ENDOTHELIAL CELL HETEROGENEITY: A CORRELATION WITH THE VON WILLEBRAND FACTOR EXPRESSION IN DISTINCT ORGANS.

by

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ABSTRACT

Endothelial cells (EC) of different organs exhibit heterogeneity in structure, function and gene expression pattern. This also extends to the pattern in which the highly endothelial-specific gene, von Willebrand Factor (VWF) is expressed. VWF is a multimeric adhesive, pro-coagulant glycoprotein involved in regulation of hemostasis and thrombosis; this protein is exclusively expressed in endothelial cells and megakaryocytes. Its two primary functions are to mediate the adhesion of platelets to the underlying endothelium and to be a carrier for the coagulation factor VIII. Deficiency in quantity and quality of the VWF protein is the cause of the most common inherited bleeding disorder, the von Willebrand Disease (VWD); on the other hand, dysregulated high levels increase the risk of thrombosis and cardiovascular disease. It was previously demonstrated that nucleotides -487 to +247 of the VWF gene function as an endothelial-specific promoter that exhibits organ-specific activity. We hypothesized that pattern of expression of transcription factors that regulate the VWF promoter may contribute to the mechanism that governs the organ-specific regulation of the VWF promoter. VWF promoter contains a binding site for GATA family of transacting factors and mutation of this site was shown to abolish the VWF promoter activity in vitro and in vivo. Several members of GATA family, including GATA2, 3 and 6 were reported to interact with the VWF promoter. We tested the hypothesis that there may be organ-specific distribution of GATA family members, which could contribute to organ-specific regulation of VWF. Immunofluorescence staining was used in various murine organs to mark the VWF and CD31 expressing EC. We also co-stained for transacting factor GATA family members GATA2, 3 and 6. Laser Capture Microdissection (LCM) was used to mark and dissect lung vessels' EC that expressed VWF. RT-PCR was used to analyze gene expression pattern in the dissected cells. Our IF and confocal microscopy demonstrated that ECs of distinct organs exhibit distinct patterns of GATA isoforms. Using IF and LCM we could positively identify, capture and isolate target cells. RT-PCR analyses of isolated target cells

demonstrated significant VWF expression in dissected EC, which was comparable to cultured EC. These data suggest that we have an organ-specific expression pattern of GATA transcription factors that may participate in the organ-specific regulation of VWF transcription. In order to gain more insight into the mechanisms that regulate VWF gene activity in vivo, I contributed to the exploration of how endothelial cells of different organs regulate VWF transcription in response to different stimuli, such as aging and hypoxia. The use of laser capture microdissection system will allow us to specifically detect the expression pattern of distinct regulatory factors that participate in the regulation of the VWF gene in EC of distinct organs not only in normal conditions but also in pathophysiological conditions. I was also involved in the project that investigates the expression of VWF in cancer cells of non-endothelial origin, since determining the mechanism of acquired VWF transcription by these cells would provide new insights towards understanding which VWF regulatory elements play a dominant role in establishing this activation.

PREFACE

The information in this thesis is the original work performed by Maria Areli Lorenzana Carrillo. Experimental design, performance and analysis of data were completed by Maria Areli Lorenzana Carrillo and her supervisor Dr. Nadia Jahroudi. Contribution studies were performed to collaborate in ongoing research projects within Dr. Jahroudi's research group, with master's student Radya Abdualla and Dr. Anahita Mojiri.

DEDICATION

This work is specially for you Grandma! You'll always be in my heart and I'll miss you forever!! Hugs and kisses all the way to heaven.

"Always remember you have within you the strength, the patience and the passion to reach for the stars to change the world."

-Harriet Tubman

"Don't be afraid to dream too big. Nothing is impossible. If you believe in yourself, you can achieve it."

-Nastia Lukin

I dedicate this work to my much-loved and amazing family, for always supporting me in all my endeavours, for being the driving force in my life and career; for showing me that there is not such thing as impossible. Without your caring love and patience, none of this would be possible. Throughout my life, you all have encourage me in my determination to find and release my potential, and to make my little contribution to our world.

"I have not failed. I've successfully discovered 10,000 things that won't work"

-Thomas Edison

"Success is not the key to happiness. Happiness is the key to success. If you love what you are doing, you will be successful."

-Albert Schweitzer

"Nothing in life is to be feared, it is only to be understood. Now is the time to understand more, so that we may fear less."

-Marie Curie

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GLOSSARY OF TERMS

ABCG2	ATP-Binding Cassette Sub-Family G Member 2
ALCAM	Activated Leukocyte Cell Adhesion Molecule
Alk1	Activin-Receptor-Like Kinase 1
AP-1	Activator Protein 1
APS	Ammonium Persulfate
AQP1	Aquaporin
ATP	Adenosine Triphosphate
AVR	Ascending Vasa Recta
BBB	Blood Brain Barrier
BSA	Bovine Serum Albumin
CD13	Cluster of Differentiation 13
CD14	Cluster of Differentiation 14
CD16	Cluster of Differentiation 16
CD31	Cluster Of Differentiation 31
CD36	Cluster of Differentiation 36
CD4	Cluster of Differentiation 4
CD44	Cluster of Differentiation 44
cDNA	Complementary Deoxyribonucleic Acid
CDw32	Cluster of Differentiation 32
ChIP	Chromatin Immunoprecipitation
CMEVC	Cardiac Microvascular Endothelial Cells
CMV	Cytomegalovirus
COUP-TFII	COUP Transcription Factor 2
COX	Cyclooxygenase
CpG	Cytosine Guanine (Linear Dinucleotide)
CRE	Cis-Regulatory Elements

CSF	Colony Stimulating Factors
CX37	Connexin 37
CX40	Connexin 40
CX43	Connexin 43
DAPI	4',6-Diamidino-2-Phenylindole
ddH_2O	Double Distilled Water
Depp	Decidual Protein Induced by Progesterone
DHSS	DNase I-Hypersensitive Site
Dll4	Delta-Like 4
DNA	Deoxyribonucleic Acid
DVR	Descending Vasa Recta
E-Cadherin	Epithelial Cadherin
EDHF	Endothelium-Derived Hyperpolarizing Factor
EDTA	Ethylenediaminetetraacetic Acid
Egr-1	Early Growth Response Protein 1
eNOS	Endothelial Nitric Oxide Synthase
EPAS	Endothelial PAS Domain Protein 1
EPCR	Endothelial Protein C Receptor
EphB4	Ephrin Type-B Receptor 4
ET-1	Endothelin-1
EtOH	Ethanol
Ets	E-Twenty-Six
FACS	Fluorescence-Activated Cell Sorting
Fc	Fragment Crystallizable Region
FITC	Fluorescein Isothiocyanate
Flk-1	Fetal Liver Kinase 1
Flt-1	Fms-Related Tyrosine Kinase 1

GATA	GATA-Binding Factor 6
GFP	Green Fluorescent Protein
GLUT-1	Glucose Transporter 1
GLUT-4	Glucose Transporter 4
GPIb-α	Glycoprotein Ib Alpha Chain
H3ac	Histone 3 Acetylated
H4ac	Histone 4 Acetylated
HATs	Histone Acetyltransferases
HCL	Hydrochloric Acid
HDACs	Histone Deacetylases
Hey	Hairy/Enhancer-Of-Split Related With YRPW
HIF1α	Hypoxia-Inducible Factor 1-Alpha
HLP	Histone-Like Protein
HMTs	Histone Methyltransferases
HPRT	Hypoxanthine-Guanine Phosphoribosyltransferase
HS	Heparan Sulfate
HUVEC	Human Umbilical Vein Endothelial Cells
ICAM	Intracellular Adhesion Molecule
IFN	Interferon
IgG	Immunoglobulin G
IL-1	Interleukin-1
KHOS	Osteosarcoma Cell Line
Lat-1	Large Neutral Amino Acid Transporter
LCM	Laser Capture Microdissection
LMVEC	Lung Microvascular Endothelial Cells
LPS	Lipopolysaccharide
Mct-1	Monocarboxylate Transporter 1

mRNA	Messenger RNA
NaOH	Sodium Hydroxide
N-Cadherin	Neural Cadherin
NFI	Nuclear Factor I
NFY	Nuclear Transcription Factor Y
NHERF-2	Na/H Exchanger Regulator Factor
NO	Nitric Oxide
NRP1	Neuropilin 1
NRP2	Neuropilin 2
NS	Non-Specific
OCT	Optimal Cutting Temperature Compound
PAF	Platelet Activating Factor
PAI	Plasminogen Activator Inhibitor
PBS	Phosphate-Buffered Saline
PECAM-1	Platelet Endothelial Cell Adhesion Molecule-1
PET	Polyethylene Terephthalate
PFA	Paraformaldehyde
PGI_2	Prostacyclin
pH	Potential of Hydrogen
PH	Pulmonary Hypertension
RNA	Ribonucleic Acid
RT-PCR	Real-Time Polymerase Chain Reaction
SAOS2	Osteosarcoma Cell Line
SDS	Sodium Dodecyl Sulfate
siRNA	Small Interfering RNA
SP1	Specificity Protein 1
TBS-T	Tris-Buffered Saline -Tween 20

TEMED	Tetramethylethylenediamine
TF	Transcription Factors
TFPI	Tissue Factor Pathway Inhibitor
Tie	Tyrosine Kinase with Immunoglobulin-Like And EGF-Like Domains
TNFα	Tumor Necrosis Factor Alpha
t-PA	Tissue-Type Plasminogen Activator
TR	Thrombin Receptor
TRP-4	Transient Receptor Potential Channel-4
TxA_2	Thromboxane
U251	Malignant Glioma Cell Line
UL-VWF	Ultralarge Von Willebrand Factor Multimers
UT-B	Urea Transporter
VCAM	Vascular Cell Adhesion Molecule
VE-Cadherin	Vascular Endothelial Cadherin
VEGF	Vascular Endothelial Growth Factor
VEGFR	Vascular Endothelial Cell-Specific Receptor
VWF	Von Willebrand Factor
VWF	Von Willebrand Disease
WPB	Weibel-Palade Bodies
YY1	Ying Yang 1
Zo	Zonula Occludens

CHAPTER 1: INTRODUCTION

The endothelium is considered a dynamic organ, which is composed of a monolayer of endothelial cells that cover the inner cellular lining of the blood vessels and lymphatics. There are approximately 1 x 10^{13} cells constituting the endothelium of an adult with a weight of practically 1 kilogram, covering a surface area of more than 1000 m^2[2-6].

The endothelium not only acts as a semi-permeable barrier that regulates the transfer of small and large molecules between the blood and the tissues, but it has a crucial role in several physiological processes such as the control of the vasomotor tone, angiogenesis, thrombosis, thrombolysis and both innate and adaptive immunity. Also, it is involved in many pathophysiological processes including atherosclerosis, hypertension, inflammatory syndromes and cancer metastasis [3-5, 7]. Aside from its barrier function mediated by the tight junctions, endothelial cells have a big array of functions as shown in Figure 1 and described below.

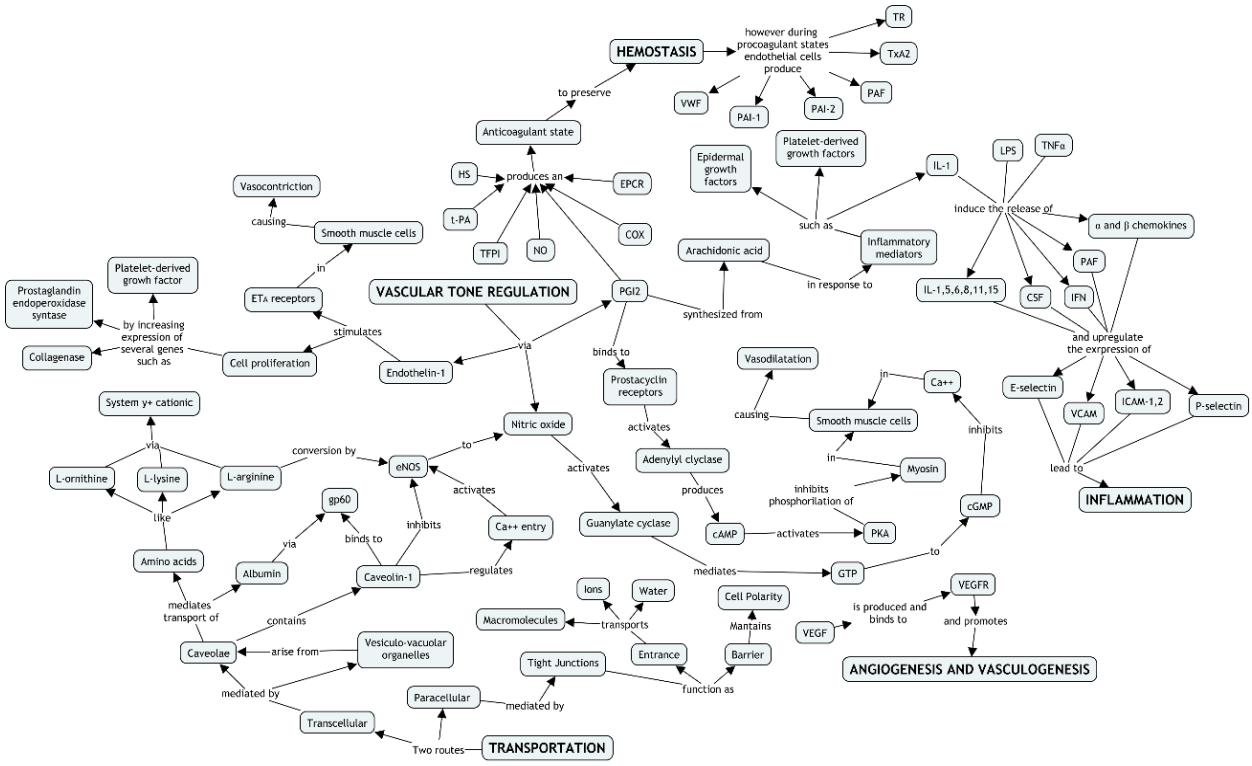

Figure 1 Schematic diagram of the endothelium functions

Endothelial cells play an important role in the transcellular and paracellular transportation pathways that mediate the transference of macromolecules, ions, and water from circulating blood to meet the metabolic requirements of the neighboring tissue cells. The paracellular pathway is mediated by the tight junctions (intercellular connections); they regulate the passage of ions, water, and some macromolecules and maintain cell polarity[4]. The transcellular pathway, on the other hand, is mediated by caveolae (invaginations of the cell membrane); albumin transport is primarily achieved by this pathway. At the same time, caveolae have different transportation systems present inside of them, such as GLUT-1 (in charge of the glucose transport) and the system y^+ cationic (that mediates the amino acid transportation, like L-arginine)[4, 8].

Caveolae not only have a function as a transportation mechanism but also are involved in other functions of the endothelial cells. Caveolin-1 is one of the major proteins present in caveolae, and in the endothelium, it regulates the nitric oxide (NO) signaling by binding to endothelial nitric oxide synthase (eNOS) and inactivating it [8]. Nitric oxide is one of the most powerful vasodilators of the blood vessels, which is generated as a result of eNOS breaking down l-arginine to l-citrulline causing the release of NO. This endothelial generated molecule not only plays a key role in the maintenance of blood pressure but is also involved in vascular remodeling, wound healing, and angiogenesis.[4] Just as NO, prostacyclin (PGI_2) is a potent and effective vasodilator that is synthesized by endothelial cells, while its main function is to inhibit platelet aggregation. Aside from NO and PGI2, endothelium-derived hyperpolarizing factor (EDHF) is another vasodilator (as yet unidentified) that is released after endothelial activation. In contrast to vasodilator, the endothelium also produces thromboxane (TxA_2), that causes both platelet aggregation and vasoconstriction. The activities of both TxA_2 and PGI_2 are essential to maintain homeostasis of the blood vessels[9]. Similar to TxA_2, endothelin-1 (ET-1) is also a vasoconstrictor expressed by endothelial cells, which stimulates the smooth muscle cells for a vessels constriction effect. Furthermore, this peptide plays a role as a stimulus for cell proliferation and hormone production [3, 4, 9].

Endothelial cells are highly involved in immune and inflammatory responses as they organize and regulate the recruitment and transference of the inflammatory cells from the blood flow into the site of injury or

infection.[3, 5, 10] Endothelial cells produce, release and respond to a diversity of cytokines, growth factors, and other mediators that function as a communication bridge between them and other immune regulatory cells, like lymphocytes and leucocytes. [4, 10] Endothelial activation is a key factor for this response. Upon activation by the stimuli of cytokines, such as tumor necrosis factor-alpha (TNFα), interleukin-1 (IL-1) and/or lipopolysaccharide (LPS), endothelial cells produce and release inflammatory mediators like IL-1,5,6,8,11,15, colony stimulating factors (CSF), α and ß chemokines, platelet activating factor (PAF), growth factors and interferons (IFN).[4, 11] There is also upregulation of the expression of the endothelial cell adhesion molecules: Intracellular adhesion molecules (ICAM-1, ICAM-2), vascular cell adhesion molecule (VCAM), P-selectin and E-selectin.[4, 5, 12] All these changes lead to the inflammation process, which includes vascular dilation, increased blood flow, endothelial cell contraction and leucocyte recruitment and extravasation.[5]

The endothelium plays a key role in hemostasis maintenance, as it secretes a variety of important molecules that regulate blood coagulation and some platelet functions.[10] Under basal conditions, endothelial cells preserve an anticoagulant state by expressing tissue factor pathway inhibitor (TFPI), tissue-type plasminogen activator (t-PA), thrombomodulin, endothelial protein C receptor (EPCR), cyclooxygenase (COX), ectonucleotidases, heparan sulfate (HS), PGI_2 and NO [4, 5, 10, 12-14]. However, during procoagulant states, endothelial cells produce von Willebrand factor (VWF), plasminogen activator inhibitor 1 and 2 (PAI-1, PAI-2), platelet activator factor (PAF), thromboxane A2 (TxA_2), thrombin receptor (TR) and thromboplastin [4, 12-14].

Endothelial cells are also involved in both vasculogenesis and angiogenesis, by producing and reacting to signals that allow them to organize the growth, development and arrangement of new blood vessels. The most important player involved in this processes is the vascular endothelial growth factor (VEGF), which is produced by a variety of cells (including endothelial cells). VEGF has a cell surface vascular endothelial cell-specific receptor (VEGFR2) that stimulates endothelial cells towards formation of new vessels[15].

ENDOTHELIAL CELL HETEROGENEITY

Developmentally, endothelial cells and hematopoietic cells arise from the same precursor cell, the hemangioblast. This precursor cell comes from the splanchnopleuric mesoderm that transforms into mesenchymal cells, which differentiate into the hemangioblast. This precursor then develops to a pre-endothelial cell, which can differentiate either into a hematopoietic cell or an endothelial cell. However, it has been previously shown that other cells, like adipose and neuronal cells, can transdifferentiate into endothelial cells and that endothelial cells can transdifferentiate into other cell lineages, like mesenchymal and intimal smooth muscle cells [4, 5, 12, 16, 17].

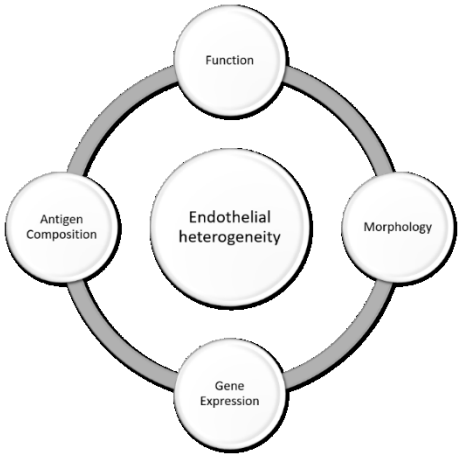

Figure 3 Characteristics from which endothelial heterogeneity has been defined.

Even though endothelial cells come from the same origin, far from being an enormous layer of indistinguishable cells, endothelial cells display both ultrastructural diversity and molecular heterogeneity (Figure 3). This phenotypical heterogeneity has been vastly described by Dr. William C. Aird in several reviews [7, 12, 18-22]. Endothelial cell phenotypes are diverse not only among different organs but even within the same organ and blood vessel type, as endothelial cells have to fulfill the highly specific functions and needs of different tissues (Figure 3) [7].

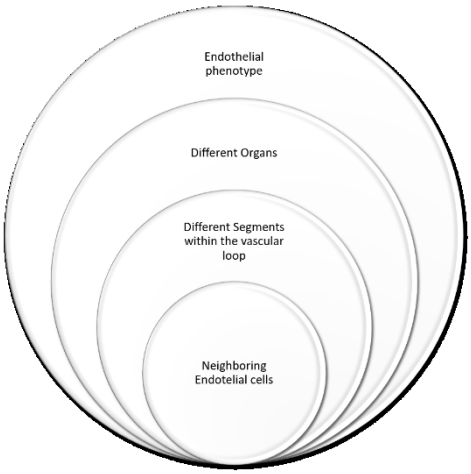

Figure 3 Levels of endothelial heterogeneity.

Morphologically, endothelial cells are approximately 10 times thicker in the aorta in comparison to capillaries and veins, and its shape can vary from very flat to cubical or rectangular[12]. On the other hand, functionally, the vasomotor tone is primarily regulated by arterioles; thermoregulation is mediated by microcirculation (specifically in the skin and bronchioles); leukocyte transmigration occurs primarily in postcapillary venules; fenestrated endothelium enables increased filtration or transendothelial transport while non-fenestrated endothelium acts as a protective barrier [12, 19, 22]. Moreover, hemostasis is regulated depending on the specific needs of the local surroundings. Endothelial cells from

different places on the vascular tree will express different anticoagulants and procoagulants to maintain the equilibrium [19]. For example, EPCR is expressed in large veins and arteries, while VWF is mostly expressed in endothelial cells from veins, and TFPI in capillary endothelium [13, 20].

BLOOD VESSEL HETEROGENEITY: ARTERIES, VEINS, AND CAPILLARIES

Arteries and veins are generated from an intricate process in which the primordial capillary plexus is influenced by genetically predetermined signals, allowing the evolution of the vasculature into diverse but interconnected blood vessels with distinct functions [23].

The main function of arteries is to carry the blood from the heart to the tissues; blood flow is controlled by the dilation and constriction of this type of vessels. Morphologically, arteries transport oxygenated blood and have thick walls to endure the high pressure of blood ejected from the heart. Genes that are preferentially expressed in arteries include ephrinB2, activin-receptor-like kinase 1 (Alk1), delta-like 4 (Dll4), neuropilin 1 (NRP1), decidual protein induced by progesterone (Depp), hairy/enhancer-of-split related with YRPW motif 1 and 2(Hey1 and Hey2), and endothelial PAS domain protein 1 (EPAS1) [18].

Alternatively, the principal occupation of veins is to transport the deoxygenated blood from the tissues back into the heart. In comparison to the arteries, they have thinner walls, low blood pressure and have one-way valves to prevent backward flow. Genetically, venous endothelial cells specifically express ephrin type-B receptor 4 (EphB4), neuropilin 2 (NRP2) and COUP transcription factor 2 (COUP-TFII) [18].

In between this two-vessel type partition, capillaries work to achieve the exchange of oxygen, nutrients, and waste between the blood and the tissues. These are tiny vessels that inter-join arteries and veins to carry out the appropriate function of the system [24]. Capillaries are the major part of the vasculature and their endothelial cells are exceptionally adapted to the underlying tissue environment [18].

MECHANISMS OF ENDOTHELIAL CELL HETEROGENEITY

It has been shown that endothelial cell's arterial-venous differentiation can be susceptible to certain level of plasticity in the case of it being required [23]. Endothelial heterogeneity has been shown to be mediated by two principal mechanisms: Microenvironment, and epigenetics [7, 22].

Firstly, endothelial cells are capable of sensing every little change of its surrounding environment, either from the inner surface, the outer surface or its junctions [22]. This allows the endothelial cells to react and respond to the stimuli to define its phenotype, and as the stimuli vary from organ to organ the heterogeneity is then generated [7]. This microenvironmental stimuli or "input" can be categorized as biomechanical and biochemical. Oxygenation, growth factors, cytokines, pH, lipoproteins, chemokines or hormones are some examples of biochemical inputs; while hemodynamic fluctuations such as shear stress or strain are included in the biomechanical stimuli [22].

Secondly, site-specific epigenetic modification plays an important role in giving heterogeneity to the endothelial cells, as these modifications, which include DNA methylation, histone modification, and chromatin remodeling are reversible and highly dynamic. Moreover, epigenetic factors have a significant role in the endothelial cell lineage determination during embryonic development [22].

Biochemical influences have a significant impact in mediating the phenotypic heterogeneity of capillaries as the microenvironment on which these beds are established vary significantly. Biomechanical fluctuations mostly impact the establishment of the arterial endothelium phenotype due to their constant exposure to flow changes; on the other hand, epigenetic modifications play major roles in establishing the distinct phenotypes of both veins and arteries [22].

ORGAN INFLUENCE ON ENDOTHELIAL HETEROGENEITY

Aside from having primary physiological differences, vessels are being constantly influenced by the environment of their specific organ, and consequently are set up in order to be best matched for their vascular bed-specific roles [23].

Cardiac endothelial cells are localized to several heart sections including the endocardium, the myocardial capillaries, and heart arteries and veins; however, the endocardium and the myocardium

show the most significance variance between their phenotypes.[18] The endocardium forms the inner layer of the heart, which is constantly perfused by the entire blood volume. Its endothelial cells are larger than any other endothelial cells and they have many microvilli projections into the lumen of the heart. On the other hand, myocardial capillaries are in close interaction with the cardiomyocytes in order to have an optimal diffusion of the nutrients and oxygen needed to promote their survival [18]. The endocardium presents deeper intercellular clefts, has an increased number of gap and tight junctions, and fewer vesicles in comparison to the myocardial capillaries[25]. Expressions of connexin 43 (CX43), connexin 40 (CX40), and connexin 37 (CX37) are found in the endocardium but not in the myocardial capillaries[26]; VWF and eNOS expressions are higher in the endocardium compared to the myocardial capillaries [27, 28], while the Receptor-like tyrosine phosphatase mu is expressed in the latter but not in the former[18, 29].

Endothelial cells have a key role and have unique features in the pulmonary circulation. Pulmonary vessels undergo the most critical postnatal changes, which forces pulmonary endothelial cells to adapt their phenotype to the biomechanical and biochemical disturbances that take place after birth [18]. Heterogeneity in the pulmonary endothelium is obvious throughout all sections of the vascular tree. For example, arterial endothelium has a higher eNOS expression and a larger NO production in comparison to the pulmonary capillaries [30]; large vessel endothelial cells contain weibel-palade bodies (WPB) and express VWF, while microvessels don't hold this organelle or express this protein within their endothelial cells [31]. On the contrary, capillary endothelium expresses more VE-cadherin, epithelial cadherin (E-cadherin), neural cadherin (N-cadherin), activated leukocyte cell adhesion molecule (ALCAM) and have the ability to bind the lectin *Griffonia simplicifolia*, which differentiates them from their counterpart in large vessels [30]. In addition, bronchial endothelial cells are more permeable, more responsive to inflammation and more angiogenic than those of the pulmonary. Bronchial microcirculation contains more cell types and has a thicker abluminal interstitium than that of pulmonary circulation[30].

In the kidney, vessels are highly specialized and display remarkable compartmentalization. Glomerular endothelial cells form the first filtration barrier. To fulfil this task, they possess fenestrae and PV-1 negative diaphragm pores[18, 23, 32]. These endothelial cells constantly synthesize glycocalyx

(proteoglycans, glycosaminoglycans, glycolipids and plasma proteins) and basement membrane, which allows them to filtrate selectively negatively charged molecules and bulk fluid [18, 23]. The glomerular endothelium is consistently positive for the specific endothelial marker PECAM-1/CD31 (Platelet Endothelial Cell Adhesion Molecule-1) and surface receptor protein CD32 expression, however, VWF expression is low and "patchy" in these endothelial cells[18]. The descending vasa arterioles (DVR) are lined by continuous non-fenestrated endothelium and the ascending veins (AVR) are lined by fenestrated endothelium. DVR specifically express the transient receptor potential channel-4 (TRP-4), Na/H exchanger regulatory factor (NHERF-2), the urea transporter (UT-B) and aquaporin (AQP1); in contrast to AVR that express PV-1. Other expression differences include higher levels of eNOS expression by the vasa recta (both DVR and AVR) compared to glomeruli [33]. Additionally, CX37 and CX40 are expressed in afferent arterioles, whereas CX43 is expressed in both afferent and efferent arterioles, and none are expressed in glomerular capillaries [34]; and finally, the vasa recta endothelium expresses claudin-10 and claudin-15, but the efferent and afferent arterioles do not [35].

The endothelium exhibits a broad range of phenotypes across the liver vasculature. At the portal venule, the endothelium is non-fenestrated and presents microvilli. The hepatic sinusoidal endothelial cells are discontinuous as they have large cytoplasmic fenestrae, exhibit gaps and a less organized basement membrane [18]. Apart from being involved in liver regeneration by producing hepatocyte growth factor and IL-6, they express ICAM-1, ICAM-2 and endoglin that play a key role in leukocyte trafficking. They also express the ET-1 receptor (ETB) that contributes to NO release and vasomotor tone regulation[36]. Sinusoidal endothelial cells are characterized by the expression of CD4, CD36, CD44, liposaccharide-binding protein receptor (CD14), aminopeptidase N (CD13), receptor 2(CDw32) and receptor III (CD16) for the Fc fragment of IgG, integrin α (1,5 and V) and β (1 and 3); however they lack expression of PECAM-1, VE-cadherin, VCAM-1, E-selectin, P-selectin and VWF [37].

Finally, the endothelium of the brain microvasculature represents the crossing point between the blood and the central nervous system. The blood-brain barrier (BBB) is characterized by its protective properties that strictly control the penetration of plasma components and circulation cells into the brain [38]. The BBB is characterized by the expression of claudin 5, occluding, Zo-1 and Zo-2 for the tight

junction determination; P-glycoprotein and ATP-binding cassette sub-family G member 2 (ABCG2) for efflux transport; and GLUT-1, monocarboxylate transporter 1 (Mct-1) and large neutral amino acid transporter (Lat-1) for nutrient transport [39].

ENDOTHELIAL CELL GENE REGULATION

An important factor that has to be considered when discussing endothelial cell heterogeneity is its' gene expression pattern and regulation as the phenotype is dictated in large part by these processes. Endothelial cell genes can be categorized per their specificity, inducibility and locality [40]. Among the endothelial cell specific genes, there are two categories: the ones that are expressed in distinct subsets of vascular beds, at the same time this can be divided into inducible (E-selectin, thrombomodulin, EPCR, eNOS and endocan) and constitutive (such as VWF, TFPI, receptor tyrosine phosphatase, ephrin B2 and ephrin B4); and the ones that are pan-endothelial and constitutive (PECAM-1 and VE-cadherin)[40].

Gene expression is tightly regulated in endothelial cells, and it mainly happens at the transcriptional level. Transcription regulation has a key role in the activation and suppression of gene expression, which is mediated by the transcriptional machinery that includes the cis-regulatory elements (CRE) and transcription factors (TF) [41]. CREs are constituted of DNA sequences with binding sites for the TF and/or regulatory molecules (such as TF co-factors) that are necessary for the initiation and maintenance of the transcription [42]. Promoters and enhancers are examples of these DNA sequences.

Apart from these regulatory elements, chromatin remodeling and histone modification mediated by several co-factors play a crucial role in gene regulation. These co-factors change the structure of the chromatin to enable or disable the recruitment of the TF or other regulatory elements into the promoter [43]. Histone modification refers to epigenetic changes such as acetylation, phosphorylation, methylation, ubiquitylation, sumoylation and isomerization. Chromatin remodeling is mediated by ATP which enhances the histone modification activity [43]. Histone acetyltransferases (HATs), histone deacetylases (HDACs), histone methyltransferases (HMTs), DNA methyltransferases and nucleosomal remodeling proteins are involved in these conformational changes that contribute to the function of transcription machinery [44].

ENDOTHELIUM-SPECIFIC PROMOTERS AND THEIR TRANSCRIPTION FACTORS.

Deciphering the molecular mechanisms that establish and preserve the endothelial-specific pattern of gene expression have important implications, like defining gene therapy agents or understanding the lineage establishment by transgenic mice development [38]. To determine these mechanisms the first step is to identify and characterize endothelial-specific promoters and their regulatory trans- and cis-acting factors [45].

Numerous endothelial-specific promoters have been identified and characterized both *in vitro* and *in vivo* including Flt-1 [46], ET-1 [47], ICAM-2[48], VE-Cadherin[49], Tie-2 [45], Flk-1 [50], eNOS[44], PECAM-1 [51] and VWF [52]. For instance, Flt-1 gene promoter is a 1-kb fragment of the 5'-flanking sequence of the flt-1 gene and is expressed in all vascular endothelial cells except for the liver [46]. VE-cadherin gene analysis showed that a 3.5kbp fragment from -2486 to +24 acts as an endothelial specific promoter that is expressed in all vascular beds except brain [53]. In addition, analysis of the eNOS gene showed that sequences from -1600 to +22 act as a 1.6kbp endothelial-specific promoter with an expression pattern that is limited to endothelial cells of brain, heart, skeletal muscle and aorta; however, if this promoter is extended to include sequences from -5200 to +28, the pattern of expression expands to endothelial cells of lung, kidney and liver [40, 44].

Despite lacking a common cis-element among all endothelial-specific genes that can bind an endothelial-specific transcription factor, endothelial-specific genes tend to share binding sites for several transcription factors that are highly enriched in the endothelium and contribute to regulation of target endothelial-specific genes expression. These include binding sites for GATA proteins, SP family members, Octamer binding site proteins, Ets family members and activator protein 1 (AP-1) [40, 44]. For example, GATA transcription factors are involved in the expression of ET-1, eNOS, Flk-1, ICAM-2, P-Selectin, PECAM-1, Utrophin-B and VWF; while SP family members regulate the expression of eNOS, Flk-1, ICAM-2, VE-Cadherin. Octamer proteins, on the other hand, are involved in the regulation of the Tie1, Tie 2 and the VWF gene; and finally, eNOS, Flk-1, Flt-1, ICAM-2, Tie1, Tie2, Utrophin-B, VE-Cadherin, and VWF require the Ets family member for their regulation [44].

Transgenic mice analyses of these promoters have shown that to orchestrate vascular bed-specific expression; several DNA regulatory elements and the promoter fragments contribute molecular markers for this vascular bed-specific transcriptional activity. However, vascular bed specificity is also related to the tissue microenvironment and it's not fixed into the genetics of endothelial cells [40]. With the goal of increased understanding of the regulatory mechanisms that control the endothelial-specific gene expression, our lab has studied the mechanisms that underline the transcriptional regulation of the gene coding for von Willebrand factor.

VON WILLEBRAND FACTOR (VWF)

The von Willebrand factor is an adhesive large multimeric plasma glycoprotein with a molecular mass of around 270 kDa and is present in blood plasma, platelets and endothelial cells [54]. VWF has two primary functions: it mediates the adhesion of platelets to the underlying endothelium through the interaction with GPIb-α and acts a carrier for the coagulation factor VIII [54]. VWF is specifically synthesized in the endothelial cells and megakaryocytes. Most of the synthesized VWF dimers or small multimers undergo basal secretion into the plasma, while the remaining are ultra large multimers (UL-VWF) that are stored either in the alpha granules of the platelets or WPBs of the endothelial cells [55]. Regulated secretion from storage organelles involves activation of WPBs and alpha granules to release the UL-VWF multimers [56].

VWF protein is considered a marker for endothelial cell heterogeneity and organ diversity as its distribution patterns vary with the size, location, and type of the vessel, which is reflective of the diverse transcriptional regulation of its gene. The VWF gene is approximately 178 kb long, is located on the short arm of the chromosome 12, contains 52 exons , and its mRNA is 8 kb long [57]. VWF expression is highly regulated by a very complex mechanism that is Intricate and influenced by many environmental as well as genetic factors outside of the VWF gene sequences [56]. Abnormalities in one of the two VWF gene alleles result into deficiencies of VWF; causing the most frequent inherited blood disorder: von Willebrand Disease (VWD) [56, 58]. On the contrary, overexpression of VWF contributes to cardiovascular complications as it can lead to a prothrombotic state and an increased risk of mortality [59]. As mention

before, VWF is best known for role in hemostasis and thrombosis; however, it has been found that VWF is also involved in other pathophysiological and physiological processes such as tumor metastasis, angiogenesis, cell proliferation and inflammatory processes [60].

VWF GENE REGULATION

The study of the highly restrictive expression of the VWF gene is a useful tool to understand the process and mechanism of endothelial cell-type-specific gene regulation. *In vitro* experiments have shown that the VWF gene sequences, from the nucleotides -487 to +246, function as an endothelial specific promoter (**Error! Reference source not found.**)[61]. This promoter contains three main regulatory regions; the core promoter, a negative regulatory region, and a positive regulatory region[61, 62].

Characterization of the endothelial specificity of the VWF promoter has resulted in the identification of some of its regulating cis-acting elements and their associated trans-acting factors. GATA-binding factor (GATA), E-Twenty-Six (Ets), nuclear transcription factor Y (NFY) (binding to site -18) and histone-H1 like protein (HLP) have been demonstrated to work as transcriptional activators[63-67]; whereas, nuclear transcription factor I (NFI), octamer-binding transcription factor (Oct-1), E4BP4 and NFY (binding to site +226) function as transcriptional repressors of the VWF promoter [1, 63, 66, 68-72].

VWF PROMOTER ACTIVITY

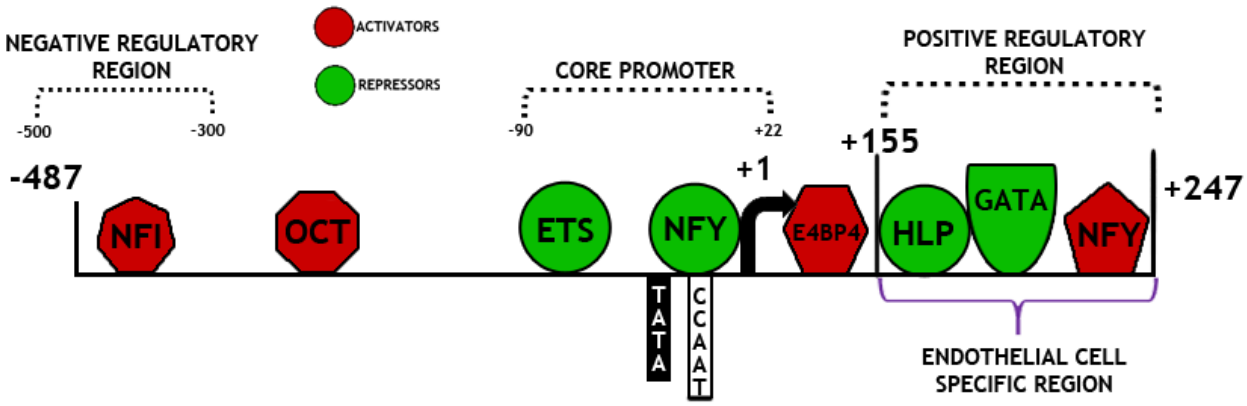

Figure 4 Von Willebrand Factor promoter with its cis-sequences and its binding transcription factors.

Multiple investigators, including my research group, have demonstrated by a series of *in vitro* experiments the VWF promoter functionality and its correlation with the activators and repressors previously mentioned.

It was demonstrated that in the -56 nucleotide there's a binding site for an Ets transacting factor that is necessary for activation of the VWF promoter and its mutation abolishes the promoter activity [64]. The region centred around nucleotide +220 in the positive region of the VWF promoter holds the binding site for the GATA family members. It has been shown by mutation analysis that the GATA binding site apart from activating the promoter, plays a critical role in the endothelial-specific expression of the VWF gene [61, 63].

As for the repressors, mutations in the binding site for Oct-1 was shown to increase the VWF promoter activation [70]. Similarly, it was demonstrated that the repressor NF1 interacts with DNA sequences spanning -440 to -470 nucleotides of the VWF promoter, mutation of the NF1 binding site in the context of the VWF promoter that included only the core promoter and negative regulatory region (sequences -487 to +155) resulted in activation of this promoter fragment in both endothelial and non-endothelial cell types [73].

Unexpectedly, NFY was discovered to perform, depending on its binding site, both as an activator and a repressor of the VWF promoter. It was demonstrated that when NFY interacts with the CCAAT sequence at the site -18 it acts as an activator; while when interacting with sequences centred around +226 it functions as a repressor of the promoter[66, 69].

Since the transcription factors involved in the regulation of VWF expression were described *in vitro,* it was important to determine the role of the VWF promoter and its regulatory regions *in vivo*.

Transgenic mice were generated with the goal of determining the -487 to +247 VWF promoter activity *in vivo* (Figure 5A). These mice exhibited endothelial specific but brain-restricted expression of the VWF promoter, showing no expression in the rest of the organs' blood vessels [52]. These results implied that other VWF gene sequences were required for the promoter activation in endothelial cells of distinct organs. Additional transgenic mice were generated to address this question. First, transgenic mice were generated that contained reporter LacZ gene under the regulation of an extended VWF promoter (spanning nucleotide -2645 to the end of the first intron) (Figure 5B). These mice showed extended VWF promoter activity primarily in the endothelial cells of veins and venules of the heart and skeletal muscle, in addition to those of the brain [74]. Later, VWF promoter sequences -487 to +247 were combined with sequences within the intron 51 of the VWF gene that are DNase I-hypersensitive (HSS), to drive LacZ gene

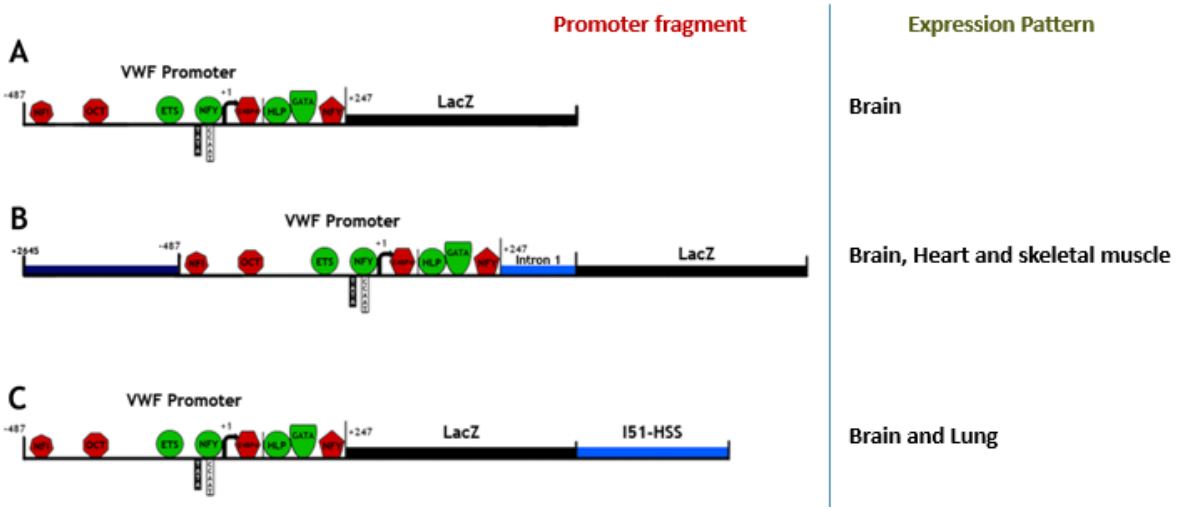

Figure 5 Von Willebrand Factor Transgene promoters.

expression in transgenic mice (Figure 5C), which resulted in the chimeric VWF promoter activation in lung and brain endothelial cells. Analysis of this Intron 51 region showed that a YY1 transcription factor interacts with this region [75].

Together, these results demonstrate that there is an organ-specific regulation of the VWF promoter; moreover, it has been shown that to maintain this organ-specific regulation, repressors play a key role in this process [1]. Several transgenic mice were generated containing the brain-specific VWF promoter (sequences -487 to +247) containing mutations in either the NFI or NFY binding site (Figure 6). Mutation of the NFI binding site resulted in the mutant promoter activation in brain, lung, and heart endothelial

cells. On the other hand, inhibition of NFY binding, as a result of the mutation in +226 region demonstrated expression of this mutant VWF promoter in brain and kidney endothelial cells. Inhibition of both NFI and NFY binding sites in combination resulted in the activation of the double mutant VWF

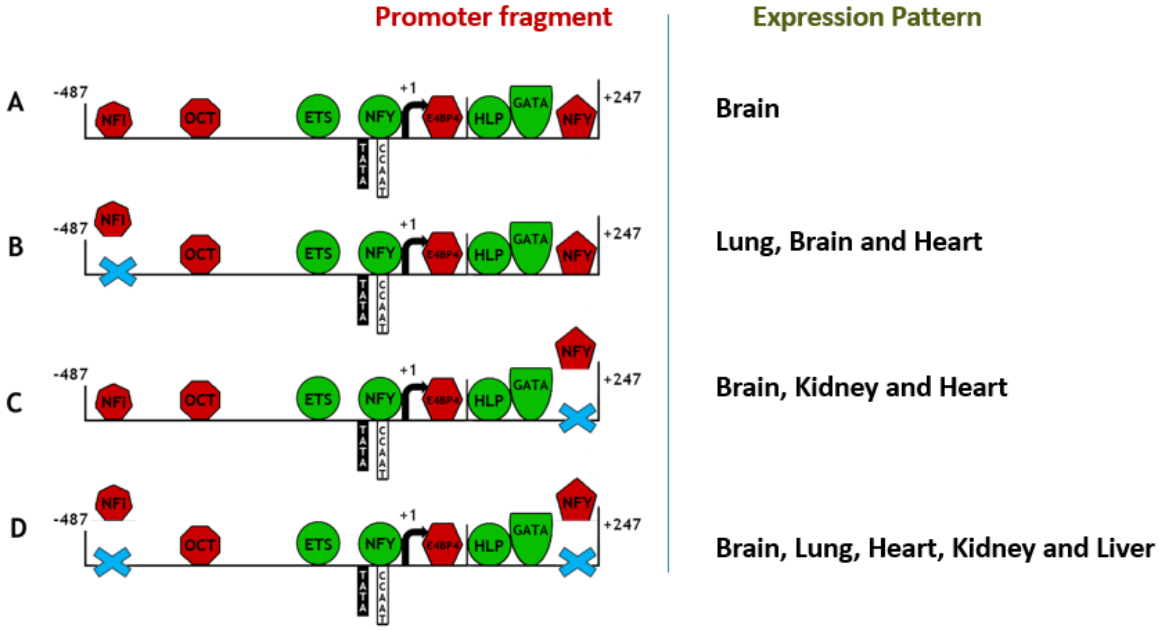

Figure 6. Expression of von Willebrand factor in mutated VWF promoter fragments.

promoter in endothelial cells of brain, lung, heart, liver and kidney [1].

Overall, the above results showed that the NFI transacting factor specifically suppresses the VWF promoter activity in the endothelial cells of lung and heart but not kidney. Further analyses demonstrated that there was an organ-specific distribution pattern of the NFI isoforms (A, B, C and X) in the vascular tree. The expression of NFIC and NFIX was most significantly detected in brain endothelial cell nuclei; NFIB and NFIA were mostly detected in the nuclei of heart endothelial cells; while NFIX was mostly detected in kidney endothelial cell nuclei; and finally, lung endothelial cell nuclei showed a higher NFIB expression[1]. Thus NF-IB was commonly expressed in both lung and heart endothelial cells, where NFI mutant VWF promoter was expressed; leading to the hypothesis that NF-IB is primarily responsible for VWF promoter repression in lung and heart endothelial cells.

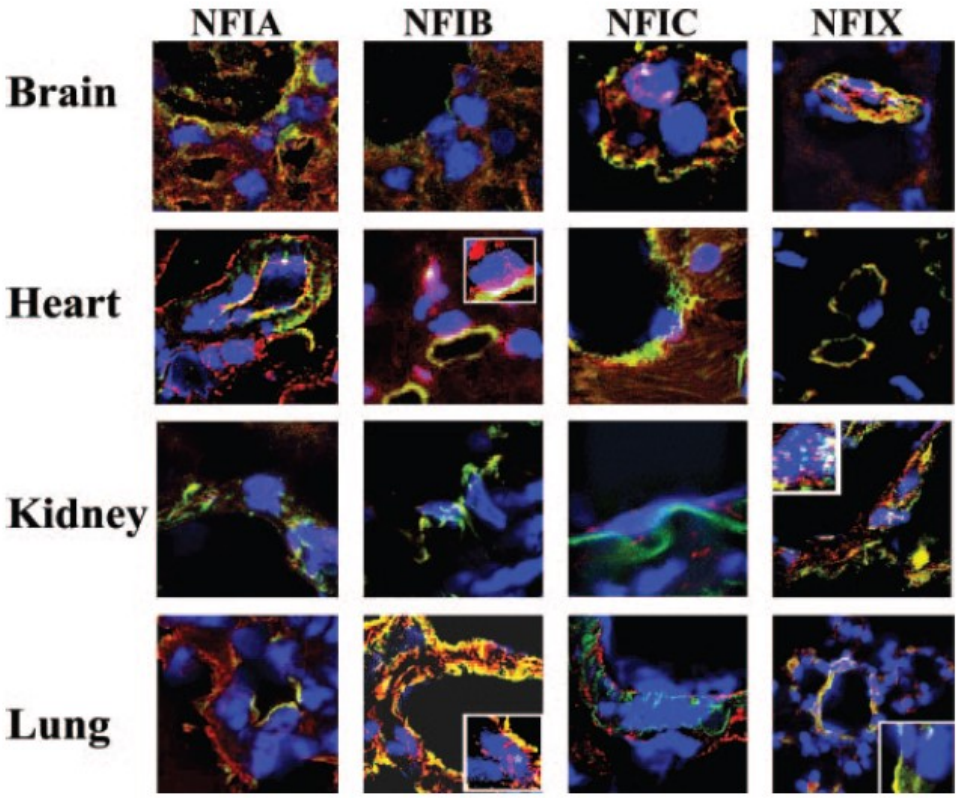

Figure 7 Figure appears precisely in this form in Nassiri et al (ATVB 2010) Repressor Function in the Regulation of von Willebrand Factor Promoter[1]

Immunofluorescent analyses of NFI isoform expression in organs of mice. Sections, 5 μm, from OCT frozen heart, kidney, lung, and brain of a control C57Bl/6 mouse that were arranged on tissue arrays were treated with antibodies specific to NFIA, NFIB, NFIC, and NFIX isoforms of NFI (red) and simultaneously with anti-PECAM (green) antibodies to detect endothelial cells. The results are representative of 2 independent experiments for each NFI antibody plus PECAM antibodies (magnification x600). The insets show magnified endothelial cell nuclei (4',6-diamidino-2-phenylindole).

Based on these results we hypothesized that the pattern of expression of transcription factors that regulate the VWF promoter may contribute to the mechanism that governs the organ-specific regulation of the VWF promoter. VWF gene regulatory transacting factors for the most part either belong to families of transacting factors, such as GATA, Ets, and NFI, or contain multi-subunits, such as NFY.

My research has been aimed at determining whether distinct members of families of these transacting factors exhibit preferential expression pattern in endothelial cells of distinct organs and whether such differential pattern may contribute to processes that regulate VWF expression in endothelial cells of different organs.

CHAPTER 2: MATERIALS AND METHODS

ORGAN RECOLLECTION

Mice were placed in dorsal position. The skin along the midline from the mandible to the pubic symphysis was cut and it was flipped laterally on each side of the incision to expose the underlying tissues and organs. The ribs were cut near their cartilage and flipped to each side of the thoracic wall laterally to expose the thoracic organs. Five organs including brain, heart, lung, liver and kidney of euthanized mice were removed and trimmed to the appropriate size.

IMMUNOFLUORESCENT ANALYSIS OF TISSUES.

OCT EMBEDDING

A 30% sucrose solution in 1X PBS was prepared and placed in 4° until tissue recollection. Organs in labeled cassettes were placed the Sucrose-PBS solution for 48 hours at 4° for fixation. Organs were taken out of solution, placed in a plastic mold, filled with OCT compound and frozen with liquid nitrogen. Organs were stored in -80°C until sectioning. OCT organs were sectioned 5 µm thick and placed on glass slides and kept in -80° until further processing.

PARAFFIN EMBEDDING

Organs in labeled cassettes were placed in 10% formalin for fixation for at least 24 hours. Formalin was drained. Cassettes were placed in 70% ethanol for 90 minutes on a shaker. Then sequentially immersed in 85% ethanol for 90 minutes, 95% ethanol for 90 minutes, 70% Butanol for 24 hours and 85% butanol for 24 hours. All of the processes were carried on the shaker. After rehydration, cassettes were immersed in paraffin wax in a 52° C heated chamber for 5 hours under vacuum. Tissues were removed from the cassettes and placed in tissue molds and filled with molten paraffin wax. Tissue molds were placed on a cold plate to harden the wax. Tissues were sectioned. Tissue blocks were cut in 5µm thick sections and placed on microscope slides

DEPARAFFINIZATION

Slides were placed in a 60°C preheated oven and baked for 2 hours. Deparaffinization was carried out by sequentially immersing the slides in 3 changes of xylene for 10 minutes each, after which was followed up by 2 minutes in 100% ethanol, 2 minutes in 95% ethanol, 2 minutes in 75% ethanol, 2 minutes in 50% ethanol and finally placed in running water for 2 minutes.

ANTIGEN RETRIEVAL

Before starting, two retrieval buffers were prepared:

1. Sodium citrate buffer: 2.94g of tri-sodium citrate (dihydrate) and 1lt of distilled water. Its pH was adjusted to 6.0 with 1N HCl and 0.5 ml of tween-20 was added. After mixed the buffer was stored at 4°C.
2. Tris-EDTA buffer: 1.21g of Tris Base, 0.37g of EDTA and 1lt of distilled water. Its pH was adjusted to 9.00 with 1N NaOH and 0.5 ml of tween-20 was added. After mixed the buffer was stored at 4°C.

For antigen retrieval, 3 ml of the retrieval buffer were mixed with 300 ml of distilled water and placed in an open container (This container was placed inside a bigger container filled up with water, which works as a water bath.). Both containers were placed in the microwave and they were heated until they reached a rolling boil (around 15 minutes). Once the buffer was in a rolling boil, the slides were placed in the boiling buffer and the microwave was set for 10 minutes. After the 10 minutes, the slides were rinsed with warm water and placed in 1X PBS buffer for further staining.

FIXATION AND PERMEABILIZATION

Both OCT sections and paraffin sections were placed in cold acetone for 5 minutes at -20°C for fixation and permeabilization, washed for 5 minutes in 1XPBS and continued with staining process.

IMMUNOFLUORESCENT STAINING

Blocking was performed for 1 hour by adding the blocking solution [5% fish gelatin (5g powder fish gelatin in 100 ml 1XPBS) and 10% serum from the species host of secondary antibody] over the tissues on the slides at room temperature in a moist chamber. Then the slides were slightly dried with a vacuum.

Primary antibody was prepared by adding the antibody (shown in Table 1) to the blocking solution and placed over the specimens and kept overnight at 4°C in a moist chamber. Next day slides were washed 3 times in high salt PBS (1.4g NaCl in 500ml 1X PBS) for 12 minutes, followed up 1 time in 1X PBS for 10 minutes. The slides were slightly dried before adding the secondary antibody. Secondary antibodies (shown in Table 2) were prepared in blocking buffer and were added to the tissues and incubated for 1 hour at room temperature in the dark. Slides were washed 3 times in high salt PBS (1.4g NaCl in 500ml 1X PBS) for 12 minutes, followed up 1 time in 1XPBS for 10 minutes. Slides were dried out, ProLong® Gold Antifade Mounting media (ThermoFisher Scientific CAT# P36934) was added on top of the tissue and a covered with a coverslip and were kept overnight at room temperature in the dark. The slides were analyzed by confocal microscopy.

Primary antibodies used for Immunofluorescence staining	
GATA2	Abcam CAT # ab173817
GATA6	Abcam CAT # ab22600
GATA3	Abcam CAT # ab106625
VWF	MyBioSource CAT # MSB2016156
VWF	Abcam CAT # ab11713
PECAM	Abcam CAT# ab28364

Table 1 Primary antibodies for IF

Secondary antibodies used for Immunofluorescence staining	
Donkey anti-sheep AlexaFluor 488	Abcam CAT # ab150177
Donkey anti-rabbit AlexaFluor 488	Invitrogen CAT # A21206
Donkey anti-rabbit AlexaFluor 594	Invitrogen CAT # A21207
DAPI	Nuclei Staining

Table 2 Secondary antibodies for IF

LASER CAPTURE MICRODISSECTION ANALYSIS

- OCT embedded tissues processing

Immediately after tissue recollection, each organ was placed in a plastic mold, filled with OCT compound and frozen with liquid nitrogen. Organs were stored in -80°C until sectioning. OCT organs were sectioned 5 µm thick in RNAse-free conditions and placed in LCM slides (Leica 11505151 Metal Frame, PET-membrane (25 x 76 mm)) and kept in -80° until further processing.

Frame Slide Sections were placed in cold 100% EtOH for 2 minutes in -20°C for fixation and permeabilization. Slides were dip rinsed with RNAse free water or kept in -80° until staining procedure.

- Immunofluorescence staining

Tissue section was brought to room temperature and marked with a PAP pen (Abcam ab2601), blocking was performed for 10 minutes by adding the blocking solution [5% fish gelatin (5g powder fish gelatin in 100 ml RNAse free 1XPBS) over the tissues on the slides at room temperature. After blocking, slides were slightly dried with a vacuum; primary antibody (Sheep polyclonal to Von Willebrand Factor FITC. CAT

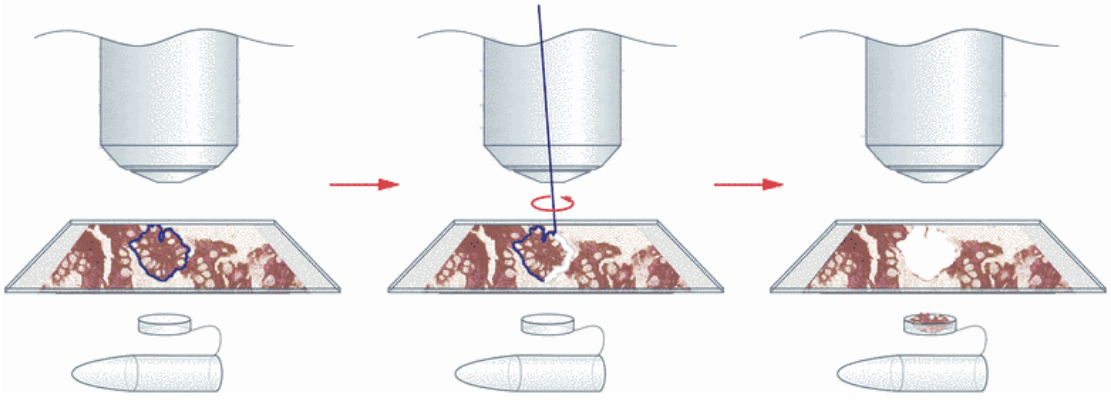

Figure 8 The Principle of laser microdissection (Leica Microsystems LMD system). (As taken from http://www.leica-microsystems.com/science-lab/laser-microdissection/an-introduction-to-laser-microdissection/)

Step 1: Define the region of interest by marking in the microscope screen. Step 2: Laser beam steered by optics along the cut line. Step 3: Specimen collection by gravity in tube cap with lysis buffer.

#ab8822) was added to blocking buffer at a 1:10 ratio + DAPI at a 1:50 ratio, added to the tissues and incubated for 15 minutes at room temperature in the dark. Primary antibody was removed and the slides

were carefully washed 2 times by dip rinsing them in RNAse free 1XPBS. Slides were left to dry for 5 minutes before cutting by microdissection.

- Microdissection process

Laser Microdissection System (Leica LMD6500) was cleaned to RNAse free conditions with RNAse away (VWR 17810-491) before the experiment was performed. Set up and microdissection was performed per manufacturer's instructions as shown in Figure 8. From each slide, 200 VWF positive stained vessels were microdissected and retrieved in 60µl of RLT Buffer (QIAGEN CAT # 79216).

- RNA extraction and cDNA generation

RNA extraction was performed with RNeasy Plus Micro Kit (QIAGEN CAT # 74034) following manufacturer's instructions. The extracted RNA was used to generate 20 µl of cDNA using QScript cDNA SuperMix (Quantabio CAT # 95048) as recommended by the manufacturer.

- Real Time – PCR analyses

The cDNA was used as a template for RT-PCR using DyNAmo ColorFlash SYBR Green qPCR kit (Thermo Fisher scientific CAT # F416). The reactions were performed in a total volume of 10 µl using the following volumes:

- 5 µl DyNAmo ColorFlash SYBR Green Master mix
- 1 µl primers
- 2 µl cDNA sample
- 2 µl distilled water

And the cycling conditions that were used are the following:

- Holding: 50°C for 2 minutes
- Holding: 95°C for 7 minutes
- 40 cycles of:
  - Denaturalization: 95°C for 10 seconds
  - Annealing: 60°C for 30 seconds
- Melt Curve

IN VITRO ANALYSIS

CELL CULTURE

- Lung Microvascular endothelial cells (LMVEC) were cultured on gelatin-coated dishes in EBM-2 Basal medium (Lonza CAT#CC-3156) and supplemented with EGM-2 MV SingleQuot Kit (Lonza CAT#CC-4147)
- Cardiac Microvascular endothelial cells (CMVEC) were cultured on gelatin-coated dishes in EBM-2 Basal medium (Lonza CAT#CC-3156) and supplemented with EGM-2 MV SingleQuot Kit (Lonza CAT#CC-4147)
- Human umbilical vein endothelial cells (HUVEC) were cultured on gelatin-coated dishes in Medium 199 (GIBCO CAT#11150-059) supplemented with 20% fetal bovine serum (GIBCO CAT#26140-079), 1% endothelial cell growth supplement (Corning CAT# CACB356006) and 1% penicillin-streptomycin.
- Osteosarcoma $SAOS_2$ were cultured in McCoy's 5A Medium (ATCC CAT#30-2007) supplemented with 10% fetal bovine serum (GIBCO CAT#26140-079) and 1% penicillin-streptomycin.
- Osteosarcoma KHOS were cultured in DMEM high glucose with L-glutamine medium (Lonza CAT#12-604) supplemented with 10% fetal bovine serum (GIBCO CAT#26140-079) and 1% penicillin-streptomycin.

Malignant Glioma U251 were cultured in DMEM high glucose with L-glutamine medium (Lonza CAT#12-604) supplemented with 10% fetal bovine serum (GIBCO CAT#26140-079) and 1% penicillin-streptomycin.

CELL TRANSFECTION

Von Willebrand factor was transiently knocked down in $SAOS_2$ and U251 cells and specific transcription factors of the HLMVEC and HCMVEC were transiently knocked down before hypoxia treatment. After achieving an 80% confluency, the cells were transfected with non-silencing or specific silencing siRNAs (shown in Table 3) twice within 48 hours, transfections were executed using Lipofectamine RNAiMAX (ThermoFisher Scientific CAT#13778-075) according to the manufacturer's protocol.

CELL TRANSDUCTION

Von Willebrand factor expression was induced in KHOS cells using VWF lentivirus. After achieving a 50% confluency, the cells were transduced with non-specific (Applied Biological Materials CAT# LVP690) or specific lentivirus (Applied Biological Materials CAT# LVP356569) according to the manufacturer's protocol. Transduced cells were isolated by puromycin selection. After full confluency, the cells and as the lentivirus contained a GFP+ marker, cells were sorted by fluorescence-activated cell sorting (FACS) and cultured until confluency for further analyses.

siRNA sequences		
VWF	5'-AACATGGAAGTCAACGTTTAT-3'	QIAGEN CAT# SI00011830
YY1	5'–ATGCCTCTCCTTTGTATATTA –3'	QIAGEN CAT#S100051926
NFIB	Sense: 5'– GGA UAG UUU UGU AAA AUC Utt –3' Antisense: 5'– AGA UUU UAC AAA ACU AUC Ctc –3'	Ambion CAT#4392420 ID: s9495
GATA6	Sense: 5'– GAC UCU ACA UGA AAC UCC Att –3' Antisense: 5'– UGG AGU UUC AUG UAG AGU Cca –3'	Ambion CAT#4392420 ID: 5607
SP1	Sense: 5'– CCA CAA GCC CAA ACA AUC Att –3' Antisense: 5'– UGA UUG UUU GGG CUU GUG Ggt –3'	Ambion CAT#4392420 ID: s13320
HIF1α	Sense: 5'– CCU CAG UGU GGG UAU AAG Att –3' Antisense : 5'– UCU UAU ACC CAC ACU GAG Gtt –3'	Ambion CAT#4392420 ID: s6541
NS	Non-specific siRNA	QIAGEN CAT# I027281

Table 3 siRNA Sequences used for endothelial cells

CELL CULTURE IN HYPOXIC CONDITIONS

LMVEC and CMVEC that were approximately 80% confluent (control, non-specific and specific siRNA), were placed in a hypoxia chamber at 1% oxygen for 1 hour and then maintained at normoxia conditions for 48 hours, after which cells were harvested with RLT buffer (QIAGEN CAT # 79216) for RNA extraction, Laemmle buffer for protein extraction or trypsinized for DNA extraction.

TRANSWELL TRANSMIGRATION ASSAY

6.5mm transwell membranes (Corning Inc. CAT#3472) in a 24 well plate were coated with 0.2% gelatin.

HUVECs were grown on the transwell to generate an intact monolayer. After HUVEC confluency, 10^4 cancer cells were added over the endothelial monolayer and incubated for two hours. Cells were then washed three times with 1X PBS and fixed with 4% paraformaldehyde for 20 minutes; after fixation membranes were quickly washed with 1X PBS and then stained for PECAM-1/CD31. Membranes were mounted with ProLong® Gold Antifade Mounting media (ThermoFisher Scientific CAT# P36934) on the slides and were quantified by confocal microscopy.

WESTERN BLOT ANALYSIS

Protein lysates were prepared and boiled for 10 minutes at 99°C. Whole cell lysate of each sample was loaded on a 6% Tris-SDS gel (Prepared as shown in Table 5, Table 6). BLUeye pre-stained protein ladder (FroggaBio CAT#PM007-0500) was loaded as a standard control. Running was performed for 1 hour at constant 100V in 1%SDS buffer and the gel was transferred overnight onto an Immobilon-P membrane (EMD Millipore CAT#IPVH00010) at constant 32V at 4°C with 1X Towbin buffer. The membrane was then blocked with 5% skimmed milk/TBS-T for 1 hour at room temperature. The primary antibodies (shown in Table 7) in blocking buffer were incubated while rotating for overnight at 4°C. The blot was washed 3 times for 10 minutes with TBS-T and then incubated for 1 hour at room temperature with the secondary antibodies. The membrane was washed 3 times for 10 minutes with TBS-T (Buffers prepared as shown in Table 4). ECL Prime Western Blotting detection reagent (GE Healthcare Amersham CAT#45-002-401) was used according to manufacturer's instructions. Membranes were exposed for development in the FluorChem FC2 Imaging System (Alpha Innotech). If necessary, the membrane was stripped with restore western blot stripping buffer (Thermo Fisher Scientific CAT#21059) per manufacturer's protocol and probed again.

Stock Buffers	
10X SDS (Running Buffer)	- 120.4 g Tris base - 576 g Glycine - 80 g SDS - 4 L ddH_2O
10X TBS (Washing Buffer)	- 350 g NaCl - 96.8 g Tris base - 4 L ddH_2O *pH 7.5*

10X Towbin (Transfer Buffer)	- 120.8 g Tris base - 576 g Glycine - 4 L ddH_2O
Working Buffers	
5% Skimmed Milk (Blocking Buffer)	- 2.5g milk - 50 ml TBS/T
1% SDS (Running Buffer)	- 400 ml 10X SDS - 3600 ml ddH_2O
1X TBS/ 0.1% Tween (Washing Buffer)	- 200 ml 10X TBS (pH 7.5) - 2 ml TWEEN - 1800 ml ddH_2O
1X Towbin (cold) (Transfer Buffer)	- 200 ml 10X Towbin - 400 ml Methanol - 1400 ml ddH_2O

Table 4 Western Blot Buffers

Separating Mix		Stacking Mix	
Tris 1.5M pH 8.8	30 ml	Tris 0.5M pH 6.8	12.5ml
SDS 10%	2ml	SDS 10%	0.5ml
ddH_2O	67 ml	ddH_2O	30.5

Table 5 Stock Gel Mix Preparation

	6 % Separating Gel	Stacking Gel
Solution Mix (Separating or Stacking)	8ml	4.4 ml
Acrylamide/BIS 30%	2ml	650 µl
Ammonium Persulfate (APS) 10%	100µl	50 µl
TEMED	10µl	10 µl

Table 6 Western Blot gel preparation

Antibodies used for Western Blot	
NFIB	Abcam CAT# ab18638
GATA6	Abcam CAT# ab22600
SP1	Abcam CAT# ab13370

HIF1α	Abcam CAT# ab85886
YY1	Abcam CAT# ab109228
VWF	DAKO CAT# A0082
Tubulin	Millipore CAT#05-829

Table 7 Western Blot Antibodies

DNA METHYLATION

Two sites of methylation located in the VWF promoter (-422 and +119) were tested in the cultured cells. DNA was extracted from the cells by QIAmp DNA mini kit (QIAGEN CAT#51304) following manufacturer's instructions. Extracted DNA (1µg) was incubated with or without restriction enzyme HpaII (ThermoFisher Scientific CAT#ER0511) and 4X Tango Buffer (ThermoFisher Scientific CAT#ER0511) for DNA digestion overnight at 37°C. Digested and undigested products were diluted 1/5 and used then as templates for RT-PCR.

RT– PCR FOR DNA METHYLATION

The DNA was used as a template for RT-PCR using DyNAmo ColorFlash SYBR Green qPCR kit (Thermo Fisher scientific CAT # F416). The reactions with two set of primers as shown in Table 8 were performed in a total volume of 10 µl using the following volumes:

- 5 µl DyNAmo ColorFlash SYBR Green Master mix
- 1 µl primers
- 2 µl cDNA sample
- 2 µl distilled water

And the cycling conditions for methylation that were used are the following:

- Holding: 50°C for 2 minutes
- Holding: 95°C for 10 minutes
- 40 cycles of:
  - Denaturation: 95°C for 15 seconds
  - Annealing: 54°C for 60 seconds
  - Extension: 72°C for 60 seconds
- Final Extension: 72° for 7 min

- Melt Curve

Primers for DNA 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

Table 8 Primers for DNA Methylation

RNA EXTRACTION

RNA was extracted by RNeasy Plus Mini Kit (QIAGEN CAT # 74134) following manufacturer's instructions. The extracted RNA was used to generate 10 µl of cDNA using QScript cDNA SuperMix (Quantabio CAT # 95048) as recommended by the manufacturer and analyzed using RT-PCR.

CHROMATIN IMMUNOPRECIPITATION (ChIP) ASSAY

10^7 cells were trypsinized, counted and resuspended in 10m of complete media. 270 µl of formaldehyde (37% solution) was added directly to the cells in suspension in culture medium (10 ml) for a final concentration of 1%. Cells were fixed at 22°C for 10 minutes and stopped by the addition of 530 µl of glycine (2.5M) to a final concentration of 0.125M. The cells were spun down for 5 min at 1000rpm at 4°C, the media was removed and the cells were washed twice by adding 10ml of cold 1XPBS. The pellets of approximately 10^7 cells were resuspended in 400µl of Buffer A, on ice, for 15 minutes. Then 25µl of 10% NP 40 was added, vortexed for 10 seconds and centrifuged at 13000 rpm for 30 seconds. The supernatant was aspired. The pellet was resuspended in 1ml of Buffer S for 10 minutes on ice. Sonication took place after that, for 1 minute on 20~40% output with VibraCell™; after 5 min, sonication was repeated for a total of 5 repeats. All processes were performed on ice.

The protein A-agarose was pre-cleaned, for every 40 µl of protein A-agarose beads, 4 µg of salmon sperm and 4 µg of BSA were added and incubated overnight at 4°C with rotation. After incubation, the beads

were spun briefly at 2500 rpm for 30 seconds, marked the volume in the tube and the supernatant was removed. Buffer D was added to bring the volume to the original volume of the beads; then add again salmon sperm and BSA (the same amount) and allow rotation for 1 to 2 hours at room temperature. Spin down the beads briefly by centrifugation (2500 rpm for 30 seconds). Wash beads with 1m Buffer D, repeat wash for 3 times.

The chromatin was pre-cleaned by diluting every 200 µl of chromatin with 1.5 ml of Buffer C and incubating it with 40 µl of protein A-agarose at 4°C for 2 hours with rotation. Then briefly spin down and collected the supernatant.

40 µl of pre-cleaned beads were incubated with 1.5 ml of pre-cleaned chromatin. 5 µg of primary antibody (shown in Table 9) or no antibody for negative control was added and incubated at 4°C with rotation overnight. Then centrifuged at 2500 rpm for 10 min at 4°C. The supernatant was removed carefully, and the beads of IP chromatin were kept.

The beads were washed for 15 minutes on a rotating platform at room temperature with 1ml of Buffer W1 for 4 times. Washing was repeated 4 times with buffers W2, W3, and W4 in that sequence.

At room temperature, the chromatin was eluted by a 15-minute incubation with 150 µl of Buffer E. 0.5 µl of RNase (10mg/ml) and NaCl (to a final concentration of 0.3M) was added to the elution and the input (Positive control, sonicated DNA), then reverse cross-linking was performed by incubating at 65°C for 4 hours, followed by the addition of 3.3 µl of 2M Tris pH 6.8, 3.3 of 5M EDTA and 1 µl of proteinase K (20 mg/ml). The mixture was incubated at 45°C for 2 hours.

Followed by purification of the elution by QIAquick PCR Purification Kit (QIAGEN CAT#28106) using manufacturer's instructions and performed RT-PCR afterward.

RT-PCR

The cDNA or DNA was used as a template for RT-PCR using DyNAmo ColorFlash SYBR Green qPCR kit (Thermo Fisher scientific CAT # F416). The reactions with two set of primers as shown in Table 10 and Table 11 were performed in a total volume of 10 µl using the following volumes:

- 5 µl DyNAmo ColorFlash SYBR Green Master mix
- 1 µl primers
- 2 µl cDNA sample
- 2 µl distilled water

And the cycling conditions that were used are the following:

- Holding: 50°C for 2 minutes
- Holding: 95°C for 10 minutes
- 40 cycles of:
  - Denaturalization: 95°C for 15 seconds
  - Annealing: 60°C for 60 seconds
- Melt Curve

Antibodies used for ChIP	
NFIB	Abcam CAT# ab18638
GATA6	Abcam CAT# ab22600
SP1	Abcam CAT# ab13370
HIF1α	Abcam CAT# ab85886
H3ac	Abcam CAT# ab47915
H4ac	Abcam CAT# ab177790
IgG	Abcam CAT# ab197767

Table 9 Antibodies used for ChIP

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'-CGC AGG GAA GAG AAG GGA AAT AAA CTGG-3'	Integrated DNA Technologies
VWF- Intron 51-reverse	5'-GAC TGA TCT TCA AGA ATT GTG GCCC-3'	Integrated DNA Technologies

Table 10 Primers used for ChIP

Primers for RT-PCR		
VWF (Human)	Hs_VWF_1_SG QuantiTect Primer Assay CAT# QT00051975	QIAGEN
HPRT (Human)	Hs_HPRT1_1_SG QuantiTect Primer Assay CAT# QT00059066	QIAGEN
VWF (Mouse)	Mm_Vwf_1_SG QuantiTect Primer Assay CAT# QT00116795	QIAGEN
HPRT (Mouse)	Mm_Hprt_1_SG QuantiTect Primer Assay CAT# QT00166768	QIAGEN

Table 11 Primers used for RT-PCR

CHAPTER 3: RESULTS

SECTION 1. GATA TRANSCRIPTION FACTOR FAMILY EXPRESSION PATTERN AND ITS CORRELATION TO THE VON WILLEBRAND FACTOR EXPRESSION IN THE HETEROGENEOUS ENDOTHELIUM.

As a first step, we chose the GATA family of transacting factors as a forerunner in our expression analyses. As mentioned before, it has been demonstrated that the VWF promoter contains a DNA motif centered around nucleotide +220 that acts a binding site for the GATA family of transacting factors. Mutation of this site abolishes the VWF promoter activity in vitro and in vivo[63]. Cultured endothelial cells express GATA2, GATA3, and GATA6 and they have been reported to interact with the VWF promoter [63, 76]. We explored the possibility that endothelial cells of brain, lung, heart, liver and kidney might exhibit organ-specific differences in GATA family members' expression patterns.

Determining the pattern of expression of the GATA transacting factors in diverse organs.

Since it has been shown that endothelial cells lose their heterogeneity when they are taken from their cellular niches. This issue makes *in vitro* assays unreliable to determine the factors that play crucial roles in giving endothelial cells their heterogeneity, making *in vivo* assays most relevant for such analyses. For this reason, immunofluorescent and confocal microscopy analyses were performed to analyze the expression pattern of the GATA family of transacting factors in VWF+ endothelial cells in various organs

of mice using antibodies to detect specific family members of GATA transacting factors (GATA2, 3 and 6) and VWF.

For these analyses, 5 µm thick sections were cut from frozen OCT blocks of brains, hearts, lungs, livers and kidneys of mice and subjected to immunofluorescence staining. Antibodies used specifically detected one of the following GATA family members, GATA2, GATA3 or GATA6 (conjugated with red

A

SUMMARY

	BRAIN	HEART	LUNG	LIVER	KIDNEY
GATA2	+++	+	++	+++++	++++
GATA3	-	++	+++	++++	-
GATA6	++	+++	-	++	++

B

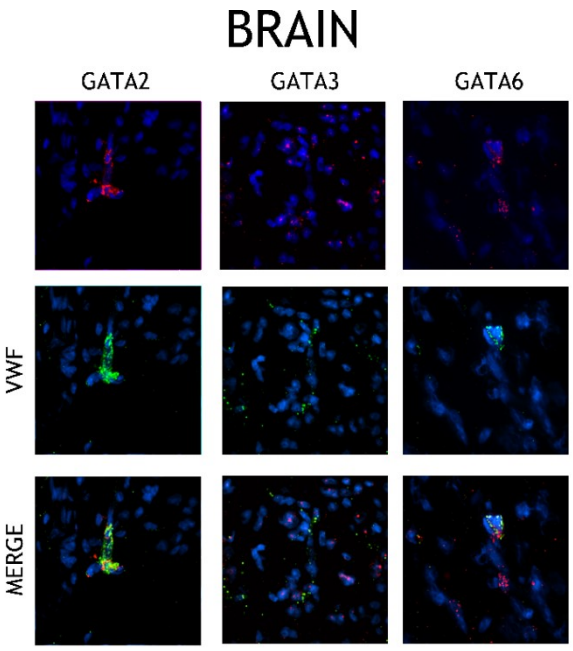

Figure 9. GATA family of transacting factors present a differential pattern of expression in distinct organs.

A: Representative quantitation of the GATA family members localizing in the nuclei of VWF positive endothelial cells. B-F: Immunofluorescent analyses of the expression of the family of GATA transacting factor in various organs of mice. 5 µm sections from OCT frozen heart, kidney, liver, lung and brain of a 3-month-old mice. Organs were arranged on arrays and were hybridized to specific antibodies to detect GATA2, GATA3 and GATA6 (shown in red) and concurrently to anti-VWF antibody (shown in green) and cell nuclei stained with DAPI (shown in blue) to detect VWF-expressing endothelial cells as described in materials and methods. The results are representative of two independent staining procedures for each GATA and VWF antibody. Pictures were taken at x400 magnification.

fluorescent dye) in combination an anti-VWF (conjugated with green fluorescent dye) antibody and DAPI

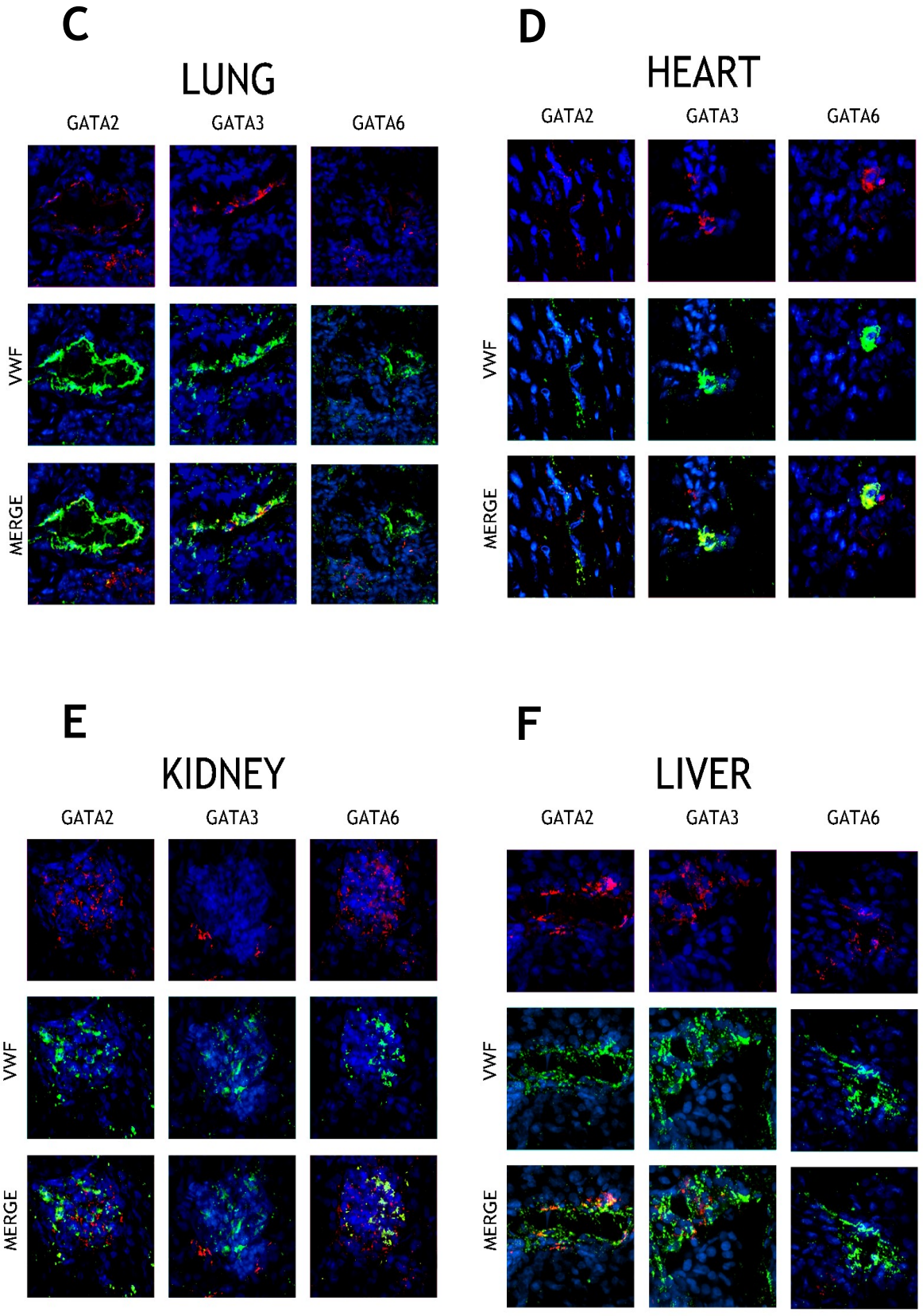

Figure 8. GATA family of transacting factors present a differential pattern of expression in distinct organs.

to stain the nuclei. The results of these analyses shown in Figure 9 demonstrated that there were significant variations in the expression patterns of GATA transacting factors family members that were detected in the nuclei of VWF+ endothelial cells. In brain (Figure 9B) the expression of GATA2, followed by GATA6 was most significantly, whereas GATA3 was barely detectable. The expression of GATA3 and GATA6 were most significantly detected in heart endothelial cell nuclei, with no detection of GATA2 (Figure 9C). Nuclear expression of GATA2 and GATA3 were predominantly detected in lung endothelial cells, with no detectable expression of GATA6 (Figure 9D). GATA2 was predominantly detected, followed by GATA3 and GATA6 in liver endothelial cell nuclei (Figure 9E). Finally, in kidney endothelial cells (Figure 9F) GATA2 was detected to the greatest extent, followed by GATA6; while no GATA3 was detected.

B

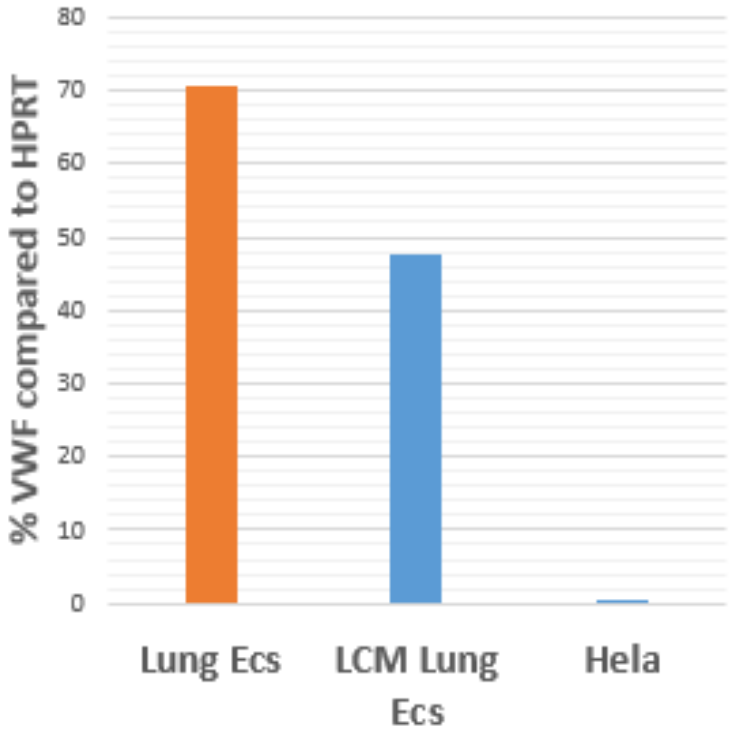

Figure 9. Immunofluorescence staining, laser capture microdissection and RT-PCR analyses.

A: Representative immunofluorescent staining with VWF antibody of mice lung tissue (as described in methods). VWF positive vessels are marked in yellow prior to microdissection. B: RT-PCR analysis of VWF+ microdissected cells were performed using specific primers. Cultured human lung microvascular endothelial cells were used as a positive control, while HeLa cells were used as a negative control.

Microdissected cells express comparable levels of VWF mRNA to cultured cells.

The results above demonstrate that each member of the GATA family of transacting factors presents a distinct pattern of expression in VWF expressing endothelial cells of each organ. However, as endothelial cells are highly heterogeneous and because the GATA transacting factors are not endothelial specific, it is difficult to determine from these immunofluorescent analyses alone how each GATA family member contributes to VWF regulation in endothelial cells of each organ. To further explore the differential expression of VWF regulatory factors in endothelial cells of distinct organs, we proceeded to use Laser Capture Microdissection (LCM). This technique will allow us to identify and isolate endothelial cells from each organ by using UV laser. Dissected endothelial cells will be used to extract RNA for further analyses to specifically detect the expression and regulation pattern of not just GATA family members, but other transacting factors that are involved in the regulation of the VWF promoter and may participate in its organ-specific activities.

As described in materials and methods, OCT-snap frozen lungs of mice and rats were cut into 5 µm thick sections and placed on LCM specific frame membrane slides. Tissues were subjected to immunofluorescence staining with an anti-VWF antibody to detect endothelial cells. Around 100 VWF+ vessels were microdissected. RT-PCR analyses of the microdissected cells demonstrated significant VWF mRNA expression, consistent with the protein expression showed after staining. VWF expression at the mRNA level was comparable to that observed in cultured lung endothelial cells, while HeLa cells used as negative control did not demonstrate significant VWF mRNA levels as shown by RT-PCR analyses using human VWF specific primers (**Error! Reference source not found.**). These results demonstrate that LCM can be used efficiently to isolate highly pure population of vascular endothelial cells with intact RNAs that are amenable to gene expression analyses. A process that is central towards achieving our proposed objective regarding the expression pattern of VWF gene regulatory transacting factors in endothelial cells of distinct organs in vivo.

SECTION 2. ENDOTHELIAL CELLS OF DISTINCT ORGANS EXHIBIT AN INCREASE OF VWF EXPRESSION LEVEL WITH AGING.

Aging has been associated with a variety of hemostasis changes that increase the risk of thrombotic disease [77, 78]. It has been previously shown that plasma levels of circulating VWF may predict some cardiovascular diseases, such as coronary heart disease [79, 80]. It has been previously demonstrated in some *in vitro* and *in vivo* studies that the expression of VWF increases with age [81-85]. For this reason, my research group looked into different organs of young and aged mice to determine whether there are differences in VWF protein expression among various organs when comparing young and aged mice. Since my main interest is to understand the mechanism by which the endothelial cells heterogeneity is governed, and endothelial cells of different organs may present heterogeneity with regard to age related changes in VWF expression, I have also contributed to this research study as follows.

Organs were harvested from young (3 months) and aged (18 months) mice as described in materials and methods and processed for western blot and immunofluorescence analyses to detect VWF. Western blot analyses were performed to determine if there was a quantitative differential expression of VWF in organs of young and aged mice as described in materials and methods. As shown in Figure 11, significantly higher levels of VWF protein were detected in lungs and livers of aged compared to young mice, demonstrating that with aging VWF protein expression is upregulated in these organs. In order to determine whether distribution pattern of the VWF expression is also altered, immunofluorescent staining was performed to determine vascular localization of VWF with regard to vessel type.

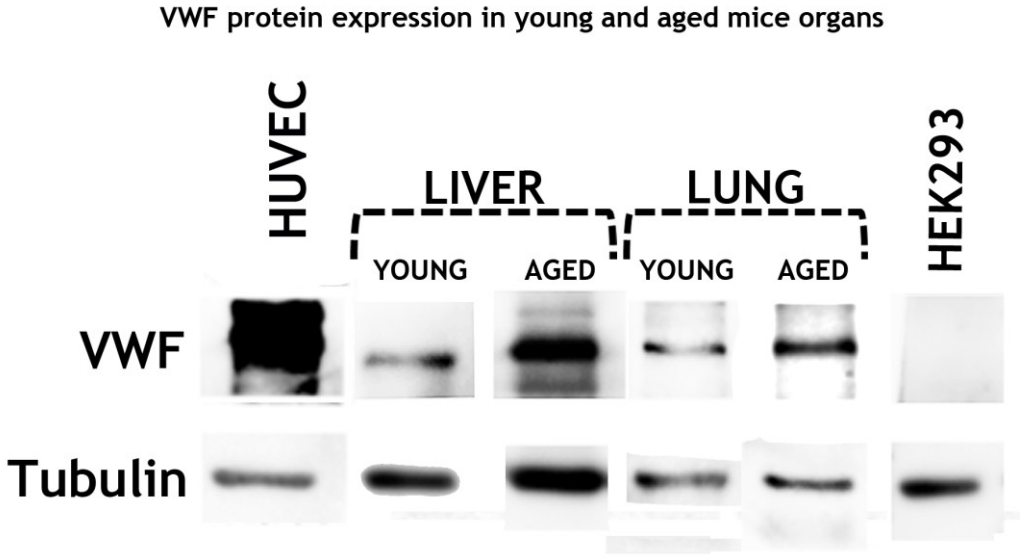

Figure 10. Western blot from young and aged liver and lung.

Western blot analyses were performed on young and aged lung and liver lysates to determine VWF expression. Tubulin was used as a loading control. Results show VWF protein expression upregulation in both aged liver and lung compared to their young counterparts. HUVEC and HEK293 were used as a positive and negative control respectively. The results are representative of 3 independent experiments for each aged and young organ.

Immunofluorescent analyses for these studies were performed using VWF and CD31/PECAM-1 (another endothelial specific marker) specific antibodies to confirm endothelial specific localization of VWF by confocal microscopy. Figure 12 shows that in both young and aged mice, CD31 expression was detected in all endothelial cells of lung and liver. In young mice, VWF colocalization with CD31 was detected specifically in endothelial cells of large vessels of both lung and liver; however, in aged mice

colocalization of VWF and CD31 was shown not only in large vessels but also in a significant number of small vessels. These results demonstrated that with aging VWF changes its distribution pattern from primarily large vessels to those of small as well as large vessels in these organs. This redistribution pattern of VWF to smaller vessels may contribute to increased thrombogenicity in these vital organs making smaller vessels susceptible to generation of platelet aggregates and potential blockage.

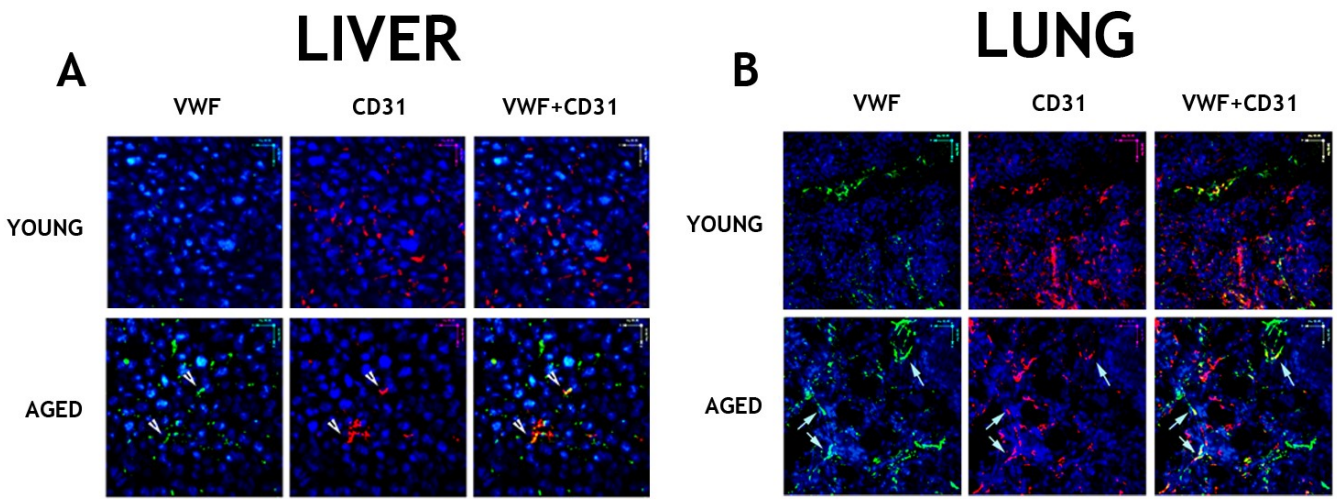

Figure 11. Immunofluorescence staining of VWF and CD31 in young and aged organs.

Immunofluorescent analyses of the expression of the VWF and CD31 in liver and lung of young and aged mice. 5µm sections from OCT frozen liver and lung of both 3-month and 18-month old mice were hybridized to specific antibodies to detect CD31/PECAM-1 (shown in red) and concurrently to anti-VWF antibody (shown in green) to detect VWF-expressing endothelial cells as described in materials and methods. The results are representative of three independent staining procedures for each organ. Pictures were taken at x200 magnification.

SECTION 3. ENDOTHELIAL CELLS OF DISTINCT ORGANS EXHIBIT HETEROGENEITY IN RESPONSE TO HYPOXIA WITH REGARD TO VON WILLEBRAND FACTOR TRANSCRIPTIONAL REGULATION.

The heterogeneity of endothelial cells regarding VWF expression also extends to how endothelial cells of different organs regulate VWF transcription in response to external stimuli. Our group has explored the response of VWF to hypoxia in endothelial cells of different organs. Based on my interest in understanding the mechanism of heterogeneity of endothelial cells with regard to VWF expression I have also contributed to these studies as follows.

Determination of the chromatin binding and participation of specific trans-acting factors in hypoxia response of VWF in lung and heart microvascular endothelial cells.

Increased VWF expression has been observed in lung microvascular endothelial cells in response to hypoxia. In control mice, VWF is primarily found in large vessels of the lung; however, it was observed that under hypoxic condition, the expression of VWF is upregulated in lung, brain, heart and liver but not in the kidney [86]. In order to gain insight into this differential regulation, I contributed to the studies which explored the mechanism of VWF expression in response to hypoxia in endothelial cells of distinct organs. This included determining which transacting factors are target of hypoxia by first determining

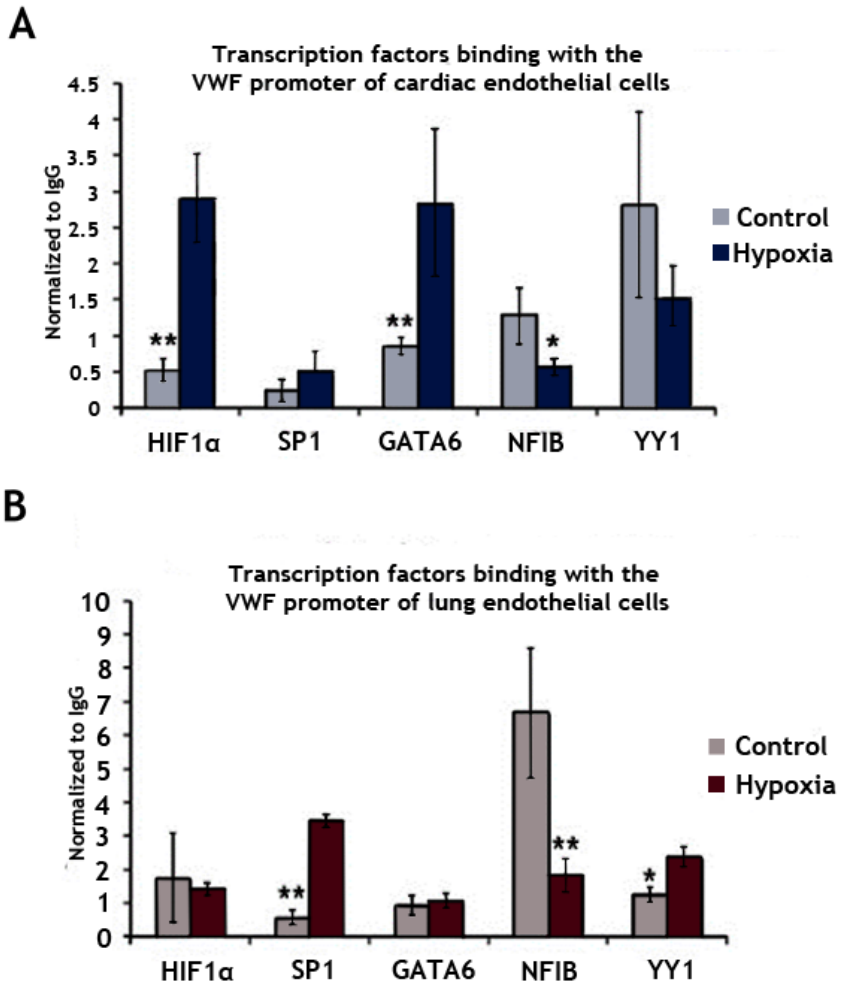

Figure 12 Chromatin binding of specific transacting factors on the VWF promoter in response to hypoxia in lung and cardiac MVEC.

ChIP assay was performed to determine associations of HIF-1α, SP1, GATA6, NFIB and YY1 with the VWF promoter in control and hypoxia treated lung and cardiac microvascular endothelial cells. Graphs represent the averages of 3-5 independent experiments for each factor (*P<0.05).

whether hypoxia exposure alters binding patterns of VWF regulatory transacting factors differentially in endothelial cells of different organs. In order to analyze the binding patterns of VWF promoter regulatory factors, including NFIB, GATA6, SP1 and YY1, as well as HIF-1α (transcription factor that is known for

mediating hypoxia response) in cardiac and lung microvascular endothelial cells (MVEC) exposed to normoxic and hypoxic conditions; ChIP was performed as described in materials and methods (Figure 13).

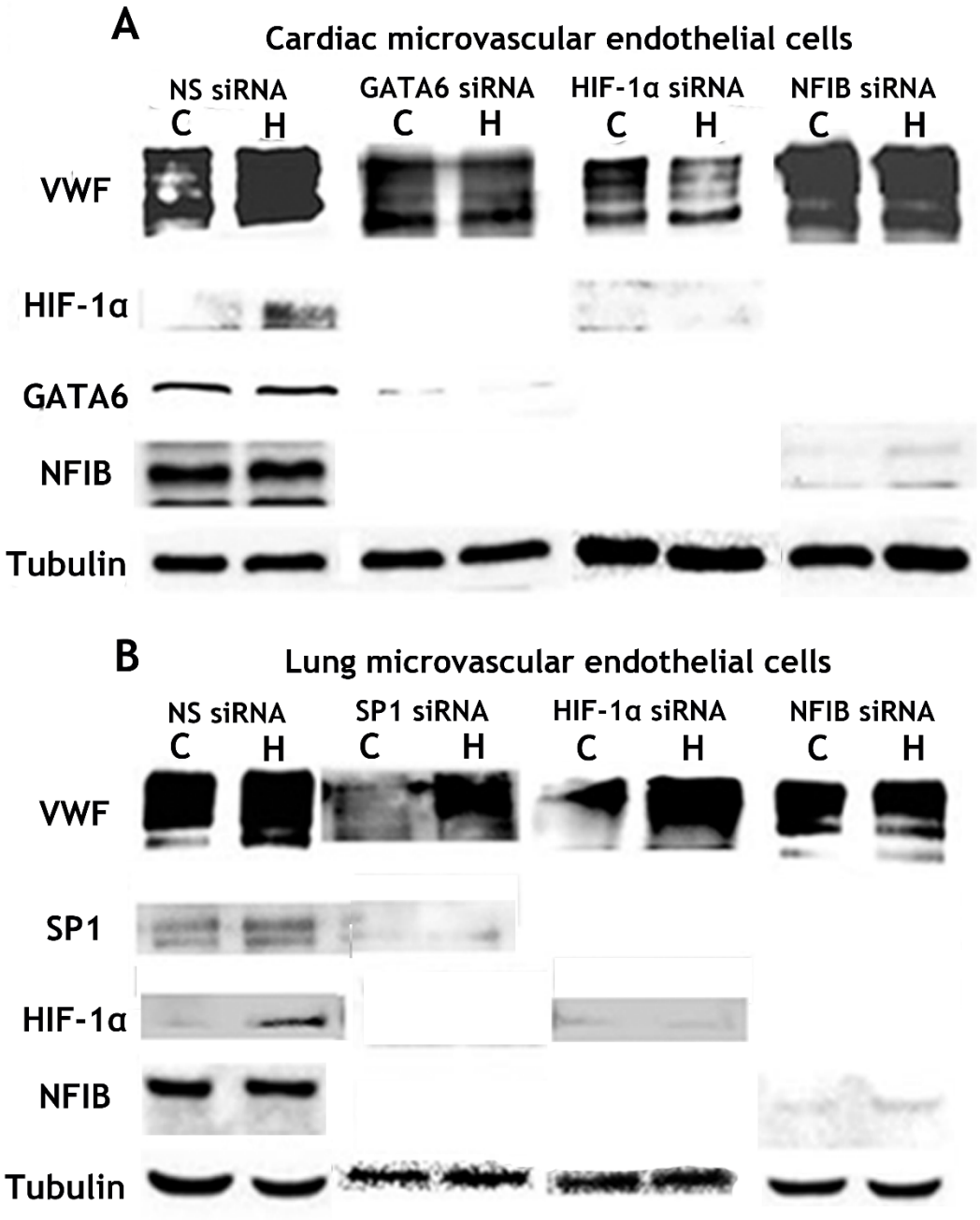

Figure 13. Transcription factors contribution in the upregulation of VWF in cardiac and lung MVECs.

Endothelial cells were transfected with either non-specific siRNA (NS siRNA) or siRNAs for NFIB, GATA6, SP1 and HIF-1α before hypoxia treatment. Western blot analyses were performed on control and hypoxia cell lysates to determine VWF expression and expression of specific siRNA targeted protein levels. The results are representative of 3 independent experiments for each siRNA transfection.

The results demonstrated that in response to hypoxia, binding of NFIB to the VWF promoter was significantly reduced in both cardiac and lung MVEC. Binding of YY1 to the I51HSS sequence (region that contribute to VWF expression in lung endothelial cells as described in introduction Figure 5C) was augmented in lung but not in cardiac MVEC. Similarly, an association of SP1 with the VWF promoter was increased in lung, but not cardiac microvascular endothelial cells. On the contrary, there were significant increases in bindings of GATA6 and HIF-1α to the VWF promoter in cardiac but not lung MVEC.

Also, to define the role of specific transacting factors in hypoxia-induced upregulation of VWF, we knocked down target transacting factors before exposing lung or heart endothelial cells to hypoxic conditions as shown in Figure 14 and described in materials and methods. To determine the protein levels of VWF and the siRNA targeted transacting factors, Western Blot analyses were performed as previously described. We observed that NFIB, GATA6, HIF-1α, SP1 were successfully knocked down by siRNA in both cardiac (Figure 14A) and lung (Figure 14B) MVECs. We determined that NFIB knockdown eliminated the VWF upregulation after hypoxia exposure in both endothelial cell types. The results also showed that

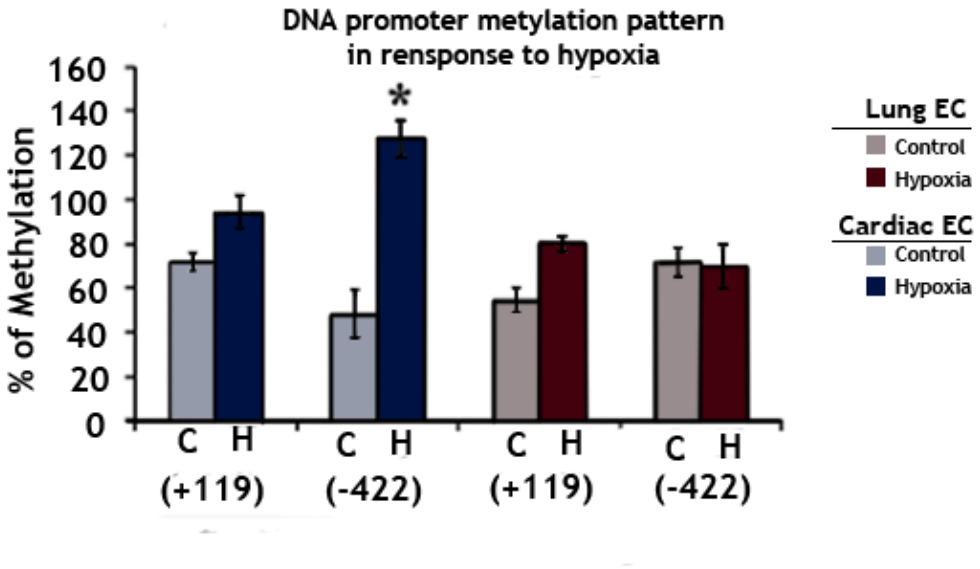

Figure 14. VWF promoter DNA methylation in response to hypoxia.

DNA retrieved from cardiac and lung microvascular endothelial cells was digested with HpaII restriction enzyme, as described in materials and methods. RT-PCR analyses were performed using VWF specific primers to determine the methylation status of the positions -422 and +119. DNA methylation analyses results are averages of 4 independent experiments for each cell type (*P<0.05).

knockdown of either GATA6 or HIF-1α inhibits the hypoxia-induced VWF upregulation in cardiac MVEC but not in lung MVEC. On the other hand, SP1 and HIF-1α knockdown abolished VWF upregulation after hypoxia exposure in lung but not cardiac MVEC.

. It has been recently demonstrated that DNA methylation of the VWF gene contributes to its transcriptional activity, as well as its mosaic pattern of expression [87, 88]. Thus, we hypothesized that the hypoxia induced VWF transcriptional regulation may be correlated to such epigenetic modifications as DNA methylation. We proceeded to determine the methylation status of the -422 and +119 specific CpG elements on the VWF promoter that are non-methylated when the VWF is expressed (Figure 15). As described in materials and methods, we used a methylation sensitive restriction enzyme and performed RT-PCR analysis to assess methylation status and levels in these CpG sites in both lung and cardiac MVECs in control and in response to hypoxia. Results showed that while lung microvascular endothelial cells didn't show any significant changes in methylation levels in either of the sites, cardiac MVEC significantly increased methylation in response to hypoxia, especially at the -422 site. Since -422 site is in close proximity to repressor NF-IB binding site, based on these results we hypothesize that specifically in cardiac endothelial cells increased methylation may interfere with the repressor NF-IB binding and consequently lead to enhanced transcriptional activity in response to hypoxia.

SECTION 4. VON WILLEBRAND FACTOR EXPRESSION BY CANCER CELLS OF NON-ENDOTHELIAL ORIGIN AND ITS FUNCTIONAL CONSEQUENCES.

It has been proposed that VWF has a role in cancer metastasis, in view of its spatial location and its highly adhesive properties. It was hypothesized that VWF facilitates extravasation of cancer cells, thus contributing to promote metastasis [89], a hypothesis consistent with some reported experimental results [90-92]. However, other investigations demonstrated that VWF acts as a pro-apoptotic agent on the tumor cells, and consequently reducing metastasis [93].

These differential observations regarding the role of VWF in cancer metastasis are credited to the belief that VWF expression has been exclusive to endothelial cells and platelets. However, it was reported that in the cultured osteosarcoma SAOS2 cell line, human colorectal SW480 cancer cells, and the

hepatocellular carcinoma (HCC) cell lines HepG2 and BEL7402, VWF protein expression has been found [94-96]. Our research group proceeded to investigate the mechanism by which cancer cells of non-endothelial cell origin may acquire VWF expression. Understanding this process would thus provide insights towards determining which VWF regulatory elements plays a dominant role in establishing its activation. Based on my interest in determining the contribution of distinct VWF regulatory elements to its transcriptional regulation, I contributed to these studies regarding VWF transcription process in cancer cells as shown below.

Cancer cells of non-endothelial origin express VWF protein.

Western blot analysis was performed to determine if there was VWF protein expression in selected patient-derived cancer cells of malignant glioma as well as osteosarcoma cancer cell lines SAOS2 and KHOS; using HUVEC and HELA as positive and negative controls. Accordingly, we found significant levels of VWF expression in the osteosarcoma SAOS2 and glioma U251, M049 cell lines, as well as in some of the patient-derived glioma cells as shown in Figure 16.

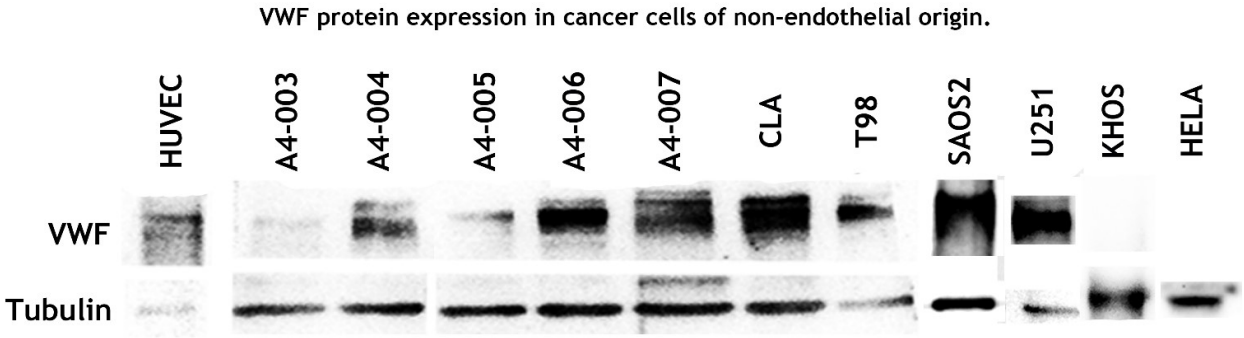

Figure 15. Some cancer cells of non-endothelial origin express VWF.

Western blot analyses were performed to detect VWF protein. Analyses were performed for osteosarcoma cell lines SAOS2 and KHOS, glioma cell lines U251, CLA and T98, several patients derived glioblastoma cells (A4-003 to A4-007); as well as HELA as a negative control and HUVEC as a positive control. Tubulin expression was used as loading control.

Knockout and overexpression of VWF in cancer cells.

To test the functional relevance of VWF in these cancer cells, VWF was either knocked down (in osteosarcoma SAOS2 and glioma U251) or overexpressed (in osteosarcoma KHOS) for further experiments.

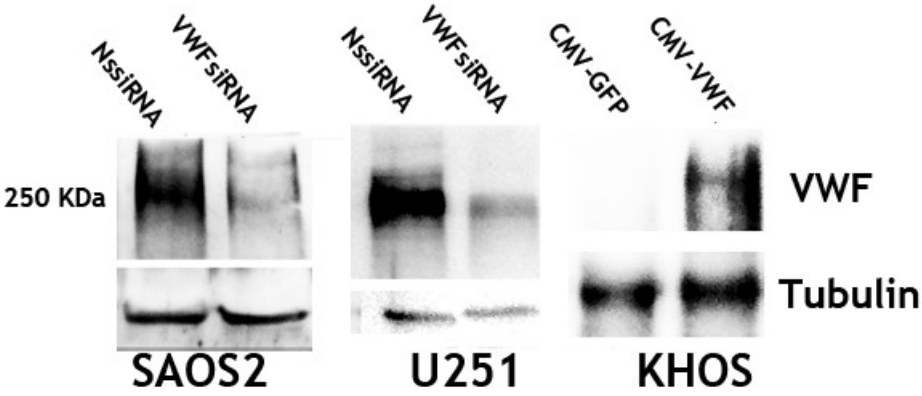

Figure 16. Western blot analyses of cancer cells with VWF overexpression and knockout treatment.

Western blot analysis demonstrating the knockdown of VWF in SAOS2 and U251 transfected with VWF siRNA compared to NS siRNA; and expression of VWF in KHOS transduced with lentiviral vectors for CMV-GFP and CMV-VWF.

As demonstrated by Western blot analyses in Figure 17, VWF expression was successfully knocked down with a specific VWF siRNA in comparison to the control NS siRNA; and on the other hand, VWF expression was effectively induced in the KHOS cell line after transduction with a VWF containing lentivirus vector but not in control transduction with a CMV lentivirus vector.

VWF expression in cancer cells increases its transmigration ability.

With the objective of determining if VWF expression increased the transmigration of the cancer cells across an endothelial barrier, a process fundamental to transmigration and consequently metastasis, we

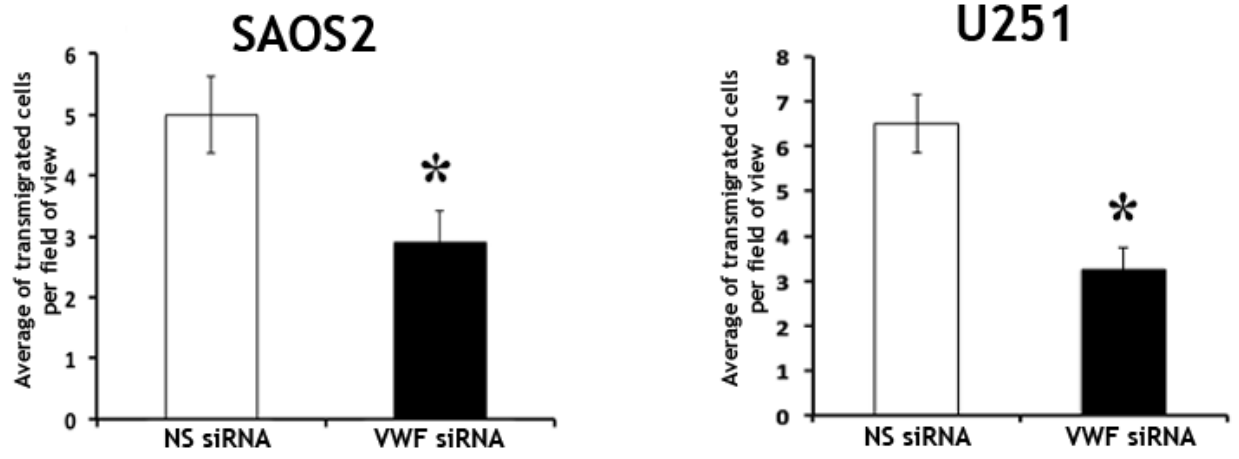

Figure 17. Cancer cells expressing VWF have an increased transmigration capacity.

Transwell assay analyses were performed to determine transmigration capacity of SAOS2 and U251 cells that were treated with NS siRNA or VWF siRNA. Cancer cells were incubated a HUVEC monolayer in a Transwell as described in materials and methods. Cells that transmigrated through the endothelial cell monolayer were quantified. Both results are quantification of 3 independent experiments (triplicate each).

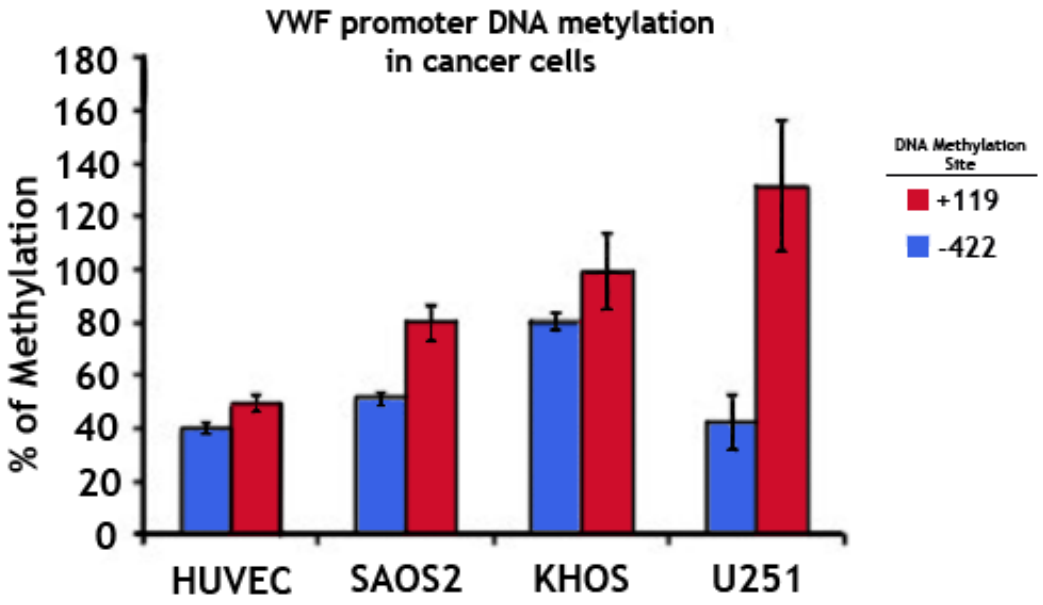

Figure 18. VWF promoter DNA methylation in cancer cells.

DNA retrieved from cancer cells (SAOS2, KHOS and U251) and HUVEC (as a positive control) was digested with HpaII restriction enzyme, as described in materials and methods. RT-PCR analyses were performed using VWF specific primers to determine the methylation status of the positions -422 and +119. DNA methylation analyses results are averages of 5 independent experiments for each cell type.

carried out a Transwell migration assay as described in materials and methods. For these experiments, GFP-expressing SAOS2, and U251 were transfected with both non-specific (NS siRNA) and VWF-specific siRNA (VWF siRNA) as explained in materials and methods. Control and transfected cells were seeded and incubated on a monolayer of endothelial cells; immunofluorescent staining and confocal microscopy were performed to detect and quantify the cancer cells that had transmigrated through the endothelial monolayer. As shown in Figure 18, the assay demonstrated that VWF expressing cancer cells transmigrated in a significantly higher rate than the VWF-knockout cancer cells. These results strongly suggested that VWF expression might confer characteristics to cancer cells that are consistent with increased metastatic potential.

Epigenetic changes of the VWF promoter in the cancer cell lines.

To gain insights into the mechanism of VWF transcription activation in the cancer cells we looked at its epigenetic modifications, specifically the DNA methylation. We proceeded to determine the methylation status of SAOS2, KHOS and U251 cells on the -422 and +119 specific CpG elements on the VWF promoter, elements that are generally non-methylated/low level methylated in endothelial cells. The result, shown in Figure 19, demonstrated that the three cancer cell types have an increased methylation on the -422 site compared to HUVEC. Nevertheless, in comparison both the VWF+ and the VWF- osteosarcoma cells didn't present any significant difference in the methylation of the -422 site; on the other hand, site +119 showed less methylation in SAOS2 compared to KHOS; moreover, U251 presented a similar methylation level to HUVEC in this site. These results suggest that increased methylation at -422 site may interfere with NF-IB repressor binding in all cancer cells, but specifically decreased methylation in +119 (potentially in combination with decreased NF-IB binding) in VWF+ expressing cancer cells is associated with VWF transcriptional activation.

CHAPTER 4: DISCUSSION

It has been well established that endothelial cells display a marked heterogeneity in structure, morphology, antigen composition and gene expression *in vivo;* however, when taken away from their microenvironment, endothelial cells lose most of their organ-specific characteristics as they suffer a phenotypic alteration [12, 18, 20].

VWF has shown to be an appropriate marker to demonstrate endothelial heterogeneity as its expression pattern varies with the size, location, and type of the vessel [27]. It was demonstrated that nucleotides from -487 to +247 within the VWF gene function as an endothelial-specific promoter *in vitro* and *in vivo* [61]. However, the *in vivo* VWF promoter (-487 to +247) activity is restricted to a subset of brain vascular endothelial cells in transgenic mice [61]. Several cis- and trans- acting factors have been identified, which function by either activating or repressing the VWF promoter activity and are involved in the VWF gene regulation. For instance, GATA, HLP, Ets and NFY (-18) act as activators, while NFI, Oct-1, E4BP4 and NFY (+226) function as repressors [1, 49, 61, 63-65, 69-73, 75, 97, 98]. Likewise, other

regions of the VWF gene have been characterized and have been shown to be necessary for the VWF to be expressed in endothelial cells of specific organs in addition to the brain. (Figure 5) [74, 75].

Transcriptional activity analyses performed by our research group, determined the importance of the repressors NFI and NFY in the organ specific regulation of the VWF promoter (+487 to -246) and showed that specific NFI isoforms participate in organ-specific repression of the VWF promoter [1]. Based on these observations we hypothesized that the pattern of expression of transcription factors that regulate the VWF promoter may contribute to the mechanism that governs its organ-specific regulation.

My primary research revolves around the role and distribution pattern of GATA family of transacting factors with regard to organ-specific regulation of the VWF promoter. We chose to start with GATA because previous studies have shown that the +220 GATA site plays a critical role in the endothelial-cell-specific expression of the VWF gene, and mutations of this site have resulted in significantly reduced activity of the VWF promoter [61]. It has been demonstrated that this transcription factor is involved in basal VWF expression; it has also been shown that the VWF promoter +220 GATA motif binds GATA-2, GATA-3 and GATA-6 members of this family of transacting factors [63]. Our results demonstrate that these transacting factors have a differential but overlapping pattern of expression in distinct organs. However, as endothelial cells are highly heterogeneous and because the GATA transacting factors are not endothelial specific, it is difficult to determine how each of them may be involved in VWF regulation. It is possible that an endothelial-organ-specific form of GATA is present (for example, GATA2, as our results show that this transcription factor is present in all the VWF positive endothelial cells of the five major organs), or even that there is a complex combination of various GATAs that would bind to the +220 motif differentially in distinct organs and activate the VWF expression.

On the other hand, our hypoxia experiment results suggested that GATA binding cis-acting element also participate in the upregulation of the VWF promoter, specifically in cardiac endothelial cells under hypoxic conditions. Our studies demonstrated that GATA6 participates in the hypoxia-upregulation of the VWF promoter activity in cardiac microvascular endothelial cells. It is possible that different GATA family members are involved in the upregulation of the VWF promoter depending on the external stimuli to which the endothelial cells are exposed. In this case, GATA6 seems to oversee the upregulation in cardiac

endothelial cells, however, it would be important to look at the rest of the GATA transcription factor to determine if they are also involved to some extent.

Nevertheless, GATA is not the only responsible factor for this upregulation, distinct transacting factors act in coordination to specifically upregulate the VWF promoter in response to hypoxia in cardiac MVECS. We observed that HIF-1α also participates in the VWF upregulation, in coordination with a reduction in NFIB binding to the promoter after hypoxia induction. In contrast, even though hypoxia also reduces NFIB binding to the VWF promoter in lung endothelial cells, our experiments showed that GATA6 and HIF are not responsible for the upregulation of the VWF promoter in lung endothelial cells but that SP1 and YY1 contribute to this process. These results were confirmed by the specific knocking down of the target transacting factors involved and analyzing their role in VWF regulation.

In addition to these observations, our research group demonstrated the role of epigenetic modification in VWF promoter activation. DNA methylation has been found to have an important role in VWF gene regulation [99]. We investigated the plausible involvement of two sites of methylation (-422 and +119) by examination of their methylation status under hypoxic conditions. We observed that even though these two CpG elements of the VWF promoter in lung MVECs don't show any significant difference in their methylation status, a significant increase in methylation at the -422 CpG site of the VWF promoter in cardiac MVECs was observed in response to hypoxia. Due to close proximity of -422 site to the binding site for NFI, it may be possible that methylation of the site diminishes the NFI binding to the VWF promoter as it was shown in our previous ChIP analyses.

In addition, it has been previously shown by our group that there is a significant change in the distribution pattern of VWF in the lungs of hypoxic mice compared to normoxic controls. Lung microvascular endothelial cells that in normal conditions do not exhibit VWF expression, suddenly showed VWF expression after exposure to hypoxia [86]. Similarly, when we analyzed the tissues of aged mice, we observed that in certain organs, including lung and liver, the distribution pattern of VWF was altered in comparison to young mice, as this endothelial specific protein expression was not only found in large vessels, but also in the microvessels of these organs. Correspondingly, western blot analyses of these organs showed an upregulation of the VWF expression with aging.

We hypothesize that these upregulations and alterations in expression patterns occur by differential expression of the transcription factors involved in the VWF gene regulation due to external stimuli, like hypoxia and aging-related influences. Since many of transcription factors that are involved in the VWF promoter regulation do not demonstrate endothelial exclusive expression pattern, it is not yet clear how they contribute to endothelial specific regulation of VWF gene in vitro or *in vivo*. For this reason, one of the major challenges that I encountered in my research, was to develop a protocol that will allow us to gain insight into the mechanism by which VWF expression is regulated in endothelial cells of blood vessels of distinct organs. As a first step towards this goal, I established a technique using laser capture microdissection to selectively isolate pure population of endothelial cells from distinct vascular beds for gene expression analyses, without exposing them to culture conditions that could lead to loss of organ-specific characteristics of the target endothelial cells.

LCM is a cutting-edge technology used to target, capture and isolate specific populations of cells from a heterogeneous tissue to perform a varied assortment of downstream analyses. The basic principle of this technology is to use UV laser to dissect target cells from a tissue mounted in a membrane slide and collect them for further processing [100]. With this technology, we were able to specifically detect and isolate VWF positive endothelial cells of distinct organs for analyses of its endothelial-specific gene expression pattern. Our results demonstrate, that after tissue processing as described in methods, we were able to detect and dissect VWF positive vessels and by RT-PCR we quantified the mRNA levels of VWF that showed an expression level that was comparable to those of cultured endothelial cells; further analyses are needed to specifically detect the expression and regulation pattern of the transacting factors that are or might be involved in the regulation of the VWF promoter.

Moreover, another useful tool to determine the mechanism by which the VWF transcription is regulated is to study how non-endothelial cells, specifically cancer cells that express basal levels of VWF, acquire their VWF expression. Understanding the role of the VWF regulatory elements in the establishment of the VWF gene activation will provide insight towards determining the mechanisms that govern the VWF transcriptional regulation.

My contribution to these mechanistic and functional studies included determining the presence of VWF in some cancer cell lines and patient-derived samples; developing knockout (SAOS2 and U251) and overexpressing (KHOS) cancer cell lines to assess VWF functionality. For example, with regard to transmigration we showed that the ratio of cancer cells that migrate across the endothelial barrier increases in VWF-positive cancer cells. Finally, to gain some understanding into the mechanism of VWF transcription, we determined the VWF promoter DNA methylation status in these cancer cells. Our results showed that osteosarcoma SAOS2 (VWF positive) have a decreased methylation of both -422 and +119 sites in the VWF promoter, compared to the KHOS (VWF negative); additionally, U251 (VFW positive) showed a significantly high levels of methylation in the site +119, while the +422 site showed similar methylation levels as HUVEC. If we interpolate these results, we could hypothesize that the increased methylation of site +119 in the VWF positive cancer cells may be involved in the recruitment of the GATA transacting factors (that has its binding site at the +220 position); this may influence the VWF promoter activation in this cancer cells. These results collectively point to an important role for GATA transacting factors in regulating VWF transcriptional activity and suggest that further investigation into the differential distribution pattern of GATA transacting factor in the vascular tree may provide insight into the potential role of this family of transacting factors in organ-specific as well as endothelial specific regulation of the VWF promoter.

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