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The mechanism of endothelial cell specific gene expression of Von Willebrand Factor in vivo

by

Marjan Nassiri

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Examining Committee

Supervisor:

Dr. Nadia Jahroudi, Department of Medicine, Division of Nephrology

Committee Members:

Dr. Manijeh Pasdar, Department of Cell Biology

Dr. Allan Murray, Department of Medicine, Division of Nephrology and Transplant Immunology

Dr. Thomas Mueller, Department of Medicine, Division of Nephrology and Transplant Immunology

Abstract

In vivo analyses of the Von Willebrand Factor (VWF) promoter previously demonstrated that a fragment spanning sequences -487 to +247 targets promoter activation to brain vascular endothelial cells. This fragment is active in all embryonic vessels of transgenic mice but in adult mice its activity is restricted to brain vascular endothelial cells, while endogenous VWF gene is expressed in vasculature of all major organs.

In this study we demonstrate that a DNase I hypersensitive (HSS) sequences in intron 51 of the VWF gene contain cis-acting elements that are necessary for the VWF gene transcription in a subset of lung endothelial cells *in vivo*.

Our results demonstrated that Nuclear Factor 1 (NF1) and Nuclear transcription Factor Y (NFY) repressors contribute to VWF organ-specific regulation. Mutation of the NF1 binding site resulted in promoter activation in lung and heart, while mutation of the repressor corresponding to a novel binding site for NFY resulted in promoter activation in kidney vasculature.

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Glossary

ACE	Angiotensin Converting Enzyme
ALCAM	Activated Leukocyte Cell adhesion Molecule
Alk 1	Activin-receptor-like kinase 1
AP-1	Activator Protein 1
AT III	Anti-Thrombin III
BBB	Blood-Brain Barrier (BBB)
COUP-TFII	COUP Transcription Factor 2
CX43	Connexin 43
Depp	Decidual protein induced by progestrone
DL4	Delta-Like 4
ELF-1	ephrin-A2 (EFNA2)
eNOS	Endothelial Nitric Oxide Synthase
EPAS 1	Endothelial PAS domain protein 1
EphB	Ephrine type-B receptor
ERG-1	CUB and zona pellucida-like domains 1 (CUZD1)
ET-1	Endothelin-1
ETS	E-twenty six (transcription factor family)
Flk-1	Fetal Liver Kinase-1
FLT1	fms-related tyrosine kinase 1
GATA	A family of transcription factors known as GATA 1 to 6
HAT	Histone Acetyltransferase
HDAC	Histone Deactylase
HEY 1	Hairy/Enhancer-of-split related with YRPW motif 1
HEY 2	Hairy/Enhancer-of-split related with YRPW motif 2
HLP	Histone H1-Like Protein
HMT	Histone Methyltransferases
ICAM-1	Inter-Cellular Adhesion Module-1
ICAM-2	Intercellular Cell Adhision Molecule-2
IL	Interleukin
KLF2	Kruppel-like transcription Factor 2
MCP-1	Monocyte Chemoattraction Protein-1
MHC II	Major Histocompatibility Complex II
NERF-2	E74-like factor 2 (ELF2)
NF1	Nuclear Factor 1
NFY	Nuclear Transcription Factor Y
NO	Nitric Oxyde
NRP1	Neurophilin 1

NRP2	Neurophilin 2
PAF	Platelet activating factor
PAI 1&2	Plasminogen activator inhibitor 1 & 2
PCAF	p300/CBP-associated factor
PCR	Polymerase Chain Reaction
PECAM-1	Platelet Endothelial Cell Adhesion Module-1
PGI2	Prostaglandin I2
ROBO4	Roundabout homologue 4
SP1	Sp1 transcription factor
TFPI	Tissue Factor Pathway Inhibitor
Tie 1	Tyrosine kinase with immunoglubolin and EGF-like domains 1
Tie 2	Tyrosine kinase with immunoglubolin and EGF-like domains 2
tPA	tissue Plasminogen Activator
TX A2/F2a	Thrombaxane A2/F2a
TXA2	Thrombaxane A2
VCAM	Vascular Cell Adhision Molecule
VE-cadherin	Vascular Endothelial - Calcium dependent adhesion molecules
VEGF	Vascular Endothelial Growth Factor
Vezf-1	Vascular endothelial zinc finger-1
VWF	Von Willebrand Factor

Chapter 1: Introduction

Endothelial cells

Endothelial cells cover the entire luminal surface of blood vessels and lymphatics. This cell layer is called endothelium. Although the shape of endothelial cells varies in different vessels, in general they are very flat; their thickness is from less than 0.1μm in capillaries and veins to 1μm in aorta. They are 10-20μm in diameter. The alignment of the nuclei of endothelial cells are as the direction of the blood flow (1;2).

The endothelium not only provide a barrier between the blood component and the tissues but also play crucial roles in many physiological and pathophysiological processes including angiogenesis, maintaining vessel toning and metastasis (3;4).

Endothelial cells selectively filter gases, fluids and various molecules across their cell membrane (5). They also participate in haemostasis function. They normally provide an antithrombotic surface for blood-tissue interaction by production of anticoagulants such as Prostaglandin I2 (PGI2), Thrombomudulin, Antithrombin III (AT III) and tissue Plasminogen Activator (tPA) (6). However in response to injury, they produce procoagulation factors such as Von Willebrand Factor (VWF), Thromboxan A2 (TxA2), Thromboplastin, Factor V, Platelet Activating Factor (PAF), and Plasminogen Activator Inhibitor 1&2 (PAI 1&2) (6).

Endothelium maintains vessels stability and integrity by production of matrix-associated proteins including fibronectin, Laminin, Collagen I-II-III-IV-VIII-XVIII and proteoglycan. These mediate the junction between endothelial cells and the subendothelium (6).

Endothelial cells play key roles in immune and inflammatory reactions by producing inflammatory mediators like Interleukin 1, 6, 8 (IL 1, 6, 8), Leukotriene B4-C4-D4-E4, Monocyte Chemoattractant Protein-1 (MCP-1) and Major Histocompatibility Complex II (MHC II) (6).They also regulate the vascular tone and therefore maintain the blood pressure and blood flow. This regulation is achieved by altering the production of vasodilator including Nitric Oxide (NO) and Prostacyclin and vasoconstrictor such as Endothelin, Thromboxane A2/F2a (TXA2/F2a), Angiotension Converting Enzyme (ACE), leukotrienes and free radicals (6).

Endothelial cells participate in the processes of angiogenesis and vasculogenesis with stimulation by growth factors such as Vascular Endothelial Growth Factor (VEGF). They can also express several factors such as insulin like growth factor, transforming growth factor and colony stimulating factor that are necessary for formation of new vessels (6).

Endothelial cell heterogeneity

Endothelial cells and hematopoetic cells arise from a precursor cell which is hemangioblast. Splanchopleuricmesoderm drives mesenchymal cells into hemangioblasts, which are the precursor of intermediate pre-endothelial cells. The pre-endothelial cells can differentiate into either hematopoetic or endothelial cells (7;8).

Although all endothelial cells have a common origin from the mesoderm, endothelial cells of different organs and vessel types have distinct phenotypes, functions and pattern of gene expression. This diversity is consistent with the function of endothelial cells, which must provide the needs of the underlying tissues. As an example, endothelial cells in kidney are fenestrated to facilitate the filtering function of the kidney (2;9) while in brain vessels, they have tight junctions that generate the blood-brain barrier (BBB) to protect the brain from fluctuations in blood components (2). In addition, endothelial cells need to survive in different environments since the blood chemistry varies in different organs. As an example, endothelial cells in lung are exposed to high amount of oxygen, whereas in kidney they are in hypoxic environment. Therefore phenotypic and functional variations of endothelial cells make them able to cope with and respond to these variations in their surroundings (2;10).

Endothelial cell diversity in arteries and veins

The diversity in endothelial cells between arterial and venous vessels is observed from the very first stages of development. During the vascular system development, the primary capillary plexus derived from angioblasts differentiates into arteries, veins and capillaries through a process that is affected by genetic as well as epigenetic factors such as blood flow and oxygen level (11).

Genes that are preferentially expressed in arteries include ephrinB2, Delta-like 4 (Dll4), Neurophilin 1 (NRP1), Hairy/enhancer -of- split related with YRPW 1 and 2 (Hey1 and Hey2), Activin-receptor-like kinase 1 (Alk1), decidual protein induced by progesterone (Depp) and endothelial PAS domain protein 1 (EPAS1). Some venous endothelial cells

express genes including Ephrin type-B receptor 4 (EphB4), neurophilin 2 (NRP2) and COUP transcription factor 2 (COUP-TFII) (11).

The details of molecular mechanisms that differentiate arterial and venous endothelial cells are still unclear; however some studies have revealed that a complex signaling pathway participate in this diversity (11). The ephrins/ Ephs system plays an important role in arterial endothelial cell differentiation, angiogenesis and cell migration during embryogenesis (12). Several members of the ephrins/ Ephs family such as EphB2, EphB3, EphB4 participate in embryonic vascular development (11). Through several mutation analyses, it was shown that the expression of ephrinB2 and EphB4 are necessary for the distinction between arterial and venous endothelial cells respectively (11).

The Notch signaling pathway acts upstream of the ephrinB2 system and it is involved in establishment of arterial endothelial cell identity. Diminishing the Notch activity results in disappearance of arterial endothelial cell markers like ephrinB2 and activation of venous markers like EphB4 (13).

VEGF, which is a proangiogenic growth factor, participates in the activation of the Notch signaling pathway. Along its role in angiogenic process, it is also a crucial factor in differentiation of endothelial cells. The identified receptors for VEGF include Fetal Liver Kinase1 (Flk-1), FMS- like tyrosine kinase 1 (FLT1) and neurophilin-1 (Nrp-1) (11;14). The knock out of Nrp1 results in defects in differentiation of arterial endothelial cells in a way that also impairs the smooth muscle formation in arteries. This indicates that the arterial specific phenotype of endothelial cells is necessary for development of smooth muscle cells in arteries during angiogenesis (11).

Genetic factors are important in endothelial cell differentiation. COUP-TFII, a member of orphan nuclear receptor family, plays a crucial role in venous endothelial cell differentiation. COUP-TFII is exclusively expressed in venous endothelial cells (15). It acts upstream of EphB4 and in mouse embryos of COUP-TFII knock out, the arterial markers such as ephrinB2 and Nrp1 are expressed in veins (11).

Epigenetic factors also have an important role in determining the endothelial cell identity. The differentiation of endothelial cells is also affected by its environment. Endothelial cells that are removed from arteries and explanted to veins or vice versa can express arteries or venous markers similar to the host vessels (11). One of the important factors in transformation of these cells is the direction of the blood flow which can up regulate venous markers and down regulate arterial markers in a reversible way (16).

The oxygen level can also determine artery and vein endothelial cell differentiation. It is shown that hypoxia stops the expression of the artery specific markers such as Dll4 and ephrin2 (17).

Organ-specific heterogeneity of endothelial cells

The diversity among endothelial cells not only exists in the endothelial cells of different types of vessels, but also observed in similar vessels of different organs. The endothelial cells of each vascular bed have their unique structure (18). This diversity could be used in targeted therapy for patients with organ specific vascular diseases (19).

In heart, the endocardial endothelial cells are large and have many microvilli. In comparison with myocardial endothelial cells, endocardial endothelial cells have deeper

intracellular clefts, more gap junctions and tight junctions (20). Connexin 43 (CX43), 40 (CX40), and 37 (CX37) are expressed in endocardial endothelial cells which are not seen in myocardial endothelial cells (21). VWF and Endothelial Nitric Oxide Synthase (eNOS) gene expressions are higher in endocardium endothelial cells in comparison with myocardial capillary endothelial cells (22;23). Receptor-like tyrosine phosphatase mu is expressed in the myocardial endothelial cells of capillaries and arteries but not in vein endothelial cells (24).

Heterogeneity in gene expression is seen in lung endothelial cells. As an example, Angiotensin-I Converting Enzyme (ACE) expression is mainly seen in alveolar capillary endothelial cells. The systemic capillary endothelial cells are only responsible for 10% of expression of this protein (25). In smaller pulmonary vessel endothelial cells, activated leukocyte cell adhesion molecule (ALCAM) is expressed (26). Alveolar capillary endothelial cells do not express VWF while endothelial cells of the larger vessels of lung express this molecule (27). The endothelial cells of bronchial vessels are more permeable and more reactive to inflammation and have more angiogenic capabilities compared to endothelial cells of pulmonary capillaries (28;29). This capacity is seen more in bronchial microvascular endothelial cells than bronchial artery endothelial cells (30). The bronchial capillaries express higher level of E-selectin, which puts them in increased state of activation in response to inhaled antigens (31).

In liver, the hepatic sinusoidal endothelial cells, which represent the capillary network of the liver, are fenestrated. Sinusoidal endothelial cells have gaps and less organized basement membrane which promote these cells to efficiently filter and transfer lipoproteins and small chylomicron between blood and hepatocytes (32). Sinusoidal endothelial cells stimulate hepatocytes regeneration by producing hepatocyte growth

factor (33). Liver is also an important site for leukocyte migration in the body via sinusoidal endothelial cells. Liver sinusoidal endothelial cells express high levels of Inter-Cellular Adhesion Molecule 1 (ICAM-1). Knock out of ICAM-1 results in low leukocyte adhesion to the endothelium of sinusoids (34). Sinusoidal endothelial cells also play a role in regulation of vasomotor toning by expressing the endothelin-1 (ET-1) receptor ETB and Nitric Oxide (NO) release (35;36).

In kidney, the glomerular endothelial cells have a fenestrated pattern that is 60 to 80 nm in diameter. They approximately cover 20% of the endothelial surface (37). VWF gene expression is confined to some human glomerular endothelial cells (27). eNOS is expressed at higher levels in endothelial cells of the renal medulla (vasa recta) than glomeruli and peritubular capillaries of the cortex (19). The gap junction protein expression is different among the renal vasculature. For instance connexin 37 and 40 which are gap junction proteins, are expressed in afferent arterioles, whereas connexin 43 is expressed in afferent and efferent arterioles (38). Claudin-10 and Claudin-15 are expressed only in vasa recta endothelial cells (39). The urea transporter UT-B1 is only expressed in endothelial cells of descending vasa recta (40).

Endothelium in diseases

The diversity among endothelial cells is also reflected in endothelial cells in different diseases. As an example, in atrial fibrillation, the level of eNOS in heart endothelium is decreased (23). Thrombomodulin and tissue factor pathway inhibitor (TFPI) expression in the endocardium of the atrium is downregulated in rapid atrial pacing in rats (41), which can be a reason for thrombosis in atrial fibrillation. In aortic stenosis,

neovascularization of the endothelial cells of the valve occurs. The endothelium of these neovesseles express Platelet Endothelial Cell Adhesion Molecule-1 (PECAM-1) (42). In obesity, diabetes and hypertension, abnormalities in vessels such as arteriolar narrowing and/or dysfunction is observed which is almost always associated with changes in the endothelial cells gene expression. As an example, diabetes and hypertension in rats are associated with downregulation of NO synthase in myocardial capillaries (43).

In portal hypertension there is underproduction of NO in liver sinusoids (44). In cirrhosis the expression of PECAM-1/CD3 and cyclooxygenase-2 is upregulated in sinusoidal endothelial cells (45;46).

The main feature in hemolytic uremic syndrome is damage of endothelium, which is typically caused by Shiga-like toxin, drugs or bacterial infection. The Shiga-toxin receptors are expressed in endothelial cells of peritubular capillaries (47).

In type 1 diabetes, VEGF, calcineurin-A-α, fractalkine and adrenomedullin expressions specifically in glomerular endothelial cells are increased (48-51).

Endothelial cell gene expression

Endothelial cells are multifunctional. To accomplish their diverse roles, they need to have a highly regulated gene expression pattern (52).

There are several genes that are preferentially or exclusively expressed in endothelial cells. They include VWF, vascular endothelial (VE)- Cadherin, eNOS, Tyrosine kinase with immunoglobulin and EGF-like domains 1 and 2 (Tie1 and Tie 2), interacellular cell

adhesion molecule-2 (ICAM-2), Flt-1/VEGF-R1 and Flk-1/VEGF-R2 (52). Expression of other endothelial-specific genes like vascular cell adhesion molecule 1 (VCAM-1) and E-selectin is induced in response to external stimuli such as inflammation (52).

Mechanisms of transcriptional regulation of these endothelial specific genes are mainly studied through identification and analysis of the promoter and other regulatory elements of the target genes.

In general the process that regulates gene expression is a complex system that includes activation of transcription machinery, interaction of general and cell-type specific transacting factors with specific DNA cis-acting elements which are identified in the 3′, distant 5′, introns as well as proximal promoter regions.

Along with the trans-acting factors and cis-acting elements, the structure of the chromatin is also very important in regulating the activation of gene expression (52). Chromatin structure can be changed by DNA methylation of the cytosine residues in CpG sites. This can prevent the interaction of the trans-acting factors with the chromatin (53). It can also be changed through modifications of histones. Such modifications include acetylation, methylation, phosphorylation, ubiquitylation, ADP-ribosylation and sumoylation of the unstructured N terminus of histones' tails (52).

Trans-acting factors play a central role in activation of gene expression by modifying chromatin structure in addition to their function as either activator or repressor of transcription. They participate in establishment of transcriptionally active chromatin structure. They can recruit co-activators with chromatin-remodeling activity to the gene. The interaction of trans-acting factors with the chromatin is not only determined by the chromatin structure, but also depends on the factors that can remodel the chromatin

structure such as DNA methytransferases, histone acetyltransferases (HATs), histone deactylases (HDACs), histone methyltransferases (HMTs) and nucleosomal remodeling proteins (52).

Identification of endothelial specific promoter

To determine the process that regulates endothelial specific gene expression, the promoters of several endothelial-specific genes have been identified both *in vitro* and *in vivo*. These include promoters for ICAM-2, VE-Cadherin, Tie-2, Flk-1/VEGF-R2, eNOS, Flk-1and VWF which will be discussed independently in later sections.

Analyses of some of these promoters *in vivo* have demonstrated organ specific activation pattern as well as endothelial specific characteristics.

Analyses of eNOS gene promoter demonstrated that a 1.6 kbp (-1600 to +22) of the 5′ flanking region functioned as endothelial-specific promoter. However this region was shown to activate gene expression in endothelial cells of brain, heart and skeletal muscle; while a larger fragment from -5200 to +28 activated the gene expression in lung, kidney, liver and spleen endothelial cells in addition to those observed with 1.6 kbp fragment (54).

Analyses of the regulatory region of Tie-2 gene in transgenic mice demonstrated that the 5′ sequences contain necessary elements for its endothelial cell activity in mice embryonic vasculature while sequences in the first intron (located 10 kbp downstream of transcription initiation site) are required for expression in endothelial cells of all vasculature in adult mice (54).

Analyses of the 3.5 kbp fragment of the human VE-Cadherin gene showed that while these sequences functioned as an endothelial specific promoter and allowed temporal expression in embryonic vessels; in adult, promoter activation was mainly detected in endothelial cells of lung, heart, ovary, spleen and kidney glomeruli. Expression in vasculature of other organs including brain, liver and skeletal muscle was insignificant (55).

In contrast analyses of ICAM-2 and Roundabout homolog 4 (Robo-4) promoters have shown that the 0.33 kb (-202 to + 44) fragment of ICAM-2 and a 3 kbp fragment of Robo-4 drive expression in endothelial cells of all organs (56).

A number of novel transcriptional regulators that are mainly expressed in endothelial cells are reported; however, target endothelial specific genes that are regulated by these factors are either limited or not known. For instance, a Kruppel-like transcription factor (KLF2), is in most part expressed in endothelium of blood vessels (57). It is hypothesized that KLF2 participates in regulation of the expression of genes which are involved in anti-thrombotic and anti-inflammatory function of endothelial cells (52). KLF2 expression is upregulated by blood flow and down regulated by pro-inflammatory cytokines (57). Over-expression of KLF2 up-regulates eNOS and thrombomodulin gene expression and suppresses the expression of VCAM-1 and E-Selectin in the presence of pro-inflammatory cytokines (58).

Vascular endothelial zinc finger-1 (Vezf-1) also is a transcription factor that its expression is limited to endothelial cells. It is shown to be expressed concomitantly with Flk-1/VEGF-R2 when angioblasts differenciate into endothelial cells. Deficit in Vezf1 results in defect in the development of vessels, endothelial cell integrity and formation (59).

DNA analyses of promoter regions of endothelial specific genes have failed to reveal the presence of common cis-acting element that may lead to characterization of a master regulator. Nevertheless, there are some cis-acting elements that are present in multiple endothelial cells-specific gene promoters. They include binding sites for E- twenty six (Ets) family members, globin transcription factor 1 (GATA), SP1, activator protein 1 (AP1) and Octamer transcription factor. These transcription factors although not endothelial specific, are necessary for expression of many endothelial specific genes (52).

GGAA/T cis-acting element is the consensus binding site for the Ets family of transacting factors. They are identified in the promoters of some endothelial specific genes (60). Ets factors such as Ets-1, Ets-2, Friend leukemia virus integration 1 (Fli-1), Elf-1, Nerf-2 and erg-1 were shown to participate in the regulation of eNOs, Flt-1/VEGF-R1, Flk-1/VEGF-R2, ICAM-2, Tie1, Tie2, Utrophin-B, VWF and VE-Cadherin (52).

Cis-acting elements that bind to the family of GATA transcription factors participate in basal expression of endothelin-1, eNOS, Flk-1/VEGF-R2, ICAM-2, P-Selectin, PECAM-1, Utrophin-B and VWF (52).

GC-rich regions known as GC boxes are identified and shown to bind to SP1 and SP3, which are zinc-finger transcription factors (61). They participate in regulating the expression of eNOS, Flk-1/VEGF-R2, ICAM-2, and VE-Cadherin genes (52).

Activated Protein 1 (AP-1) site (consensus TGASTCA) is also important in the expression of endothelial specific genes including Endothelin-1, eNOS, Utrophin-B and Notch4 (52).

The arterial endothelial cell specific expression of Notch 4 is regulated by AP-1 (62). Treatment of non-endothelial cells with angiogenic factors was shown to activate AP1 and induce Notch 4 expression (62).

Another commonly expressed transcription factor that has binding sites (sequences ATGCAAAT) in several endothelial specific genes is Octamer transcription factor which is a member of POU protein family. POU is a family of proteins that contain homeodomains which are important in transcription factors that participate in developmental regulation (63).

Endothelial cell gene regulation *in vivo*

Under *in vivo* conditions, endothelial cells demonstrate heterogeneity in structure and function (64). Endothelial cell heterogeneity is also mediated by different local microenvironments. When endothelial cells are removed from their native environment, they undergo major phenotypic transformation (65). Therefore, the results from *in vitro* studies must be confirmed by *in vivo* analyses.

Many studies have explored the function of endothelial cell-specific promoters in transgenic mice. Various promoter fragments of a number of endothelial specific genes were fused to reporter molecules such as LacZ or Luciferase and the expression of the reporter gene was analyzed in various organs in transgenic mice. Most of these investigations have shown that analyzed promoter regions of endothelial specific genes carry information for expression in distinct subsets of endothelial cells or vascular beds

(55;66-71). Although it is established that distinct regions of these genes are necessary for targeting to specific endothelial subset, the mechanism and regulators of this differential expression is not yet known. To gain insight into the regulatory mechanisms that regulates endothelial-specific gene expression we have studied the mechanism of transcriptional regulation of VWF.

Von Willebrand Factor (VWF)

Von Willebrand Factor is a large adhesive glycoprotein, about 20,000 kDa that is exclusively expressed in endothelial cells and megacaryocytes both *in vivo* and *in vitro*. Therefore, the expression pattern of VWF can be used as a marker to identify endothelial cells from other cell types (72). The VWF gene is located in human chromosome 12 at 12p13.2. It has 52 exons and spans about 180 kb (73).

VWF is necessary for normal haemostases. It mediates the adhesion of platelets to damaged subendothelia and also acts as a carrier and stabilizer for blood clotting factor VIII therefore increasing its half life in the circulation (72).

Deregulation of production of VWF can lead to low or high levels of this protein.

Qualitative and quantitative deficiencies of VWF lead to Von Willebrand disease which is the most common inherited bleeding disorder. Patients who have Von Willebrand disease have severe bleeding disorder since they have defect both in platelet plug formation in the site of injury and in stability and levels of circulating factor VIII (74;75).

High levels of VWF can cause cardiovascular disease and morbidity and increase mortality in general population (76).

As mentioned earlier, VWF is only expressed in two highly specialized cell types, endothelial cells and megakaryocytes. Identification of the important regulatory elements that participate in VWF gene expression will help us to identify the factors that could participate in regulating endothelial-specific gene expression. In addition, determining the mechanism of transcriptional regulation of VWF could provide clues about the molecular nature of diseases that result from deregulated expression of VWF leading to too low or too high VWF levels.

Characterization of VWF promoter and its regulation in vitro

DNA sequences located at the 5′ regions of genes comprise promoters that generally regulate gene transcription. Thus identification and characterization of VWF promoter is fundamental to studying its mechanism of transcription regulation.

To identify the promoter of VWF gene, a number of deletion and mutation analyses were performed. These analyses resulted in identification of a region of the VWF gene spanning sequences -487 to +247 that functions as an endothelial-specific promoter in cell culture (74). A number of cis and trans-acting regulatory factors that positively and negatively regulate the activity of this promoter fragment have since been identified **(Figure 1)**.

Trans-acting factors GATA6, Nuclear Transcription Factor Y (NFY) (interacting with the consensus CCAAT sequence at position -18 to -14), a histone H1-like protein (HLP) and Ets function as positive regulators of the VWF promoter. Nuclear factor 1 (NF1), NFY

(binding to a novel sequence CCGNNNCCC at position +226 to +234) and Octamer transcription factor 1 (Oct1) function as negative regulators of the VWF promoter (74;77-82).

NF1 transacting factor interacts with negative regulatory cis-acting element that comprises nucleotide sequences -440 to -470 region of the VWF gene. The NFY interacts with two cis-acting elements; a CCAAT consensus sequence located at -18 to -14, and a novel binding site located at +226 to +234. NFY interacting with the consensus CCAAT element functions as an activator while when interacting with the novel binding site (+226 to +234) it functions as a repressor. The activating and repressive function of NFY is regulated by its interaction with histone modifying cofactors P300/CBP- associated factor (PCAF) and HDAC respectively (77;83;84).

Mutation analyses of NF1 and NFY repressor binding sites demonstrated that suppression of the promoter activity in non-endothelial cells did not change by independently mutating either element. For example mutation of NF1 or NFY had no major effect on the VWF promoter activity in cell culture; however mutation in both repressor sites simultaneously resulted in partial activation of gene expression in non-endothelial cells in culture (85).

Organ-restricted activity of von Willebrand factor promoter

Endogenous VWF gene expression starts early in vascular development but in a limited subset of endothelial cells (86). This variation in VWF level is also seen in the

vasculature of adult mammals (87). Understanding the molecular mechanisms which causes the diversity of the VWF level can help us understand the molecular mechanisms that program the endothelial cells diversity.

To identify the role of the VWF promoter *in vivo*, the human VWF promoter sequences -487 to +247 were fused to the Escherichia coli LacZ reporter gene and this construct was used to generate transgenic mice. X-Gal staining analysis of the frozen sections of the transgenic mice demonstrated expression of the VWF-LacZ transgene in almost all embryonic vessels of transgenic mice; however, in adult mice, LacZ expression was restricted to a subset of the brain vasculature (67). These results revealed that the sequences -487 to + 247 of the VWF gene although endothelial specific are not sufficient for the activation in endothelial cells of the vasculature of all organs. DNA sequences distal or proximal to the -487 to+247 regions most likely are required to direct the activation of the promoter in endothelial cells of other organs. These results provided us with an opportunity to investigate the molecular mechanism of the diversity among endothelial cells.

According to these observations, we proposed to determine: i) What are the other regions that are necessary for activation of the VWF gene in other organs; ii) Do the repressors play a role in organ specific activity of the promoter during development. In another word, what represses the activity of the promoter during development.

Chapter 2: Materials and Methods

Generation of transgenic mice

Transgenic mice and plasmids used for their derivation were generated prior to initiation of my project which was primarily focused on the analyses of the obtained transgenic mice.

Following approach was used to generate two plasmids that contained hypersensitive sequences of intron 51 located either at 5′ or 3′ relative to VWF promoter driving the expression of LacZ gene. These plasmids were referred to an HSS-VWF-LacZ (intron 51 sequences located at 5′) and VWF-lacZ-HSS (intron 51 sequences located at 3′).

For generation of HSS-VWF-LacZ transgene the fragment spanning nucleotides 231-613 of Eco RI fragment 38 of the VWF gene (88) was amplified by PCR using 3′-HGH-K plasmid as the template, and the following primers: 5′ CCGTCGACGGTACCGAGGGTTGAGCCTCCGTGTT-3′ (forward), and 5′GCCGTCGAC GGTCTGACATGTCCTCTTCTCTATGG3′ (reverse). The forward primer contains Sal I and Asp 718 enzyme restriction sites followed by intron 51 sequences, while the reverse primer contains the Sal I enzyme restriction site followed by intron 51 sequences. The PCR fragment generated by these primers were digested with Sal I enzyme and cloned upstream of the plasmid VWF LacZ (67) containing a single Sal I site upstream of the VWF promoter. Clones containing the intron 51 sequences in the sense orientation were selected and verified by sequence analysis. The resulting plasmids

contained intron 51 sequences 213-613 (wild type or mutant) immediately followed by VWF promoter sequences –487 to +247 and LacZ gene as shown in Figure 6A.

To generate VWF-LacZ-HSS plasmid the fragment 213-613 of intron 51 generated as described above for generation of plasmid HSS-VWF-LacZ, except that the forward primer contained Asp718 site followed by intron 51 sequences, while the reverse primer contained Asp718 followed by Sal I and intron 51 sequences. The resulting PCR fragment was digested with ASP718 and cloned into the Asp 718 site following the poly adenylation signal at 3′ end of the LacZ gene in the VWF-LacZ plasmid. Clones containing the intron 51 sequences in the sense orientation were selected and verified by sequence analysis. The resulting plasmid contained VWF promoter sequences -487 to +247 followed by LacZ gene and poly A signal and VWF intron 51 sequences 213-613.
Plasmid LacZK ^{NF1}, containing 3 bp substitution mutations in the NF1 binding site was generated by replacing the Hind III fragment of LacZK that spans sequences -487 to -90 sequences of the VWF, with the similar Hind III fragment from plasmid HGHK^{rm3} (39) which also spans VWF sequences -487 to -90, but contains the desired mutation in NF1 binding site.

Plasmids LacZK ^{NFY} and LacZK$^{NF1\text{-}NFY}$ were generated as follows: The VWF promoter fragment -90 to +247 was amplified using primers that corresponded to sequences -90 to -60 (with an additional 9 bp sequence containing the Hind III site); and +213 to +247 with base substitution mutations at +226 to +228 (CCG to GAA) as well as an additional 9 bp sequence containing the Pst I site. The plasmid LacZK was digested with Hind III and Pst I and sequences corresponding to VWF fragment -487 to + 247 were removed. The PCR generated VWF sequences -90 to +247 containing the mutation in NFY binding site were then ligated to the Hind III/Pst I digested LacZK fragment. This resulted in

generation of a plasmid containing VWF sequences -90 to +247 with mutation in NFY binding site. This plasmid was then linearized with Hind III, followed by insertion of Hind III fragments containing either wild type VWF sequences -487 to -90 (obtained from LacZK) to generate plasmid LacZK^{NFY}, or NF1 mutant VWF sequences -487 to -90 (obtained from LacZK^{NF1}) to generate plasmid LacZK$^{NF1\text{-}NFY}$. Sequences of all plasmids were confirmed.

DNA fragments containing HSS sequences, VWF-LacZ and poly A were isolated and purified from the plasmids HSS-VWF-LacZ and VWF-LacZ-HSS by digestion with ASP718 (for plasmid HSS-VWF-LacZ) or Sal I (for plasmid VWF-LacZ-HSS). Fragments were used for microinjection to generate C57BL/6 transgenic mice by Ozgene (Bentley, WA, Australia) and University of Alberta transgenic mice facility respectively. In addition DNA fragment containing VWF-LacZ transgene was used to generate transgenic mice containing VWF-LacZ gene in C57BL/6 mice by Ozgene (Bentley, WA, Australia). DNA fragments containing VWF-LacZ and poly A were isolated from plasmids LacZK, LacZK^{NF1}, LacZK^{NFY} and LacZK$^{NF1\text{-}NFY}$ were generated by Sal I digestions of these plasmids, purified and used for microinjection to generate C57BL/6 transgenic mice by Ozgene (Bentley, WA, Australia).

The founder transgenic mice were bred with wild type C57BL/6 to generate heterozygote transgenic progenies. Presence of the transgene was determined using specific primers to detect LacZ gene from mouse tail biopsies prepared by the staff of animal facility Health Science Laboratory Animal Services (HSLAS) at University of Alberta. All animal housing and experimentation were approved by the Health Sciences Animal Policy and Welfare Committee at the University of Alberta. Two-three independent lines of heterozygote transgenic mice were analyzed for LacZ expression

Organ preparation

1) Mice were placed in dorsal position on the narcolepsy table.
2) The skin along the midline from the mandible to the pubic symphysis was cut.
3) Skin was flipped laterally on each side of the incision to expose the underlying tissues and organs.
4) The ribs were cut near their cartilage and flipped to each side of the thoracic wall laterally to expose the thoracic organs.
5) Six organs including brain, heart, lung, liver, intestine and kidney of euthanized mice were removed and trimmed to the appropriate size. Samples from each organ were frozen in liquid Nitrogen for RNA analyses and were processed for immunohistochemistry and immunofluorescence confocal microscopy as described below.

RNA preparation and real-time polymerase chain reaction (PCR) analyses

1) RNA was prepared from frozen organs (heart, lung, liver kidney and brain) using Qiagen RNeasy Mini Kit Cat. # 74104 as recommended by manufacturer.
2) 1 microgram of RNA was used to generate cDNA using Invitrogen SuperScript® Reverse Transcriptase (Carlsbad, CA, Cat. # 12371-019) as recommended by manufacturer.
3) The cDNA was diluted (1:50) and 10 micro liters was used as template for Real-time PCR analyses using power SYBR PCR Master Mix (Cat. # 4367659 Biosystems, Foster Green CA). The PCR reactions were carried out in a total volume of 25

microlitre using 0.25 microlitre of each primer (stock concentration 10 picomol/microlitre). Cycling conditions used were as follows:

- 50^{o}C 2 minutes
- 95^{o}C 10 minutes
- 95^{o}C 15 seconds
- 60^{o}C 1 minute

4) Number of cycles ran were 40 and dissociation curve were run on all samples.
5) Tie 2, VWF and LacZ were normalized to GAPDH by generating an arbitrary standard curve generated from cycle threshold (Ct) values of GAPDH

Preparation of tissues for immunohistochemical analyses

Tissues were formalin fixed and paraffin embedded as follows (89;90):

1) Each specimen was put in a cassette with its paper label specimen number which was written with 4B pencil.
2) Cassettes were placed in 10% formalin for temporary rinse.
3) Formalin was discarded and cassettes were drained well.
4) Cassettes were processed in 70% Ethanol for 90 minutes on shaker (sudden transfer of tissues in higher concentration of ethanol may cause distortion of tissues).
5) Then cassettes were sequentially processed in 85% Ethanol for 90 minutes, 95% Ethanol for 90 minutes, 70% Butanol for 24 hours and 85% Butanol for 24 hours. All incubations were carried out on shaker.
6) Cassettes were placed either in absolute Butanol for long term storage or continued for paraffin wax impregnation.
7) Cassettes were immersed in paraffin wax under vacuum, in a heated chamber at 52 degree Celsius for five hours.

8) Tissues were removed from the cassettes and placed in tissue molds of the appropriate size, which was then filled with molten wax from the wax dispenser.
9) Tissue molds were placed on the refrigerated cold plate to harden the wax.
10) Tissue blocks were cut in 5 micron in thickness and transferred onto microscope slides (Fisherbrand Colorfrost/Plus Catalogue No: 12-550-17), air dried in room temperature for one hour and placed in 37 degree oven overnight .

Immunohistochemistry staining

1) Sections were de-paraffinized and rehydrated as follows:
    a. 30 minutes in 58 degree oven.
    b. 2x5 minutes in Xylene (Fisher Scientific Catalogue No. 180860010)
    c. 2x5 minutes in 100% Ethanol
    d. 1x5 minutes in deionized water
2) Slides were placed in 1x wash buffer (Dakocytomation Catalogue No: S3006) for 30 minutes.
3) Slides were transferred into humidifier chamber.
4) Slides were removed from the wash buffer. Around the specimens were wiped by Kimwipe to remove any remaining liquid.
5) Sections were bordered with PAP Pen.
6) Slides were covered with 3% Peroxidase block (Fisher Scientific Catalogue No. H324-500) for 10 minutes.
7) Slides were rinsed gently with 1x wash buffer.
8) Slides were incubated for 10 minutes in Protein Block (Dakocytomation Catalogue No. X0909).
9) Slides were shaken in the air to remove the Protein block without rinsing.

10) Primary Antibody (monoclonal to beta-galactosidase from abcam Catalogue No. ab 116-100) was diluted 1:500 with antibody diluent (DakoCytomation Catalogue No. S0809).

11) Negative Control mouse IgG1 (DakoCytomation Catalogue No. X0931) was diluted in antibody diluent (DakoCytomation Catalogue No. S0809).

12) Each specimen together with a positive control and a negative control were incubated in 200µL diluted primary and an isotype control antibody for 2 hours.

13) Slides were washed extensively by 1x wash buffer.

14) Envision+ R system/ Labelled polymer-HRP Anti mouse (DakoCytomation Catalogue No. K4000) was applied to cover the entire specimen for 30±1 minutes.

15) Slides were rinsed extensively with 1x wash buffer.

16) Slides were gently shaken in the air to remove the layer of wash buffer.

17) SIGMA FAST DAB (3,3′-Diaminobenzidine) tablet (Catalogue No.D-4293) was dissolved in 5 ml Dionized water by vortexing .

18) Slides were covered with 300µL of the solution.

19) Slides were carefully monitored and washed extensively with 1x wash buffer as soon as the color of the positive control slide turned brown (this step took about 30 seconds).

20) Slides were placed in tap water for 1 hour.

21) Slides were incubated in methyl green (DakoCytomation S1962) for 20 minutes.

22) Slides were washed in running tap water to remove excessive green-blue color.

23) Slides were placed in the following solutions:

    a. Three minutes in 95% Ethanol

    b. Three minutes Xylene

24) Slides were coversliped (Fisher Scientific Catalogue No.12-541B) with cytoseal (Fisher Scientific Catalogue No. 8310-16) and air- dried overnight.

Immunoflourescent staining

1) Tissues were retrieved from mice and washed with PBS.
2) Tissues were cut into cubes of approximately 3 mm.
3) Tissues were then soaked in 30% sucrose in water overnight at 4C.
4) After sucrose tissues were washed in PBS three times and then placed into OCT.
5) Tissues were frozen over liquid nitrogen and then stored at -80C until sectioning.
6) Cryosections were made on a Leica (Wetzlar, Germany CM 3050S) cryostat at 5 mm slices.
7) Sections were placed on Fisher Colorfrost positively charged slides (Catalogue No. 12-550-17)
8) Sections were fixed in cold acetone for 10 minutes and then dried overnight at 4C.
9) Sections were then washed in buffer (PBS+0.05% Triton X-100) three times, five minutes at room temperature.
10) Sections were blocked with buffer plus 3% donkey serum for 1 hour at room temperature.
11) Primary antibody was added at the dilution of 1:100 and allowed to incubate overnight at 4C in humidified chamber.
12) Slides were then washed three times, five minutes in buffer.
13) Secondary antibody, Molecular Probes (Carlsbad, CA) Alexafluor 488 nm or 594 nm, at 1:2000 dilution was added for 1 hour at room temperature in the dark.
14) Slides were washed three times, five minutes in buffer at room temperature with occasional agitation.
15) Slides were allowed to dry and mounted with 20 micro litre Prolong Antifade Gold from Molecular Probes.
16) Slides were observed on a Zeiss LSM510 confocal microscope.
17) Antibodies used were as follows:

Primary antibodies:

- Rabbit anti-beta galactosidase (Catalogue No. G1041-41 US Biological)
- Rat anti mouse cd31 PECAM (clone 390; Pharmingen, San Diego, CA)
- Polyclonal rabbit antibodies specifically detecting NF1A (Catalogue No. ARP32714-P050 Aviva System Biology, San Diego, CA), NF1B (Catalogue No. ARP32716-T100 Aviva System Biology, San Diego, CA), NF1C (Catalogue No. P100810-100 Aviva System Biology, San Diego), NF1X (Catalogue No. ARP32717-P050 Aviva System Biology, San Diego)
- Goat polyclonal anti-mouse PECAM antibody (Catalogue No. sc-1506 Santa Cruz Biotechnology Inc., Santa Cruz, CA).

Secondary antibodies:

- Goat anti-rabbit Alexa 488 and 594 (Molecular Probes)
- Goat anti-rat cy3 (Jackson ImmunoResearch Laboratories, West Grove, PA)

Chapter 3: Results

Background

Related work carried out in the lab prior to initiation of my project.

VWF promoter sequences –487 to +247 functions as an endothelial-specific promoter in cell culture (Figure 1). However, in adult transgenic mice, this VWF promoter fragment is activated only in the brain vascular endothelial cells, while endogenous VWF gene is expressed in vasculature of all major organs. To identify additional regions of the VWF gene that may participate in its regulation, DNase I hypersensitive assay was used. Considering that DNA regulatory elements are usually positioned in open chromatin structures that are more accessible to DNase I digestion, this approach provides a tool to scan large genomic regions for presence of potential cis-acting elements. Since the analysis of 5′ region of the VWF gene revealed limited pattern of promoter activity *in vivo,* the 3′ region of the gene was investigated for the presence of regulatory element(s). These analyses that were previously performed in our lab had led to identification of a DNase I hypersensitive site in intron 51 region of the VWF gene. These hypersensitive sequences were shown to contain a functional binding site for transcription factor YY1 (Figure2) (91). A 380 bp fragment encompassing this DNase I hypersensitive region (referred to as HSS-I51) was subsequently cloned upstream of the homologous VWF promoter (sequences –487 to +247) or a heterologous SV40 promoter fused to a reporter gene. The activity of the resulting plasmids were determined in endothelial and non-endothelial cells. The results demonstrated that the hypersensitive sequences activated the

heterologous SV40 promoter in endothelial cells however, no significant effect was observed on homologous VWF promoter (91). Based on these results we hypothesized that the intron 51 sequences may have an endothelial-specific enhancing activity but this effect may only be demonstrated on a promoter that is not active by itself in endothelial cells. We hypothesized that since VWF promoter (sequences –487 to +247) is active in endothelial cells in culture the effect of the enhancer on the homologous VWF promoter in cell culture may be obscured. Based on this reasoning we hypothesized that these sequences may extend the activation pattern of the VWF promoter *in vivo*. Thus, presence of the HSS-I51 may result in promoter activation in endothelial cells of other organs in addition to brain in transgenic mice.

The second part of our studies was based on the observation that while the activity of the VWF promoter fragment in adult transgenic mice was restricted to a subset of brain vascular endothelial cells, almost all embryonic vascular endothelial cells supported the activity of the promoter. This observation led to design the hypothesis that in the absence of additional VWF DNA sequences, the promoter activity of sequences -487 to +247 is repressed during development from embryonic to adult tissues in all organs except brain.

In the first part of this study we tested the above hypothesis as described below.

Role of intron 51 HSS in VWF promoter activity *in vivo*

To determine whether the 380 bp DNA region (HSS-I51) that encompasses the DNase I hypersensitive participates in regulation of VWF transcription *in vivo*, transgenic mice were generated harboring a LacZ transgene driven by the VWF promoter (sequences –

487 to +247) and the I51-HSS sequences [spanning nucleotides 38/231 to 38/613] positioned either upstream of the VWF promoter or downstream of the LacZ gene (transgenes HSS-VWF-LacZ and VWF-LacZ-HSS) previously in the lab as described in Materials and Methods. The resulting plasmids were used by Ozgene Company (Australia) and University of Alberta Transgenic Facility to generate 2-3 transgenic founder lines for each transgene in C57BL/6 mice strain. As control we also regenerated transgenic mice containing the VWF promoter (sequences –487 to +247) fused to LacZ gene (designated VWF-LacZ). Transgenic founders were maintained and bred with wild type C57BL/6 by HSLAS facility at the University of Alberta to generate hereozygous F1. Transgenic progenies were identified by PCR analyses of DNA from mice tails biopsies (carried out by HSLAS staff) to detect the presence of LacZ transgene. We analyzed 2-3 independent lines of each transgenic mouse containing the HSS-51 (HSS-VWF-LacZ and VWF-LacZ-HSS) and one line of the VWF-LacZ, using immunohistochemistry and immunofluorescence to detect LacZ gene products in various organs. We analyzed one founder line, and at least two F1 generations of each line, for each transgene. Organs from transgenic and non-transgenic littermates were harvested and fixed in formalin or cryoperserved in 30% sucrose. Tissue arrays were prepared (by Histobest Company, Edmonton, Alberta) from formalin treated organs and subjected to immunohistochemistry with anti-β−galactosidase specific antibody. The use of tissue arrays in which sections of various organs were placed on a single slide allows for samples to be simultaneously processed under a similar condition, thus minimizing inter-experimental staining variability.

Positive immunoperoxidase staining for β−galactosidase was shown almost exclusively in the vasculatures of lung and brain (Figure 3) for both HSS-VWF-LacZ and VWF-LacZ-HSS. In the lung, vessels of the alveolar walls, especially in the parenchyma near

the pleura, were most commonly positive. Specific staining of the endothelial cells of lung and brain tissues of the transgenic lines with control IgG antibody, and non-transgenic littermates with the anti-β−galactosidase antibody was not observed (Figure 4). Also consistent with our previous report, there was no LacZ detection in the lung of transgenic mice (VWF-LacZ) that contained VWF promoter (-487 to +247) in the absence of the HSS-I51 sequences while expression was observed in the brain vasculature of these animals (Figure 4).

To determine the cellular origin of the LacZ expression in the lung vasculature, immunofluorescent analysis was performed on OCT frozen organs harvested from one line of VWF-LacZ-HSS using specific antibodies to detect LacZ gene product (shown as green) and PECAM (shown as red, a marker for endothelial cells) and slides were subjected to confocal fluorescence microscopy. Overlapping windows (yellow) demonstrated co-localization of both PECAM and β−galactosidase in brain and lung endothelial cells (Figure 5). Similarly LacZ expression was not detected in endothelial cells of other organs tested. Some β−galactosidase staining was observed in colon (non-endothelial cells) of one line of HSS-VWF-LacZ and kidney (non-endothelial cells) of one line of VWF-LacZ-HSS. These were attributed to ectopic expression, possibly due to transgene integration site, since these were not observed in more than one transgenic line each. There was no other detection of β−galactosidase in endothelial or non-endothelial cells of other organs tested. Also there was no expression detected in the megakaryocytes.(91)

The results demonstrated that LacZ transgene was expressed only in the brain and lung vasculatures of these mice and that the expression was restricted to endothelial cells of these organs. Since the VWF promoter sequence -487 to +247 was previously shown to target activation in brain vascular endothelial cells, these results demonstrate that the

lung-endothelial-specific activation of the chimeric promoter, containing I51-HSS sequences and sequences -487 to +247, is due to the regulatory function of the I51-HSS sequences. These results supports the hypothesis that additional regions of VWF gene in combination with sequences –487 to +247 are required to extended the transcriptional activation of the VWF promoter to endothelial cells of multiple organs. Specifically a regulatory region in intron 51 is necessary for activation in lung endothelial cell.

These results were published in: "Kleinschmidt AM, Nassiri M, Stitt MS, Wasserloos K, Watkins SC, Pitt BR, et al. Sequences in intron 51 of the von Willebrand factor gene target promoter activation to a subset of lung endothelial cells in transgenic mice. J BIOL CHEM 2008 Feb 1;283(5):2741-50".

To test the second part of our hypothesis we explored whether repressor elements that constituted the binding sites for NF1 and NFY participate in organ-specific regulation of the VWF promoter as described below.

Role of NF1 and NFY in VWF promoter activity *in vivo*

To determine whether repressors NF1 and NFY participate in organ-restricted activity of the VWF promoter *in vivo*, we generated transgenic mice harboring LacZ gene under the regulation of the VWF promoters containing mutations in either NF1 binding site [designated $LacZK^{NF1}$ (Figure 6B)], NFY binding site [designated $LacZK^{NFY}$ (Figure 6C)] or both [designated $LacZK^{NF1\text{-}NFY}$ (Figure 6D)]. We also used the VWF-LacZ transgenic mice (here designated LacZK) containing the wild type VWF promoter that was described above (Figure 6A). The VWF promoter in all transgenes constituted the VWF

gene sequences -487 to +247 and the mutations consisted of the three base substitutions that were previously used to explore the functions of NF1 and NFY repressors binding sites *in vitro* (77;78;81). We obtained two-three independent lines of mice carrying each transgene. Major organs were harvested from transgenic mice LacZK (one line), LacZK^{NF1} (three independent lines), LacZK^{NFY} and LacZK$^{NF1\text{-}NFY}$ (two independent lines each) and subjected to RNA analyses using quantitative real-time PCR (RT-PCR) to detect LacZ mRNA, and immunohistochemistry to detect LacZ protein product. We also performed analyses of Tie2 and VWF endogenous mRNA in all organs of the 8 transgenic mice to determine the pattern of endogenous VWF expression and whether it strictly correlates with the level of vascularization. These analyses (presented as averages of 8 mice for each organ) demonstrated that Tie2 expression was highest in the lung followed by kidney, heart, brain and liver. The highest level of VWF expression was detected in the lung followed by heart, brain, liver and kidney (Figure 7A).

(i) RNA analyses of LacZ transgene in transgenic mice

The RNA analyses of the LacZK (containing the wild type promoter) transgenic mouse performed as described in Materials and Methods demonstrated that LacZ mRNA was detected in the brain but also in kidney (Figure 7B). The expression in the kidney appears to be the result of ectopic expression in this specific line since immunohistochemistry analysis demonstrated LacZ expression only in tubular cells in a limited region of kidney. This ectopic expression was not observed in any other previously analysed lines of LacZK. In contrast to LacZK transgenic mouse, in three lines of LacZK^{NF1}, the highest level of LacZ mRNA was detectable in the lung, followed by the heart and to a lesser degree in the liver and in the brain. Although the level of expression was variable among

the three lines (potentially reflecting transgene variation in integration site and copy number), the pattern of expression was similar (Figure 8). RNA analyses of the two lines of LacZK^{NFY} demonstrated the expression of LacZ mRNA in the kidneys and hearts of transgenic lines as well as brain. In one line expression in liver and lung was also detected (Figure 9A). Results of RNA analyses of the organs from two lines of double mutant LacZK$^{NF1\text{-}NFY}$ demonstrated the expression of LacZ mRNA in all five organs tested (Figure 9B).

Together these results suggest that in contrast to the wild type VWF promoter that is activated only in brain, mutation of the repressor elements in the promoter results in promoter activation in other organs. In addition the pattern of activation appears to differ depending on the mutation, with activation in kidney observed in NFY mutant but not in NF1 mutant promoter.

(ii) Immunohistochemical detection of LacZ protein expression in transgenic mice

To determine pattern of LacZ expression at the protein levels, tissue arrays were prepared from formalin treated organs and subjected to immunohistochemistry with anti-β−galactosidase specific antibody. In these analyses sections of various organs were placed on a single slide thus allowing multiple tissue samples to be simultaneously processed under similar conditions and hence minimizing inter-experimental staining variability. The immunohistochemistry analyses demonstrated that consistent with previous reports (67;92), LacZ expression was detected in the brain vasculature but not

heart, lung or liver of the transgenic mouse LacZK (Figure 10 panel LacZK). In the kidney, expression in all vasculature was undetectable. In contrast, analyses of $LacZK^{NF1}$ transgenic mice demonstrated significant LacZ expression in the vasculature of lung and heart, in addition to the brain. Similar results were obtained for all three lines and results for one line is shown (Figure 10 panel $LacZK^{NF1}$). Analyses of $LacZK^{NFY}$ transgenic mice (shown for one line) demonstrated that expression of LacZ was detected in the brain and kidney vasculature in both lines (Figure 10 panel $LacZK^{NFY}$). In one line some expression in heart and lung vasculature was also detected but the levels were significantly less than that observed in hearts and lungs of $LacK^{NF1}$. Analysis of $LacZK^{NF1\text{-}NFY}$ transgenic mice demonstrated that LacZ expression was detected in the vasculature of brain, heart, lung, kidney and liver in both lines (Figure 10 panel $LacZK^{NF1\text{-}NFY}$). Thus, combined mutation of both NF1 and NFY repressors binding sites results in an expression pattern that constitutes an overlap of expression patterns observed with individual mutations. In addition, expression was detected in the livers of double mutant transgenic mice, which was not observed with either of the individual mutants, suggesting a synergistic effect of the mutations in both NF1 and NFY repressor.

Determining the pattern of NF1 isoforms

The results of the mutation analyses described above demonstrated that the NF1-binding site mutation specifically activates VWF promoter in the lung and heart vasculature but not kidney, and that mutation of NFY binding site is required to activate the promoter in the kidney. These results strongly suggest that NF1 transacting factor specifically represses VWF promoter in the lung and heart but not kidney vasculature. There are four known NF1 isoforms designated as A, B, C and X. Thus, we explored whether endothelial cells of lung, heart and kidney, as well as those of brain exhibit differences in

expression pattern of specific NF1 isoforms. Since endothelial cells that are grown in culture lose their organ-specific characteristics and any differences observed in cultured cells may not accurately reflect *in vivo* differences we chose to perform immunoflourescence/confocal microscopy analyses of mouse organ sections using antibodies to detect specific NF1 isoforms and PECAM as a marker for endothelial cells in various organs.

For these analyses OCT frozen sections of organs (arranged in an array) were subjected to immunoflourescence analyses using anti-PECAM antibody (conjugated to green fluorescent dye) to identify endothelial cells in combination with antibodies (conjugated to red fluorescent dye) that specifically detect either A, B, C or X isoforms of NF1 as described in Materials and Methods. DAPI staining was used to detect nuclei and determine the presence or absence of NF1 in the nuclei. The results of these analyses shown in Figure 11 demonstrated that there were significant variations in the expression of specific NF1 isoforms that were detected in the nuclei of endothelial cells in the four organs studied. In the brain, expression of NFIC and NFIX were clearly detected in the nuclei of endothelial cells while NFIA and NFIB were barely detected. In the endothelial cells of the heart predominantly NFIB followed by NFIA were detected while NFIX and NFIC were undetectable. In the lung endothelial cells, the most significant NFI isoform detected was NFIB while NFIA, C or X were not detectable. In the kidney endothelial cells, NFIX was predominantly detected with significantly lower levels of NFIC and no NFIB or A. These results demonstrate that in lung, heart and kidney, the endothelial cells express distinct non-overlapping isoforms of NF1. Specifically, NFIX is the major isoform expressed in the kidney and NFIB is the major isoform expressed in the lung and heart. Based on the observations that mutation of NF1 binding site activates the VWF promoter in lung and heart but not kidney we hypothesize that the B isoform of NF1 but

not X may function as a repressor of VWF promoter. These results however do not exclude the possibility that C and A isoform may also function as repressors of VWF. These results are submitted for publication in a manuscript entitled: “Repressors NF1 and NFY participate in organ specific regulation of VWF promoter activity in transgenic mice”.

Chapter 4: Discussion

Under *in vivo* conditions, endothelial cells display marked heterogeneity in structure and function. Local microenvironment is a major participating factor in establishment endothelial cell heterogeneity, to an extent that when they are removed from their native environment and grown in culture, endothelial cells undergo phenotypic drift and lose many of their organ specific characteristics (2;19).

In vitro analyses of the VWF promoter have provided information with regard to identification of cis-acting elements and trans-acting factors that interact with the VWF promoter, and their role in endothelial-cell type specific regulation of this promoter (77;78;81;82;93). To date, almost all of the trans-acting factors and their cognate binding sites that are characterized as VWF transcriptional regulators, are localized to VWF promoter sequences -487 to +247. Although these sequences function as endothelial-specific promoter *in vitro* (74), *in vivo* analyses demonstrated that the activity of the -487 to +247 VWF promoter is restricted to a subset of brain vascular endothelial cells in transgenic mice, while in embryonic vessels near uniform activity in almost all vasculature was observed (67). In addition, endogenous VWF gene is expressed in endothelial cells of almost all organs, although at variable levels and not uniformly in all vessels of each organ. Together, these observations suggested that additional regions of the VWF gene (outside sequences–487 to +247) are required to obtain activation in endothelial cells of other organs in addition to brain. This is supported by the report that extension of this VWF region to include additional 5′ and 3′ sequences (-2182 to the end

of the first intron) resulted in promoter activation in endothelial cells of heart and smooth muscle cells as well as brain (67;94).

Previous reports have demonstrated that DNA regulatory elements are located in introns and 3′ regions of some genes, as well as the 5′ proximal and distal sequences to the transcription initiation site. Endothelial- specific enhancer for several genes including Tie-2, flk1 and GATA2 were identified down stream of transcription initiation site, ranging in location from 3′ region of intron 1 to intron 4 (66;95).

Since the analysis of the 5′ region of the VWF gene showed limited pattern of promoter activity *in vivo*, we explored whether potential regulatory elements could be located at the 3′ region of the gene. DNase I hypersensitive assay was used and a DNase I hypersensitive site in intron 51 region was identified. Analysis of this region showed that a YY1 transcription factor interacts with this region (91). Thus we hypothesized that I51-HSS sequences may participate in activation of VWF promoter *in vivo* in endothelial cell types where sequences -487 to +247 alone are not sufficient for promoter activity.

To test this hypothesis we generated transgenic mice containing the intron 51 DNase I hypersenstive site (I51-HSS) sequences placed either upstream or downstream of the VWF promoter sequences -487 to +247 (HSS-VWF-LacZ and VWF-LacZ-HSS) that regulated the expression of the LacZ transgene.

Since generation of a transgene that mimicked endogenous VWF gene with regard to the position of HSS-I51 relative to the promoter was not feasible, we chose to place these sequences upstream of the VWF promoter (HSS-VWF-LacZ) for one set of analysis. Alternatively since I51-HSS is located at the 3′ region of the VWF gene and its location with regard to being positioned downstream vs. upstream of transcription initiation site

may influence its function we also generated transgene that contained I51-HSS positioned downstream of the VWF promoter (VWF-LacZ-HSS) for another set of analysis.

Analysis of the transgenic mice demonstrated that independent of positioning; I51-HSS sequences activated the VWF promoter in a subset of lung endothelial cells as well as brain microvascular endothelial cells as was expected from the activity of the VWF promoter sequences -487 to +247. The expression pattern of the LacZ transgenes in lungs of these transgenic mice was heterogeneous and in large part was restricted to a subset of endothelial cells in the parenchyma near the pleura. This likely reflects the requirement for additional VWF gene sequences to mimic endogenous VWF expression pattern. It may also partly reflect the heterogenous nature of endogenous VWF gene expression in lung. Reports of endogenous VWF gene expression in human and mouse have demonstrated that VWF expression in lung is not uniformly observed in all endothelial cells (22;96). However, *in vivo* analyses of the VWF promoter sequences -487 to +247 demonstrated non-uniform activation pattern in the brain vasculature, with a subset of endothelial cells expressing the fused LacZ gene. Thus, suggesting the distinct region of the VWF gene identified to date may contain minimal necessary elements for transcriptional activation in vasculature of specific organs, but additional sequences may also contribute to expression in a broader range of endothelial cells in vasculature of these organs. The narrow range of activation by I51HSS that is specific to the lung, suggests that elements that are necessary for VWF promoter activation in vasculature of all organs are not clustered together and that sequences for activation in various organs may be scattered throughout the VWF gene. This hypothesis is consistent with the observation that the additional 5′ and intron 1 sequences also resulted in activation only in two additional organs, heart and muscle. Alternatively, the narrow specificity may reflect detection of partial function of these sequences when studied out of their natural context.

The I51HSS sequences in their native position in relation to the promoter could have additional function that may not have been detected by these analyses.

Based on the observation that -487 to +247 sequences are active in embryonic vessels but not in adult organs except brain, we hypothesized that in transition from embryo to adult, the activity of this VWF promoter fragment (in the absence of additional DNA sequences) was repressed in the vasculature of all organs except brain and that one or more of the trans-acting factors which were identified as repressors of the VWF promoter may participate in this process. We tested this hypothesis by determining the *in vivo* activation pattern of the VWF promoter sequences -487 to +247 containing mutations (individually and in combination) in two repressor cis-acting elements, namely NF1 and a novel NFY binding site. Our analyses demonstrated that while the wild type promoter is activated exclusively in brain vascular endothelial cells, mutation in NF1 binding site resulted in promoter activation in lung and heart vasculature; mutation in the novel NFY binding site resulted in promoter activation in kidney vasculature; and double mutations in both NF1 and NFY binding sites resulted in promoter activation in lung, heart, kidney and liver , as well as brain vasculature. These results demonstrated that the NF1 and NFY repressors participate in the mechanism that regulate organ-specific activation pattern of the VWF promoter sequences –487 to +247 *in vivo* and is consistent with our hypothesis that repressors inhibit VWF promoter activity during development from embryonic to adult state. These results suggest that NF1 participates in VWF promoter repression specifically in lung and heart, while NFY participates in its repression in kidney vasculature. Both NF1 and NFY appear to participate in repression in the liver, since only a double mutation resulted in promoter activation in these organs. In addition to their participation in organ-specific pattern of VWF promoter activity, repressors NF1 and NFY may also quantitatively regulate the level of VWF promoter activity. However, this

cannot be determined by analyses of standard transgenic mice since various transgenic mice, even distinct lines generated for each transgene may express the transgenes at different copy numbers and they may be incorporated in varying integration sites. This may contribute more significantly to a quantitative level of expression compared to qualitative (i.e. pattern demonstrating absence or presence in specific organ vasculature) thus rendering a comparative quantitative analyses at best ambiguous. Similar expression pattern obtained in two to three lines of each transgenic mouse (regardless of quantitative level) however is a strong evidence of qualitative effects of mutations on the promoter activity. Future analyses using HPRT-targeted transgenic mice will be the best indication of any quantitative effects of these mutations.

The two repressors elements that we have studied NFY and NF1 were shown to function as both repressor and activator of transcription depending on the binding sequence, gene and/or cell type. To our knowledge a repressive function for NFY specifically in endothelial cells has only been reported with respect to VWF promoter. However, a repressive function for NF1 with regard to regulation of tPA gene (an endothelial-specific gene) promoter was previously reported (97). A specific DNA-NF1 complex with NF1 binding sequence of tPA promoter was demonstrated only when extracts from lung and brain were used. Such NF1-DNA complexes were not detected in extracts prepared from kidney or liver in these studies (97). It was hypothesized that NF1 repression of tPA occurs differentially in endothelial cells of different organs, specifically in lung and brain but not in kidney and liver. Our analysis of the NF1 mutation in the VWF promoter also suggests that NF1 repression of the VWF promoter occurs in lung and heart but not kidney or liver, although repression in liver may require combined action of NFY and NF1. Four distinct NF1 genes and numerous splice variants of these four genes (NF1A, NF1B, NF1C and NF1X) in murine, human and other vertebrates are detected (98).

Various NF1 gene products have distinct but overlapping patterns of expression in different organs and tissues and are differentially expressed during development (98). The exact NF1 isoform that interacts with the VWF or tPA is not yet determined, so it may be that an endothelial-organ-specific form of NF1 is present in brain, lung and heart (functioning as repressor of some endothelial-specific genes such as VWF and tPA) that is not present in kidney and possibly liver.

Based on the observations that extended VWF promoter (VWF2 spanning -2182 to the end of the first intron) results in promoter activation in the heart (94); and that addition of intron 51 sequences results in promoter activation in lung (91), we hypothesize that activators (not yet identified) that interact with VWF2 sequences (outside -487 to +247 region) in heart; and activators that interact with intron 51 sequences in the lung, overcome the repressor function of NF1 specifically in these organs. We further hypothesize that as yet other uncharacterized regions of the VWF gene are required to overcome inhibitory function of NFY in the kidney, and both NF1 and NFY in liver, and potentially other organs. Other identified repressors in the VWF promoter sequences –487 to +247 are Oct1 (79) and EBP4 (93) which were shown to participate in cell type specific regulation of the VWF promoter in cell culture analyses, however their *in vivo* function in transgenic mice are not yet determined. A module describing our hypothesis of de-repressor leading to promoter activation in distinct organs is shown in figure 12.

The activation of the wild type VWF promoter sequences –487 to +247 in the brain vasculature may suggest that either NF1 and/or NFY do not function as repressors of the VWF in the brain, or that the –487 to +247 sequences contain regulatory elements that overcome the inhibitory function of these repressors specifically in the brain. We hypothesize that VWF gene activation may involve a mechanism of "de-repression", where regulators interacting with distinct regions of VWF gene function to overcome the

inhibitory effects of repressors NF1 and NFY and that the activities of such regulators may be modulated differentially in distinct organs.

Figures

Figure 1.

Proximal VWF promoter schematically presented with positive (green) and negative (red) transacting factors. T and C represent TATA and CCAAT elements respectively. +1 represents transcription start site at the beginning of exon 1. Endothelial-specific region represents the sequences that are necessary for the enndothelial-specific activation of the promoter in cell culture.

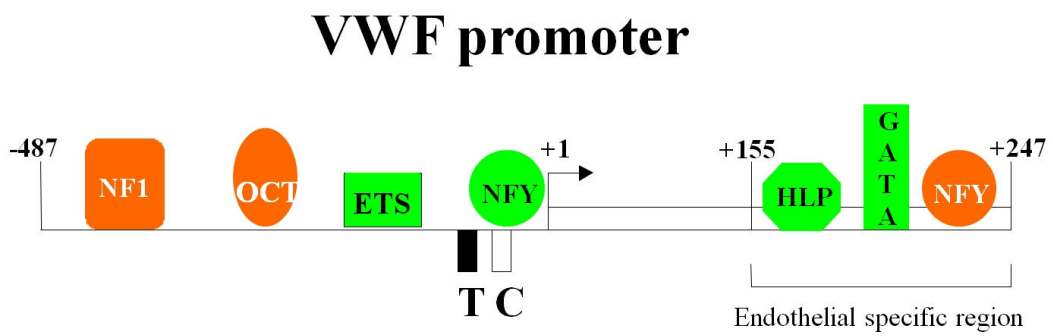


Figure 2.

VWF gene and the position of identified DNase I hypersensitive site relative is shown. Exons 51 and 52 are shown in the enlarged section and the unfilled region of the rectangle depicting exon 52 represents 3′ untranslated region. The 5′ untranslated region containing part of the VWF proximal promoter (sequences 1 to +247) is shown in blue.

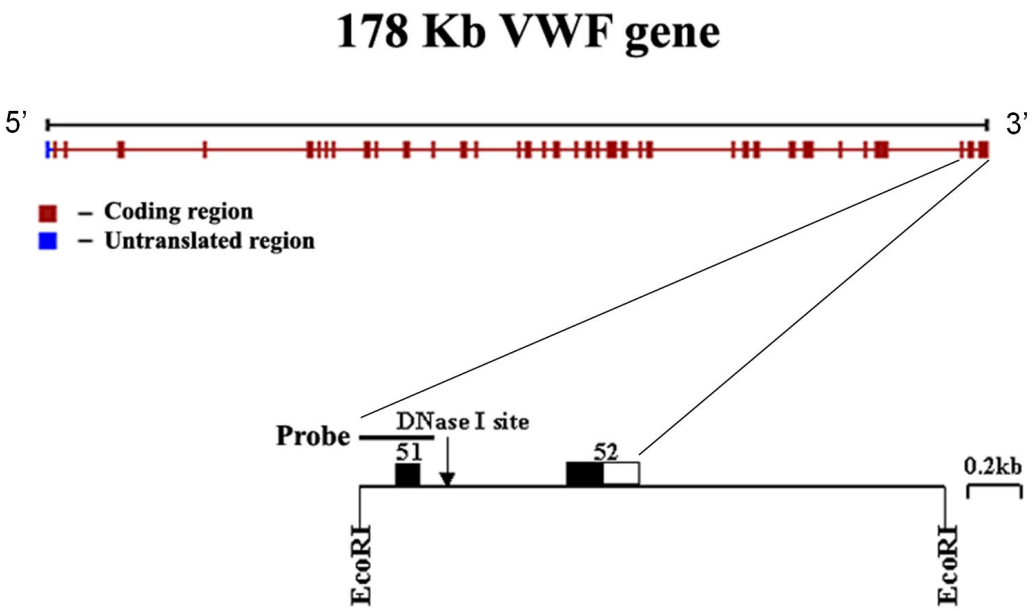


Figure 3.

Hypersensitive region of the VWF gene intron 51 when placed upstream of the VWF promoter targets the promoter activity to the lung vasculature in transgenic mice. (A) Schematic representation of the transgenes containing the VWF promoter and LacZ gene (VWF-LacZ), VWF-LacZ with intron 51 HSS sequences (fragment spanning nucleotides 231-613 of Eco RI fragment 38 of the VWF gene) located upstream of VWF promoter (HSS-VWF-LacZ), and VWF-LacZ with intron 51 HSS sequences located downstream of LacZ gene (VWF-LacZ-HSS). Sequences containing polyadenylation signal are represented as pA (B) Sections from indicated organs of an adult F1 generation of one transgenic line of HSS-VWF-LacZ were immunostained with the antibody specific to LacZ. The arrows show representative vessels expressing LacZ (magnification 400X).

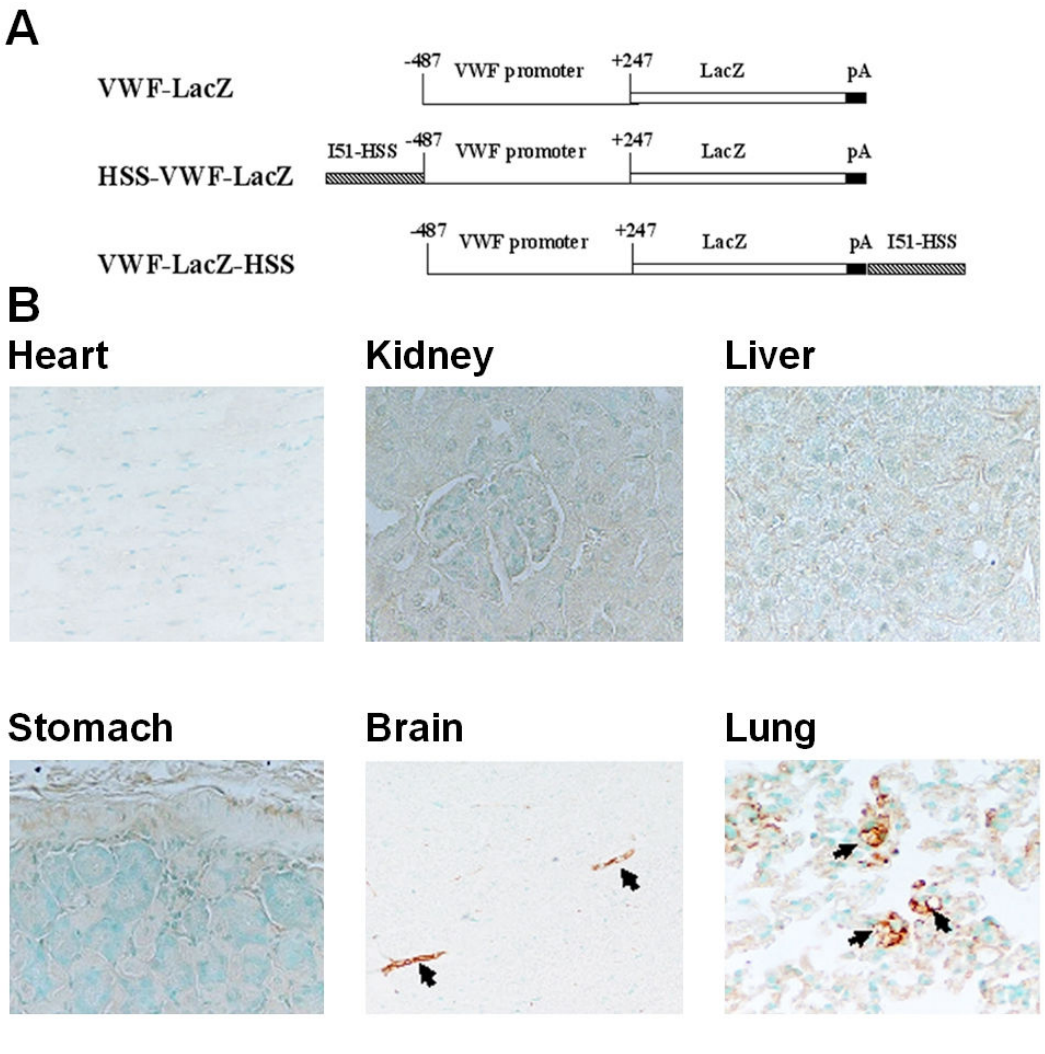


Figure 4.

Hypersensitive region of the VWF gene intron 51 when placed downstream of the VWF promoter also targets the promoter activity to the lung vasculature in transgenic mice. Sections from lung and brain of the transgenic mice (F1 generations) HSS-VWF-LacZ (A), VWF-LacZ-HSS (B), VWF-LacZ (C) and a non-transgenic littermate (E) were immunostained with an antibody specific to LacZ. The lung and brain sections of HSS-VWF-LacZ transgenic mouse were also immunostained with an isotype matched IgG antibody used as negative control (D). The arrows show representative vessels expressing LacZ (magnification 600X).

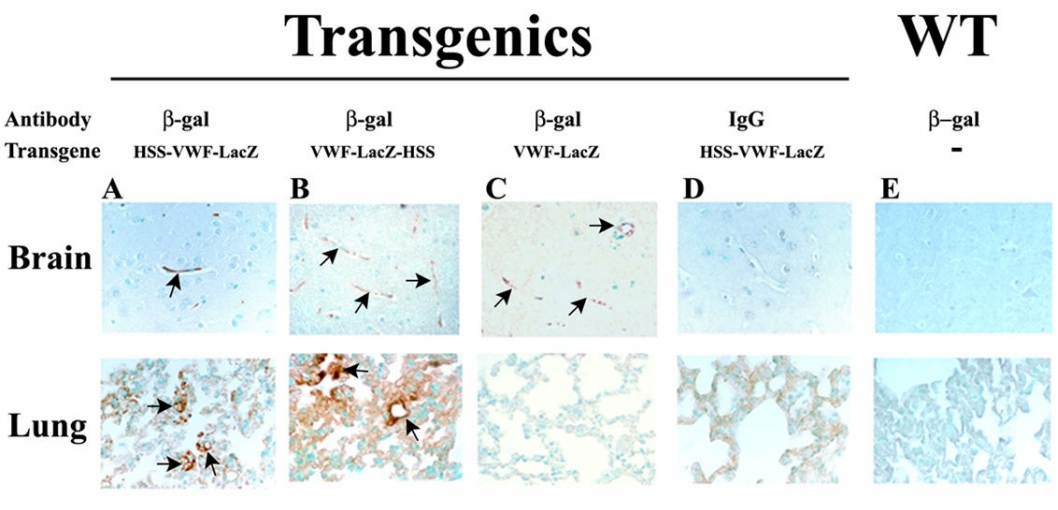


Figure 5.

Confocal microscopy analysis of LacZ and PECAM (Red) showing overlapping LacZ and PCAM expression in brain and lung endothelial cells of a VWF-LacZ-HSS transgenic mouse line 1132.

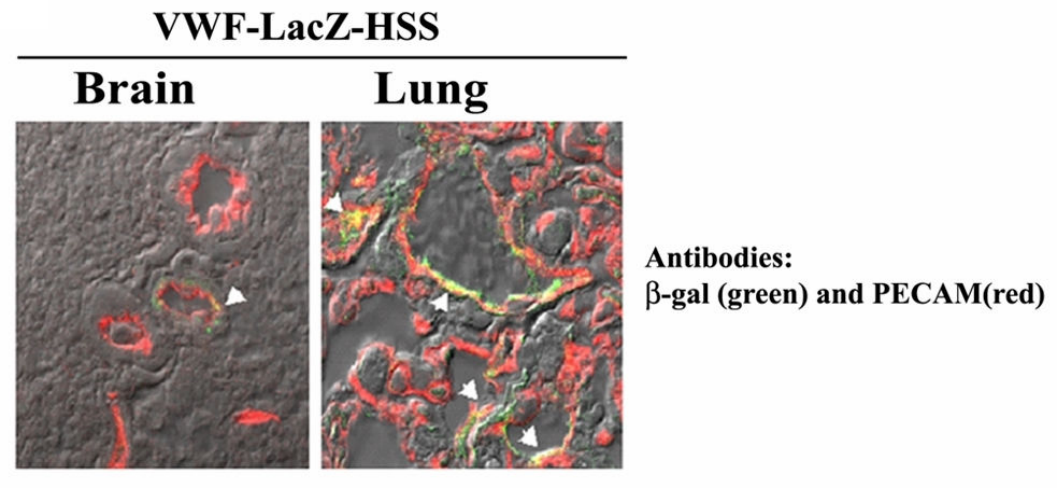


Figure 6.

Schematic representation of the wild type and mutant VWF promoter sequences –487 to +247 that were fused to LacZ gene. Transacting factors functioning as repressors (NF1, Oct-1, and NF-Y shown by ovals) and activators (Ets, NF-Y and GATA6 shown as rectangles) are shown. Binding sequences of NF1 and two NF-Y binding sites, one functioning as an activator (when interacting with the CCAAT sequence) and the other functioning as a repressor when binding to the novel CCGCAGCCC sequence are shown. Solid rectangle represented as T indicates the TATA element. Arrow designated +1 represents transcription start site. (A) Wild type VWF promoter is represented. (B-D) Mutant VWF promoters with mutations in NF1 (B), NF-Y(C) and combined mutation of both NF1 and NF-Y (D) are represented. Base substitution mutation in NF1 and NFY binding sites are underlined.

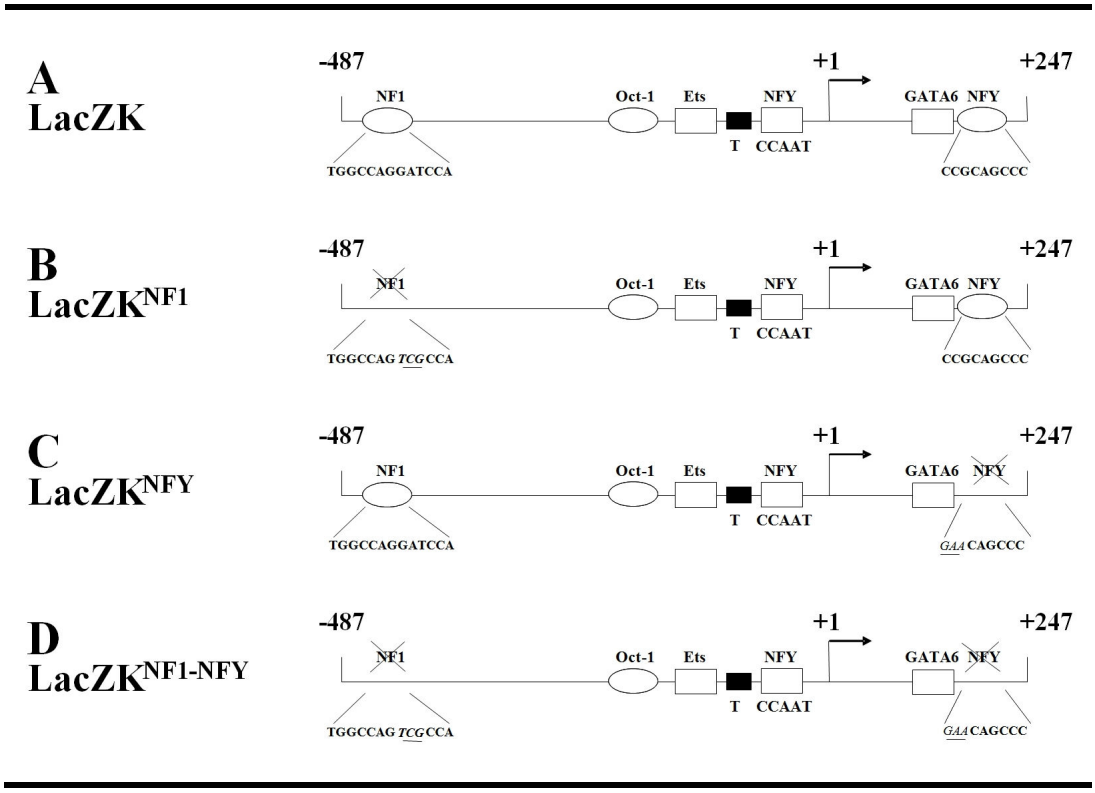


Figure 7.

Real-time PCR analyses of LacZ transgene mRNA in LacZK transgenic mice and endogenous VWF and tie2 mRNA in various organs of all transgenic mice. (A) RNAs (1μg) prepared from various organs of transgenic mice were subjected to reverse transcription and real-time PCR analyses to detect endogenous VWF, tie2 and GAPDH. The graph represents the relative levels of VWF and Tie2 normalized to GAPDH. Results represent the averages of RNA from 8 mice (one of each line of transgenic mice for LacZK, LacZKNF1, LacZKNFY and LacZKNF1-NFY) for each organ. (B) RNAs (1μg) prepared from various organs of LacZK transgenic mice (F2) were subjected to reverse transcription and real-time PCR analyses to detect LacZ transgene and endogenous GAPDH for normalization. Results represent the averages of RNA from 2 mice for each organ.

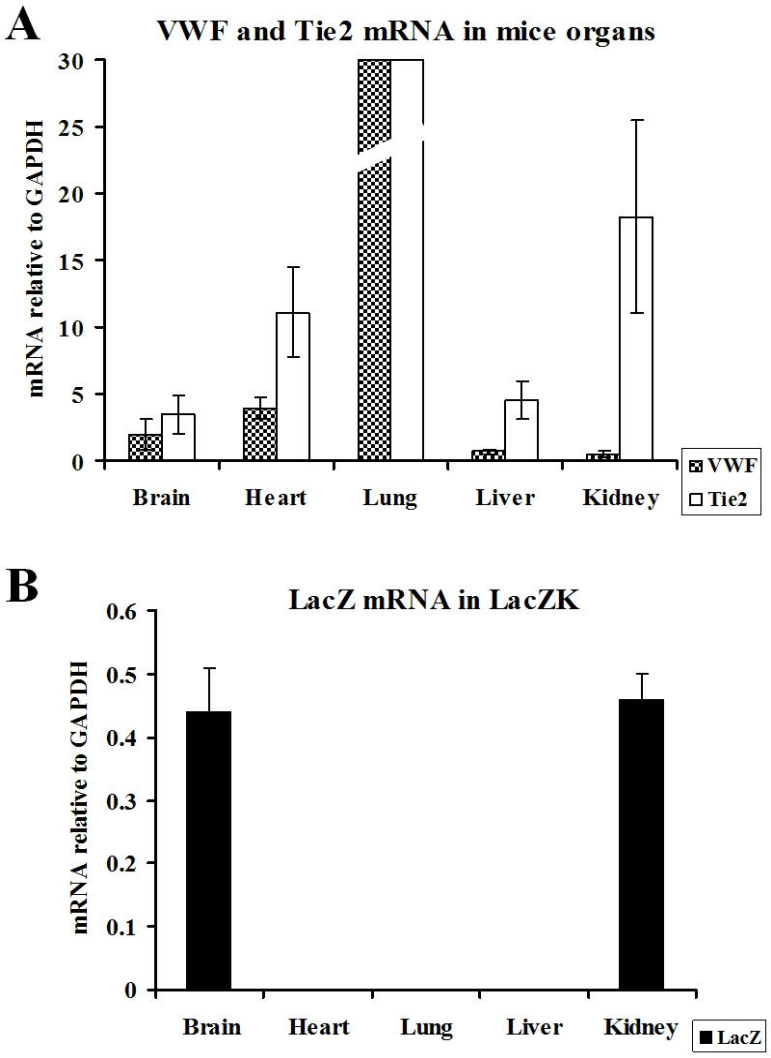


Figure 8.

Real-time PCR analyses of LacZ transgene mRNA in LacZK^{NF1}, transgenic mice. RNAs (1μg) prepared from various organs of 3 independent lines of LacZKNF1 transgenic mice (F2) were subjected to reverse transcription and real-time PCR analyses to detect LacZ transgene and endogenous GAPDH for normalization. Results represent the averages of RNA from 2 mice for each organ.

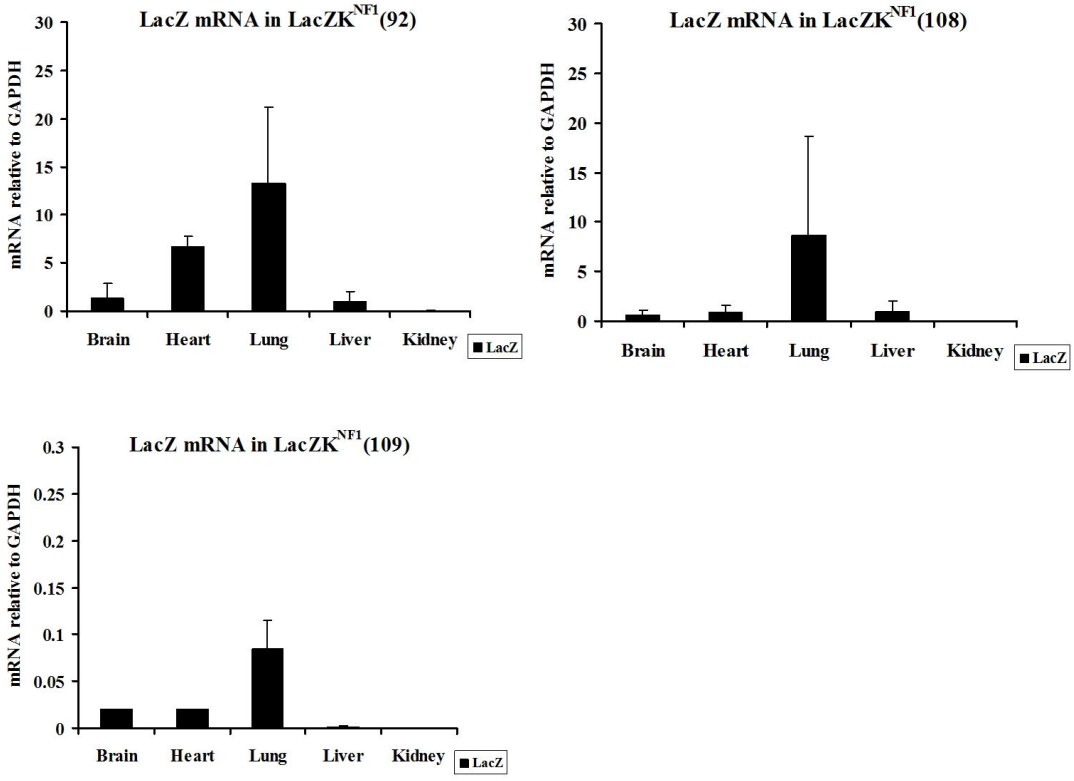


Figure 9.

Real-time PCR analyses of LacZ transgene mRNA in LacZKNFY and LacZKNF1-NFY transgenic mice. RNAs (1μg) prepared from various organs of 2 independent lines of LacZKNFY transgenic mice (A) and 2 independent lines of LacZKNF1-NFY transgenic mice (B) were subjected to reverse transcription and real-time PCR analyses to detect LacZ transgene and endogenous GAPDH for normalization. The mice used for LacZKNFY for each line were F2, while the mice used for LacZKNF1-NFY included one F2 and one founder line. The transgenic mice designated line 94 is the founder line that could not be bred. Results represent the averages of RNA from 2 mice for each organ of the F2 (line 7) and two independent RNA preparation from the single line 94 mouse.

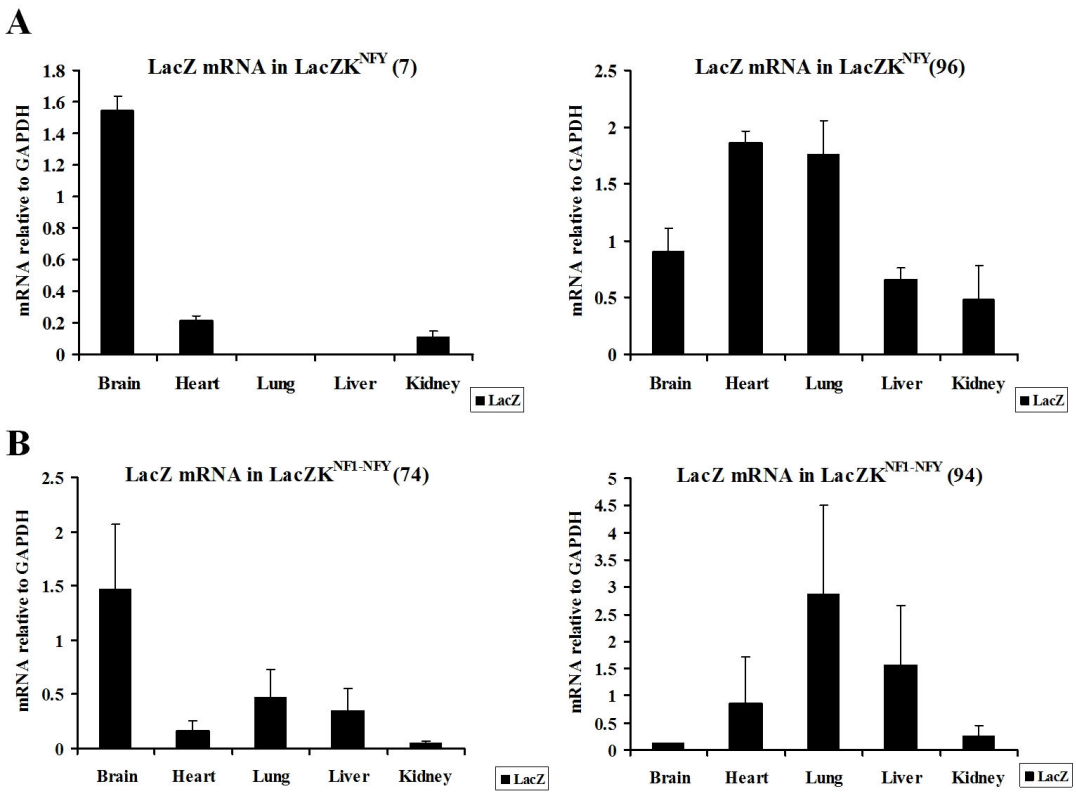


Figure 10.

Immunohistochemical analyses of LacZ expression in various organs of transgenic mice. Sections (5 μm) from formalin embedded heart, kidney, liver, lung and brain of the four transgenic mice (F1 generations) LacZK, LacZK^{NF1}, LacZK^{NFY} and LacZK$^{NF1\text{-}NFY}$ on tissue arrays were immunostained with anti-β-galactosidase antibody as previously described (magnifications 400X).

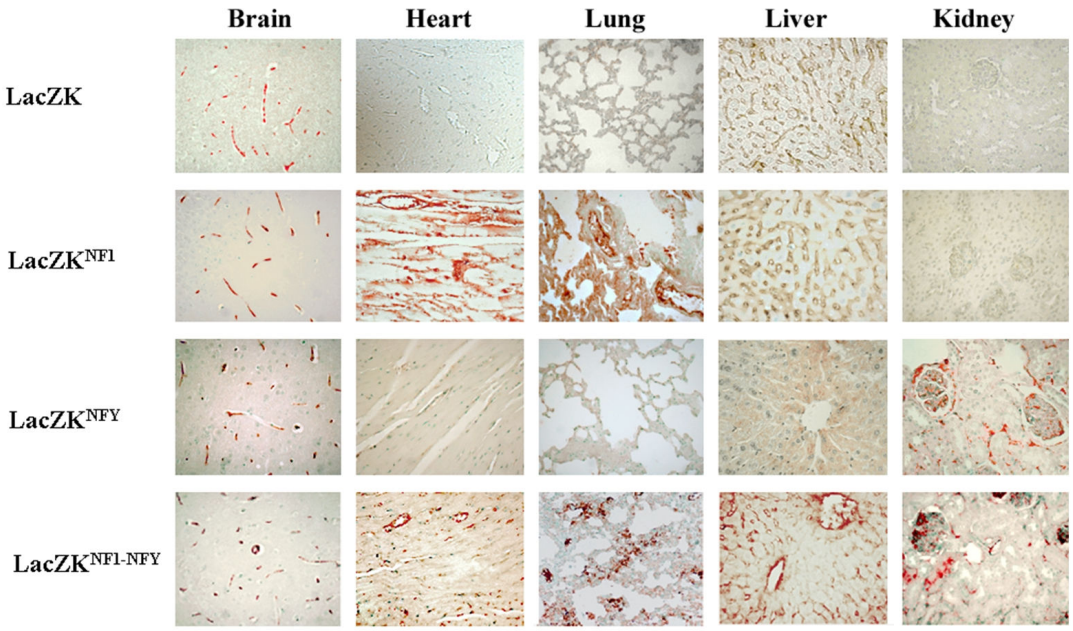


Figure 11.

Immunofluorescent analyses of NF1 isoforms expression in various organs of mice. Sections (5 μm) from OCT frozen heart, kidney, lung and brain of a control C57Bl/6 mice that were arranged on tissue arrays were hybridized to specific antibodies to detect NF1-A, NF1-B, NF1-C and NF1-X isoforms of NF1 (all shown in red) and simultaneously to anti-PCAM antibody (shown in green) to detect endothelial cells as described in materials and methods. The results are representative of two independent hybridizations for each NF1 plus PECAM antibody (magnifications 600X). The insets demonstrate the magnified nuclei of the endothelial cells.

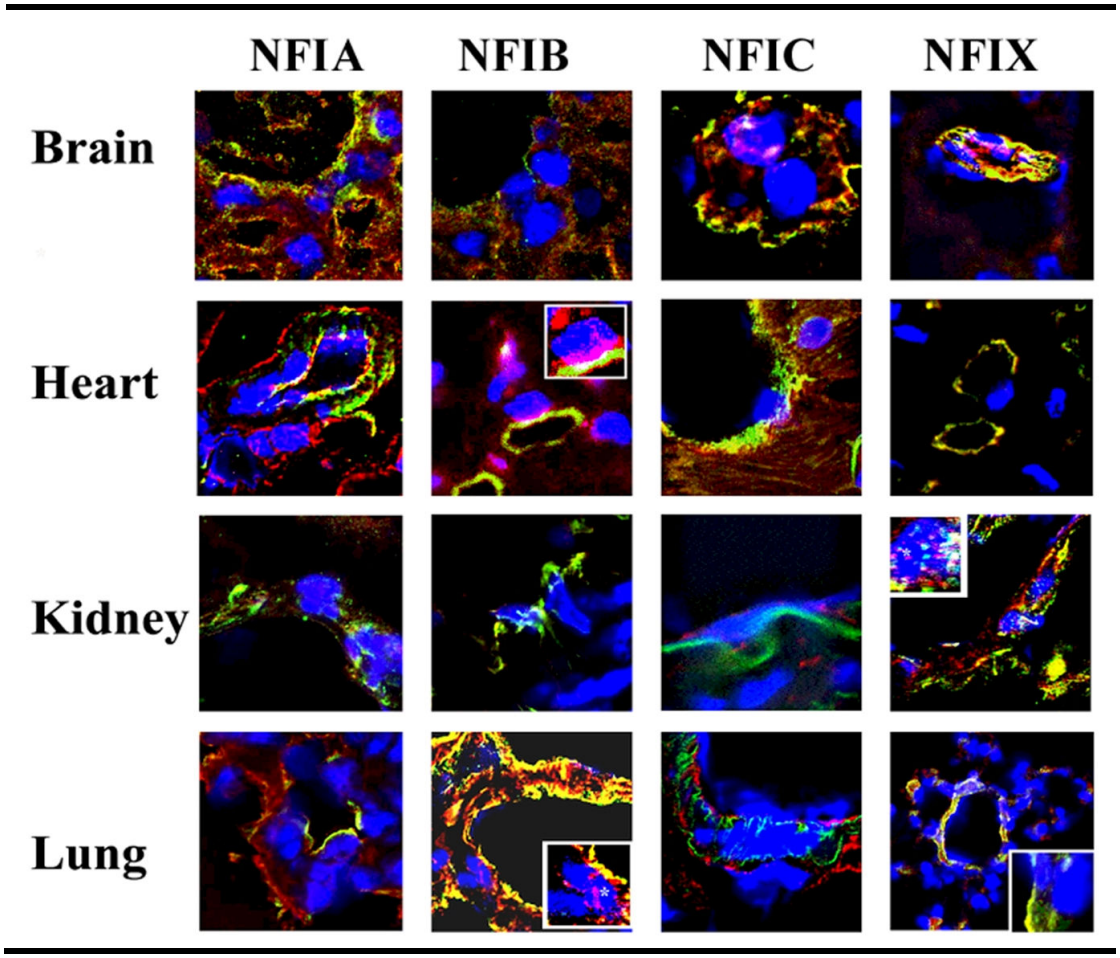
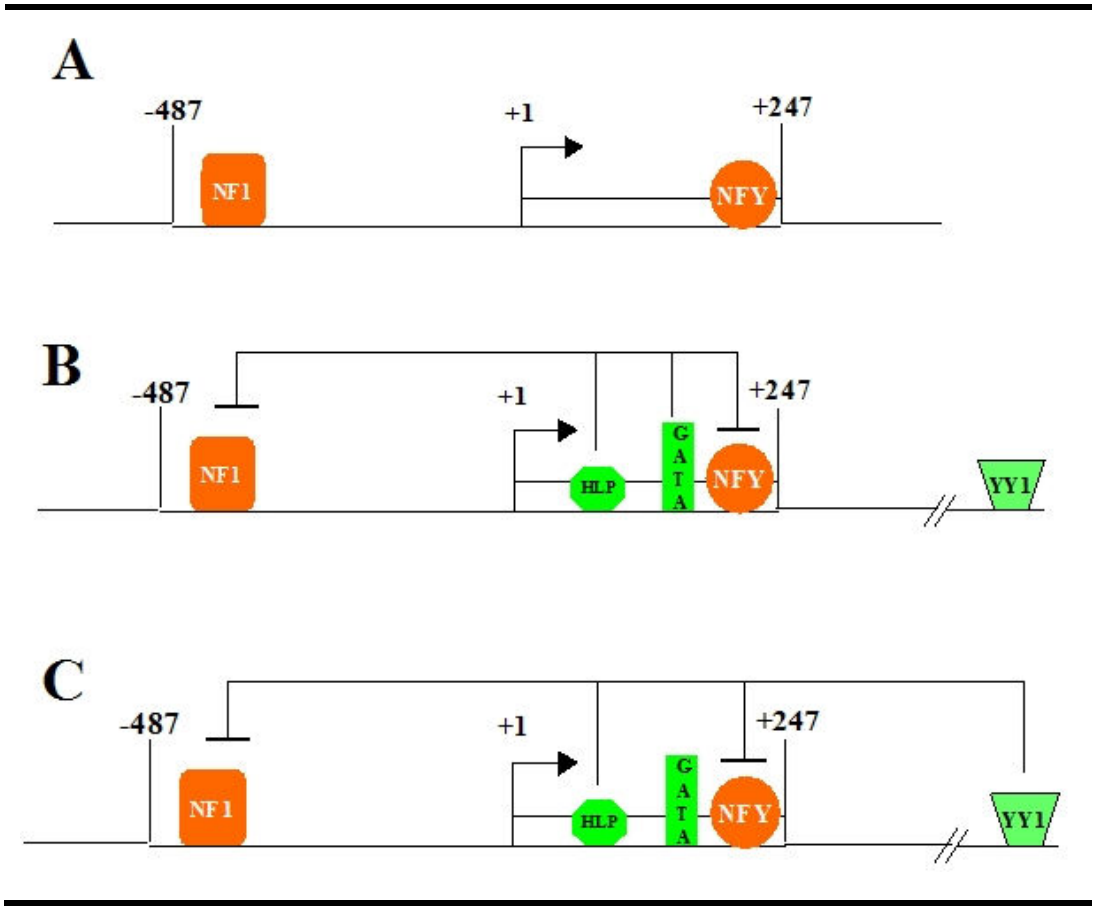


Figure 12.

Model describing the hypothesis tested in this proposal. (A) NF1 and NFY function as repressors and inhibit promoter activity in all cell types. (B) In brain endothelial cells factors that interact with –487 to +247 sequences, including GATA6 and HLP cooperate to inhibit repressors' activities. (C) In other endothelial cell types, for instance in lung, a different cohort of factors are necessary to overcome inhibitory function of repressors. These factors interact with VWF sequences outside –487 to +247, but may cooperate with one or more of factors that interact with –487 to +247 (for example HLP). For instance, sequences in intron 51 that interact with YY1 are necessary for promoter activation in lung. We hypothesize that differential modification of factors in endothelial cells of different organs could contribute to this process. For instance GATA6 may be modified in an endothelial-specific manner only in brain but not lung endothelial cells, thus rendering it an activator that in conjunction with HLP overcomes the repression of NFY and NF1 in the brain but not lung endothelial cells. In lung however, similar situation may exist with regard to YY1, thus leading to cooperation of YY1 with HLP to achieve activation in lung endothelium.



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