795
LacZ	QIAGEN - QuantiTect Primer Assay Cat. no.: QT00371630
HPRT	QIAGEN - QuantiTect Primer Assay Cat. no.: QT00166768
Primers for ChIP	
VWF- proximity promoter-forward	5'–CCT TCA TCT TTA GCC GAT CCA–3' Integrated DNA Technologies
VWF- proximity promoter-reverse	5'–GAC TCC AGG GAA GTT GAG AAA–3' Integrated DNA Technologies
VWF- Intron 51-forward	5'-CGCAGGGAAGAGAAGGGAAATAAACTGG-3'
VWF- Intron 51-reverse	5'-GACTGATCTTCAAGAATTGTGGCCC-3'
Primers for Methylation	
-422 site-forward	5'–GCC AGG ACC GGA TCC TT–3' Integrated DNA Technologies
-422 site-reverse	5'– CAC CTC AAC CTG AGC CAA TTT –3' Integrated DNA Technologies
+119 site -forward	5'– AGC TAT TGT GGT GGG AAA GG –3' Integrated DNA Technologies
+119 site -reverse	5'– GCT CAA TCA GGT CTG CTA CA –3'

	Integrated DNA Technologies
siRNA-sequences	
YY1-siRNA	5'–ATGCCTCTCCTTTGTATATTA –3' QIAGEN - Hs_YY1_3 FlexiTube siRNA Cat. no.: SI00051926
NFIB-siRNA	Sense: 5'– GGA UAG UUU UGU AAA AUC Utt –3' Antisense: 5'– AGA UUU UAC AAA ACU AUC Ctc –3' Ambion Cat. no.: 4392420 siRNA ID: s9495
GATA6-siRNA	Sense: 5'– GAC UCU ACA UGA AAC UCC Att –3' Antisense: 5'– UGG AGU UUC AUG UAG AGU Cca –3' Ambion Cat. no.: 4392420 siRNA ID: s5607
SP1-siRNA	Sense: 5'– CCA CAA GCC CAA ACA AUC Att –3' Antisense: 5'– UGA UUG UUU GGG CUU GUG Ggt –3' Ambion Cat. no.: 4392420 siRNA ID: s13320
HIF1α-siRNA	Sense: 5'– CCU CAG UGU GGG UAU AAG Att –3' Antisense: 5'– UCU UAU ACC CAC ACU GAG Gtt –3' Ambion Cat. no.: 4390824 siRNA ID: s6541

RESULTS

Endothelial cells of lung, heart and kidney exhibit heterogeneity with regard to *VWF* expression in response to hypoxia

To determine whether cultured endothelial cells of various organs mimic their counterparts *in vivo*, we explored *VWF* response to hypoxia in cultured human cardiac and kidney glomerular, as well as lung microvasculature. These endothelial cells were exposed to hypoxia for 1 hour followed by 48 hours normoxia as previously described [86]. Consistent with the previously reported *in vivo* observations, the results demonstrated a significant *VWF* mRNA upregulation in cultured human lung and cardiac, but not kidney glomerular MVEC in response to hypoxia (Fig. 3-1A). We have previously reported that YY1 mediates hypoxia-induced *VWF* upregulation in human lung MVEC and siRNA-mediated *YY1* knockdown abolishes this process [86]. Thus we proceeded to determine whether YY1 was necessary for hypoxia-induced *VWF* upregulation in human cardiac MVEC, as it was in lung MVEC. *YY1* knockdown assay was performed using specific siRNA as previously described in methods for lung MVEC. Cardiac MVEC transfected with YY1 siRNA, or a non-specific siRNA (NSsiRNA) as control, were exposed to hypoxia as described above and *VWF* mRNA levels were determined in control and hypoxia treated cells. The results demonstrated that *YY1* knock down did not alter hypoxia response of the *VWF* gene in cardiac endothelial cells (Fig. 3-1B). These results demonstrated that unlike lung MVEC, cardiac MVEC do not require YY1 to upregulate *VWF* in response to hypoxia.

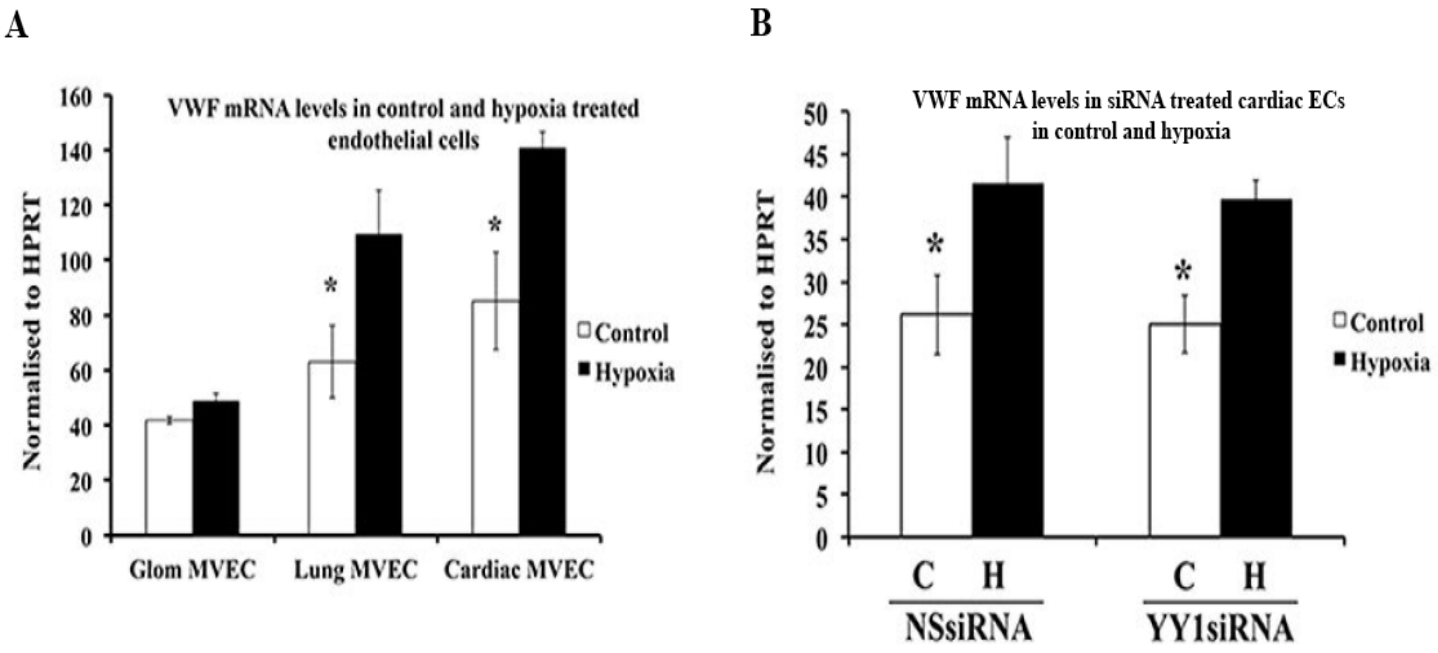


Figure 3-1. Analysis of *VWF* expression in control and hypoxia treated human cardiac, lung and glomeruli endothelial cells.

(A) Glomeruli (Glom), lung and cardiac microvascular endothelial cells (MVEC) were either maintained as control or exposed to hypoxia (1% oxygen for 1 hour). Cells were then maintained in normoxia for 48 hours, after which RNA were collected and subjected to quantitative RT-PCR to detect endogenous *VWF* and *HPRT* mRNAs. (B) Human cardiac MVEC were transfected with non-specific siRNA (NSsiRNA) or YY1 specific siRNA (YY1siRNA) prior to hypoxia treatment and *VWF* mRNA detection as described for (A). The graphs represent the levels of *VWF* mRNA normalized to *HPRT* and are the average of three-four independent experiments (*P<0.05).

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

We proceeded to determine the hypoxia response of the *VWF* proximal promoter 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 *VWF* proximal promoter sequences - 487 to +247 (AdLacZK), or proximal promoter and I51HSS sequences (AdLacZKHSS), fused to *LacZ* gene (Fig. 3-2A). The adenoviral vectors, were similar to that previously described to generate LacZK and VWF-LacZ-HSS transgenic mice respectively [59, 63]. Cardiac and lung, as well as kidney glomerular MVEC were transduced with these adenoviral vectors and then maintained as control 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 *LacZ* transgene mRNA levels. Results demonstrated that human cardiac MVEC that were transduced with AdLacZK exhibited a significantly increased level of *LacZ* mRNA in response to hypoxia (Fig. 3-2B), while human lung MVEC that were transduced with AdLacZKHSS, but not AdLacZK, exhibited this response (Fig. 3-2C). These results demonstrate that while I51HSS enhancer sequences are required for hypoxia response of the *VWF* promoter in lung, they are not required for this response in cardiac endothelial cells. Additionally the results demonstrate that the elements necessary to mediate hypoxia induction of the *VWF* promoter in cardiac endothelial cells are located within -487 to +247 sequences. In kidney glomeruli endothelial cells neither AdLacZK nor AdLacZK-HSS51 were responsive to hypoxia

(Fig. 3-2D), which was consistent with the lack of hypoxia response of endogenous *VWF* gene in cultured human glomerular endothelial cells, as well as in mouse kidney *in vivo*. As controls we transduced a human non-endothelial cell type, namely HFF1, with these adenoviruses and exposed them to hypoxia; and also examined hypoxia response of AdCMVLacZ (an adenovirus containing *LacZ* gene under the regulation of ubiquitous cytomegalovirus promoter) that was transduced into all cell types studied. The results of these analyses demonstrated that there was no expression of *LacZ* in HFF1 transduced with AdLacK or AdLacZKHSS, neither before nor after hypoxia treatment (Fig. 3-2D). These data demonstrated that *VWF* regulatory sequences in the context of adenoviral vectors maintain their cell type specific activities in response to hypoxia. Also the level of *LacZ* expression did not change in various endothelial cells transduced by AdCMVLacZ after hypoxia (Fig. 3-2E), demonstrating that the observed hypoxia response was due to *VWF* regulatory sequences.

Collectively these analyses demonstrate that the organ-specific pattern of *VWF* expression in response to hypoxia is maintained and reflected in cultured MVEC of corresponding organs. Additionally the results suggest that differential *VWF* elements are required for hypoxia induction of the *VWF* expression in heart versus lung. Lung specific I51HSS enhancer region of the *VWF* gene is necessary for hypoxia-induced *VWF* upregulation in the lung MVEC, whereas *VWF* proximal promoter sequences -487 to +247 are sufficient to induce hypoxia-response in cardiac MVEC.

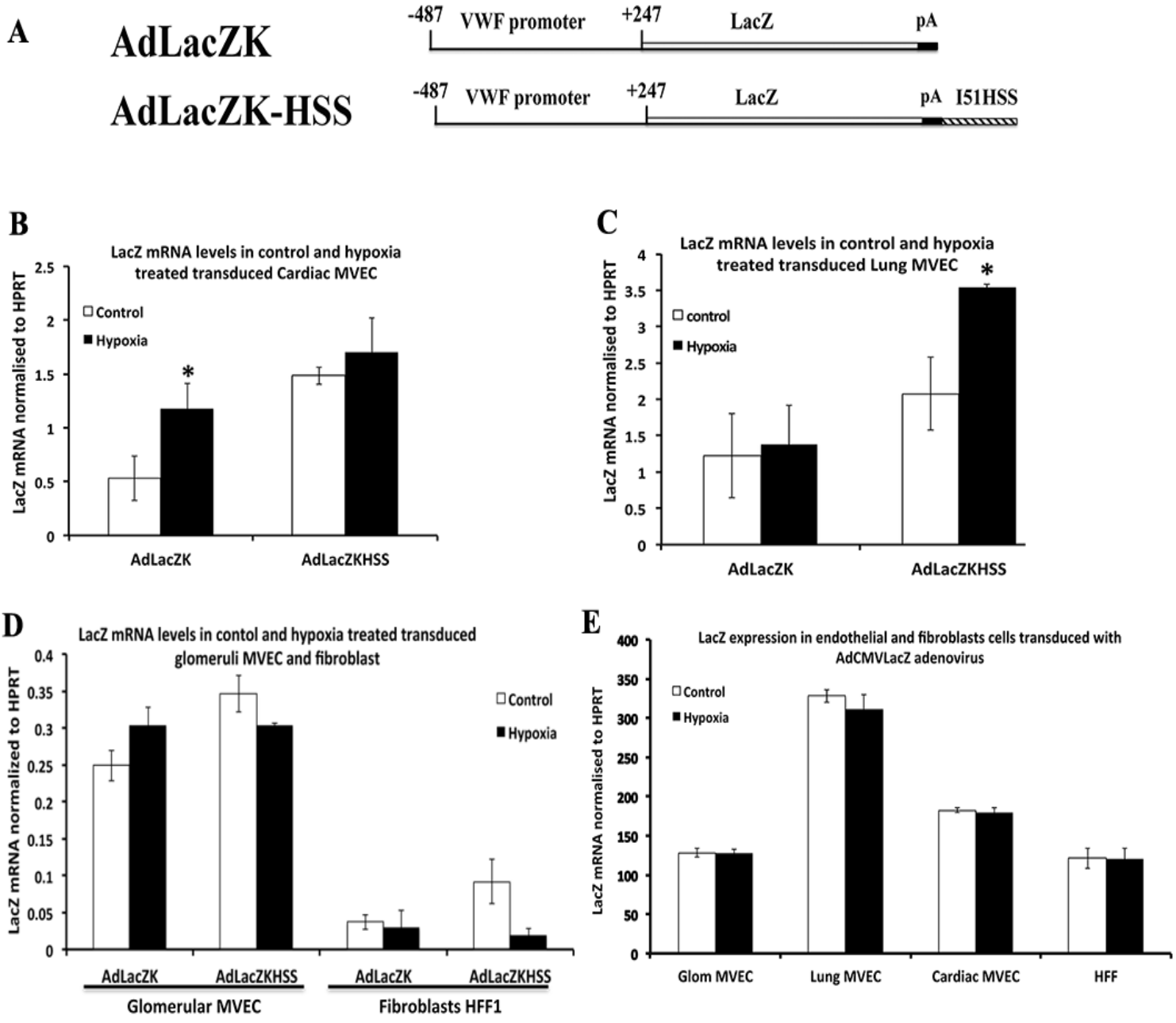


Figure 3-2. Analyses of *LacZ* expression in various transfected endothelial cells and fibroblast in control and hypoxia treated cells. (A) Schematic representations of the transgenes containing *VWF* promoter and regulatory sequences as well as *LacZ*, incorporated into adenoviral vectors AdLacZK and AdLacZKHSS. Human cardiac, lung and glomeruli MVEC, as well as human fibroblasts (HFF1), were transduced with either AdLacZK or AdLacZKHSS as described in materials and methods, and 48 hours after transduction they were maintained as control or exposed to hypoxia as described for Figure

1. (B-D) Graphs represent the levels of RT-PCR detected *LacZ* mRNA normalized to HPRT. (E) All MVEC and HFF1 cell types were transduced with AdCMVLacZ as described in methods and subjected to hypoxia treatment and *LacZ* mRNA analyses as described above. Results are the averages of three independent experiments for each cell type (*P<0.05).

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

Deletion analyses using adenoviral vectors demonstrated that *VWF* proximal sequences -487 to +247 are sufficient for mediation of the hypoxia response in cardiac MVEC, while lung MVEC require I51HSS in addition to the proximal region. These results suggested that factors interacting with sequences -487 to +247 mediate hypoxia induction of the *VWF* in cardiac MVEC. To test this hypothesis we first explored and compared the binding patterns of a number of transacting factors that were previously shown to participate in *VWF* promoter regulation, or have a potential binding site on the *VWF* promoter, as well as HIF-1α (a well known mediator of hypoxia response, although its binding site was not detected on the *VWF* proximal sequences), in control and hypoxia exposed cardiac and lung MVEC. *VWF* regulatory factors included, NFIB (a repressor of *VWF* [55, 153]), GATA6 (which we and others have shown to function as activator of the *VWF* promoter [39, 49, 57]), SP1 (which has a potential binding site on the *VWF* promoter [39]) and YY1 [63, 86]. For these analyses, chromatin immunoprecipitation were performed using target transacting factors specific antibodies, and primers for PCR that amplified *VWF* proximal regulatory sequences, or I51HSS sequences specifically for YY1, as previously described [86]. The results demonstrated that in response to hypoxia, association of the repressor NFIB with the *VWF* promoter proximal sequences was significantly reduced in both cardiac and lung MVEC (Fig. 3-3A-C). However, a distinctly different pattern of association of transacting factors that function as activators were observed among the two cell types

in response to hypoxia. Association of YY1 with the I51HSS sequences was increased in lung (as previously reported) but not in cardiac MVEC in response to hypoxia (Fig. 3-3B and C). Also a significant increase in association of Sp1 in lung but not cardiac MVEC was observed (Fig. 3-3B and C). In contrast, in response to hypoxia, a significant increase in association of GATA6 and HIF-1α was observed in cardiac but not lung MVEC (Fig. 3-3B and C).

Next to directly determine the role of each transacting factor in hypoxia-induced upregulation of the *VWF*, we used specific siRNA to knockdown target transacting factors prior to hypoxia treatment, and then determined the hypoxia response of the *VWF* in cardiac and lung MVEC. Western blot analyses were used to determine protein levels of VWF and siRNA targeted transacting factors. In both human cardiac and lung MVEC, we observed that *NFIB* knockdown (using NFIB siRNA) abolished the hypoxia-induced upregulation of *VWF* (Fig. 3-3D). These results are consistent with the hypothesis that hypoxia exposure somehow "lifts" the repression imposed via NFIB, by 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 knock down of SP1 and HIF-1α in lung MVEC. Although we had not observed increased recruitment of HIF-1α to the *VWF* promoter in lung MVEC, we could not exclude the possibility that this highly hypoxia responsive activator may regulate *VWF* response indirectly, and not necessarily through recruitment to the *VWF* promoter. The results demonstrated that the knockdown of either *GATA6* or *HIF-1* with specific siRNA lead to inhibition of

hypoxia-induced *VWF* upregulation in cardiac MVEC (Fig. 3-3E). However, knockdown of neither *HIF-1α*, nor *SP1* inhibited the hypoxia induction of *VWF* in lung MVEC (Fig. 3-3F).

These results collectively demonstrate that GATA6 and HIF-1α participate in *VWF* upregulation in response to hypoxia specifically in cardiac but not lung MVEC, while as previously reported YY1 specifically mediates hypoxia response in lung but not as shown above (Fig. 3-1) in cardiac MVEC. Although we have not identified a HIF-1α binding sequence (HRE) on the *VWF* promoter, these results in combination with ChIP analyses, suggests that HIF-1α may be recruited to the *VWF* promoter, specifically in cardiac MVEC, through interaction with partners that directly bind to the *VWF* promoter sequences. The results of *HIF-1α* knockdown in lung MVEC was consistent with a lack of recruitment of this factor to the *VWF* promoter as demonstrated by ChIP. However, the result of *SP1* knockdown was unexpected and indicates that although ChIP analyses demonstrates enhanced binding of this factor to the *VWF* promoter in lung MVEC, knockdown experiments do not support a role for SP1 in mediating hypoxia induction of *VWF*.

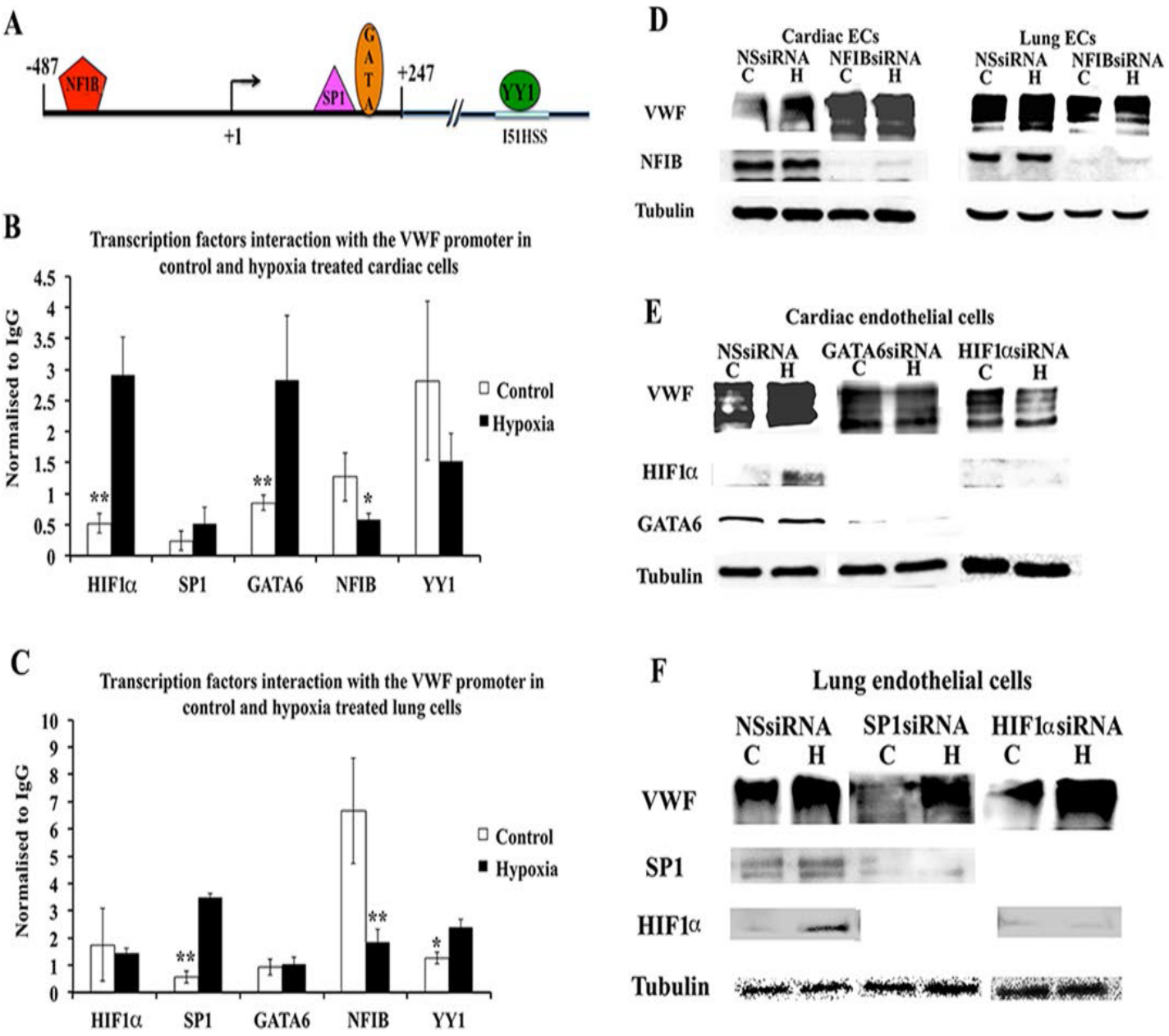


Figure 3-3. Determination of the chromatin binding and participation of specific transacting factors in hypoxia response of *VWF* in lung and heart MVEC. (A) Schematic representation of *VWF* regulatory sequences and corresponding transacting factors. (B-C) Chromatin Immunoprecipitations (ChIP) were performed to determine associations of HIF-1α, SP1, GATA6, NFIB and YY1 with the *VWF* regulatory sequences in control and hypoxia treated lung and heart MVEC. Graphs represent the averages of 3-5 independent experiments for each factor (*P<0.05). (D-F). Human cardiac and lung MVEC were transfected with either non-specific siRNA (NSsiRNA) or

siRNAs that specifically targeted (D) NFIB (NFIBsiRNA), (E) GATA6 (GATA6siRNA) and HIF-1α (HIF1αsiRNA) or (F) SP1 (SP1siRNA) prior to hypoxia exposure. Western blot analyses were performed on control and hypoxia treated cells to determine *VWF* expression and expression of specific siRNA targeted protein levels. The results are representative of 3 independent experiments for each siRNA transfection.

Epigenetic modification of the *VWF* promoter in response to hypoxia

VWF transcriptional regulation has been shown to be correlated with chromatin modifications [57]. We have previously reported that endothelial specific regulation of *VWF* transcription, as well as its induction in response to irradiation, are correlated with increased acetylation of histones, specifically that of histone H4 [57, 101]. To determine whether hypoxia induction also alters acetylation pattern of *VWF* associated histones, we performed similar chromatin immunoprecipitation analyses using antibodies specific to acetylated histones H3 and H4. The results demonstrated a significant increase in association of acetylated histone H4 with the *VWF* promoter in response to hypoxia exposure of both lung and cardiac MVEC (Fig. 3-4A and B). These results demonstrate that hypoxia-induced upregulation of *VWF* transcription, irrespective of the transcriptional machinery that is invoked in different endothelial cells, leads to increased acetylation of 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 expression [66]. Recent data have revealed that DNA methylation of the *VWF* gene is another key factor in regulating its cell type specific, as well as mosaic pattern of expression [67, 154]. The *VWF* promoter contains 8 CpG sites that are targets for methylation, and these sites are hypermethylated in non-endothelial cells, while hypomethylated (for the most part) in endothelial cells. Thus, we proceeded to determine whether hypoxia-induced *VWF* upregulation in lung and cardiac MVEC is associated with alterations in 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 [154]. For these analyses we used methylation sensitive restriction enzymes and PCR analyses as described in methods. Results demonstrated that in cardiac MVEC, specifically site -422 exhibits significantly increased methylation in response to hypoxia, while there were no significant changes in methylation pattern of either of the two sites in human lung MVEC in response to hypoxia (Fig. 3-4C). Dinucleotide CpG in site -422 is within the proximity of NFIB binding site (-442 to -471), and we hypothesize that methylation of this dinucleotide may interfere with NFIB repressor association with the *VWF* promoter, thus contributing to increased transcription.

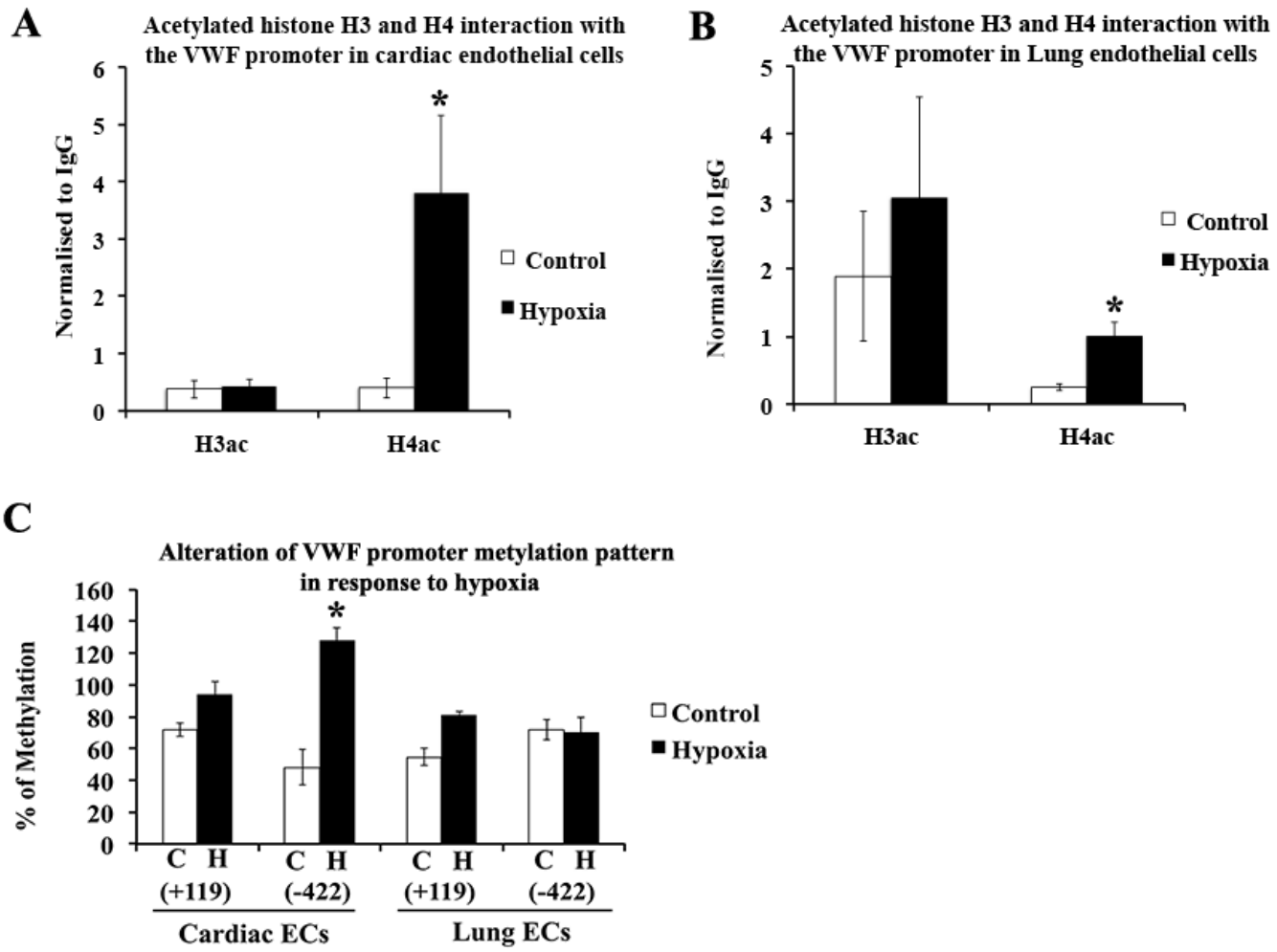
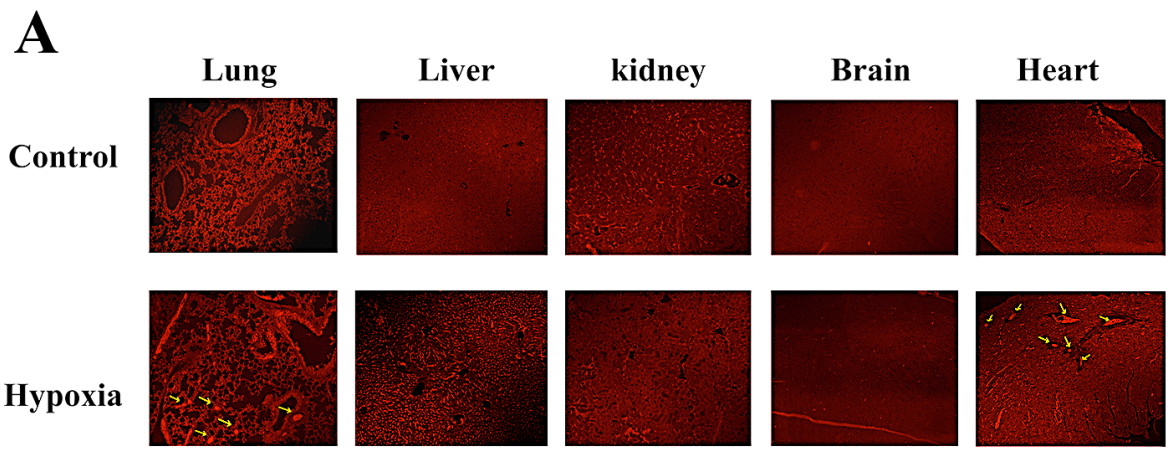


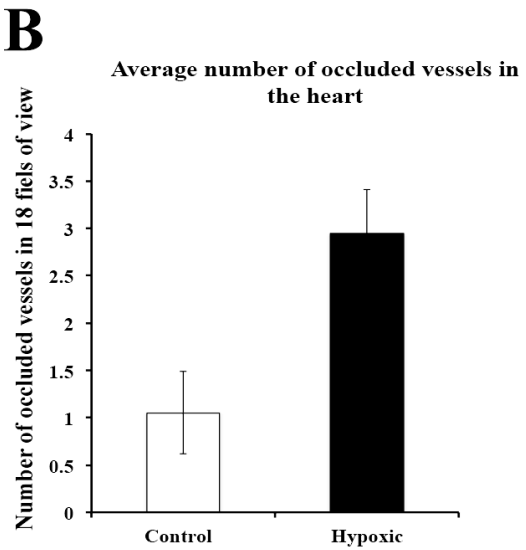
Figure 3-4. Histone acetylation and DNA methylation analyses of the *VWF* promoter in control and hypoxia treated cardiac and lung MVEC. (A-B) Control and hypoxia treated lung and heart MVEC were subjected to ChIP analyses to determine association of acetylated histone H3 and H4 with the *VWF* promoter. (C) Isolated DNA from human cardiac and lung MVEC were subjected to digestion with methylation sensitive restriction enzymes. Digested DNA was subjected to quantitative-PCR analyses using *VWF* promoter specific primers to determine relative methylation status of the CpG dinucleotide at positions -422 and +119 as described in methods. The results for ChIP and methylation analyses are averages of 4 independent experiments for each cell type (*P<0.05).

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

Increased levels of high molecular weight VWF has been associated with increased thrombosis [155, 156]. Thus we explored whether increased VWF in organs of mice exposed to hypoxia is associated with thrombus development. The analyses were performed on organs of hypoxic mice that were previously reported [86]. All major organs of control and hypoxic mice were analyzed for platelets aggregate formation in their vascular beds, using antibody to platelets-specific marker CD41 in immunofluorescence (IF) staining analysis. Significant platelets aggregates were detected specifically in hypoxic heart vessels, and also to a lesser extents in the lung, and arguably in the liver, but not in brain vasculature (Fig. 3-5A). Quantification of occluded vessels (50-80% occlusion) in the heart demonstrated that hypoxia exposure results in a significantly higher number of vessels with platelets aggregates leading to full or partial lumen occlusion (Fig. 3-5B). Consistent with the lack of kidney MVEC response to hypoxia with regard to *VWF* expression, platelets aggregates were not detected in kidney vasculature (Fig. 3-5A). To determine whether platelets aggregates colocalized with VWF expression at target vessels, we performed double staining using VWF and CD41 specific antibodies, followed by confocal microscopy analyses. The results demonstrated the presence of VWF in the platelets aggregates observed in heart and lung vessels of hypoxic mice (Fig. 3-5C and D). These data suggest that platelets aggregates are potentially representative of thrombi. Since fibrinogen

deposits is reported to be associated with thrombus formation, we explored the presence of fibrinogen in the platelets aggregates detected in the hearts of hypoxic mice. We performed triple staining IF to detect platelets, VWF and fibrinogen and demonstrated their colocalization (Fig. 3-6A). To obtain a clear histological representation of any morphological changes that may occur in heart vasculature of hypoxia treated mice, we performed immunohistochemistry (IHC) assay using Tricom staining. IHC demonstrated that in vessels of hypoxic heart endothelial cells were swollen and media thickness was increased. Smooth muscle cells nuclear hypertrophy (nucleomegaly) and perivascular edema were also observed. Microthrombosis and aggregation of erythrocyte in lumina was significant (Fig. 3-6B). Together these results demonstrate that hypoxia-induced *VWF* upregulation specifically in the heart, and to a lesser extent in the lung, of mice is associated with thrombus formation.


A
Lung
Liver
kidney
Brain
Heart
Control
Hypoxia


B
Average number of occluded vessels in the heart
Number of occluded vessels in 18 fiels of view
4
3.5
3
2.5
2
1.5
1
0.5
0
Control
Hypoxic

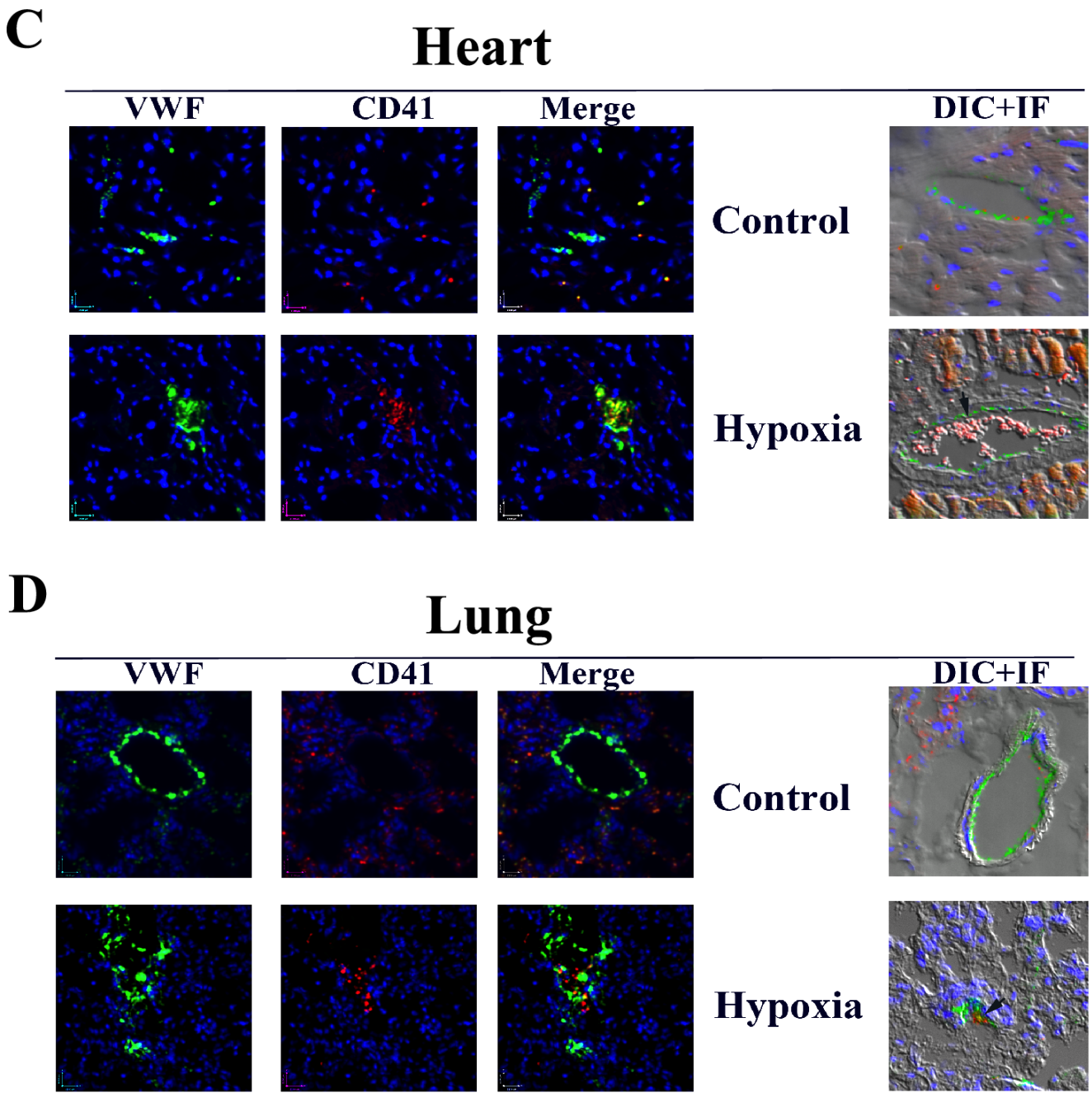


Figure 3-5. Determining the presence of platelets aggregates in organs of control and hypoxic mice.

(A) Frozen sections (5μm) of organs from control and hypoxia-exposed mice were subjected to immunofluorescence (IF) analyses using CD41 (platelets marker) specific antibody (red) as described in 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. (C and D) IF and confocal microscopy analyses of heart and lung were performed using double staining for CD41 (red) and VWF (green) as described in methods. Platelets (red) aggregates were detected in vascular areas demonstrating 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 heart and lungs of control mice. Right panel represents phase contrast (DIC) picture superimposed with IF staining for VWF (green) and platelets (red) of heart and lung vessels. Nuclei are detected by DAPI staining (blue). Results are representative of 2 independent immunostaining experiments with different controls or hypoxia treated mice.

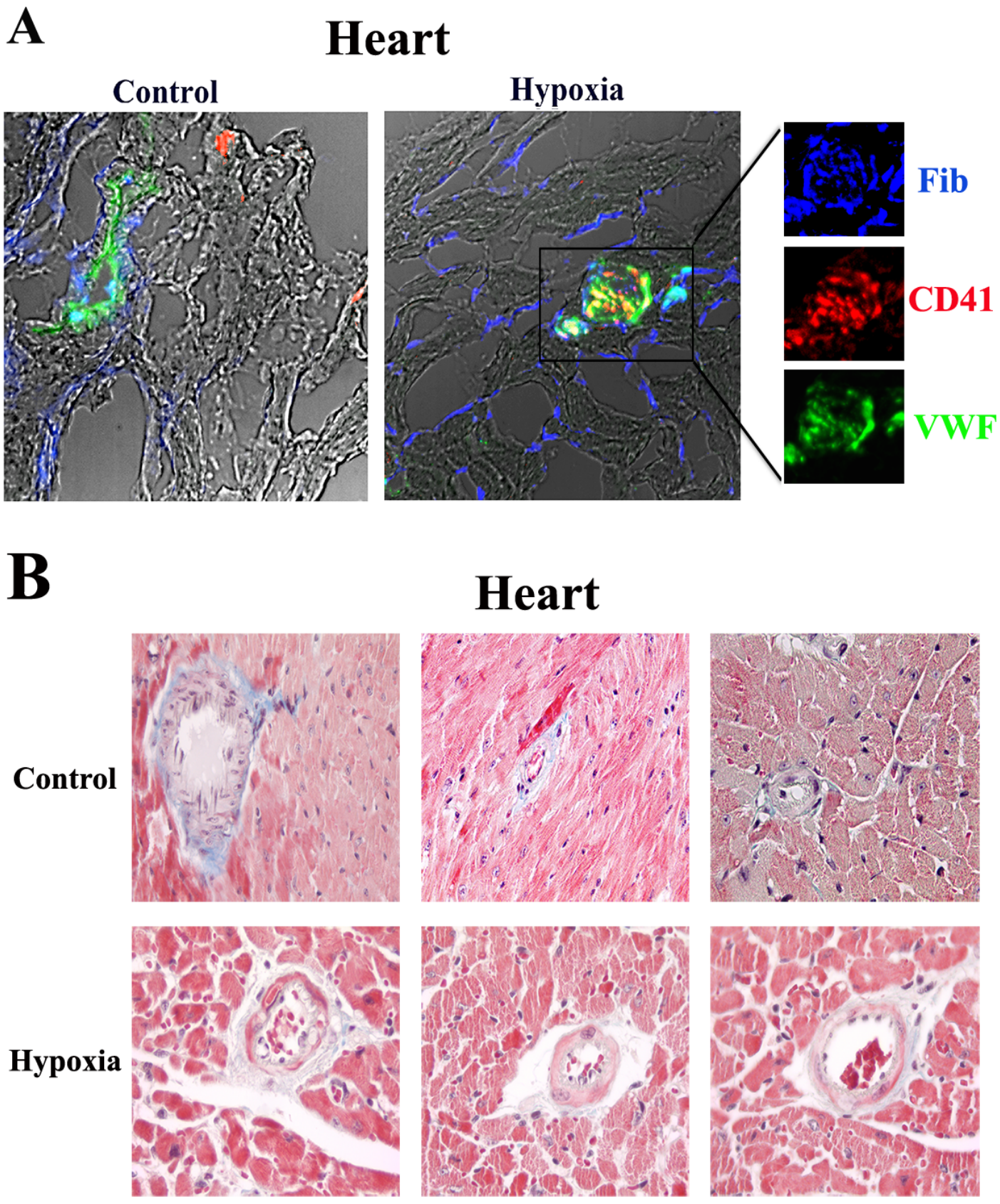

A
Heart
Control
Hypoxia
Fib
CD41
VWF
B
Heart
Control
Hypoxia

Figure 3-6. Thrombus formation and morphological changes in hearts of hypoxic mice

(A) Hearts frozen sections (5μm) from control and hypoxia-exposed mice were subjected to IF analyses using triple staining for CD41 (red), VWF (green), and fibrinogen (blue) specific antibodies as described in methods. Confocal microscopy analyses demonstrated fibrinogen deposits (blue), characterizing thrombus formation, colocalizing with platelet aggregates (red) and VWF (green) in the phase contrast overlay. (B) Tricom staining of control and hypoxic mice hearts demonstrating thickening of vessels walls, microthrombosis and aggregation of erythrocyte in lumina of hypoxic mice (arrows) but not control.

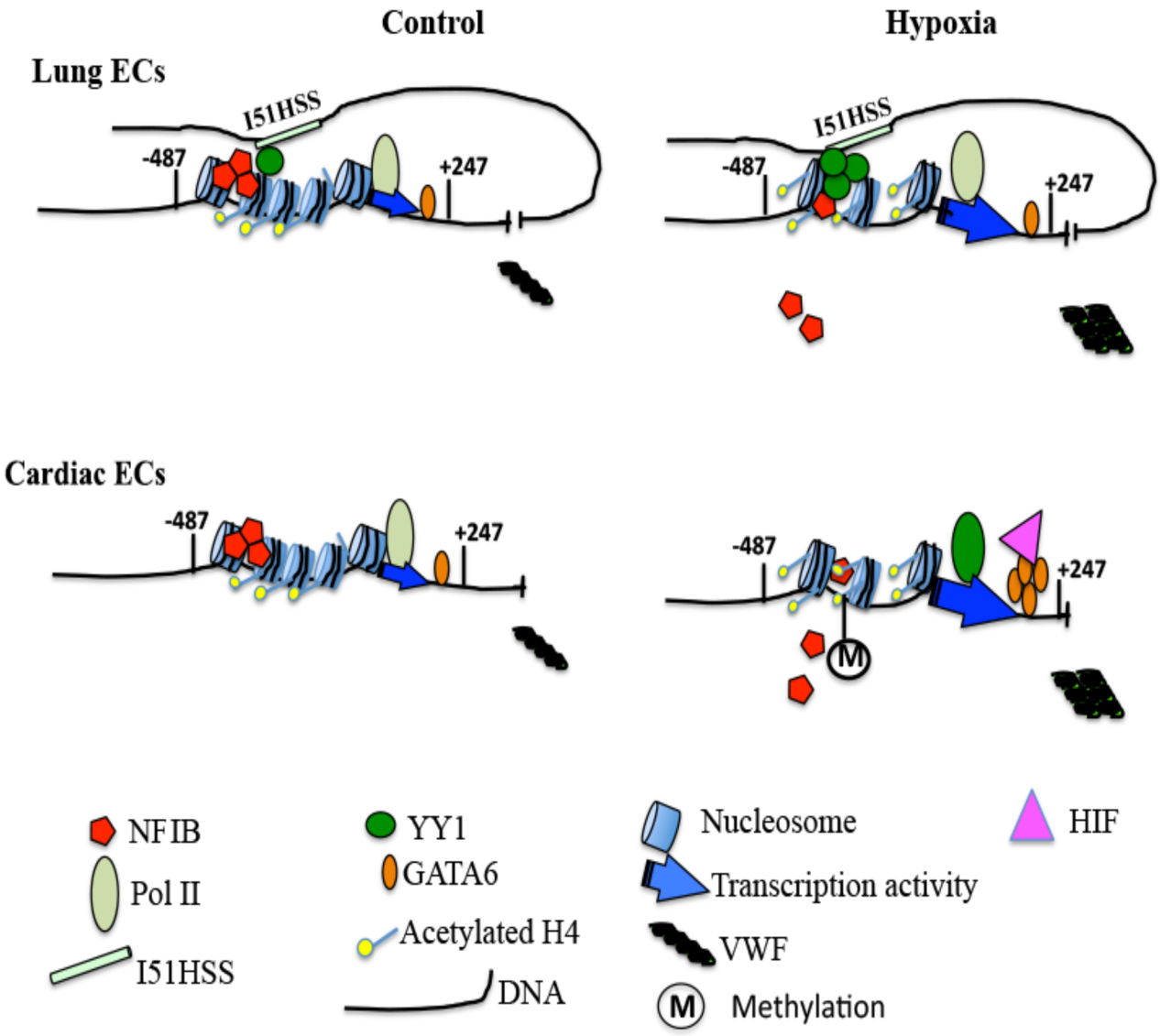


Figure 3-7. Schematic representation of a model describing the mechanisms of hypoxia-induced *VWF* upregulation in heart and lung MVEC. We propose the following model to describe the events leading to *VWF* upregulation in response to hypoxia in lung compared to heart MVEC. In both cell types, hypoxia results in dissociation of repressor NFIB from the *VWF* promoter and increased acetylation of

histone H4 that is associated with the promoter, leading to enhanced transcription. However, in lung endothelial cells, hypoxia also results in increased association of YY1 with a lung specific enhancer located in intron 51 (I51HSS). The I51HSS is brought in proximity to the NFIB binding proximal promoter sequence through chromatin looping as previously shown. Thus we hypothesize that increased YY1 binding may contribute to dissociation of NFIB. In contrast, in cardiac MVEC, YY1 and I51HSS sequences are not involved in hypoxia-induced upregulation of *VWF*, while specifically increased association of GATA6 with the *VWF* promoter, as well as recruitment of HIF-1α, are participating factors. GATA6 and HIF-1α recruitment may contribute to decreased association of NFIB. Furthermore in cardiac MVEC specifically, hypoxia leads to increased methylation of a CpG dinucleotide located at close proximity to the NFIB binding site, which may also hinder NFIB binding. We conclude that a focal event leading to *VWF* upregulation in response to hypoxia is inhibition of NFIB association with the *VWF* promoter. However in different endothelial cells, distinct pathways and specific activators are invoked which converge on this event, i.e. dissociation of NFIB.

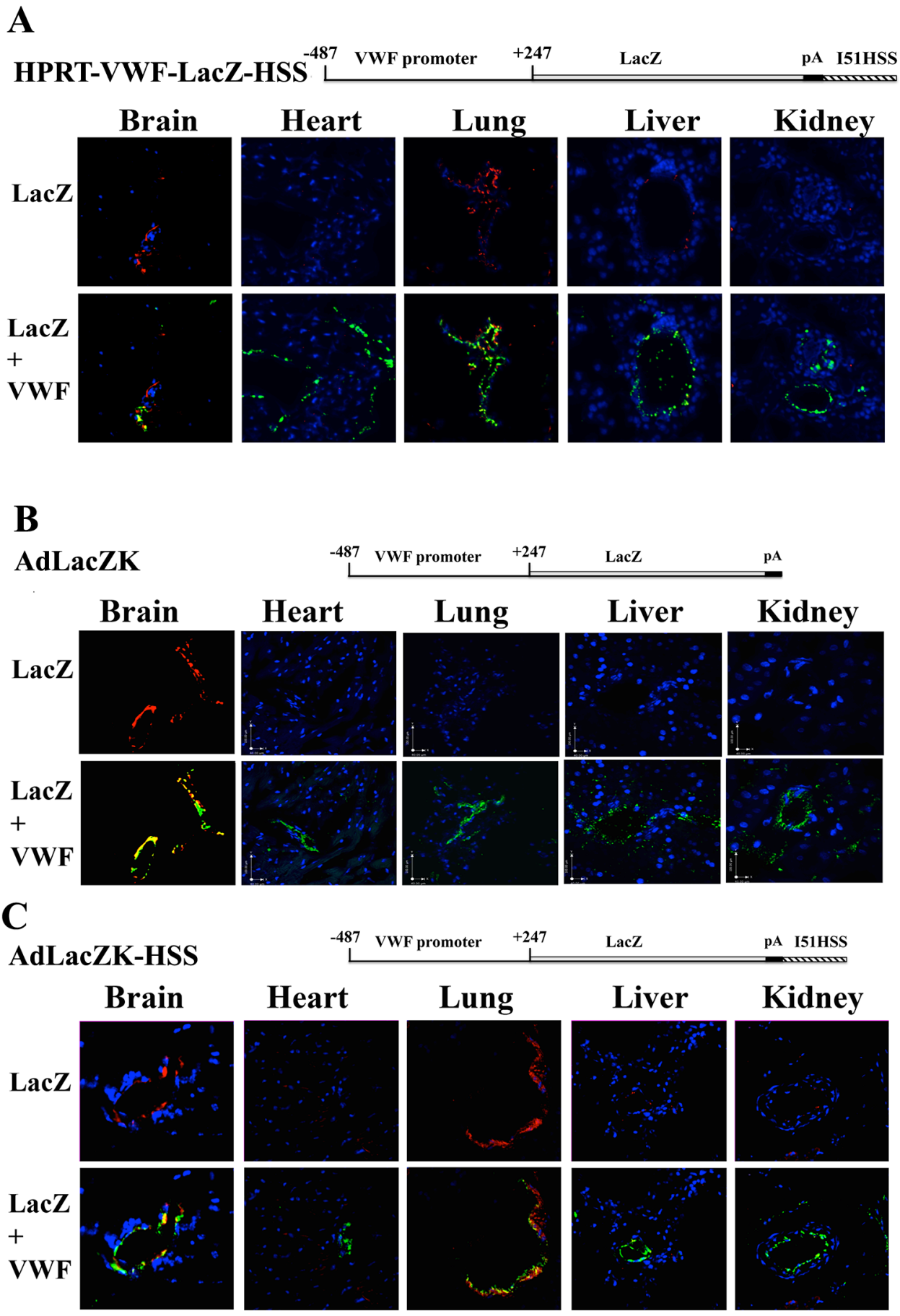

A
HPRT-VWF-LacZ-HSS
-487
VWF promoter
+247
LacZ
pA
I51HSS
Brain
Heart
Lung
Liver
Kidney
LacZ
LacZ
+
VWF
B
AdLacZK
-487
VWF promoter
+247
LacZ
pA
Brain
Heart
Lung
Liver
Kidney
LacZ
LacZ
+
VWF
C
AdLacZK-HSS
-487
VWF promoter
+247
LacZ
pA
I51HSS
Brain
Heart
Lung
Liver
Kidney
LacZ
LacZ
+
VWF

Figure 3-1S. *LacZ* expression patterns in *HPRT* targeted transgenic mice and in mice transduced with adenoviral vectors. Schematic representation of transgenes containing *VWF* regulatory sequences and *LacZ* are shown at the top of each panel. Polyadenylation site is shown as pA. (A) OCT frozen sections (5 μm) of brain, heart, lung, liver and kidneys were prepared from HPRT-targeted transgenic mice harboring transgene VWF-LacZ-HSS. Organ sections were subjected to double IF staining and confocal microscopy analyses to detect transgene LacZ (red) and endogenous VWF (green). Panels demonstrating LacZ expression and colocalization of LacZ and VWF (yellow) are shown. DAPI staining was used to detect nuclei (blue). The results are representative of three independent staining for each organ. (B-C) Mice were transduced with adenoviral vectors (B) AdLacZK and (C) AdLacZKHSS as described in methods. OCT frozen sections of organs were subjected to double IF staining and confocal microscopy analyses to detect LacZ and endogenous VWF as described for (A). The results are representative of three independent transductions for each adenoviral vector and two staining for each organ.

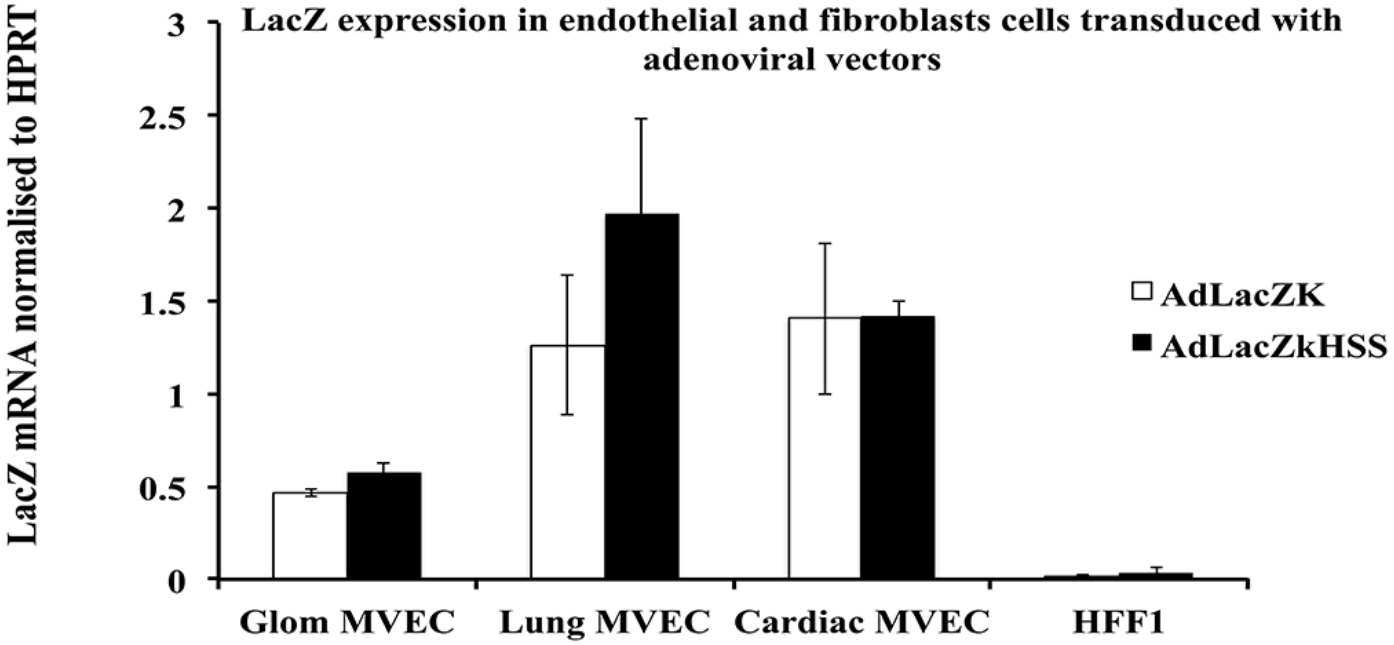


Figure 3-2S. Analyses of *LacZ* expression in various MVEC and fibroblast transduced with adenoviral vectors. Glomeruli (Glom), lung and cardiac MVEC as well as HFF1 were transduced with AdLacZK and AdLacZKHSS as described in methods. 48 hours post-transduction RNA were prepared and subjected to quantitative RT-PCR analyses to detect *LacZ* mRNA and endogenous HPRT. The graph represents the levels of *LacZ* mRNA normalized to *HPRT* and is the average of three independent transductions for each adenoviral vector in each cell type.

DISCUSSION

We have previously shown that hypoxia results in *de novo* expression of *VWF* in lung MVEC; as well as its general upregulation in endothelial cells of lung, heart, liver and brain but not kidney *in vivo* [86]. Here we explored and compared the molecular mechanisms that participate in hypoxia-induced *VWF* upregulation in heart and lung MVEC, and its functional consequences with regard to thrombogenecity. Our analyses demonstrated that the observed *in vivo* organ-specific pattern of *VWF* gene regulation in response to hypoxia is reflected in endothelial cells of corresponding organs that are cultured in vitro, demonstrating that differential hypoxia response of endothelial cells in distinct vascular beds, regarding *VWF* regulation, is at least to some extent independent of microenvironment.

Previous analyses of the mechanism of hypoxia-induced *VWF* upregulation specifically in lung endothelial cells demonstrated that transcription factor YY1 participates in this process [86]. Here we demonstrated that while YY1 did not participate in hypoxia induction of *VWF* in cardiac MVEC, GATA6 and HIF-1α participated in this process. Conversely, neither GATA6 nor HIF-1α participated in hypoxia induction of *VWF* in lung MVEC. Consistent with this observation, GATA6 was reported to play a protective role in lung with regard to pulmonary hypertension, and was reduced specifically in pulmonary endothelial cells in hypoxia-exposed mice [157].

In contrast, NFIB, which is a repressor of *VWF* promoter [55, 153], participated in mediating hypoxia response in both cardiac and lung MVEC. In addition, in both cell types, hypoxia-induced transcription was concomitant with increased acetylation

of histone H4, consistent with previous results demonstrating that both endothelial – specific and irradiation-induced activation of the *VWF* promoter are specifically associated with increased acetylation of histone H4 [57, 101]. However, specifically in cardiac MVEC hypoxia response was associated with increased methylation of a CpG site within proximity of the NFIB binding site. Based on these observations we propose the following model to describe hypoxia induction of *VWF* in cardiac and lung endothelial cells (Fig. 3-7). Hypoxia response is centered on "lifting" the repression exerted on the *VWF* promoter through decreased association of repressor NFIB with the *VWF* promoter, leading to increased acetylation of histone H4, and consequently increased transcription. However, 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, while in cardiac MVEC increased association of GATA6 with the *VWF* promoter may be the mediator of this process. Additionally, increased methylation at CpG site located within the proximity of NFIB binding site may also contribute to the process, by interfering with NFIB binding. The observed role of HIF-1α in cardiac but not lung endothelial cells, and a lack of detectable HIF-1α binding site on the *VWF* promoter, suggests that participation of HIF-1α may occur as a result of its recruitment to the promoter through association with other factors, potentially GATA6. Our result demonstrated that despite certain common elements of hypoxia response of *VWF* in lung and cardiac MVEC, these two cell types employ distinct molecular pathways for this process.

While our results demonstrate that *in vivo* heterogeneity of endothelial cells regarding hypoxia-induction of *VWF* is maintained when corresponding endothelial cells are cultured *in vitro*, It is well established that many organ-specific characteristics of endothelial cells are lost once these cells are removed from their native microenvironment [158]. This includes organ-specific activity of various *VWF* promoter sequences. For instance, proximal *VWF* promoter sequences -487 to +247 exhibit endothelial specific promoter activity and is expressed in a diverse range of endothelial cell types maintained in culture, however *in vivo* these sequences function as an endothelial specific promoter exclusively in brain endothelial cells [39, 59, 159]. Similarly, a fragment containing proximal promoter and I51HSS is active in various endothelial cells in culture, but *in vivo* it only functions as promoter in endothelial cells of lung and brain [63]. Using *HPRT* targeted transgenic mice we have demonstrated that these organ-specific characteristics are independent of gene copy number and integration site (supplementary Fig. 3-S1A). We also explored the activation pattern of adenoviral vectors that carry these sequences (AdLacZK and AdLacZKHSS) *in vivo* and demonstrated that they exhibit similar organ specific activation pattern (supplementary Fig. 3-S1B and C). These results demonstrate that organ-specific activities of these promoters are maintained *in vivo* even when transgenes are expressed somatically and episomally. The adenoviral vectors carrying *VWF* regulatory sequences thus provide useful tools for targeting endothelial cells of specific organs for somatic expression of desired molecules, including anti thrombotic factors for therapeutic purposes. This organ-specific expression pattern that was highly maintained *in vivo* was not observed *in vitro*. The adenoviral vectors, similar to

previously analyzed plasmids carrying *VWF* regulatory sequences, did not exhibit a pattern of activation that reflected their *in vivo* organ specificity (supplementary Fig. 3-2S). However, they demonstrated organ-specific activity regarding response to hypoxia as discussed above. Our results are consistent with previous reports demonstrating that heterogeneity of endothelial cells with regard to external stimuli, specifically inflammatory stimuli including TNFα, IL-I and lipopolysaccharide are maintained *in vitro* [160].

Demonstration of heterogeneity in response of endothelial cells to hypoxia-induced regulation of *VWF* gene raised the question of whether this process has *in vivo* functional consequences with regard to thrombosis. VWF is a procoagulant protein and a major contributor to thrombus initiation and formation. Excessive circulating levels of VWF protein are an independent risk factor for cardiovascular mortality [131]. Increased plasma sodium concentration was recently shown to be a stimulus for increased VWF production, leading to increased generation of microthrombi [92]. Analysis of clinical data from Atherosclerosis Risk in Communities demonstrated an association of VWF levels with the risk of stroke [92]. Thus we analyzed various organs of control and hypoxia exposed mice for the presence of thrombi in various vascular beds. Our results demonstrated presence of significant platelets aggregates that included VWF and fibrin in heart and lung vasculature, but not in the kidney of hypoxia-exposed mice. These results were consistent with significant endogenous *VWF* upregulation in cardiac and lung, and no upregulation in kidney. Although significant *VWF* upregulation was also observed in liver and brain of hypoxic mice, we did not observe detectable thrombi in vasculature of the brain. Platelets aggregates

in liver were barely detectable. The effect of hypoxia on endothelial cells is not restricted to *VWF*, and increased levels of other adhesion molecules including E-selectin and P-selectin have also been demonstrated [161]. Hypoxia was shown to result in increased tissue factor expression by leukocytes and microvesicles, which when tethered to activated endothelial cells will contribute to increased thrombogenecity [161]. Furthermore, hypoxia exposure is shown to down-regulate anti-thrombotic factors such as thrombomodulin, thus also contributing to increased thrombogenicity [162]. We hypothesize that the heterogeneity of endothelial cells' response to hypoxia may also extend to these other pro and/or anti –thrombotic molecules and thus contribute to differential thrombus formation in vasculature of different organ. Additionally other thrombus prevention systems, such as generation of ADAMS13, may be differentially activated and/or functioning in vascular beds of distinct organs in response to hypoxia. This may also contribute to differential thrombus formation in vasculature of different organs; i.e. inhibition of thrombus formation in brain and liver, even in the presence of increased VWF levels. Potential chromatin binding levels of transacting factors which function as activators or repressors of the VWF gene regulation in lung and heart endothelial cells under control and hypoxic conditions are demonstrated in the chart below.

Chromatin binding levels of VWF promoter regulator

		Control	Hypoxia
	YY1	(+)	(+++)
Lung ECs	Sp1	-	-
	NFI	(++)	(+)
	HIF-1α	-	-
	GATA6	-	-
Heart ECs	YY1	-	-
	Sp1	-	-
	NFI	(++)	(+)
	HIF-1α	-	(+++)
	GATA6	(+)	(+++)

CHAPTER 4

VON WILLEBRAND FACTOR EXPRESSION BY CANCER CELLS OF NON-ENDOTHELIAL ORIGIN AND ITS FUNCTIONAL CONSEQUENCES

INTRODUCTION

Von Willebrand Factor (VWF) is a procoagulant protein with an expression pattern that is highly restricted to endothelial cells and megakaryocytes, and commonly used as a marker of endothelial cells [122]. It functions as a mediator of platelets-endothelial/subendothelial adhesion to promote platelets aggregate formation [163-165]. VWF also functions as a carrier for factor VIII, thus increasing its half-life in the circulation [166]. The main function of VWF is long known to be hemostatic regulation. However, VWF has been shown to also participate in immune response, inflammation, angiogenesis and cancer metastasis [28, 167, 168].

Considering the highly adhesive properties of VWF, and its cellular expression pattern in megakaryocytes and endothelial cells, a role for VWF in cancer metastasis has been postulated and explored. It was hypothesized that VWF participates in adhesion of cancer cells to platelets and endothelial surfaces thus facilitating extravasation and promoting metastasis [84]. A role for platelets in cancer metastasis is well established. Tumor cells associate with platelets in circulation and form heteroaggregates, which is proposed to either protect tumor cells from immune surveillance thus increasing their circulatory half-life, and/or contribute to the metastatic process through association of

heteroaggregates with the vascular endothelium [85, 169]. Thus, VWF as a major participant in promoting platelet aggregation /endothelium interactions, presents itself as a highly likely candidate to promote metastasis. Consistent with this hypothesis, anti-VWF antibodies were shown to decrease metastatic activities of some cancer cell lines in mice, and inhibited adhesion of a colon cancer cell line to endothelial cells in a co-culture adhesion assay [170, 171]. Additionally, VWF fibers in tumor vasculature were shown to mediate platelet aggregation and contribute to melanoma metastasis [118].

In contrast, analyses of tumor cell metastasis in VWF deficient mice clearly demonstrated that VWF deficiency significantly enhanced tumor metastasis [167]. Further investigations demonstrated that VWF exerts a pro-apoptotic effect on the tumor cells, thus leading to tumor cell death and consequently reduced metastasis [114, 172]. However, clinical studies exploring levels of VWF in cancer patients and specifically those with von Willebrand disease (VWD) have presented a picture that is more consistent with a potentially pro-metastatic role for VWF [84]. Increased levels of plasma VWF have been consistently demonstrated in patients with colorectal, breast, prostate, ovarian, and other types of malignancies and higher VWF levels were detected in cancers with distant metastasis [84, 115, 117, 173].

Discordant observations regarding the role of VWF in cancer metastasis may be, at least partly, attributed to the focus of investigations on endothelial cells and platelets as the source of VWF. Since *VWF* expression was long believed to be an exclusive property of these two cell types (endothelial cells and megakaryocytes), the possibility that *VWF* may also be expressed in cancer cells (of non-endothelial origin) has been generally unexplored. However, there have been few reports of VWF protein detection in cancer

cells of non-endothelial origin. VWF protein detection was reported in the cultured osteosarcoma SAOS2 cell line, human colorectal SW480 cancer cells, and recently in two hepatocellular carcinoma (HCC) cell lines HepG2 and BEL7402 [119-121]. In these studies, increased levels of VWF in osteosarcoma and hepatocarcinoma tumour tissues in situ were demonstrated and were shown to be associated with increased metastasis and clinicopathologic staging [119, 121]. Increased VWF levels were not associated with increased vascular density [119], suggesting that increased VWF expression may have a cellular origin that is distinct from vascular endothelial cells.

Based on these reports we explored whether some cancer cells of non-endothelial origin, including malignant glioma as well as osteosarcoma SAOS2, acquire *de novo* transcription of *VWF* gene and determined the consequences with regard to tumour cell adhesion and extravasation. We also explored the alteration in transcriptional regulatory mechanisms that are associated with activation of *VWF* gene transcription in cancer cells, and demonstrated the presence of *VWF* expressing cancer cells in patients' tumour samples of malignant glioma and osteosarcoma. Our results indicate that cancer cells that acquire *de novo VWF* expression have increased endothelium adhesion, and extravasation potential, which is conducive to increased metastasis.

MATERIALS AND METHODS

Cell culture

Human umbilical vein endothelial cells (HUVEC) and HEK293 were maintained as previously described [57]. Fibroblast, MDC1 and HeLa were grown in DMEM supplemental with 10% fetal calf serum. Primary renal Proximal Tubule

Epithelial cells (PTEC) were obtained from ATCC and maintained according to the manufacturers protocol. Osteosarcoma SAOS2 (Lonza), KHOS (Lonza) and malignant glioma [U251and M049 (gift from Dr. Godbout)] were cultured using McCoy's 5A (for SAOS2) and DMEM (KHOS and malignant glioma) media with supplements of 15% FBS (for SAOS2) and 10% FBS (for KHOS and malignant glioma). Media for all cells were supplemented with 1% Penicillin-Streptomycin.

RNA and protein analyses

RNA was extracted (Qiagen) from cultured cells (malignant glioma, osteosarcoma and PTEC) and subjected to quantitative reverse transcription-polymerase chain reaction to detect *VWF* and *HPRT*. Primers for RT-PCR were obtained from Qiagen and used as previously described [55]. Western blot analyses were performed for protein detection, using cell lysates prepared from cancer cells, HEK 293, fibroblast and MDC1 cells as previously described [174]. Proteins (50 μg) were loaded on a 6% SDS-PAGE gel and transferred to nitrocellulose membranes overnight, blocked and immunostained with a human specific anti-VWF antibody (Dako Omnis, Denmark) as described [174].

Immunofluorescence staining of cultured cells and tissues

Cultured cells were fixed with 4% PFA, blocked with 10% goat anti serum, washed with 1X PBS, and stained with sheep FITC preconjugated anti-VWF antibody (Abcam-ab8822), as previously described (Mojiri, et al). Slides containing sectioned paraffin embedded biopsies of normal human brain (gift of Dr. Eisenstat), normal bone (distal humeral resection bone samples, Folio Biosciences, Powell, OH), osteosarcoma

(provided by Dr. Sergi) and malignant glioma (provided by Dr. Eisenstat) tumor biopsies [Glioblastoma, Asterocytoma-pilocytic, Glioma WHO II] were processed for deparaffinization and antigen retrieval. The use of patient biopsy samples was approved by the Health Research Ethics Board at the University of Alberta. Briefly, slides were baked at 60^0C for two hours and then deparaffinized with xylene for 3X 10 minutes each, followed by dehydration with ethanol starting at 100% and ending at 50%. Antigen retrieval was done by boiling the slides in sodium citrate for 10 minutes. Slides were blocked with 10% donkey sera for 1 hour at room temperature followed by incubation with primary antibodies against CD31 (Dako Omnis, Denmark) and VWF (sheep FITC preconjugated anti-VWF antibody). Primary antibodies incubation was carried out at 4^oC overnight followed by incubation with secondary antibody (Cy3 conjugated anti-rabbit antibody, Invitrogen, Burlington, ON) against CD31. Slides were mounted in mounting media containing DAPI (Invitrogen, Burlington, ON).

Chromatin immunoprecipitation assay

Chromatin immunoprecipitations (ChIP) were performed using antibodies to specifically determine interactions of NFIB, GATA6, acetylated histones H3 and H4, HDAC2, PCAF with the *VWF* promoter. Anti IgG antibody was used as negative control. All antibodies were purchased from Abcam Inc. (Abcam, Cambridge, MA, USA). ChIP assays were performed, using primers for PCR that specifically amplify *VWF* proximal promoter sequences (human VWF NF1-binding region), as previously described [174].

DNA methylation analysis

The two potential methylation sites (cytosines in CpG dinucleotide) located in the *VWF* promoter (-422 and +119) were tested for methylation using OneStep qMethyl kit (Zymo Research, Irvine, CA), according to the company's manual. The sequences of primers are noted in (Table 4-1). Briefly, DNA extracted from cells (1 μg) was either digested or not with methylation sensitive restriction enzyme Hpall. Digested and undigested products were used as templates for quantitative PCR. Two sets of primers were used to amplify DNA sequences centered around the target CpG dinucleotide sequences. If the sites in the promoter DNA sequences are methylated they will be protected from digestion and amplified, otherwise no or little amplification products is detected.

Generation of *VWF*-depleted cancer cells

To knock down *VWF* in cells both siRNA and shRNA were used. For transient *VWF* knock down, cells were transfected twice within 48 hours with siRNA [(10 nmol/L) non-silencing or *VWF* specific silencing] using Lipofectin 2000 (Thermofisher) transfection reagent according to the manufacturer's protocol. The VWF specific siRNA used was Hs_VWF_4 Target Sequence:
5'-AACATGGAAGTCAACGTTTAT-3' (QIAGEN Cat. no.: SI00011830).
The non-specific siRNA used was ALL Stars Neg. Control siRNA (QIAGEN Cat. no.: I027281).

For stable knock down of *VWF,* shRNAs in lentiviral vectors were used. Cells were transduced with lentiviruses containing either VWFshRNA (sc-36828-v Santa Cruz

Biotechnology, Santa Cruz, CA), or non-specific NSshRNA (sc-108080 Santa Cruz Biotechnology, Santa Cruz, CA). Stably transduced cells were isolated by puromycin selection. To visualize cancer cells, prior to shRNA transduction, cells were stably transduced with a GFP/luciferase expressing lentivirus (provided by Dr. Lewis) and GFP+ cells were selected by fluorescence-activated cell sorting (FACS) analysis. Transductions of all lentiviral vectors were performed according to the manufacturer's protocol.

Cancer cell adhesion to HUVEC monolayer under static and shear flow conditions

Cancer cells were labeled with cytoplasmic staining reagent (Cell Tracker-Invitrogen) for visualization according to the manufacturer's protocol. In static condition, 10^5 cells (non- transfected, or transfected with either VWFsiRNA or NSsiRNA) were incubated on top of a monolayer of HUVEC cells for 30 min. in 35mm cell culture dishes. Cells were washed with PBS and were trypsinized for FACS analyses. Cancer cells were detected by cytoplasmic green fluorescence from Cell Tracker using a Becton Dickinson (San Jose, CA) FACS and quantified using CellQuest software. Similar analyses were performed using cancer cells that were pre-incubated with freshly isolated human platelets for 20 min. prior to adding to the HUVEC monolayer. After 30 min. incubation, non-adhered cells were removed and fixed to proceed with immunofluorescence staining using cd42b (marker for platelets) as described above. Laminar flow adhesion assays were performed as previously described [174] except that platelet-cancer mixture was passed over endothelial monolayer. Platelet-cancer cells

heteroaggregate interactions with endothelial monolayer under sheer flow were visualized, captured using CCD camera, and quantified as previously described [174].

The Ex Ovo Chick Embryo assay

GFP+ cancer cells (10^5) non-transduced, transduced with VWFshRNA or NSshRNA were injected intravenously into a vein of the chick chorioallantoic membrane (CAM). Endothelial cells within the CAM were marked by intravenous injection of Lectin-Rhodamine/Fluorescein. Cancer cells were assessed 8 hours post injection for their intra or extravascular localization (extravasated) and quantified as previously described [175].

Mouse experimental lung extravasation assay

GFP+ cancer cells ($8.7x10^5$) transduced with VWFshRNA or NSshRNA were injected into the tail vein of immunocompromised mice (NOD *scid* gamma 005557, Jackson laboratory). Mice were euthanized 24 hours post injection and lungs were analyzed for extravasated cells by intravital microscopy as previously described [175]. GFP+ cancer cells in the lung were quantified. The Health Sciences Animal Policy and Welfare Committee at the University of Alberta approved all animal housing and experimentation.

Trans-well assay

Membranes in 24 well Transwell culture dishes (Corning Transwell, Corning Inc. Corning, NY) were coated with 2% gelatin. HUVECs were grown on the Transwell to generate an intact monolayer. GFP+ cancer cells (10^4) transfected with VWFsiRNA or NSsiRNA were added to the endothelial monolayer and incubated for two hours. Cells

were washed and fixed with 4% PFA, then stained for CD31 as endothelial cell marker. Membranes were detached from the wells and mounted in mounting media on the slides. The number of green cancer cells, which transmigrated through the endothelial monolayer and the membrane pore were quantified.

Statistical analyses

Data are given as mean (SD) and statistical analyses used the paired *t* test. Statistically significant changes ($p<0.05$) are marked by asterisks (*)

RESULTS

VWF is expressed in some cancer cells of non-endothelial cell origin.

To determine whether *VWF* is expressed in cancer cells, we screened a variety of malignant glioma cell lines, as well as two osteosarcoma cell lines SAOS2 and KHOS to detect *VWF* mRNA and protein. Various levels of *VWF* mRNAs were detected by quantitative RT-PCR in malignant glioma and SAOS2 cell lines, but not in KHOS or proximal tubular epithelial cells (PTEC, used as negative control) (Fig. 4-1A). RNA from human umbilical vein endothelial cells (HUVEC) was used as positive control, but due to significantly higher levels of *VWF* mRNA in HUVEC, it is not represented in the graph. VWF protein expression was detected by Western blot analysis in selected malignant glioma cancer cells, as well as in SAOS2, and HUVEC (positive control); but not in KHOS and other primary and established cell lines of non-endothelial origin that were used as negative control (Fig. 4-1B). Cytoplasmic VWF expression was also demonstrated by immunofluorescence staining in SAOS2 and a representative malignant glioma cell line M049, but not KHOS (Fig. 4-1C). These results demonstrated that some cancer cell lines of non-endothelial origin acquired the ability to express *VWF* at the RNA and protein levels.

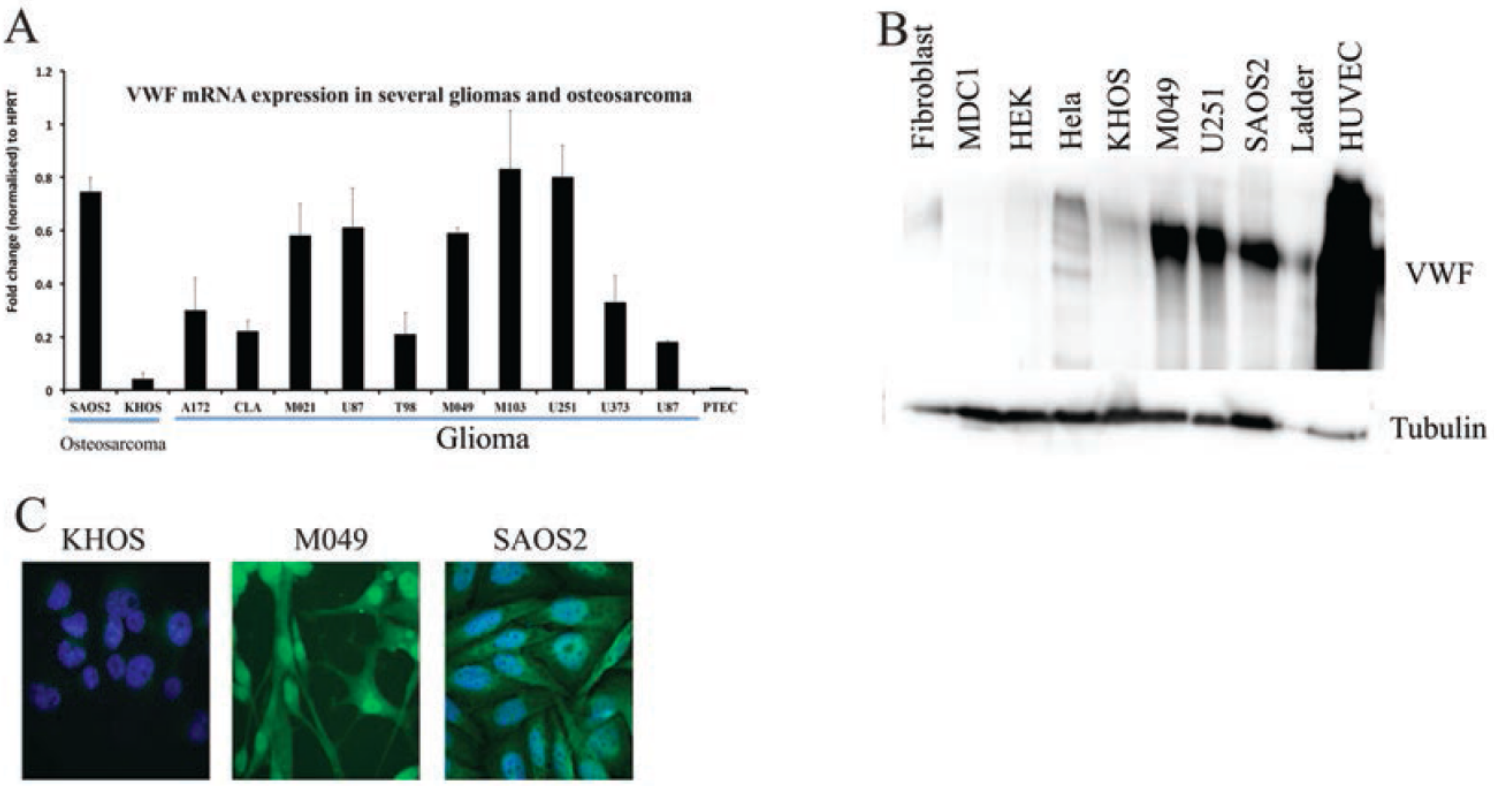


Figure 4-1. *VWF* is expressed in some cancer cell lines of non-endothelial origin.

(A) Quantitative RT-PCR analyses were performed to detect *VWF* mRNA expression in osteosarcoma cell lines SAOS2 and KHOS as well as several malignant glioma cell lines (on the chart from A172 to U87). Proximal tubular epithelial cells (PTEC) were used as negative control. The levels of *VWF* mRNA were normalized to HPRT. (**B**) Western blot analyses using human VWF specific antibody was performed to detect VWF protein. Cell lysate from two osteosarcoma cell lines SAOS2 and KHOS, two of the malignant glioma cell lines (M049 and U251), and several other non-endothelial cell types including HEK 293 (HEK), HeLa, human primary fibroblast (Fibroblast) and primary dendritic cell (MDC1), as well as human umbilical vein endothelial cells (HUVEC, used as positive control) were used for these analyses. Tubulin expression was used as loading control. (**C**) KHOS and SAOS2 and malignant glioma M049 cell lines were subjected to immunofluorescence staining to detect VWF (green). DAPI staining (blue) marked the nucleus (20X magnification). Results are representative of 3-4 independent experiments.

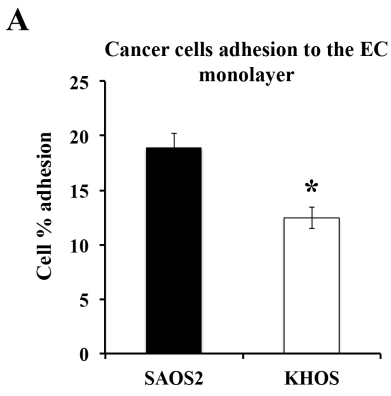
Functional consequences of *VWF* expression by cancer cells with regard to endothelial and platelet adhesion

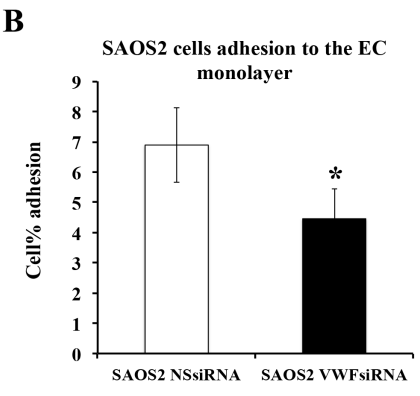
To determine whether *VWF* expression influences endothelium-adhesion capability of the cancer cells, *VWF* expressing (SAOS2) and non-expressing (KHOS) osteosarcoma cell lines were treated with cytosol staining fluorescent dye (Cell tracker-green) for visualization, and equal numbers of cells were incubated on the monolayer of endothelial cells. Adhesions of cancer cells to endothelial monolayers were determined by immunofluorescence (IF) staining and fluorescent activated cell sorting (FACS) analyses as described in methods. The results demonstrated that SAOS2 exhibited significantly higher endothelium-adhesion capacity compared to KHOS (Fig. 4-2A). To determine whether *VWF* expression by SAOS2 contributed to its higher endothelial adhesiveness SAOS2 were transfected with either VWF-specific (VWF siRNA) or control non-specific siRNA (NSsiRNA) prior to incubation on monolayer of endothelial cells. Effective silencing of *VWF* in SAOS2 with VWF-specific siRNA was confirmed by western blot analysis (Fig. 4-2C). The results demonstrated a significant reduction in endothelial monolayer adhesiveness of SAOS2, when these cells were transfected with VWF-specific siRNA compared to control (Fig. 4-2B).

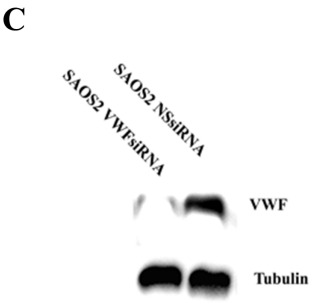
To test the hypothesis that *VWF* expression by cancer cells also contributes to enhanced cancer cell-platelet aggregate formation and increased association of heteroaggregates with endothelial monolayer, we incubated fluorescently labeled SAOS2 and KHOS with freshly isolated platelets for 20 minutes prior to perfusion on a monolayer of endothelial cells (HUVECs). After 30 minutes, endothelial cells were washed, fixed and immunofluorescence staining performed for platelet activated marker

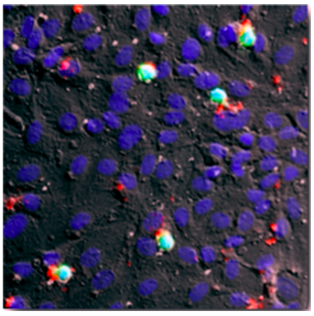
cd42b. Adhered cancer cells were quantified and results indicated that more SAOS2–platelets mixtures were adhered to the monolayer of endothelial cells compared to KHOS-platelets mixture (Fig. 4-2D, E).

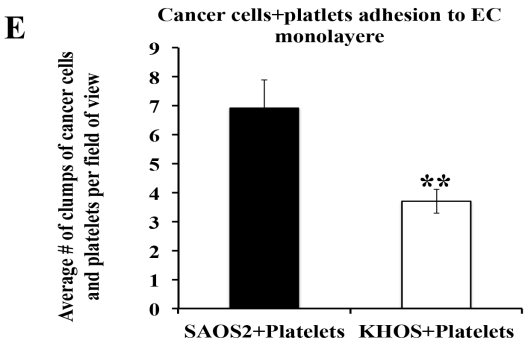
To explore the adhesion ability of cancer cells in a more physiologically relevant condition we induced shear flow and let cancer cells-platelet mixtures flow on top of the endothelial monolayer for 10 minutes using a laminar shear flow chamber. We observed that under flow, SAOS2 cells form clumps (cancer cell-platelet heteroaggregates) that are larger in size and number compared to those formed by KHOS. Treatment of SAOS2 with VWF-specific siRNA resulted in a significant decrease in number as well as the size of the clumps (Fig. 4-2F-J). These results demonstrated that *VWF* expression by cancer cells promotes their interaction with platelets and adherence to endothelial cells.

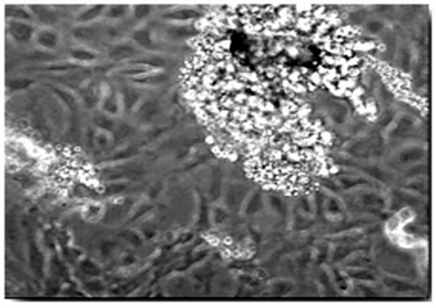




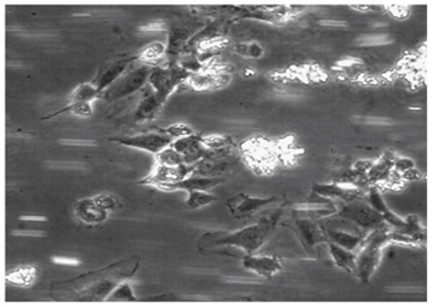




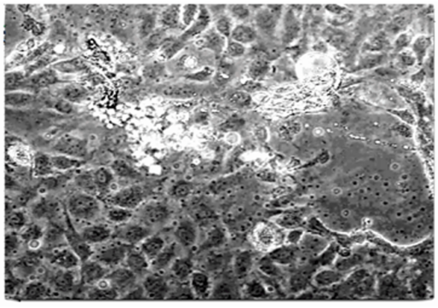




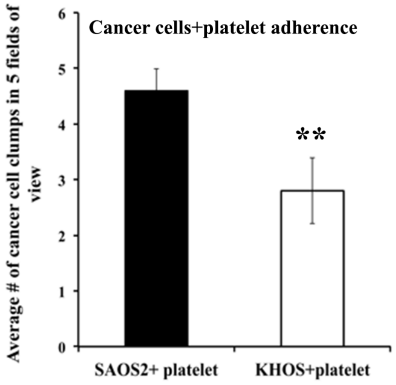
SAOS2 NSsiRNA + platelets



KHOS + plateles



SAOS2 VWFsiRNA + platelets



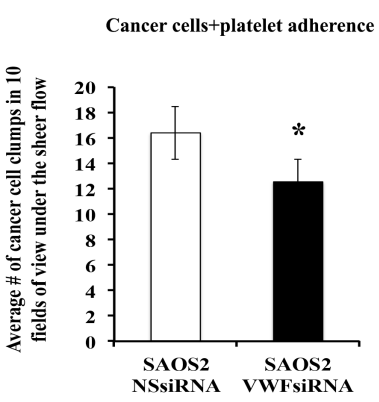


Figure 4-2. *VWF* expressing cancer cells demonstrate increased adhesion to the endothelial cells and platelet under static condition and shear flow.

(**A and B**) Cancer cells [KHOS, SAOS2, SAOS2 transfected with non specific siRNA (NSsiRNA) and *VWF* specific siRNA (VWFsiRNA)] were labelled using fluorescent CellTracker (green) and incubated on HUVEC monolayer. Percentages of adhered cells were determined by flow cytometry. (C) Western blot analysis demonstrating the knock down of *VWF* in SAOS2 transfected with VWFsiRNA compared to NSsiRNA. (**D and E)** Fluorescently labeled SAOS2 and KHOS cells (green) were incubated with freshly isolated platelets for 20 min prior to adding to the monolayer of HUVEC. Adhered cells were fixed and stained using antibody cd42b, a marker for activated platelets. (**D**) A representative picture of cancer cells (green) in association of platelets (red) on the monolayer of HUVEC (blue shows nuclei stained with DAPI) is shown in. (**E**) Clumps of cancer cells and platelets (heteroaggregates) were counted in 5 fields of view and presented as bar graphs. (**F-J**) Using parallel-plate laminar flow adhesion assay, fluorescently labelled cancer cells (KHOS and SAOS2 as well as transfected with NSsiRNA or VWFsiRNA) that were incubated with freshly isolated platelets were perfused over the HUVEC monolayer at shear flow 1 dyne/cm2 for the 10 min. Cancer cell–platelet heteroaggregates clumps in representative fields of views are shown in **F-H** and were quantified in 10 fields of view for each and presented in bar graphs **I** and **J**. Results for adhesion assays are representative of minimum of 3 independent experiments (triplicate each) for both static and shear flow.

Functional consequences of *VWF* expression by cancer cells with regard to transmigration

To determine whether increased endothelial-adhesiveness of VWF-expressing cancer cells leads to increased transmigration across an endothelial barrier, we performed the Transwell migration assay as described in methods. For these analyses SAOS2 cells were transduced with a lentivirus carrying GFP for visualization. GFP-SAOS2 that were maintained as control, transfected with NSsiRNA, or VWF-specific siRNA were seeded on a monolayer of HUVECs in a Transwell culture dish. Following 2 hours of incubation the transwell-membrane containing the endothelial monolayer and cancer cells were washed, removed and subjected to immunofluorescence analyses and confocal microscopy to detect and quantify the number of cancer cells that had transmigrated through the endothelial monolayer. The results demonstrated that a significantly higher number of *VWF* expressing cancer cells (control SAOS2-GFP or SAOS2-GFP transfected with NSsiRNA) had transmigrated through the endothelial barrier compared to SAOS2-GFP cells that were transfected with VWF-specific siRNA (Fig. 4-3). The results demonstrated that acquiring *VWF* expression leads to enhanced transmigration of cancer cells.

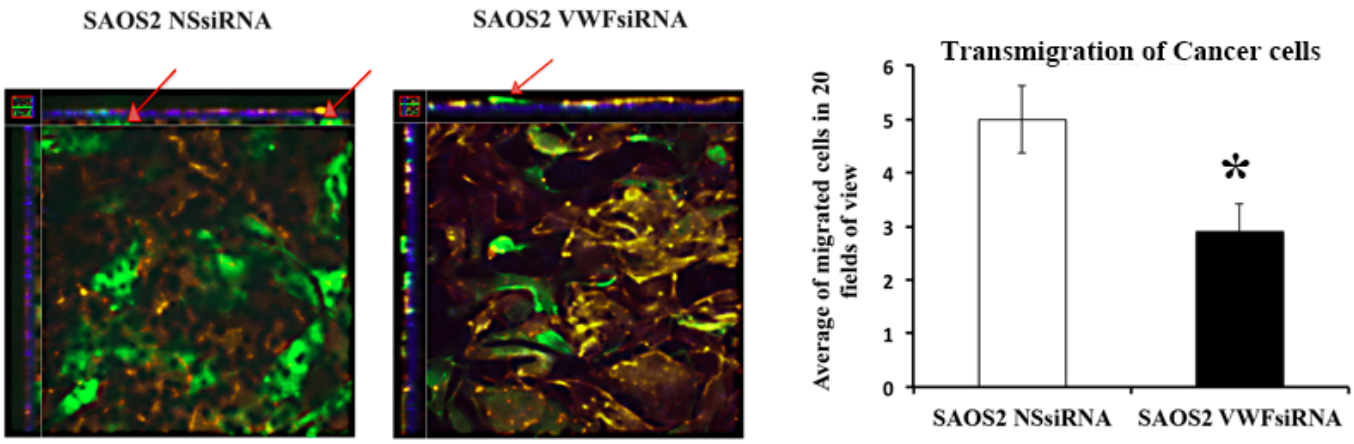


Figure 4-3. *VWF* expressing cancer cells demonstrate increased transmigration. Transwell assay analyses were performed to determine the transmigration capacity of SAOS2 cells that were transfected with NSsiRNA or VWFsiRNA. Cells were incubated on the intact monolayer of HUVEC for two hours and numbers of cancer cells (green) that were able to transmigrate through endothelial cell monolayer (red) and move to other side of the membrane (blue) were quantified (shown in bar graphs). Representative snapshot pictures of confocal microscopy showing cancer cells that were transmigrated (under the blue membrane shown by arrows in NSsiRNA transfected SAOS2) or not (above the blue membrane shown by arrow in VWFsiRNA transfected SAOS2). Results are averages of 3 independent experiments (each in triplicate).

VWF expression promotes cancer cell extravasation

To determine whether increased endothelial-adhesiveness and transmigration ability of VWF-expressing cancer cells translates into increased potential for extravasation, we performed an ex ovo chicken chorioallantoic membrane (CAM) assay. For these analyses, SAOS2-GFP cells were transduced with either VWF-specific shRNA (VWFshRNA) or control-nonspecific shRNA (NSshRNA) and injected into the vitelline vein of ex ovo chicken embryos. Eight hours post injection, the embryo vasculatures were labeled with Rhodamine-Lectin and cancer cells extravasation was determined by intravital microscopy analyses as previously reported [175]. The results demonstrated that SAOS2-GFP cells that were either non-transduced or transduced with NSshRNA migrated intravascularly and passed the endothelium to seed extravascular stroma and start dividing; whereas SAOS2-GFP cells transduced with VWFshRNA were trapped inside the vessels and demonstrated significantly lower number of extravasated cells (Fig. 4-4A-D). Similar experiments were performed with malignant glioma cells, which also demonstrated a significant reduction in extravasation as a result of *VWF* knockdown (Fig. 4-1S.Supplementary data). These results strongly support the hypothesis that *VWF* expression confers a significantly enhanced extravasation potential to cancer cells of diverse origins such as osteosarcoma and malignant glioma. Further support for this hypothesis was provided by the results of *in vivo* mouse extravasation analyses, in which SAOS2-GFP expressing cells, transduced with either VWFshRNA or NSshRNA, were injected into the tail vein of immunodeficient mice, and mice lungs were analyzed for the presence of cancer cells 24 hours post injection, using an immunofluorescence assay. These analyses demonstrated that the number of SAOS2 transduced with NSshRNA, was

significantly higher than that of cells transduced with VWFshRNA in the lungs of injected mice (Fig. 4-4E-G). These results further support the hypothesis that acquiring *VWF* expression confers an enhanced extravasation capability to cancer cells.

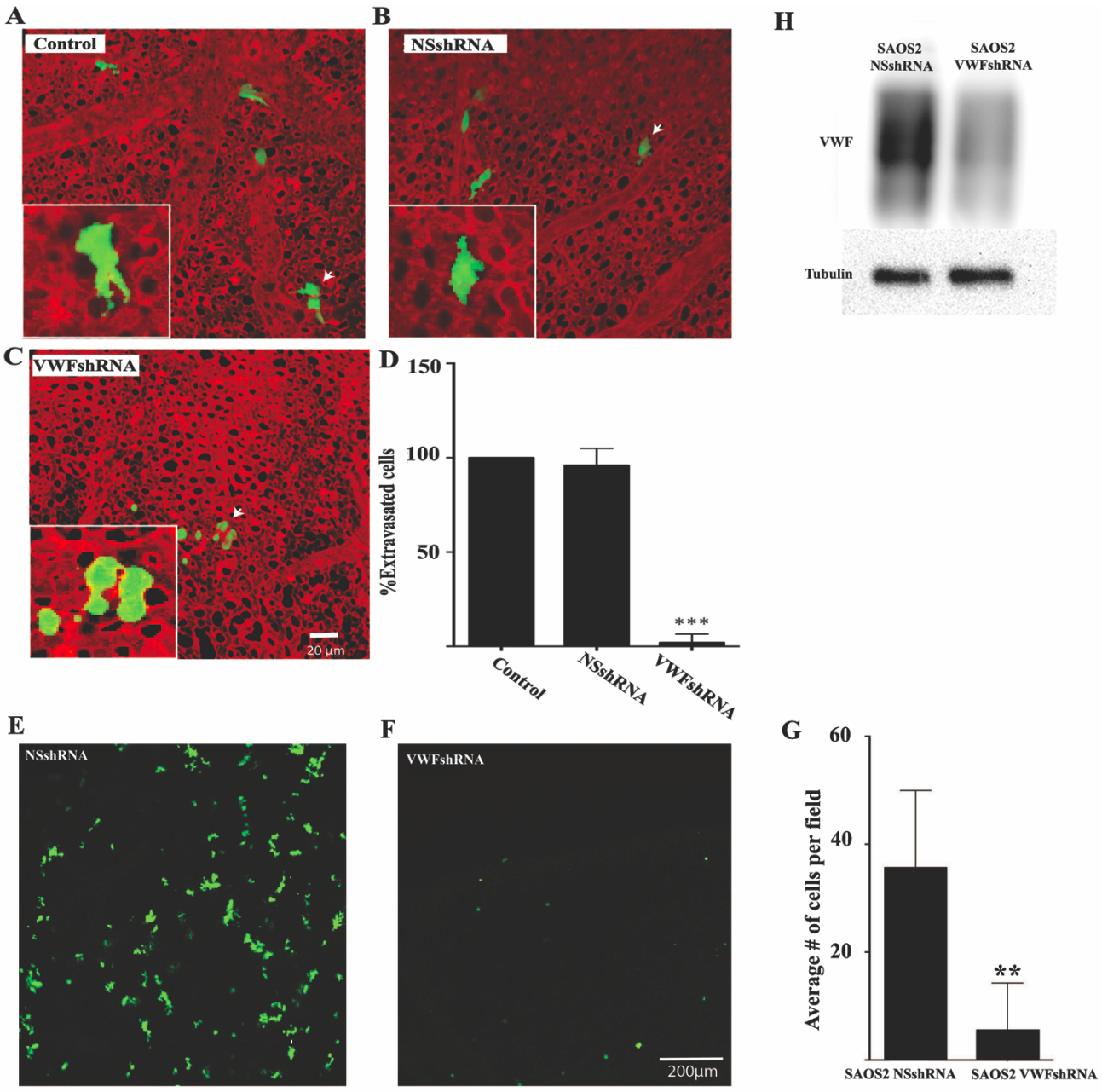


Figure 4-4. *VWF* expression by SAOS2 results in enhanced extravasation. GFP expressing SAOS2 were stably transfected with shRNA targeted specifically against *VWF* (VWFshRNA) or a non-specific shRNA (NSshRNA). (**H**) Western blot analysis confirmed *VWF* knockdown in SAOS2 cells expressing VWFshRNA. (**A-D**) Control (NSshRNA) and *VWF* knockdown (VWFshRNA) SAOS2 cells were injected

intravenously into the veins of 12dpf (12 day post fertilization) chick embryos. Eight hours post injection embryos' vasculatures were labeled with Rhodamin-Lectin (red) and cancer cells (green) were quantified for their extra or intra vascular localization. Representative images of (**A**) control (non-transduced GFP expressing SAOS2), (**B**) NSshRNA and (**C**) VWFshRNA transduced SAOS2, 8 hours post injections are shown. Panels **A-C** show 3D rendering of the cells and the vasculature. Insets show single optical sections of selected (white arrows) cells. (**D**) Quantification (n=6) of percentage of extravasated control, NSshRNA and VWFshRNA expressing SAOS2. (**E-G**) GFP expressing SAOS2 that were transfected with NSshRNA or VWFshRNA was injected into the tail vein of immunodeficient mice and cancer cell localization in the lung were determined after 24 hours. Representative images of mice lungs with GFP positive cancer cells (green) expressing are shown in **E** and **F**. Quantification of extravasated cancer cells in the lungs of mice (n=4) are shown in **G**.

Mechanism of *de novo* activation of *VWF* expression in cancer cells

Normally *VWF* expression is exclusively restricted to endothelial cells and megakaryocytes. Thus, to determine whether expression of *VWF* in the SAOS2 cell line was indicative of acquiring an endothelial cell phenotype, we explored the expression pattern of other endothelial-specific genes in the two osteosarcomas cell lines SAOS2 and KHOS. The results showed that, except for *VWF*, other endothelial cell-specific genes analyzed were either not detectable or were similarly expressed in the two cell types (Fig. 4-2S Supplementary data). These results suggest that the *VWF* expression by SAOS2 is not a consequence of a general phenotypic shift of these cells towards acquiring an endothelial cell phenotype. However, acquiring *VWF* expression by cancer cells of non-endothelial/megakaryocyte origin suggests an alteration in the gene regulatory mechanisms that should otherwise inhibit *VWF* expression in these cells.

Previous analyses of transcriptional regulation of the *VWF* gene have demonstrated participation of a number of transcription activators (GATA transacting factors, Ets, Histone H1-like protein, NFY interacting with CCAAT elements) and repressors (NFI, Oct1, NFY interacting with a non-consensus sequence) (Fig. 4-5A), as well as chromatin modifications and DNA methylation [39, 41, 42, 44-47, 57, 67, 154, 174, 176]. Thus to explore the mechanism of transcriptional activation of the *VWF* gene in cancer cells, we explored the presence and VWF-chromatin binding patterns of these regulatory transacting factors in the VWF- expressing (SAOS2) and -non-expressing (KHOS) osteosarcoma cell lines. Comparative RNA analyses demonstrated that the expression levels of trans-acting factors that function as activators of *VWF* (ETS, GATA2 and 6) were not significantly different between the two cell types. Also expression levels

for two of the subunits that comprise NFY, namely NF-YA and C were not altered, while the levels of the other subunit NF-YB were increased in SAOS2 compared to KHOS (Fig. 4-4SA Supplementary data). Since NFY can function as either activator or repressor of *VWF*, we could not determine the correlation of this increased NF-YB level to *VWF* expression; however similar levels of expression of other factors that were shown to strictly function as activators in the two cell types suggests that acquiring *VWF* expression is not generally correlated to increased levels of transacting factors that function as activators of *VWF* promoter. Analyses of the expression levels of various NFIs transacting factor that strictly function as a repressors of *VWF* promoter demonstrated that while NFIA, C and X were expressed at higher levels in SAOS2, NFIB levels were significantly decreased in SAOS2 compared to KHOS (Fig. 4-4SB and C Supplementary data). These results suggeste that decreased levels of repressor NFIB might correlate with increased *VWF* expression in SAOS2.

To determine whether increased *VWF* expression is correlated with altered association of these transcription factors with the *VWF* promoter we performed chromatin immunoprecipitation (ChIP) analyses. Consistent with lower levels of NFIB in SAOS2, chromatin immunoprecipitation analyses also demonstrated a significant decrease in binding of NFIB to the *VWF* regulatory sequences in SAOS2 compared to KHOS (Fig. 4-5B). Using a pan NFI antibody that does not discriminate among the four of NFIs (A, B, C and X), chromatin immunoprecipitation analyses demonstrated that in general NFI interaction with the *VWF* promoter was significantly reduced in SAOS2 compared to KHOS. These results suggest that despite higher levels of NFIA, C and X in SAOS2, the NFI interaction with the *VWF* promoter in SAOS2 is significantly reduced compared to

KHOS. ChIP analyses of GATA6 association with the *VWF* promoter demonstrated that despite similar levels of this transacting factor in the two cell types, significantly higher levels of GATA6 were associated with the *VWF* promoter in SAOS2 compared to KHOS. The results demonstrate that *VWF* expression in SAOS2 is associated with decreased expression and interaction with the repressor NFIB, as well as increased association of GATA6 with the *VWF* promoter, when compared to KHOS (Fig. 4-5B).

To gain further insight into the mechanism of *VWF* transcription, we determined the correlation of *VWF* expression with epigenetic modifications of the *VWF* promoter in SAOS2 and KHOS. We have previously demonstrated that *VWF* promoter in endothelial and non-endothelial cells is differentially associated with histone deacetylase HDACs and that the net result is increased acetylation of histone H4 that is associated with the active *VWF* promoter [57]. Thus to determine whether *VWF* expression in SAOS2 is associated with similar *VWF* chromatin modifications, we performed ChIP analyses to determine association of HDAC, PCAF and acetylated histones H3 and H4 with the *VWF* chromatin encompassing the promoter region. The results demonstrate that while the level of PCAF association with the *VWF* promoter is similar between the two cell types, HDAC association is significantly decreased in SAOS2 compared to KHOS (Fig. 4-5C). Furthermore, specifically association of acetylated histone H4 (but not that of Histone H3) was significantly higher in SAOS2 compared to KHOS (Fig. 4-5D). These results are highly consistent with those previously reported for *VWF* expression in endothelial cells, and demonstrate that acquiring *VWF* expression in SAOS2 is accompanied by decreased association of histone deacetylase HDACs and subsequently increased acetylation of promoter associated histone H4.

To explore association of *VWF* expression with the methylation pattern of the *VWF* promoter sequences, we determined the methylation status of two specific CG elements located at -422 and +119 that were reported to be non-methylated specifically in endothelial cells. Quantitative PCR were performed on the DNA isolated from SAOS2 and KHOS, as well as malignant glioma U251 (a malignant glioma cell line that also expresses *VWF*) and HUVECs, which were subjected to digestion by methylation sensitive restriction enzymes. Comparison of methylation status of -422 site demonstrated that all three cancer cell types (SAOS2, KHOS and U251) exhibit increased methylation of this site compared to HUVECs (Fig. 4-5E). However, when comparing KHOS and SAOS2, there were no significant differences in methylation status of -422 site between these two cell types. In contrast site +119 exhibited significantly less methylation in SAOS2 compared to KHOS (Fig. 4-5E). Methylation levels for this site was similar to that observed in HUVECs. *VWF* expressing U251 malignant glioma cells also exhibited methylation levels at +119 site that were similar to HUVEC (Fig. 4-5E). These results are consistent with decreased methylation, specifically at site +119 being associated with the *VWF* promoter activation in cells that express *VWF*.

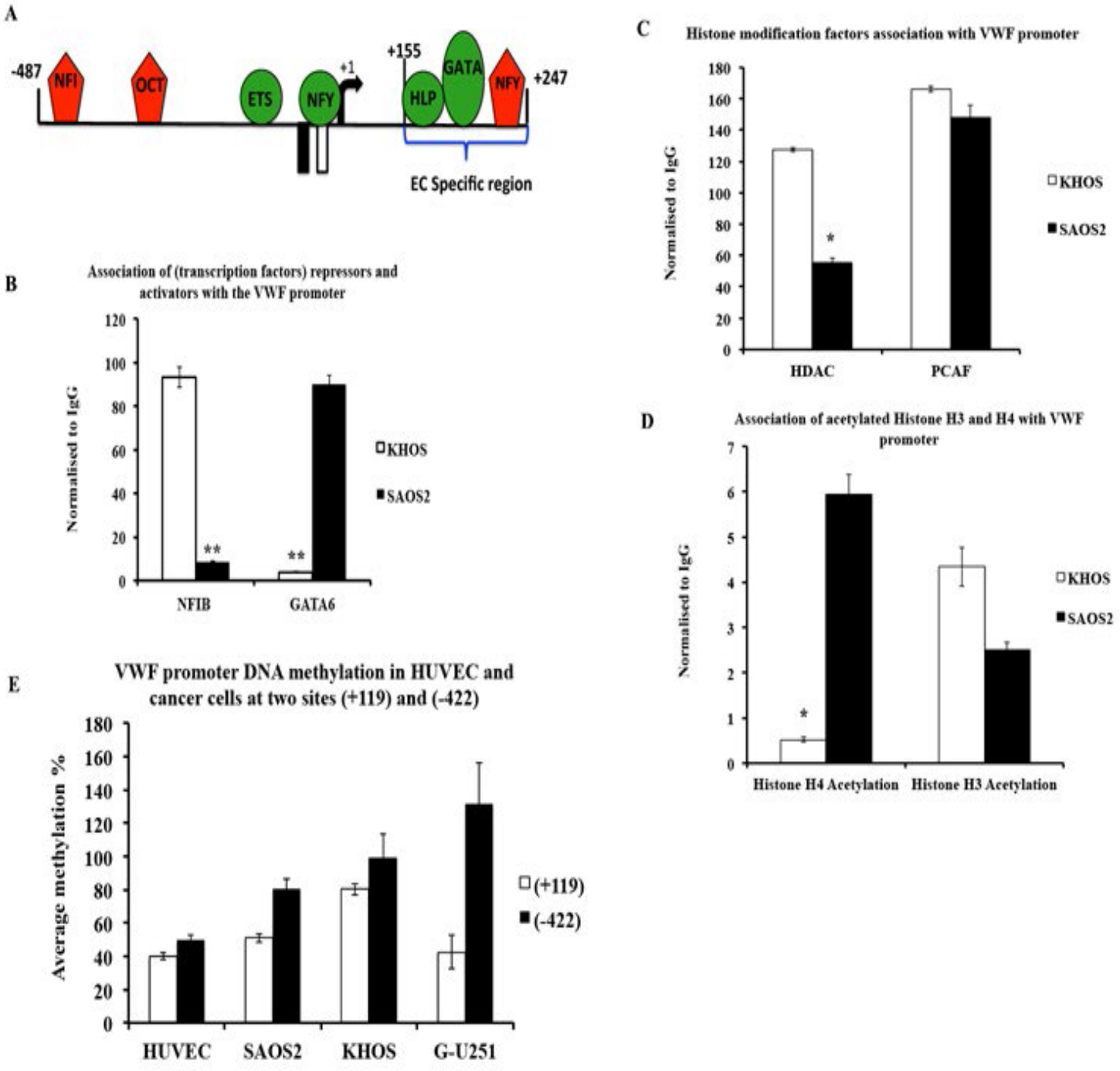


Figure 4-5. Transcription factors association and epigenetic modifications of the *VWF* promoter in VWF-expressing and –non-expressing cancer cells. (**A**) Schematic representation of *VWF* promoter and transcription factors that positively (green) and negatively (red) regulate the promoter activity. (**B-D**) Chromatin immunoprecipitations (ChIP) analyses of SAOS2 and KHOS

were performed (n=3 for each factor) to determine association of (**B**) NFIB and GATA6, (**C**) histone modifying cofactors PCAF and HDAC1, and (**D**) acetylated histones H3 and H4. (**E**) DNA isolated from cancer cells (SAOS2, KHOS, and glioma G-U251) and HUVEC (as a positive control for *VWF* expressing endothelial cells) were subjected to digestion with methylation sensitive restriction enzymes and subjected to quantitative-PCR analyses (using *VWF* promoter specific primers as described in methods) to determine relative methylation status of the CG nucleotides at positions -422 and +119. The results are averages of 5 independent experiments.

Sub populations of cancer cells in patients with osteosarcoma and malignant glioma tumor express *VWF*

To further probe the physiological relevance of the *in vitro* and *in vivo* results regarding the functional consequences of *VWF* expression by cancer cells, we analyzed one patient's sample of osteosarcoma and three patients' samples of brain tumors [Glioblastoma, Asterocytoma-pilocytic, Glioma WHO II] for *VWF* expression. We performed immunofluorescence staining on tumor samples as well as normal brain and bone tissues using antibodies against endothelial marker CD31 and VWF protein. Our results demonstrate that in normal brain and bone tissues, all cells that express VWF (green), also express CD31 (red), indicating that all the *VWF* expressing cells detected are of endothelial origin (Fig. 4-6A and C). However, in all tumor samples we observed some cells that only express VWF (green) but not CD31, demonstrating that they are not of endothelial origin and most likely they represent tumor cells that acquired *VWF* expression (Fig. 4-6B, D, E, F white arrows). Although these analyses were performed on only a few tumor samples and statistical significance will require analyses of significantly more patients 'samples, they underscore the physiological relevance of *VWF* expression by cancer cells since they demonstrate that this phenomenon is not restricted to cancer cell lines that are grown in culture.

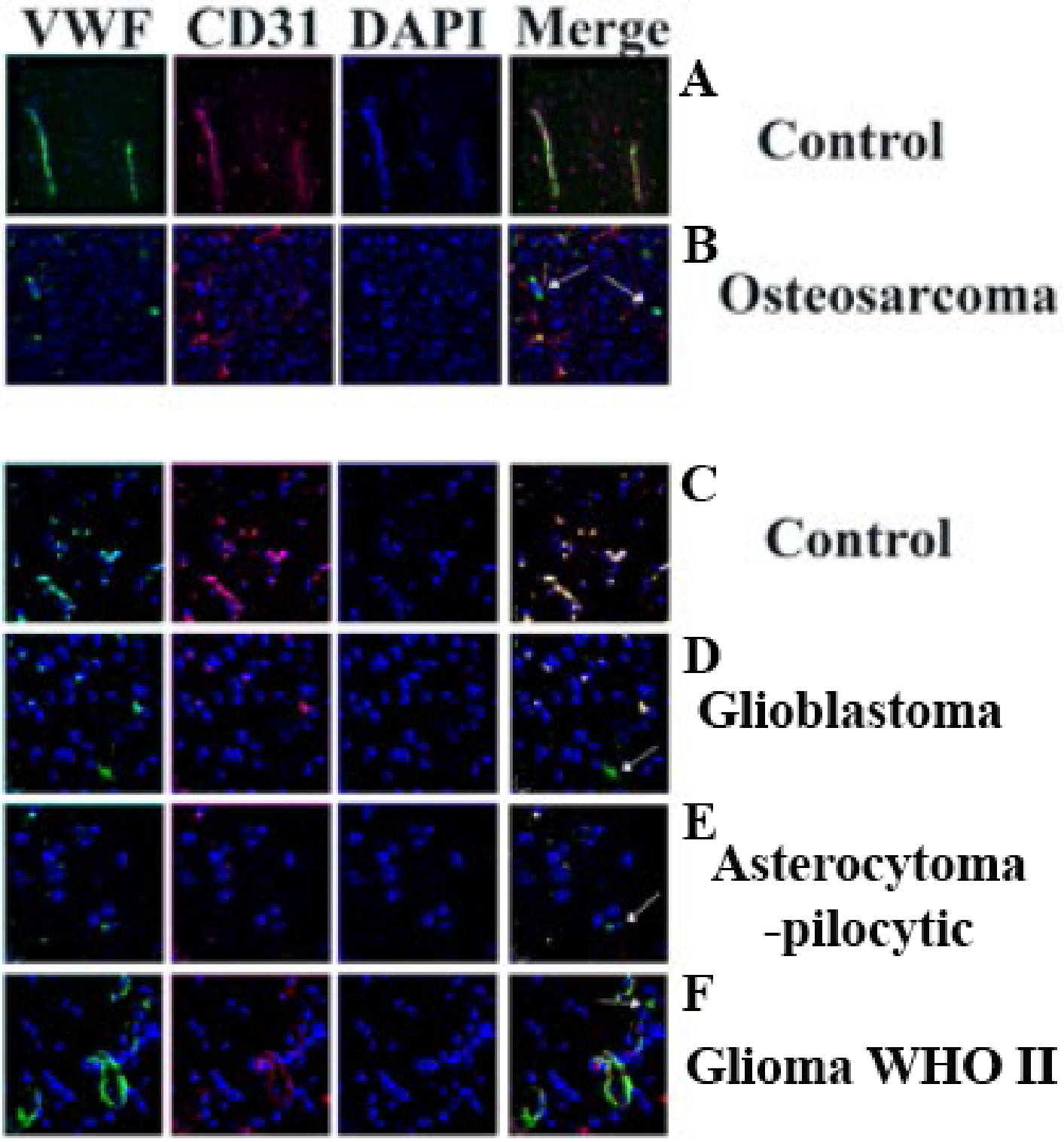


Figure 4-6. Detection of *VWF* expressing cancer cells in osteosarcoma and malignant glioma patients' tumor biopsies. Confocal microscopy and immunofluorescence staining

for VWF (green) and endothelial cell specific protein CD31 (red) were performed in (**A**) normal bone, (**B**) an osteosarcoma tumor sample, (**C**) control brain, and (**D-F**) three independent malignant glioma tumor samples Glioblastoma, Asterocytoma-pilocytic, Glioma WHO II]. Colocalization of VWF and CD31 (yellow) was observed in all samples, demonstrating *VWF* expression by vascular endothelial cells in all samples. However, in tumor samples, but not controls, a few *VWF* expressing cells that did not exhibit colocalization with CD31, demonstrating their non-endothelial origin, were also detected (shown by arrows). Blue represents DAPI stained nuclei. Results are representative of two independent staining for each sample.

DISCUSSION

Commonly used expression of the *VWF* as a marker for endothelial cells is reflective of its highly restrictive regulation and exclusive expression in endothelial cell and megakaryocytes. Thus, previous reports of its detection in some cancer cell lines, including osteosarcoma SAOS2 [119-121], that are of neither endothelial nor megakaryocytic origin presents a unique opportunity to determine how and why this endothelial specific gene is activated in some cancer cells. We analyzed a number of malignant glioma as well as osteosarcoma (SAOS2 and KHOS) cell lines for *VWF* expression. Our analyses demonstrated that *VWF* RNA and proteins were detected in a number of malignant glioma cell lines, as well as osteosarcoma SAOS2, while not detectable in another osteosarcoma cell line KHOS as previously reported [119]. These results combined with previous reports of VWF detection in human colorectal SW480 cancer cells [120], and two hepatocellular carcinoma (HCC) cell lines HepG2 and BEL7402 [121], demonstrate that acquiring *VWF* expression by subpopulations of cancer cells is not restricted to one or two cell lines and thus may not be attributed to a phenotypic artifact of cultured cells. This conclusion is supported by immunofluorescence confocal microscopy analyses of few patient malignant glioma and an osteosarcoma tumor samples that demonstrate the presence of VWF positive and CD31 negative cells, thus confirming that in situ tumors contain cells of non-endothelial origin that express *VWF*. Collectively the results demonstrate that a subpopulation of non-endothelial cancer cells, from various origin, acquire *de novo* expression of *VWF*.

To determine the functional consequences of *VWF* expression by cancer cells we chose to focus on the pair of osteosarcoma cells, SAOS2 and KHOS, as *VWF* expressing

and non-expressing cancer cells of closely related and similar origin. We compared their adhesion ability to endothelial cell monolayer under static condition, shear flow, and in absence and presence of platelets. Our analyses demonstrated that in all these processes osteosarcoma cells that express *VWF*, namely SAOS2 exhibited increased association with the endothelial cell monolayer, and formed larger and more adhesive platelet-cancer cell heteroaggregates. Furthermore, *VWF* knockdown of SAOS2 significantly interfered with its endothelial cell adhesiveness, in the presence or absence of platelets.

The results demonstrated that acquiring *VWF* expression confers an increased endothelial and platelet adhesion capacity to the cancer cells, which may contribute to an increased metastatic potential. The metastatic process also involves migration and extravasation of cancer cells from the blood stream and "seeding" of target organs. *In vitro* migration assays of SAOS2 and KHOS demonstrated an increased migration capability for SAOS2 compared to KHOS, which was significantly reduced by *VWF* knockdown. Furthermore, *in vivo* analyses of extravasation potential of these cells demonstrated a significantly higher rate of extravasation for SAOS2 compared to KHOS in CAM assay, as well as in mice extravasation assay. Direct involvement of VWF in extravasation of SAOS2 was demonstrated by significant reduction in extravasation as a result of *VWF* knock down in SAOS2. We also performed the CAM assay on a *VWF* expressing malignant glioma cells with or without *VWF* knock down and the results demonstrated a significant reduction in extravasation potential of the *VWF* knocked-down malignant glioma compared to that of control. Based on these results we propose that acquiring *VWF* expression confers a metastatic advantage to subpopulations of cancer cells, by enhancing their platelet interaction, adhesion capacity, migration, and

extravasation capability. We propose that this hypothesis may bring together seemingly contradictory evidence regarding the role of VWF in cancer metastasis.

On one hand the use of *VWF* knock out mice to investigate cancer cell metastasis clearly demonstrated an anti-metastatic role for VWF, which was shown to exert an apoptotic effect on cancer cells [114, 167]. On the other hand the use of VWF antibodies were shown to decrease metastatic activities of some cancer cell lines in mice, and inhibited adhesion of a colon cancer cell line to endothelial cells in a co-culture adhesion assay [170, 171]. These observations may be reconciled if we consider a distinct role for VWF when expressed by endothelial cells compared to that acquired by cancer cells. We hypothesize that cancer cells that do not express *VWF* may be susceptible to pro-apoptotic effect of external VWF. Thus such cancer cells will have enhanced survival and metastasis if presented to *VWF* knock out mice. Furthermore, if a population of cancer cells expresses *VWF* and this contributes to their metastatic role, they will continue to metastasize in the *VWF* knockout mice. However, in experiments in which anti-VWF antibodies were introduced in mice, these antibodies will exert their effect not only on VWF from endothelial and megakaryocytes, but also on the subpopulation of cancer cells which may be expressing *VWF*. Thus, interfering with VWF function in *VWF* positive cancer cells may be the mechanism by which metastatic process was affected in experiments that involved anti-VWF antibody.

We explored the mechanism by which *VWF* expression is acquired in cancer cells. Previous analyses of the *VWF* transcriptional regulation had led to identification of a number of cis- and transacting factors that regulate *VWF* transcription. Although an endothelial-specific master regulator has not been identified, several repressors and

activators that in combination participate in *VWF* transcription regulation are identified [39, 41, 42, 44-47, 57]. In addition, a distinct role for chromatin modification and DNA methylation were also demonstrated in *VWF* transcriptional regulation [57, 67, 154]. Based on this information, we explored the role of various components of the *VWF* transcriptional regulatory system and demonstrated that *VWF* expression in osteosarcoma cancer cells SAOS2 was associated with a significant decreased binding of the repressor NFI to the *VWF* promoter; while binding of the activator GATA6 to the promoter was significantly enhanced. Furthermore, chromatin modification corresponding to increased levels of acetylated histone H4 that was associated with the *VWF* promoter was observed, and DNA methylation associated with specific region in the promoter was reduced.

These results were highly consistent and similar to what was observed for active *VWF* promoter in endothelial cells compared to non-endothelial cells. Analyses of the *VWF* promoter in a *VWF* expressing malignant glioma cell generated similar results (i.e, increased GATA6 binding, reduced NFI binding and increased acetylation of histone H4 integrin alphaV beta3. These observations suggests that irrespective of the cellular origin, and potentially distinct upstream events that may be invoked, downstream events that converge on *VWF* gene are similar and target NFI, GATA6, histone H4 acetylation and DNA methylation. These results provide strong evidence for the role of these regulatory components in establishing *VWF* transcription and present them as potential targets for future development of therapies towards regulation of *VWF* expression.

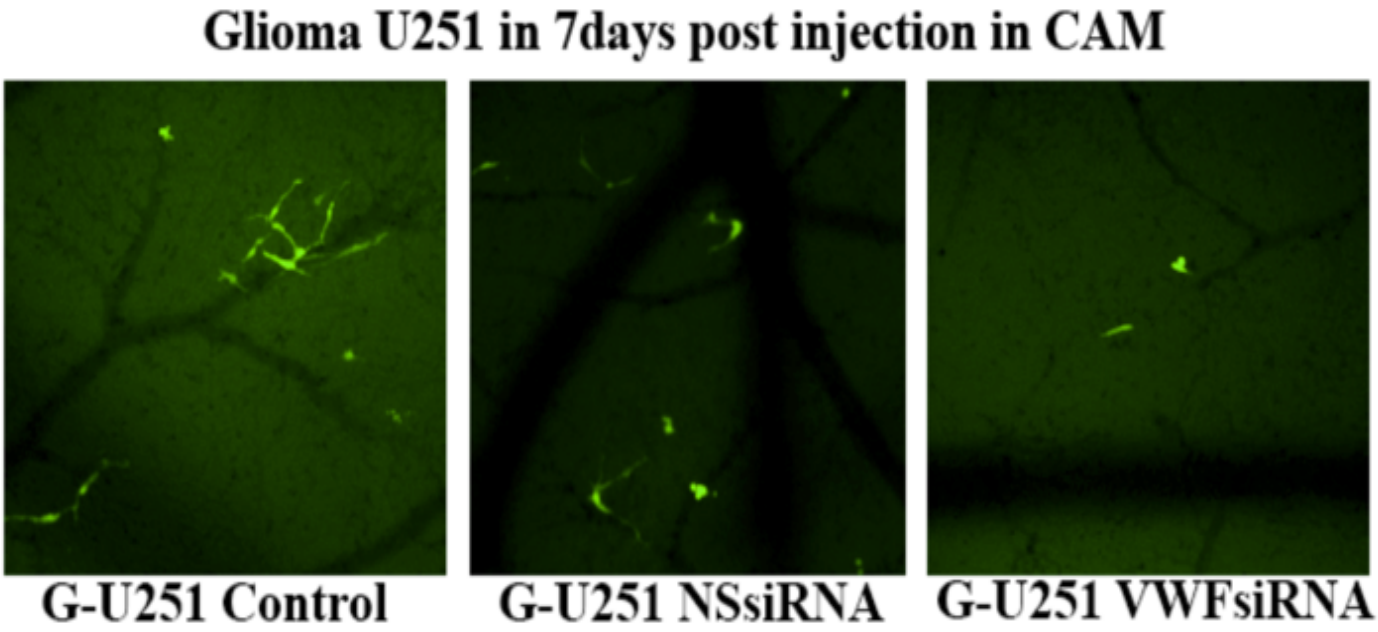


Figure 4-1S. Representative image from CAM assay of U251 (GFP+) cells. Control malignant gliomaU251 (left panel), malignant glioma U251 transfected with either non-specific siRNA (middle panel), or with VWFsiRNA (right panel) were injected into the vein of chicken embryo. Embryos were analyzed and imaged to detect cancer cells (green) 7days after injection.

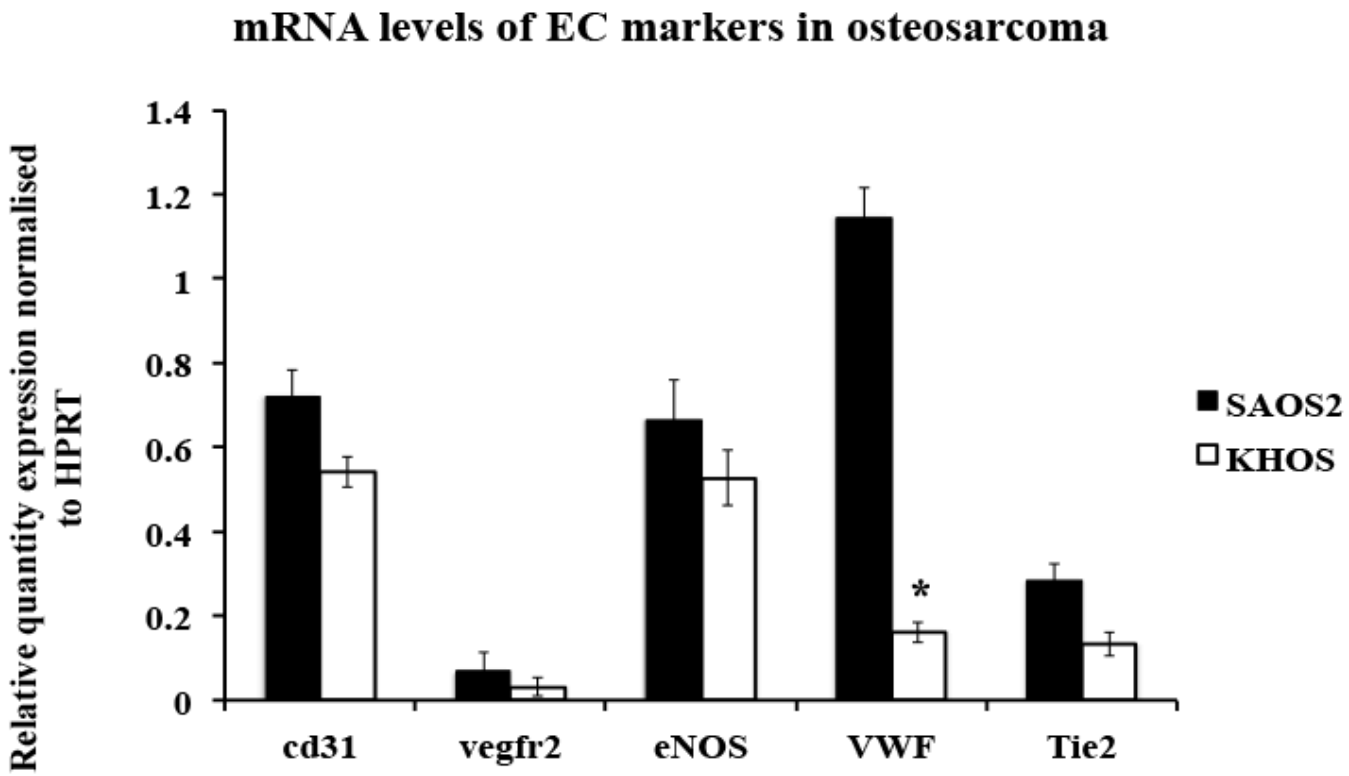
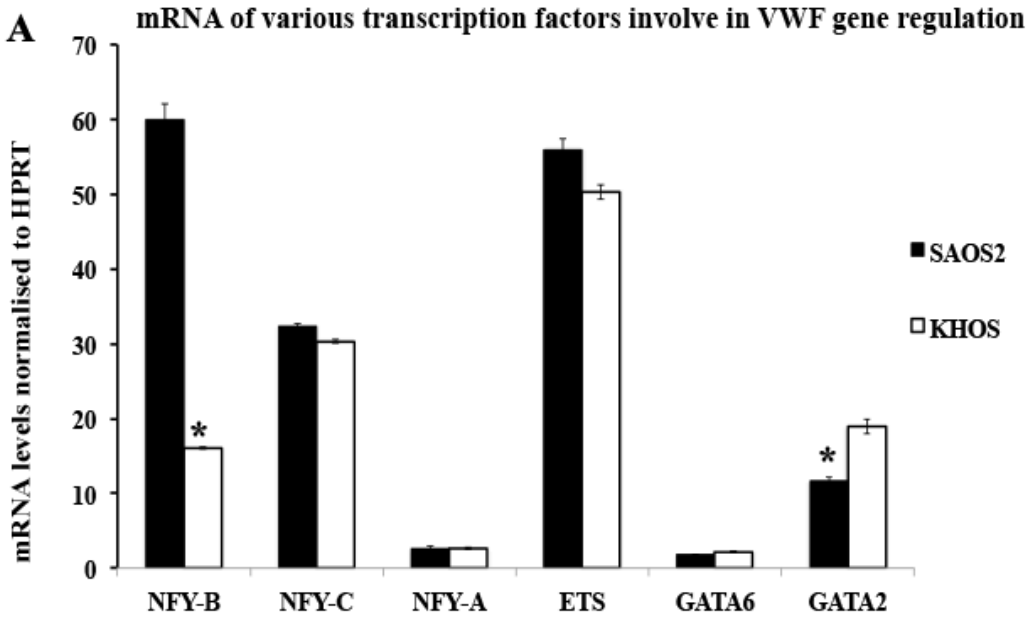
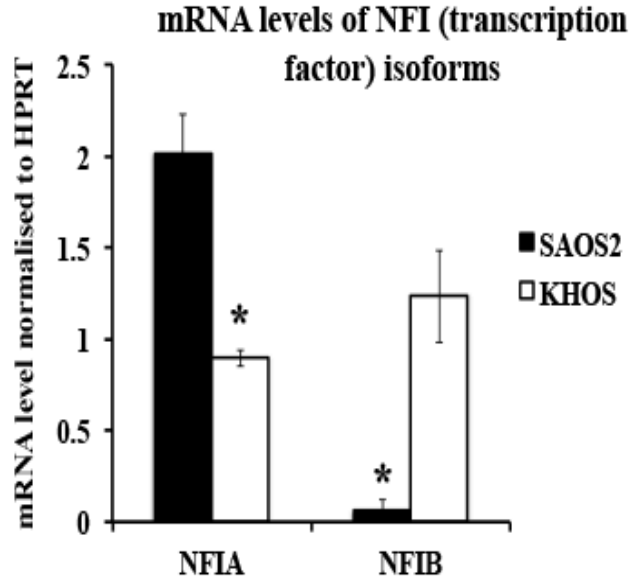


Figure 4-2S. The mRNA levels of several endothelial specific genes in osteosarcoma SAOS2 and KHOS cell lines. Quantitative RT-PCR was performed to determine the mRNA levels of *CD31, VEGFR2, eNOS, VWF* and *Tie 2* in the two osteosarcoma cell lines.


A
mRNA of various transcription factors involve in VWF gene regulation
mRNA levels normalised to HPRT
70
60
50
40
30
20
10
0
SAOS2
KHOS
NFY-B
NFY-C
NFY-A
ETS
GATA6
GATA2


B
mRNA levels of NFI (transcription factor) isoforms
mRNA level normalised to HPRT
2.5
2
1.5
1
0.5
0
SAOS2
KHOS
NFIA
NFIB

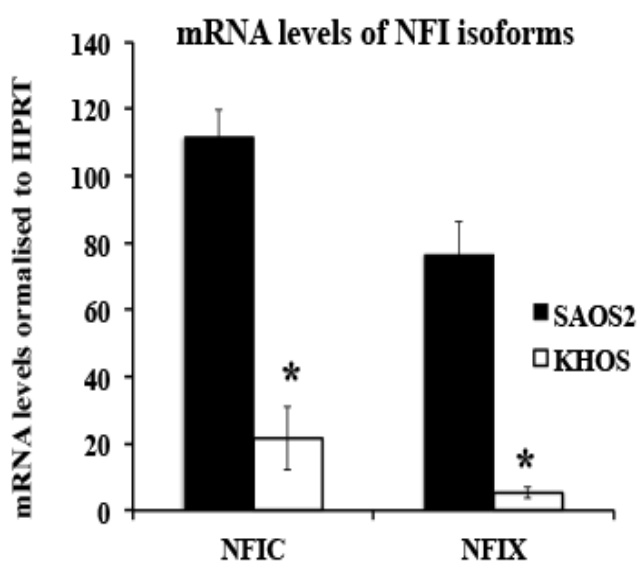

C
mRNA levels of NFI isoforms
mRNA levels ormalised to HPRT
140
120
100
80
60
40
20
0
SAOS2
KHOS
NFIC
NFIX

Figure 4-3S. The mRNA levels of various transacting factors in osteosarcoma SAOS2 and KHOS cell lines. (**A**) The mRNA levels of NFY subunits (NFY-A, B and C) are shown. NFY functions as both activator and repressor depending on its binding site on the *VWF* promoter. Also shown are the mRNA levels for ETS, GATA2 and 6 transacting factors which function as activators. (**B and C**) The mRNA levels of repressor NFI isoforms A, B, C and X are shown.

CHAPTER 5

SUMMARY

Hemostasis factors are responsible for maintaining the fluid phase of blood circulation under physiological conditions, while causing immediate clot formation at the site of vascular injury [177]. The role of VWF-mediated platelet activation and adhesion to the subendothelial matrix, as well as its protective effect on factor VIII, are central to maintaining normal hemostasis or, in unfavorable circumstances, to causing abnormal pathological thrombogenesis [178].

The plasma levels of VWF are determined mainly by *VWF* gene transcription and/or release of VWF from WPBs in endothelial cells. A small percentage of VWF protein is also expressed by megakaryocytes and is stored in alpha granules in platelets, which are released upon platelet activation. Increased levels of plasma VWF have been reported to originate from activated endothelial cells in response to fluctuations in blood components (such as sugar, salt, chemokine and cytokines among others) or diseases/physiological insults (cancers, inflammation, irradiation, bacterial infection and toxins). However, it is not clear whether increased levels can occur as a result of transcriptional upregulation, nor have the molecular mechanisms involved in many physiological/pathophysiological events been determined.

In this thesis, we have explored the mechanisms, and functional consequences, of *VWF* response to hypoxia *in vivo* in a mouse model of hypoxia-induced pulmonary hypertension, and *in vitro* in human cultured endothelial cells. We have demonstrated that some cancer cells of non-endothelial origin acquire *de novo* expression of *VWF*.

We have also explored the molecular mechanism that leads to *VWF* expression in these cancer cells and its functional consequences with respect to metastasis. Our major findings are as follows:

1. Hypoxia led to significantly increased *VWF* mRNA expression in all major organs, except kidney.

2. In the hypoxic lung, *de novo* expression of *VWF* was detected in small microvessels, while in control mice, *VWF* expression was predominantly limited to large vessels of the lung.

3. Hypoxia induced-upregulation of *VWF* in the lung endothelial cells was associated with increased binding of the YY1 transacting factor to its cognate binding site (intron 51 region), concomitant with decreased binding of the NFIB repressor to the promoter.

4. Hypoxia induced-upregulation of *VWF* in the cardiac endothelial cells was associated with increased binding of GATA6, as well as HIF1α, to the *VWF* promoter, concomitant with decreased binding of the NFIB repressor to the promoter.

5. In both heart and lung endothelial cells hypoxia-induced upregulation of *VWF* was associated with epigenetic modifications of *VWF* chromatin.

6. Hypoxia-induced *VWF* increased the recruitment of platelets on the released VWF strings from endothelial cells under shear stress.

7. Thrombus formation was detected in the lung and heart microvasculature in hypoxic mice, but not in other organs.

8. Organ-specificity of various wild type and mutant *VWF* promoter fragments were maintained in an adenovirus delivery system.

9. *De novo* expression of *VWF* occurs in some cancer cells of non-endothelial origin.

10. *VWF* transcriptional activation in cancer cells was associated with increased binding of GATA6 and decreased binding of the NFIB repressor to the promoter, concomitant with epigenetic modifications.

11. *VWF* expressions in cancer cells increased their adhesion to endothelial cells and platelets, as well as their endothelial monolayer transmigration.

12. Cancer cells that acquired *VWF* expression exhibited increased extravasation *in vitro, ex ovo,* and *in vivo*.

VARIATION IN PLASMA LEVELS OF VWF

Due to their unique location in the lining of blood vessels, endothelial cells are found in all organs. Endothelial cells adapt to a wide range of physiological conditions specific for each organ. This adaptation is consistent with their heterogeneity in phenotypes, functions and gene expression profiles. Accordingly, *VWF* expression also presents a heterogeneic pattern throughout the vasculature of the body, which potentially implies heterogeneity of *VWF* promoter regulation.

A wide diversity in the levels of VWF protein has shown in the normal population. However, a significant quantitative or qualitative deficiency of VWF causes an inherited bleeding disorder named von Willebrand disease, VWD [179,

180]. Several studies have shown that nearly 60% of the total variation in the level of plasma VWF is related to genetic factors [181], and many studies have investigated the cellular and molecular bases of VWF with regard to VWD [180]. As the roles of activators and repressors in *VWF* promoter regulation were determined, it can be hypothesized that polymorphic expression of *VWF* could arise either through mutations in their binding sites, or via the modifications of the transacting factors themselves, which could inhibit their binding and /or function on the *VWF* promoter.

Conversely, excessive plasma levels of VWF are an independent risk factor for cardiovascular complications [110]. Increased expression of *VWF* in the lung, specifically, was shown to be associated with endothelial dysfunction, increased inflammatory responses, vascular remodeling and subsequent lung injury [182]. Increased circulating plasma levels of VWF multimer in patients with pulmonary hypertensions are associated with short-term survival and have been suggested to be a predictor in pulmonary arterial hypertension [110, 113].

We investigated the molecular basis of differential regulation of the *VWF* gene in response to hypoxia in lung and heart endothelial cells. Our studies demonstrated the role of the intron 51enhancer in *VWF* upregulation in response to hypoxia, as well as in basal level expression, specifically, in lung endothelial cells. Such elements in the *VWF* gene may also potentially influence variations in plasma levels of VWF protein in the normal population.

HYPOXIA-INDUCED *VWF* UPREGULATION IN LUNG ENDOTHELIAL CELLS

Our analyses demonstrated that hypoxia upregulates *VWF* mRNA levels in all organs except the kidney, and that cultured endothelial cells *in vitro* reflect this *in vivo* response to hypoxia. Furthermore, our analyses demonstrated that while both lung and heart endothelial cells exhibit *VWF* upregulation in response to hypoxia, they employ distinct molecular mechanism for this process. We have investigated the molecular mechanisms of hypoxia-induced *VWF* upregulation in lung endothelial cells both *in vitro* and *in vivo* (Chapter 2). In brief, our analyses demonstrated that YY1, which interacts with the lung-specific enhancer of the *VWF* gene that is located in intron 51, and NFIB, which interacts with the upstream promoter sequences, are major participants in the *VWF* response to hypoxia. While YY1 functions as an activator, NFIB functions as a repressor of the *VWF* promoter and previous studies demonstrated the formation of a chromatin loop in the *VWF* gene, which brings the distal YY1 binding sequences to the close proximity of the NFIB binding sequence [63].

In our papers [86] and (Mojiri et al 2016- submitted manuscript), we demonstrated that *VWF* upregulation in lung endothelial cells is a result of a series of events in response to hypoxia, which leads to decreased binding of NFIB repressor and increased binding of the YY1 activator to the *VWF* promoter. This was concomitant with epigenetic modifications, specifically the acetylation of promoter-associated histone H4, which is consistent with transcriptional activation. We hypothesized that as a result of chromatin looping, increased YY1 binding may

somehow influence the association of NFIB with the *VWF* promoter, leading to decreased NFIB binding, and subsequently increased transcriptional activation.

Chromatin undergoes rearrangements in the deposition of histones in order to acquire changes towards gene activation or silencing, depending on the type of signals they receive [183]. YY1 was shown to recruit various chromatin modifying enzymes to its DNA binding site, and these enzymes induce epigenetic changes in histone marks that lead to the architectural rearrangement of nucleosomes, resulting in the activation or repression of target genes [184, 185]. It has been shown that N-terminal histone tail modifications are critical in regulating gene expression. PRMT1, a histone H4 (Arg3)-specific methyltransferase, was recruited to the bound YY1 at the YY1-DNA-binding site to add a methyl group to the H4 tail [185], which then allowed further histone modifications and resulting in chromatin activity through open configuration. We observed that in human lung endothelial cells, concomitant with increased YY1 binding, there was increased acetylation of histone H4 associated with the *VWF* promoter in response to hypoxia. This is consistent with a potential function of YY1 in mediating *VWF* chromatin remodeling associated with transcriptional activation.

YY1 not only has a direct effect as an activator or repressor, but it also induces epigenetic changes that influence the expression of its target genes by modifying the accessibility of other transcription factors to promoter sequences in those genes [186]. This function of YY1 is also consistent with our hypothesis that increased YY1 binding may somehow influence NFIB binding, leading to decreased

association of this repressor with the *VWF* promoter, and as a result increase transcriptional activity.

Aside from the ability of YY1 to influence histone modifications, YY1 can also displace bound transcription factors or compete with them for binding sites on the promoters of genes [187]. Using constructs in which the promoter contains binding sequences for transcription factors NFI, YY1 and TATA box {in this order} fused to CAT (reporter genes), YY1 protein has been found to significantly increase the expression of CAT. This effect is due to its ability to induce DNA bending, which alters the affinity of the transcription apparatus towards potential transcription, whereas relocation of the YY1-binding sequence upstream of the NFI-binding sequence {YY1NFITATA} abolished increased CAT expression [187]. Therefore, it is possible that loop formation, by bringing YY1 into the proximity of NFIB, may physically adjust the binding of the NFIB, and may consequently regulate *VWF* promoter activity in lung endothelial cells.

The role of YY1 in hypoxia induced upregulation of *VWF* in lung endothelial cells was firmly established in our study, by demonstrating that inhibition of YY1 expression through siRNA treatment abolished hypoxia-induction of *VWF*. Increased association of YY1 with intron 51 sequences was also concomitant with increased YY1 translocation from cytoplasm to the nucleus. However, the molecular mechanisms of these responses remain to be determined. For instance, it is not known whether increased YY1 association with the *VWF* gene alters the epigenetic modifications in the *VWF* promoter and/or intron 51. Also, it is not clear whether the

increased YYI localization in the nucleus in response to hypoxia is due to post-translational modifications of YYI.

HYPOXIA-INDUCED *VWF* UPREGULATION IN HEART COMPARED TO LUNG ENDOTHELIAL CELLS

Our experimental evidence documented the significance of the vascular bed signaling in the differential regulation of genes, such as *VWF*, in endothelial cells. Our analyses of the response of cardiac endothelial cells to hypoxia demonstrated that in these cells, as in lung endothelial cells, hypoxia leads to decreased NFI binding. In contrast to lung endothelial cells, however, YY1 or intron51 sequences do not participate in this process. This was clearly established by demonstrating a lack of effect of YY1 specific siRNA on hypoxia induction of *VWF* in cardiac endothelial cells and a lack of alteration in the association of YY1 with *VWF* chromatin, and in a deletion analysis that demonstrated intron 51 sequences were not required for a hypoxia response. In contrast, we demonstrated that in cardiac endothelial cells GATA6 and HIF1α, interactions with the downstream *VWF* promoter were required for hypoxia-induced *VWF* upregulation. The participation of these two factors was specific to cardiac endothelial cells, since we showed that neither of them was involved in hypoxia-response of *VWF* in lung endothelial cells.

The differential role of YY1 as a regulator of gene expression in the endothelial cells of various organs has been previously reported. YY1 regulates occludin expression (mediated by the Sp family of transcription factors) differently in endothelial cells of different organs. For example, YY1 induced occludin expression

in brain endothelial cells by binding to the F0.1 region of the promoter, leading to the maintenance of tight junctions, whereas YY1 bound to the F5 region of the same promoter in lung endothelial cells and did not activate the occludin gene, indicating that YY1 operates differently in the regulation of a specific gene in the same type of cells in different organs [188]. This observation is supporting our results in which YY1 regulates hypoxia-induction of *VWF* in the lung but not in heart endothelial cells.

However, in cardiac endothelial cells, as in lung endothelial cells, hypoxia-induced *VWF* upregulation was concurrent with increased acetylation of promoter-associated histone H4. Thus a picture of *VWF* response to hypoxia arises from these analyses; it suggests two distinct mechanisms leading to a common event. We present a model {Fig. (5-1) or (3-8)}, which proposes that two separate upstream events, one occurring in the lung and one in the heart, are invoked by hypoxia: the first (in the lung) involves YY1 and the second (in the heart) involves GATA6 and HIF activations. These upstream processes lead to decreased NFI binding and increased acetylation of *VWF* promoter-associated histone H4 in both cell types. Others have shown that post-transcriptional modifications of NFI, such as phosphorylation, could affect NFI binding [65]. Therefore, other mechanisms could be involved in the decreased binding of the NFIB repressor on the *VWF* promoter in response to hypoxia.

The role of GATA binding at the (+220) site has been shown in the activation level of the *VWF* 3kb promoter (spanning the sequences from -2182 to the end of the first intron) in transgenic mice [49], as well as in *VWF* promoter -487 to +247 activation in cultured endothelial cells [39]. Mutation of the (+220) site led to lower levels of VWF 3Kb-LacZ-transgene activity in transgenic mice, but preserved *LacZ*

expression patterns in vessels of the brain, heart and skeletal muscle of mice compared to those in control transgenic mice harbouring constructs with the wild-type promoter.

Our results suggested that GATA binding cis-acting elements, in addition to participating in regulating the basal level of *VWF* promoter activity in general, also participate in hypoxia induction of the *VWF* promoter specifically in cardiac endothelial cells. This organ-specific function of the GATA binding site may at least partly depend on the particular GATA family member that could interact with the cis-acting element. While GATA2, 3 and 6 were shown to interact with the *VWF* +220 site [39, 59], our analyses have shown that GATA6 in particular participates in hypoxia-induction of the *VWF* promoter in cardiac endothelial cells.

Although our analyses demonstrated an organ-specific mechanism of *VWF* response to hypoxia that is reflected in cultured endothelial cells, we cannot exclude the additional impact or signaling from the organs' microenvironment on endothelial cells in this event. Therefore, additional *in vivo* investigations are required to confirm the dominant role of GATA6, HIF, and YY1 as well as NFI repressor in increased expression of *VWF* in response to hypoxia in the heart and lung *in vivo*.

Our analyses of the hypoxia-exposed transgenic mice that contain the *VWF* upstream promoter (sequences -487 to +247) and intron 51 HSS fused to *LacZ* gene demonstrated that while the transgene in control mice was exclusively expressed in lung and brain endothelial cells, in hypoxic mice transgene expression was detected in heart endothelial cells as well. We propose that our model, which suggests increased GATA6 binding and decreased NFIB binding as participatory events in hypoxia-induced *VWF* upregulation in the heart, can explain this observation (Fig. 5-1).

We propose that under control conditions, the interaction of NFIB with the brain-lung specific transgene *VWF* promoter sequences inhibits its activity in heart endothelial cells. However, when exposed to hypoxia, events leading to decreased-NFIB binding (including increased GATA6 binding) may result in exogenous *VWF* promoter activation in heart endothelial cells. Based on this model we would predict that the same process also induces activation of brain-specific *VWF* promoter sequences in the heart endothelial cells of transgenic mice harbouring the *LacZ* gene fused to these sequences (-487 to +247) in response to hypoxia, since both GATA6 and NFIB binding sites are located within these *VWF* promoter sequences. A potential control model for this proposed experiment would be a transgenic mouse carrying the mutation of the GATA binding site of the 734 bp *VWF* promoter.

Among the differences in mechanisms of the hypoxia-response of *VWF* in the heart compared to lung was the role of HIF. Hypoxia induction of many genes has been reported to be regulated by HIF [189]. HIF is a transcription factor that consists of an HIF-a subunit that interacts with a b subunit, which is generally sequestered by VHL, which in response to hypoxia is degraded and releases the b subunit to form a dimer. Once the dimer is formed, it interacts with HIF binding sequences known as HRE, which are located in the promoter of hypoxia-responsive genes. Our analyses did not demonstrate the presence of HRE in the *VWF* promoter; however, since HIFs are also reported to exert their effects through association with other transacting factors [190], we could not exclude a role for HIF in hypoxia-induction of *VWF*.

The results of our analyses, which demonstrated that HIF participated in hypoxia-induction of *VWF* in heart but not lung endothelial cells, in addition to

revealing a lack of HRE on the *VWF* promoter, strongly suggest a role for HIF that does not involve a direct interaction with the *VWF* promoter sequences. This organ-specific participation of HIF further underscores distinct organ-specific processes that are invoked in response to hypoxia in endothelial cells, and that may contribute/influence activities of *VWF* regulatory transacting factors.

Increased expression of members of the GATA family by HIF-1α has been previously shown [191]. In addition to demonstrating that HIF-1α inhibition through siRNA treatment abolishes hypoxia-induction of the *VWF* promoter specifically in cardiac endothelial cells, ChIP analysis of hypoxia-treated human cardiac endothelial cells indicated significantly increased binding of HIF-1α to the *VWF* promoter. We hypothesize that there may be a potential interaction of GATA6 and HIF-1α, which may result in GATA6 stabilization/and or increased transactivation, and may explain recruitment of HIF-1α to the *VWF* promoter via GATA6.

In addition to changes in acetylation of histone H4, which demonstrate the role of epigenetic modification in *VWF* promoter activation, another marker of epigenetic modification, DNA methylation, has been found to participate in *VWF* gene regulation. There are eight CpG dinucleotide sites capable of being methylated in the *VWF* promoter, and their methylation is associated with the level of *VWF* promoter activity [154] [67]. It has been generally accepted that increased DNA methylation is accompanied by histone modification [192], which could reveal or mask transcription factor binding sites on the promoter. We have investigated the methylation status of two important sites (-422 and +119) in heart and lung endothelial cells. While we did

not observe any significant changes in the lung, there were significant alterations in DNA methylation on *VWF* promoter in endothelial cells in the heart.

In comparing the methylation pattern of two CpG sites in the *VWF* promoter in control and hypoxia-treated cardiac endothelial cells, we observed significantly increased methylation at the -422 site in the *VWF* promoter under hypoxic conditions. Based on the close proximity of NFI binding site (position -463 to -450) to this methylation site, we hypothesize that increased DNA methylation at the -422 site may interfere with the accessibility of NFIB to the chromatin for binding, leading to decreased association as demonstrated by ChIP analysis. Our results demonstrating that there was no significant change in methylation at the +119 site suggest that this site remained available for increased binding of GATA6 in response to hypoxia.

Although increased methylation is generally associated with decreased activation, hypoxia-induced changes in the methylation pattern of the *VWF* promoter (increased methylation at site -422) appear to be aimed at interfering with the association of the repressor (NFIB) with the DNA, while the region that mediates the association of the activator (GATA6) is not altered. Thus this site-selective increased methylation leading to decreased repressor association is consistent with increased transcription. This evidence represents another level of regulation at the epigenetic level, which also underscores the fact that the regulation of the endothelial response to hypoxia is organ-specific. Furthermore, it would be of interest to investigate the other 6 potential sites of methylation in the *VWF* promoter in the heart and lung endothelial cells in response to hypoxia (Fig. 5-1).

Our recent work has indicated that the regulation of *VWF* transcription in response to hypoxia is organ-specific. Therefore, it would be interesting to determine the regulatory cis- or trans-acting elements that contribute to *VWF* expression in the endothelium of the liver and brain, in addition to determining the mechanism that inhibits this process in kidney endothelium.

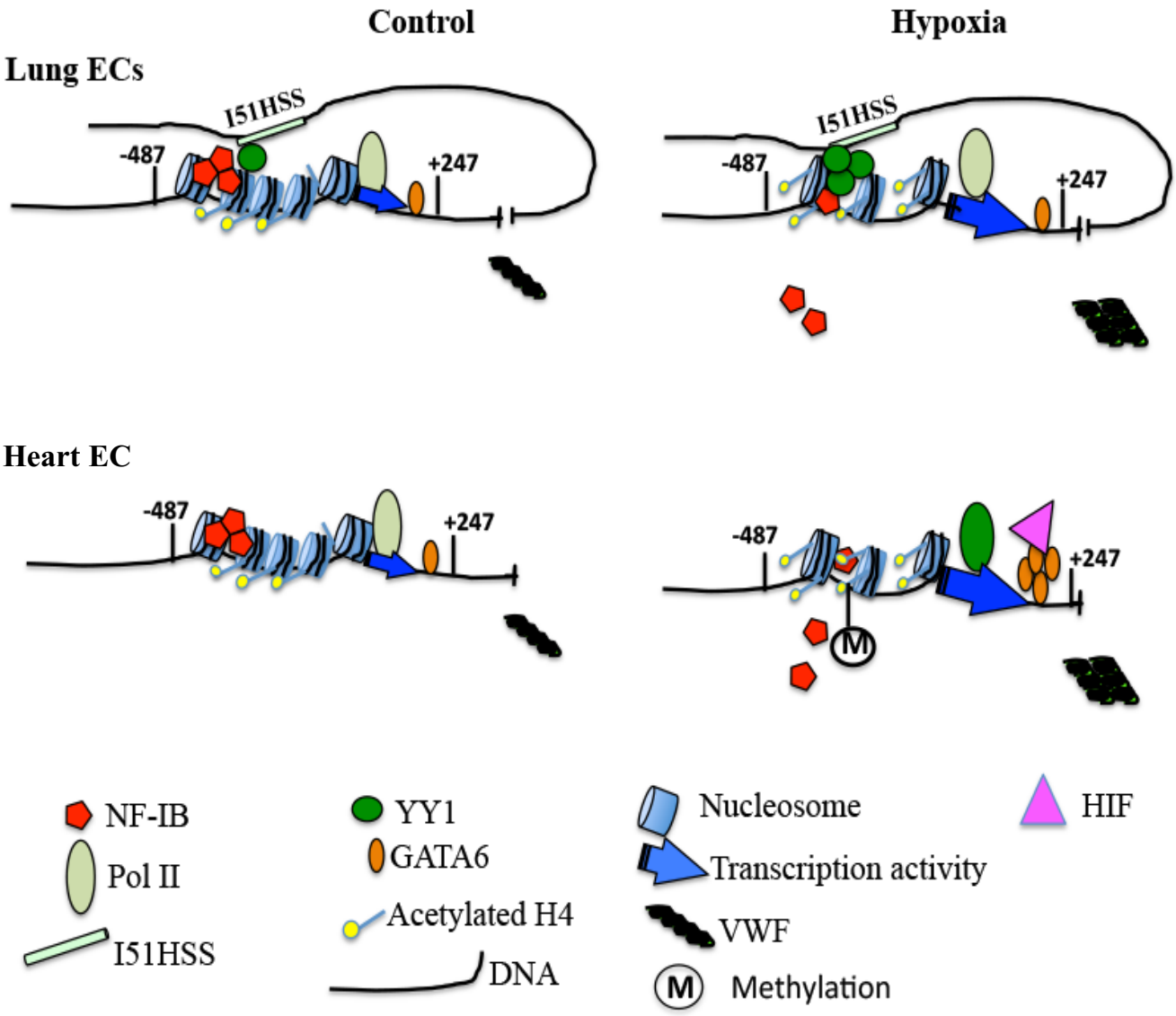


Figure 5-1. Schematic representation of a model describing the mechanisms of hypoxia-induced *VWF* upregulation in heart and lung MVEC. We propose the following model to describe the events leading to *VWF* upregulation in response to hypoxia in lung compared to heart MVEC. In both cell types, hypoxia results in the dissociation of the NFIB repressor from the *VWF* promoter, and in increased acetylation of histone H4 associated with the promoter, leading to enhanced transcription. However, in lung endothelial cells, hypoxia also results in increased association of YY1 with a lung specific enhancer located in intron 51 (I51HSS). The I51HSS is brought to the proximity

of the NFIB binding proximal promoter sequence through chromatin looping, as previously shown. Thus we hypothesize that increased YY1 binding may contribute to dissociation of NFIB. In contrast, in cardiac MVEC, YY1 and I51HSS sequences are not involved in hypoxia-induced upregulation of *VWF*, while specifically increased association of GATA6 with the *VWF* promoter, as well as recruitment of HIF, are participating factors. GATA6 and HIF recruitment may contribute to decreased association of NFIB with the promoter sequences. Furthermore, in the specific case of cardiac MVEC, hypoxia leads to increased methylation of a CpG dinucleotide located at close proximity to the NFIB binding site, which may also hinder NFIB binding. We conclude that the focal event leading to *VWF* upregulation in response to hypoxia is the inhibition of the association between NFIB and the *VWF* promoter. However, in different endothelial cells, distinct pathways and specific activators which converge on this event, i.e. the dissociation of NFIB, are invoked.

HYPOXIA-INDUCED *VWF* UPREGULATION IS ASSOCIATED WITH PLATELET AGGREGATION AND THROMBOGENESIS

We have shown that *VWF* mRNA levels in all major organs (except kidney) increased significantly in response to hypoxia. In human lung microvascular cultured cells, we have demonstrated that increased *VWF* expression in response to hypoxia leads to the generation of VWF strings that are decorated with numerous perfused platelets under shear stress conditions.

The critical role of VWF protein in thrombus formation in arteries and veins has been documented by many studies. Population studies have revealed increased plasma levels of VWF in patients with atherothrombotic complications or diseases associated with endothelial dysfunctions [72, 193].

When injuries were induced in the arteries of VWF-deficient mice, not only was there a significant delay in platelet adhesion and thrombus formation, but the thrombus structure observed was special. It appeared that these thrombi were lacking the cohesive mesh structure of VWF, resulting in less compact and more porous clots [194]. VWF binding to platelet receptors GPIb alpha and alpha(IIb)beta(3) is crucial and required for normal hemostasis under shear stress [14]; however, thrombus formation in VWF deficient mice (even with a significant delay) indicated the role of other players in thrombus formation. It has been shown that GPIb alpha mediates the recruitment of platelets onto the VWF network, which leads to further binding of the platelet integrin alpha(IIb)beta(3). The interaction of the latter with VWF initiates irreversible signals for platelet activation that lead to platelet aggregation at the site of vascular injury [195].

In addition, a role for VWF in venous thrombosis has been shown. This is not only attributable to the function of VWF as a protector of Factor VIII, which is shown to increase predisposition to venous thrombosis, but also as a direct contribution of VWF to establishment of venous thrombus formation that was shown in mice model lacking VWF [196, 197]. In the event of venous thrombosis, however, it appeared that *VWF* deficient mice showed significantly reduced thrombus formation independent of GPIb alpha and alpha(IIb)beta(3) receptors [197]. Furthermore, it has been shown that the role of VWF in thrombus formation in veins is independent on the presence of factor VIII [198] [199]. Recent studies also have shown the association of neutrophil extracellular traps (NET) with VWF in developing vein thrombosis [200]. Considering the demonstrated roles of VWF in platelet adhesion, inflammation, neutrophil and leukocyte recruitment, the overexpression of *VWF* in lung and heart vasculature could potentially mediate these processes which together, may initiate endothelial dysfunction and injury [113].

We analyzed the lung and heart vessels of control and hypoxia-exposed mice for the presence of potential thrombi. The results of immunofluorescence and immunohistochemistry analyses of various organs demonstrated that hypoxia treatment not only upregulated and released VWF from activated endothelial cells, but also enhanced the aggregation and potential occlusion of the vessels specifically in the heart and lung. Furthermore, colocalization of platelets, VWF and fibrinogen (signs of thrombogenesis) were observed in the heart and lung vessels. While the lack of platelet aggregate detection in the kidneys of hypoxic mice was consistent with a lack of observed *VWF* upregulation, we did not detect thrombus formation in the

vasculature of brain and liver either, even though increased *VWF* mRNA levels were detected in these organs in response to hypoxia. Potentially, the lack of occlusion in kidney could be an organ-specific prevention mechanism against thrombus formation with a different threshold upon hypoxia.

For example, the presence of a significantly hypoxic microenvironment has been shown in the medulla layer of the kidney in normal mice, while at the same time, *VWF* expression was significantly higher in the medulla versus the cortex of the kidney [89, 92, 201] under normal conditions. This observation, could potentially link physiologically present hypoxic conditions in kidney medulla with the high density of VWF protein in this region of the kidneys.

It has been shown, furthermore, that podocytes express significant levels of ADAMTS13 and deposit it in the basement membrane of glomeruli in the kidney, which could be a protective mechanism from unnecessary thrombus generation in this structure [103-105, 202]. Although hypoxia per se does not lead to increased *VWF* expression in kidney, other stimuli may do so. Thus the elevated presence of VWF cleaving ADAMTS13 in glomeruli may indicate the specific need for protecting these kidney functional units from potential thrombus formation. Similar anti-thrombotic processes may be preferentially invoked in brain and liver in an organ-specific manner, thus protecting vasculature of these organs from platelet aggregation and thrombus formation. The correlation between a lack of thrombus formation and high levels of local ADAMTS13 expression could potentially be explored by studying thrombotic microangiopathy in TTP patients, who are either lacking ADAMTS13 or produce antibodies against ADAMTS13. In TTP syndrome, the kidney is one of the

primary organs in which thrombotic microangiopathy manifestations with ULVWF are found in the microvasculature [203]. Further damage was also identified in the microvasculature of brain and heart in TTP patients, which suggested that ADAMTS13 also contributes to prevention of thrombus formation under normal condition specifically in the heart and brain.

Tissue-specific differences in endothelial cell phenotype also influence WPB maturation, size, population and the secretion of these organelles based on distinct physiologic conditions [204]. Indeed, the dynamic ranges of WPBs not only determine the plasma levels of VWF, but also regulate pro-thrombotic and pro-inflammatory factors in the specific sites of vasculature, which reflect the pathophysiology of cardiovascular complications [204].

We have shown that thrombus formation is a potentially pathophysiological consequence in the heart and lung vasculature in response to hypoxia. Among several factors that have been identified as contributing to the development of thrombi, a main role can be ascribed to VWF protein. Strong evidence from the population of VWD patients has shown that there is a considerable protective effect against thrombogenesis or cardiovascular complications which is associated with arterial thrombotic events [205].

VWF protein and platelets normally travel in the blood circulation; however, under certain circumstances, which are regulated by endothelial cells, blood components and shear flow, they initiate thrombogenesis. Whether cardiac endothelial cells are more sensitive to the hypoxia than those of the lung, why thrombus formation

is not observed in liver and brain of hypoxic mice despite increased-VWF levels, and what the underlying mechanisms mediate these differences remain to be determined.

SOMATIC TARGETING OF ENDOTHELIAL CELLS OF VARIOUS ORGANS USING ORGAN-SPECIFIC *VWF* PROMOTER FRAGMENTS

Our results demonstrated the organ-specific response of endothelial cells to stimuli such as hypoxia, with potentially organ-specific pathophysiological consequences, i.e thrombus formation. These results are consistent with the well-established organ-specificity and heterogeneity of endothelial cell function, which is also the underlying basis for the focal nature of many vascular diseases. Thus, developing tools that permit the selective targeting of endothelial cells of specific organs somatically is highly advantageous for exploring the mechanisms of, and developing potential therapeutic approaches against, diseases that affect the vasculature of specific organs.

We used three different methods (standard transgenic mice, HPRT-transgenic mice, and an adenovirus delivery system) and demonstrated that organ specific information for *VWF* expression is maintained mainly in the *VWF* promoter sequences, which enable them to function whether or not they integrate into the chromatin structure.

We previously demonstrated that various wild type and mutant *VWF* promoter fragments exhibit organ-restricted activation pattern in addition to endothelial cell-

specific activity. A proximal promoter fragment spanning sequences -487 to +247, containing the binding sites for several transacting factors including NFI and NFY (function as repressors), exhibited brain endothelial-specific activity [39, 41, 42, 57, 59]. Addition of a DNase 1 hypersensitive region from intron 51 sequences of the *VWF* gene conferred lung endothelial cell activity (in addition to brain) to the proximal promoter sequences [63]. Mutation of the NFI binding site resulted in proximal promoter activation in lung and heart endothelial cells, while mutation of the repressor NFY binding site resulted in promoter activation in kidney endothelial cells in addition to those in the brain [55]. A double mutation in the NFI and NFY binding sites of both repressors resulted in promoter activation in endothelial cells in the heart, lung, kidney and liver, as well as in brain endothelial cells [55].

Generating HPRT mice using the above information resulted in organ-specific effects, as demonstrated in Fig. 5-2. In *Hprt* -VWF-LacZ-HSS mice, which contain the *VWF* promoter sequences -487 to +247 plus the intron 51 lung-specific enhancer region, *LacZ* expression was demonstrated in the endothelial cells of brain and lung but not in other organs (Fig. 5-2A). In *Hprt* -LacZK^{NFI} mice containing *VWF* promoter sequences -487 to +247 with mutations in the NFI binding site, *LacZ* expression was observed specifically in endothelial cells in the brain, heart and lung (Fig. 5-2B). In *Hprt* -LacZK$^{NFI\text{-}NFY}$ mice, containing *VWF* promoter sequences -487 to +247 with mutations in the binding sites of two repressors (NFI and NFY), *LacZ* expression was observed in the endothelial cells of five major organs tested (Fig. 5-2C). Colocalization of LacZ (red) with endogenous VWF (green) protein (merge panels Fig. 5-2) confirmed the endothelial specificities of all of the analyzed promoter fragments.

The observed expression pattern of VWF-LacZ transgenes in *HPRT* transgenic mice were consistent with that previously reported for standard transgenic mice. This demonstrates that organ-specific activities of various *VWF* promoter fragments are inherent to the genetic constituencies of the promoters and independent of transgene copy number or chromosomal locus influences.

The fact that the activities of various *VWF* promoters are organ-specific suggest that these sequences may present an opportunity for developing targeting vectors for manipulation of endothelial cells of specific organs. However, the observed organ-specificities were demonstrated in transgenic mice, and observed organ-specificity may be the result of germ line integration of the transgenes. The maximal utility of the *VWF* promoters as targeting vectors requires demonstration of their cell type and organ-specific activities when expressed somatically. Generation of adenoviral vectors that were used in deletion analyses to characterize hypoxia-responsive regions of the *VWF* promoter in cardiac versus lung endothelial cells provided an opportunity to address this question. The generated adenoviral vectors contained *LacZ* gene fused to either a brain-specific (sequences -487 to +247) or brain-lung specific (sequences -487 to +247 plus Intron51 HSS sequences) *VWF* promoter, referred to as AdLacZK and AdLacZKHSS, respectively.

In addition to these two adenoviruses, which we discussed in Chapter 3, we also generated an adenoviral vector containing a *LacZ* gene fused to the *VWF* promoter sequences -487 to +247 containing a mutation in the repressor NFY binding site, which we referred to as AdLacZK^{NFY}. These mutant *VWF* promoter sequences in mice were activated specifically in kidney endothelial cells, in addition to those of the brain. The

three adenoviral-vectors were administered via tail vein, or carotid artery, injection into mice (as described in Chapter 3), and their patterns of expression were determined. The results demonstrated that all three adenoviral vectors exhibited an organ- and cell type-specific activation pattern that mimicked the activities of the corresponding sequences in transgenic mice.

Administration of AdLacZK led to *LacZ* expression in endothelial cells of brain, while AdLacZKHSS activated *LacZ* expression in endothelial cells of lung and brain, and AdLacZK^{NFY} showed *LacZ* expression in the endothelial cells of brain and kidney (Fig. 5-2D). Thus the organ-specific activities of the various *VWF* promoter fragments are maintained *in vivo* even when delivered somatically; this suggests that these promoter-fragments are a useful tool for cell type and organ-restricted endothelial cell targeted delivery of desired molecules.

However, the organ-specific activities of these adenoviral vectors were not maintained in cultured cells. There were no preferential expression of the adenoviral vectors in cultured endothelial cells of various organs. All three vectors were expressed in the endothelial cells of the lung, brain and kidney. We have shown that the organ specificity of the hypoxia-response of the *VWF* promoters was maintained in cultured primary endothelial cells from various organs. These results demonstrate that the loss of an organ-specific microenvironment in cell culture leads to the loss of organ-specific activities of the *VWF* promoters under basal conditions, while still maintaining an organ-specific response to hypoxia. Regardless of the *in vitro* results, our *in vivo* studies demonstrated that VWF-promoter elements embedded in

adenoviruses could potentially be used as valuable tools for the somatic delivery of desired molecules in the targeted endothelial cells of various organs.

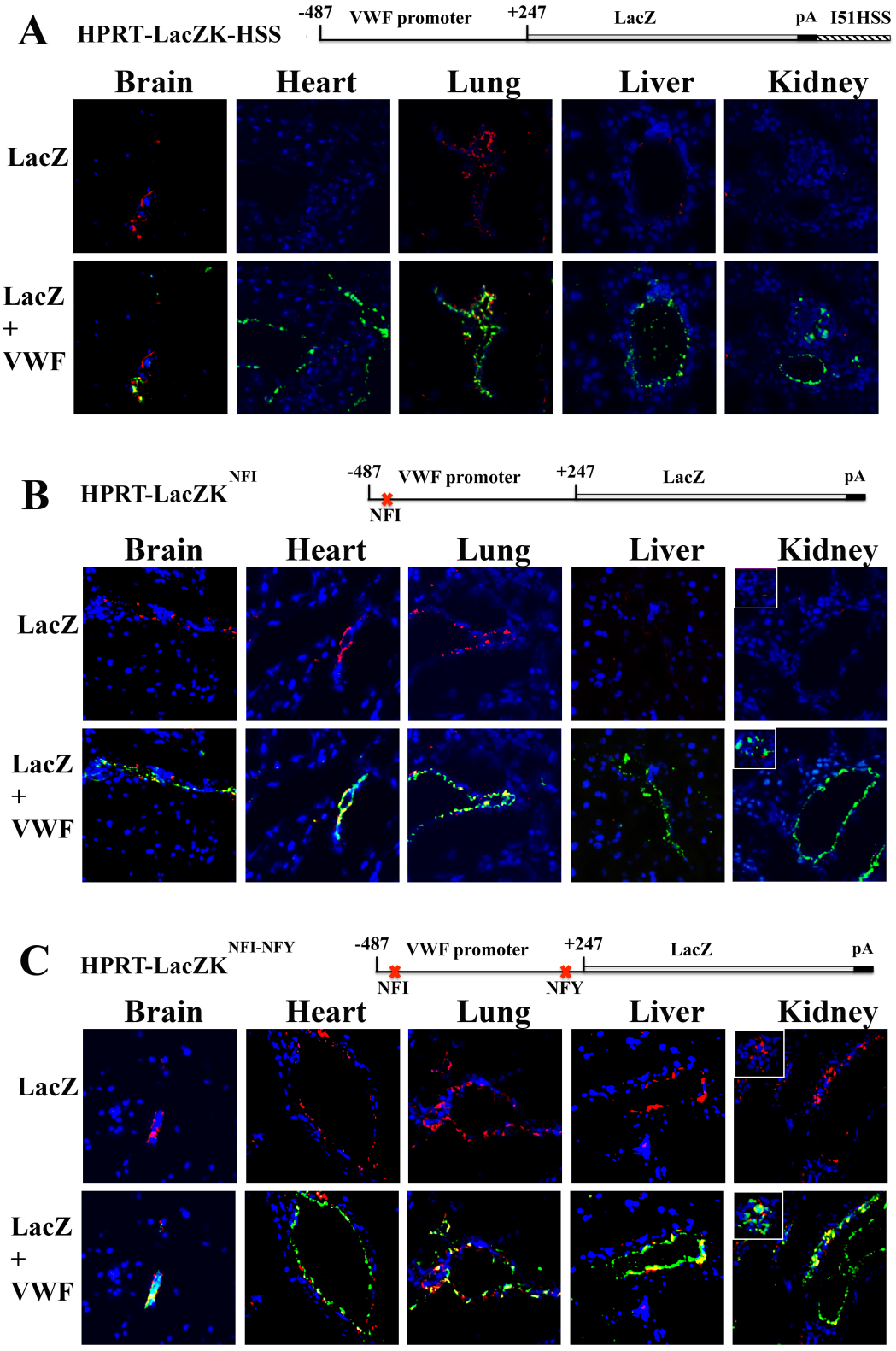

A
HPRT-LacZK-HSS
-487
VWF promoter
+247
LacZ
pA
I51HSS
Brain
Heart
Lung
Liver
Kidney
LacZ
LacZ + VWF
B
HPRT-LacZK
NFI
-487
VWF promoter
+247
LacZ
pA
NFI
Brain
Heart
Lung
Liver
Kidney
LacZ
LacZ + VWF
C
HPRT-LacZK
NFI-NFY
-487
VWF promoter
+247
LacZ
pA
NFI
NFY
Brain
Heart
Lung
Liver
Kidney
LacZ
LacZ + VWF

Figure 5-2: *LacZ* expression patterns in *HPRT* targeted transgenic mice.
OCT frozen sections (5 μm) of brain, heart, lung, liver and kidneys were prepared from HPRT-targeted transgenic mice harboring transgenes (A) LacZKHSS, (B) LacZK^{NFI}, and (C) LacZK$^{NF1\text{-}NFY}$. The schematic representations of the transgenes are shown at the top of each panel. Organ sections were subjected to double IF staining and confocal microscopy analyses to detect transgene LacZ (red) and endogenous VWF (green). Panels demonstrating LacZ expression and colocalization of LacZ and VWF (yellow) are shown. DAPI staining was used to detect nuclei (blue). The results are representative of three independent stainings for each organ.

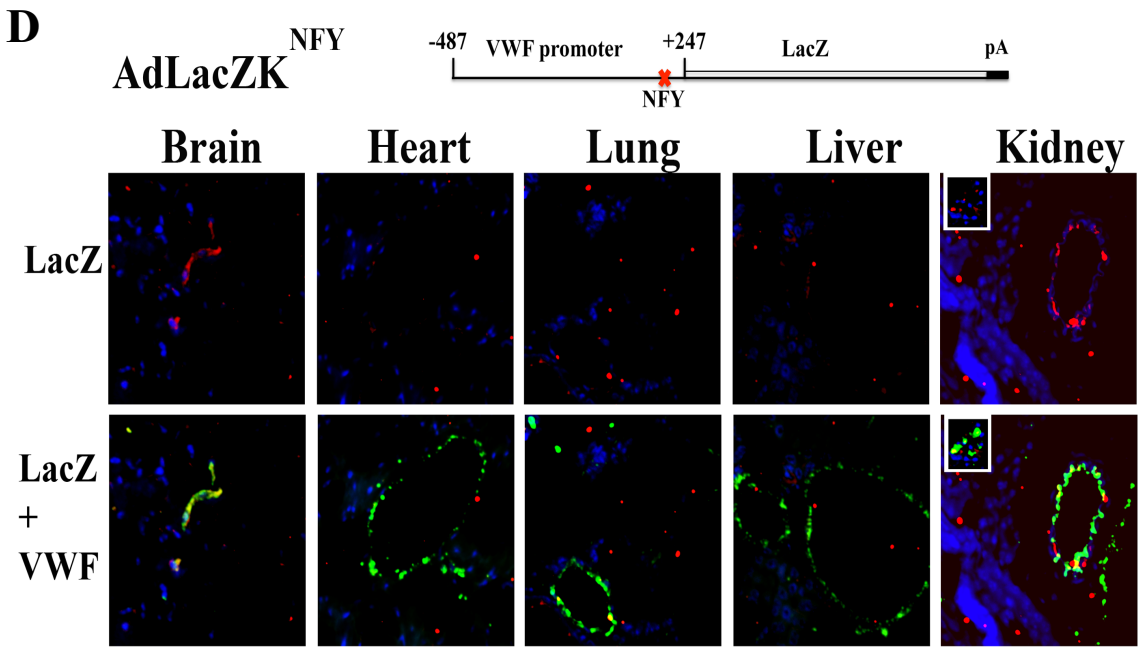


Figure 5-2D: LacZ expression patterns in mice transduced with AdLacZK^{NFY}

Mice were transduced with adenoviral vectors AdLacZK^{NFY}, as described in methods (above). OCT frozen sections of organs were subjected to double IF staining and confocal microscopy analyses to detect LacZ and endogenous VWF protein, as described for the *HPRT* transgene. The schematic representation of the transgene in each adenoviral vector is shown at the top of each panel. The results are representative of three independent transductions for adenoviral vector and two staining for each organ.

DE NOVO EXPRESSION OF *VWF* IN CANCER CELLS

VWF protein is a pro-coagulant, glycosylated protein with an expression pattern that is highly restricted to endothelial cells and megakaryocytes. Excessive circulating levels of VWF with signs of thrombosis are frequently present in patients with various cancers. Aberrant platelet activation and aggregation mediated by VWF have been shown to induce intravascular coagulation in progressive stages of metastasis [206]. The multimeric conformation of the VWF provides it with the flexibility to bind to several ligands on tumor cells [207]. VWF binding to tumor cells could arrest them in the microvasculature and further facilitate extravasation [208]. It is shown that platelets can coat cancer cells and mask them from immune surveillance, which could not only prolong their survival in blood circulation but also induce entrapment of cancer cells in capillaries leading to potential metastasis. This entrapment can occur as a result of formation of migratory microthrombosis that contain cancer cell platelet heteroaggregates with contribution of plasma VWF, platelets and activated endothelial cells especially in capillaries [169]. In addition VWF function as pro-inflammatory molecule also contributes to cancer progression and metastasis. Inflammation results in recruitment of leukocytes, which lead to increased vascular permeability through decreased endothelial adherence junction. Pro-inflammatory cytokines contribute to this process by inducing endothelial actin remodeling. Therefore, increased vascular permeability facilitates transmigration of cancer cells through vascular wall leading to extravasation and cancer metastasis. VWF proinflmmatory function thus also contributes to increased metastasis. Increased plasma levels of VWF in inflammatory diseases are described [209]. It was reported that melanoma cells take advantage of released ultra large VWF fibers to trigger

accumulation of other inflammatory factors via recruited platelets [210-212] and leukocytes [213, 214] at the site of cancer cell adhesion. The local deposition of VWF fibers and activated platelets at the site of activated endothelial cells plus recruited leukocytes are shown to facilitate vascular permeability, tumor cells transmigration and extravasations [118].

Considering the highly adhesive properties of VWF and its cellular expression pattern in megakaryocytes and endothelial cells, a link between VWF and cancer metastasis has been postulated and supported by experimental studies [171, 207]. However, analyses of tumor cell metastasis in VWF-deficient mice clearly demonstrated that VWF deficiency significantly enhanced tumor metastasis. These studies have shown that VWF induced apoptosis in cancer cells, thereby limiting the number of cancer cells that could metastasize [114, 167]. These discordant observations regarding the role of VWF in cancer may be attributed, at least partly, to the focus of investigations on endothelial cells and platelets as a source of VWF protein.

We have shown in Chapter 4 that several cancer cell lines of non-endothelial origin acquired *VWF* expression. Alterations in the binding of transcription factors as well as chromatin modifications of the *VWF* gene in SAOS2 osteosarcoma cells has been shown to favor the gene activation that occurs in SAOS2 but not in KHOS osteosarcoma cells, in which the *VWF* gene is silent. Furthermore, ChIP analyses also demonstrated that *VWF* expression specifically in SAOS2 is associated with the decreased binding of NFI and increased binding of the GATA6 transacting factor to

the *VWF* promoter, processes that are known to be consistent with *VWF* gene activation in endothelial cells.

We hypothesize that the changing of transcription factor binding, together with epigenetic modifications, fundamentally alters the promoter status from silent to active mode in SAOS2 cells but not in KHOS cells. It has been documented that modifications of the DNA (e.g. methylation) and histone tails (e.g. acetylation) can potentially change chromatin structure to either a condensed or a loose structure, which in turn causes either the masking or the exposure, respectively, of transcription factor binding sites [66].

The changes in the methylation pattern discussed above also correlate with *VWF* promoter activity [154]. Analyses of the methylation pattern of *VWF* in osteosarcoma cellshave demonstrated significantly decreased methylation at both (-422 and +119) sites in SAOS2 (VWF+) compared to those in KHOS; this could result in the activation of the *VWF* gene in the former (SAOS2) cells but not the latter. Furthermore, comparing this pattern in SAOS2 to HUVEC demonstrated almost equal methylation of the (+119) site, but increased methylation of the (-422) site in SAOS2 cells. Increased methylation in position -422 is reminiscent of the similar increased methylation at this site that was observed in hypoxia-exposed cardiac endothelial cells. In both cases this may favour decreased binding of the repressor NFI to its closely positioned binding site. Decreased methylation at +119 may facilitate the recruitment of the GATA activator to its closely positioned binding site (position +220). While the methylation status of the promoter can significantly affect promoter activity, some

studies have shown that methylation is not the only factor that activates genes in SAOS2 cells.

It has been demonstrated that the *Fas* gene is highly expressed in SAOS2 cells while, at the same time, it is highly methylated. Moreover, further treatment with a demethylating agent did not change *Fas* expression in SAOS2 cells [215]. These results suggest that the *de novo* activation of *VWF* expression may be an outcome of various processes and alterations of transcription factors, which modify their function and interaction with the *VWF* gene. We have demonstrated increased association between acetylated histone H4, as well as decreased HDAC, and the *VWF* promoter in SAOS2 (VWF+) cells when compared to KHOS cells. These chromatin modifications could potentially explain the low level of NFI repressor binding, in addition to increased activator GATA6 binding, to the *VWF* promoter in SAOS2; they may consequently explain *VWF* promoter activation in this cell line.

In physiological situations, multimeric forms of VWF protein are released from activated/injured endothelial cells; they then either adhere to endothelial cells or are circulated in the blood and consequently cleared by ADAMTS13. In a mouse model of skin melanoma, it was demonstrated that VWF fibers released from activated endothelial cells within the microvessels of tumors could induce thrombogenesis and facilitate the extravasation of cancer cells [118]. It was shown that melanoma cells take advantage of released ultra large VWF fibers to localize other inflammatory factors via recruited platelets [210-212] and leukocytes [213, 214] at the site. This local deposition of VWF fibers, activated platelets and recruited leukocytes has been shown to facilitate vascular permeability and the transmigration and extravasation of

tumor cells [118]. In addition, other cancer cells express receptors that bind to platelets [216] [217] or VWF [207, 218], thus coating cancer cells and allowing them to escape from the immune system [85].

We observed that *VWF* gene activation in several malignant glioma and osteosarcoma cell lines is associated with the increased ability of these cells to adhere to endothelial cells; this may have functional consequences with regard to cancer cell interaction with the vascular wall. Our results have indicated not only that some cancer cells acquired *VWF* expression, but also that the expression of VWF facilitated their transmigration through endothelial cells, thus potentially contributing to enhanced extravasation.

The extravasation of cancer cells occurs mainly within capillaries, where they have direct contact with endothelial cells. This initial step is critical for successful extravasation and further metastatic steps [219, 220]. Our data have indicated that the expression of *VWF* in SAOS2 (VWF+) cells increased their adhesion to the endothelium as well as to platelets, which facilitated the extravasation of cancer cells from capillary walls toward the extravascular stroma, as demonstrated in an *ex ovo* chicken embryonic model (CAM assay), and *in vivo* in a mouse lung metastasis assay. All of these processes were inhibited as a result of *VWF* knockdown in SAOS2.

Studying the initial steps of metastasis in the early hours of cancer cells circulation in the blood for different cancer cells has indicated that extravasation mainly occurs within 24 hours after I.V. injection of the cells. If cancer cells are arrested within the vascular network and are not extravasated during this initial 24 hour period, they do not survive and are disposed of through blood circulation [221,

222]. Our analyses demonstrated that significant numbers of knockout-VWF SAOS2 cells died within 24 hours of injection into chick embryos and mice; this stood in contrast to the behavior of control cells, which were able to extravasate from the vessels within the same period of time after injection. Specifically, in our experimental metastasis mouse model, the difference in the number of extravasated *VWF*+ compared to *VWF* knock down SAOS2 cells, which were detected based on the GFP+ cells, was highly significant in the mice lungs.

Tumor metastasis involves a complex process of interactions between host and tumor cells. While a significant number of cells are detached from primary tumors and have the potential to proceed towards metastasis, the rate of metastasis is very low; hence this is considered to be an inefficient process [219]. Therefore, cells that have acquired a specific ability or trait that empower them to execute the necessary steps of the metastatic pathway are able to initiate secondary tumor lesions. In Chapter 4, we have demonstrated a potential pro-metastatic role for *VWF* expression in SAOS2 cancer cells, which was abolished when we used specific siRNA against *VWF* in these cells. Specifically, we have shown the increased adhesion of cancer cells to endothelial cells and their transmigration from endothelial cells, as well as their enhanced interaction with platelets. However, we did not delineate the molecular bases of these processes. Therefore, whether VWF protein increases adhesion of cancer cells to the endothelium by binding to integrin alphaV beta3 or by activating the endothelial cells to release other adhesion molecules or possibly affecting endothelial junctions is not yet known and requires further study.

Since VWF is normally expressed in endothelial cells and megakaryocytes, using a therapeutic approach against VWF would unfortunately target both cancer cells and endothelial cells. However, a large-scale screening of cancer cells from various types of tissues could provide valuable information in implementing VWF as a potential marker for metastasis in cancer patients. Investigation of *VWF* expression in isolated circulating cancer cells (CCC) would provide the answer to whether VWF can potentially be used as a marker for early diagnosis of cancer metastasis. Methods to interfere with *VWF* expression by either genetic or pharmacological means to decrease or abrogate extravasation and, eventually, metastasis, could be potential targets for investigation.

GENERAL CONCLUSION

VWF protein is a multimeric glycosylated protein which is normally expressed in endothelial cells and megakaryocytes. The *VWF* expression pattern is representative of endothelial heterogeneity and thus potential heterogenic transcriptional regulation of this gene in various microenvironments. In this thesis, we have provided evidence to demonstrate that *VWF* gene expression in response to hypoxia is regulated in an organ-specific manner, with organ-specific functional consequences, such as increased thrombus formation. We also demonstrated that cancer cells of non-endothelial origin acquire *de novo* activation of *VWF* transcription, which contributes to their increased extravasation ability; this may play a role in increased metastatic potential of these cancer cells. Furthermore, determination of the molecular mechanisms that contribute to the *de novo* activation of *VWF* gene in cancer

cells and upregulation of *VWF* in response to hypoxia in endothelial cells provided insights that will be helpful in determining which specific regulatory factors and epigenetic modifications are major participants in regulating *VWF* gene transcription. This will aid in identifying potential targets for the development of therapeutic approaches.

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