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The regulatory role of eNOS-derived nitric oxide on transcription in endothelial cells: Impact of S-nitrosylation on β-catenin signaling

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The regulatory role of eNOS-derived nitric oxide
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Impact of S-nitrosylation on β-catenin signaling

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RÉSUMÉ

Les cellules endothéliales forment une couche semi-perméable entre le sang et les organes. La prolifération, la migration et la polarisation des cellules endothéliales sont essentielles à la formation de nouveaux vaisseaux à partir de vaisseaux préexistants, soit l'angiogenèse. Le facteur de croissance de l'endothélium vasculaire (VEGF) peut activer la synthase endothéliale du monoxyde d'azote (eNOS) et induire la production de monoxyde d'azote (NO) nécessaire pour la régulation de la perméabilité vasculaire et l'angiogenèse. β-caténine est une composante essentielle du complexe des jonctions d'ancrage ainsi qu'un régulateur majeur de la voie de signalisation de Wnt/β-caténine dans laquelle elle se joint au facteur de transcription TCF/LEF et module l'expression de nombreux gènes, dont certains sont impliqués dans l'angiogenèse. La S-nitrosylation (SNO) est un mécanisme de régulation post-traductionnel des protéines par l'ajout d'un groupement nitroso au niveau de résidus cystéines. Le NO produit par eNOS peut induire la S-nitrosylation de la β–caténine au niveau des jonctions intercellulaires et moduler la perméabilité de l'endothélium. Il a d'ailleurs été montré que le NO peut contrôler l'expression génique par la transcription. Le but de cette thèse est d'établir le rôle du NO au sein de la transcription des cellules endothéliales, spécifiquement au niveau de l'activité de β-caténine.

Le premier objectif était de déterminer si la SNO de la β-caténine affecte son activité transcriptionnelle. Nous avons montré que le NO inhibe l'activité transcriptionnelle de β-caténine ainsi que la prolifération des cellules endothéliales induites par l'activation de la voie Wnt/β-caténine. Il est intéressant de constater que le VEGF, qui induit la production de NO via eNOS, réprime l'expression de *AXIN2* qui est un gène cible de Wnt s'exprimant suite à la

stimulation par Wnt3a et ce, dépendamment de eNOS. Nous avons identifié que la cystéine 466 de la β-caténine est un résidu essentiel à la modulation répressive de son activité transcriptionnelle par le NO. Lorsqu'il est nitrosylé, ce résidu est responsable de la perturbation du complexe de transcription formé de β-caténine et TCF-4 ce qui inhibe la prolifération des cellules endothéliales induite par la stimulation par Wnt3a.

Puisque le NO affecte la transcription, nous avons réalisé l'analyse du transcriptome afin d'obtenir une vue d'ensemble du rôle du NO dans l'activité transcriptionnelle des cellules endothéliales. L'analyse différentielle de l'expression des gènes de cellules endothéliales montre que la répression de eNOS par siRNA augmente l'expression de gènes impliqués au niveau de la polarisation tels que : *PARD3A, PARD3B, PKCZ, CRB1 et TJ3*. Cette analyse suggère que le NO peut réguler la polarisation des cellules et a permis d'identifier des gènes responsables de l'intégrité des cellules endothéliales et de la réponse immunitaire. De plus, l'analyse de voies de signalisation par KEGG montre que certains gènes modulés par l'ablation de eNOS sont enrichis dans de nombreuses voies de signalisation, notamment Ras et Notch qui sont importantes lors de la migration cellulaire et la différenciation des cellules de têtes et de tronc (tip/stalk). Le regroupement des gènes exprimés chez les cellules traitées au VEGF (déplétées de eNOS ou non) révèle que le NO peut affecter l'expression de gènes contribuant au processus angiogénique, dont l'attraction chimiotactique.

Notre étude montre que le NO module la transcription des cellules endothéliales et régule l'expression des gènes impliqués dans l'angiogenèse et la fonction endothéliale.

Mots clés : Monoxyde d'azote; cellule endothéliale; signalisation de Wnt/β-caténine; activité transcriptionnelle; analyse bio-informatique

ABSTRACT

Endothelial cells form a semi-permeable layer between blood and the rest of tissues. The proliferation, migration and polarization of endothelial cells are critical for angiogenesis, a process of the formation of new blood vessel from pre-existing ones. Vascular endothelial growth factor (VEGF) can activate endothelial nitric oxide synthase (eNOS) to induce the production of nitric oxide (NO), which is critical for vascular permeability and angiogenesis. β-catenin is an essential component of the adherens junction as well as Wnt/β-catenin signaling pathway and it binds T-cell factor (TCF)/lymphoid enhancer factor, regulating expression of numerous genes including those involved in angiogenesis. S-nitrosylation (SNO) is a mechanism used by NO to regulate protein activity by adding a nitroso group to cysteine residues. eNOS derived-NO is capable to induce SNO of β-catenin at cell-cell junction and modulate endothelial permeability. Additionally, NO has been implicated in the transcriptional control of gene expression. Therefore, the goals of our studies were to investigate the regulatory roles of NO on transcription in endothelial cells, in particular to the modulation of the transcriptional activity of β-catenin.

The objective of the first study is to investigate whether the SNO of β-catenin affect its transcriptional activity. We found that NO inhibits β-catenin transcriptional activity and endothelial cell proliferation induced by activation of Wnt/β-catenin signaling. Interestingly, VEGF, which can activate eNOS to produce NO in endothelial cells, repressed Wnt3a-induced expression of Wnt target gene *AXIN2* in an eNOS-dependent manner. Moreover, we identified that Cys466 on β-catenin is a critical residue for the repressive effects of NO on β-catenin transcriptional activity. Furthermore, we showed that Cys466 is responsible for the disruption

of β-catenin/TCF4 transcriptional complex, and NO-dependant inhibition of Wnt3a-simulated endothelial cell proliferation.

Given the known effects of NO on transcription, whole transcriptome sequencing was performed in order to understand the transcriptional regulation of NO in endothelial cells. By analyzing gene differential expression in cells transfected with control and eNOS siRNA, we show that eNOS knockdown upregulates the expression of genes involved in cell polarization, such as *PARD3A*, *PARD3B*, *PKCZ*, *CRB1* and *TJ3*. The up-regulation of these genes was confirmed by qRT-PCR analysis, suggesting that NO may regulate cell polarization. The analysis also showed that genes regulated by eNOS knockdown were involved in endothelial cell integrity and immune response. In addition, KEGG signaling pathway analysis showed that genes regulated by eNOS were enriched in many signal pathways including Ras signaling, which are important for endothelial cell migration. Moreover, clustering of differentially expressed genes in VEGF-treated cells and VEGF-treated eNOS-depleted cells revealed that NO may affect expression of genes in angiogenesis in response to VEGF, including those genes involved in chemotaxis.

Our studies show that NO affects transcription in endothelial cells and regulates expression of genes involved in angiogenesis and endothelial cell function.

Keywords: nitric oxide; endothelial cell; Wnt/β-catenin signaling; transcriptional activity; bioinformatic analysis

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ABBREVIATIONS

Akt/PKB : Protein kinase B

Ang-1/-2 : Angiopoietin-1/-2

ARM(s) : Armadillo(s)

APC : Adenomatous polyposis coli

aPKC : Atypical protein kinase C

BAEC : Bovine aortic endothelial cells

BBB : Blood brain barrier

Bcl-9 : B-cell CLL/lymphoma 9 protein

bFGF : Basic fibroblast growth factor

BH4 : Tetrahydrobiopterin

β-TrCP1: Beta-transducin repeat containing protein 1

CaM: Calmodulin

cGMP : Cyclic guanosine 3', 5'-monophosphate

CKIα : Casein kinase Iα

CRD : Cysteine rich domain

CT-CM : Control-conditioned medium (control for Wnt-conditioned medium)

Cx : Connexin

DAG : Diacylglycerol

DKK1 : Dickkopf Wnt signaling pathway inhibitor 1

Dvl : Dishevelled

EC(s) : Endothelial cell(s)

eNOS : Endothelial nitric oxide synthase

ECM : Extracellular matrix

EGF : Epidermal growth factor

EPC : Endothelial progenitor cell

ERK : Extracellular signal-regulated kinase

FAD : Flavin adenine dinucleotide

FGF : Fibroblast growth factor

FMN : Flavin mononucleotide

Fz : Frizzled

GM-CSF : Granulocyte macrophage colony stimulating factor

GSK3β : Glycogen synthase kinase 3 β

GSNO : S-nitrosoglutathione

GSNOR : S-Nitrosoglutathione reductase

HIF-1 : Hypoxia-inducible factor-1

HMG : High mobility group

HSP90 : Heat shock protein 90

HUVECs : Human umbilical vein endothelial cells

Ig : Immunoglobulin

iNOS : Inducible nitric oxide synthase

IL-6, -8 : Interleukin -6, -8

MMPs : Matrix metalloproteinases

nNOS : Neuronal nitric oxide synthase

IP3 : Inositol trisphosphate

IQGAP1 : Ras GTPase-activating-like protein

JAMs : Junctional adhesion molecules

JNK : c-Jun N-terminal kinases

KO : Knockout

Krm : Kremen

LDL : Low-density lipoprotein

LEF : Lymphoid enhancer factor

LRP5/6 : LDL receptor-related proteins 5 and 6

MAPK : Mitogen-activated protein kinase

MMTS : Methyl methanethiosulfonate

NADPH : Nicotinamide adenine dinucleotide phosphate

NES : Nuclear export signal

NLS : Nuclear localization signal

NO : Nitric oxide

NPC : Nuclear pore complex

Nrps : Neuropilins

ODQ : 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one

PAF : Platelet-activating factor

PAK : p21 activated kinases

PAR : Partitioning-defective

PI3K : Phosphatidylinositol 3-kinase

PIP2 : Phosphatidylinositol 4,5-bisphosphate

PKC/G : Protein kinase C/G

PLC : Phospholipase C

PP2A : Protein phosphatase 2A

Rac1 : Ras-related C3 botulinum toxin substrate 1

ROS : Reactive oxygen species

RTK : Receptor tyrosine kinase

RT-qPCR : Reverse transcription quantitative polymerase chain reaction

SFK : Src family kinase

sGC : Soluble guanylate cyclase

SH2 : Src Homology 2

SNO : S-nitrosylation

siRNA : Small interfering RNA

TCF4 : T cell factor 4

TGF-β : Transforming growth factor-β

VCAM-1 : Vascular cell adhesion molecule -1

VEGF : Vascular endothelial growth factor

VEGFR : Vascular endothelial growth factor receptor

VE-cadherin : Vascular endothelial cadherin

VSMC : Vascular smooth muscle cells

WIF : Wnt inhibitory factor

Wnt3a-CM : Wnt3a conditioned medium

WT : Wild type

ZO(s) : Zonula occluden(s)

Dedicated to my family and all my friends

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Chapter I Introduction

I.1. Vasculature, endothelium and angiogenesis

I.1.1 Vascular structure

The vascular system is a complex network hierarchically composed of arteries, veins and capillaries. Blood vessels form a closed tubular circulation system that transports blood and oxygen throughout the body; they are therefore crucial for the development of organs and tissue repair in human (1, 2).

Large vessels, including arteries and veins, are made up of a trilaminate tissue architecture composed of the *tunica intima*, *tunica media* and *tunica externa* (Figure I.1). The innermost layer, the *tunica intima*, is lined with endothelial cells, and the subendothelial layer underlying them, called the extracellular matrix (ECM), is a highly organized network of proteins and other macromolecules important for cell adhesion, migration and differentiation (3). The second layer, the *tunica media,* is separated from the tunica intima by an elastic lamina, a connective tissue sheet formed by smooth muscle cells (4). The outermost and densest layer is the *tunica externa*, which is made up of connective tissues and also contains nerves, fibroblasts, the ECM and capillaries (5). The capillaries are composed of a layer of endothelial cells and connective tissues, connecting arterioles and venules (6).

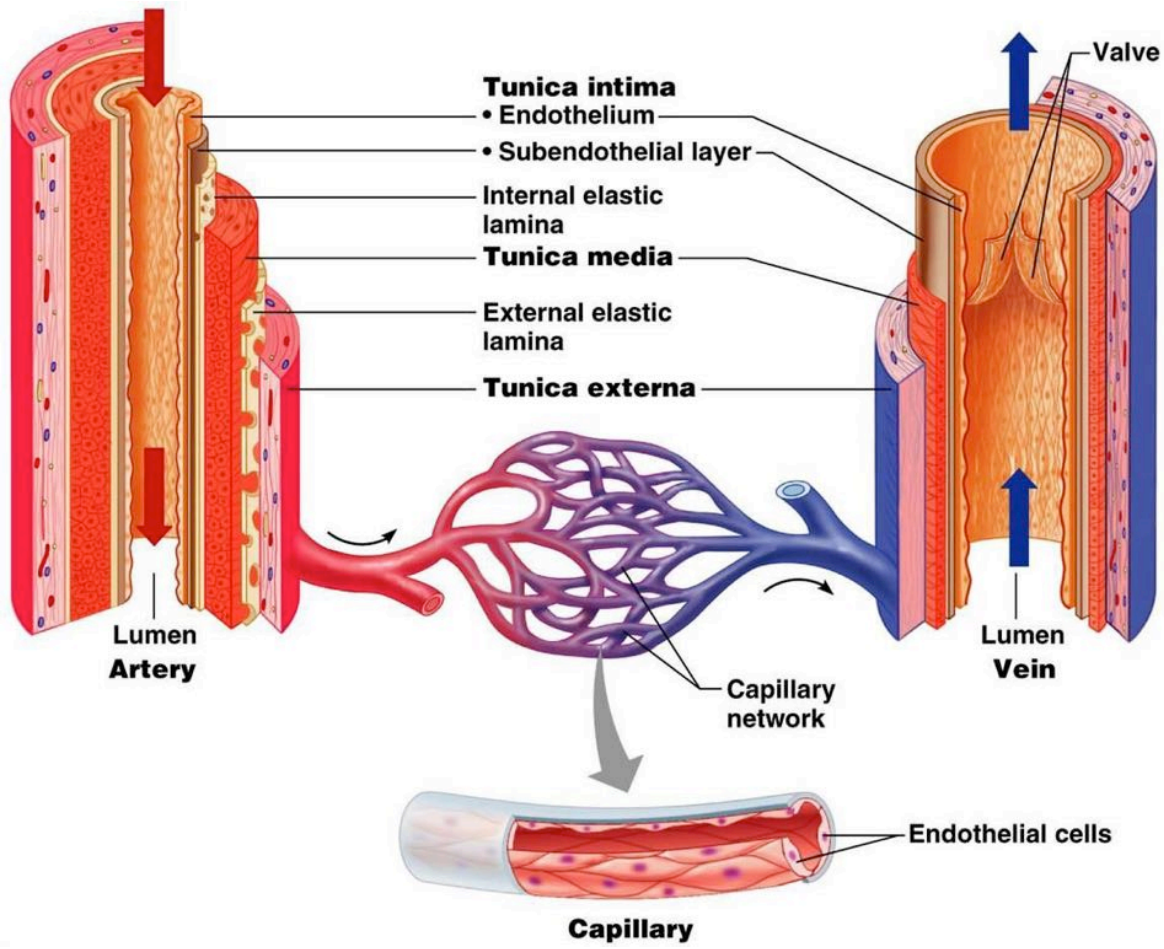

Figure I.1. Structure of blood vessels.

The circulatory system is a complex network of veins, arteries and capillaries. Arteries and veins contain three layers: tunica intima, tunica media and tunica adventitia. Capillaries consist of a layer of endothelial cells and connective tissue. Adapted from reference (7).

I.1.2 Endothelium and endothelial cells

The endothelium is a type of epithelium that lines the interior of blood and lymphatic vessels (Figure I.1). It forms a semi-permeable barrier between blood or lymph in the lumen and the surrounding tissues to control the passage of immune cells and metabolites. The importance of the endothelium was first recognized in relation to effect on vascular tone through the secretion of molecules that relax or contract vessels to regulate the vascular tone (7).

Endothelial cells that make up the endothelium originate from angioblasts during embryonic development. Angioblasts are derived from the mesoderm, a layer of cells that also give rise to hematopoietic cells, connective tissues, and muscles. In addition, endothelial progenitor cells (EPCs) can home into neovessels sites and differentiate into endothelial cells in adults.

In the human body, approximately $1x10^{13}$ endothelial cells form the endothelium that lines the entire vascular system (8). In most adult tissues, the turnover rate of endothelial cells is slow, ranging from a couple of months to years, depending on their localization in different tissues (8).

Endothelial cells regulate a variety of functions, including vascular smooth muscle tone, host defence reactions, angiogenesis and vascular homeostasis. Endothelial cells interact with smooth muscle cells in the vascular wall; alternatively, they can also interact with cells and molecules in circulating blood. One of the most well-known functions of endothelial cells is the regulation of vascular tone such as making certain adjustments to adapt with the local environment in a finely controlled mechanism. For example, endothelial cells are able to sense the shear stress generated by blood flow and pass this signal on to the surrounding tissues and smooth muscle cells, thereby regulating the diameter of blood vessels to adapt to blood flow. This vasomotion is

important for the balance of tissue oxygen and metabolic supply, as well as for the remodeling of the vascular structure (9). Another important function of endothelial cells is to provide a barrier between blood and vascular tissues by acting as a semi-permeable layer.

I.1.3 Vasculogenesis and angiogenesis

Vasculogenesis and angiogenesis are two distinct mechanisms for the formation of the vascular network in the embryo. Vasculogenesis gives rise to the heart and the primitive vascular plexus inside the embryo, while angiogenesis is responsible for the remodeling and expansion of this network.

I.1.3.1 Vasculogenesis

Vasculogenesis is defined as the *de novo* formation of blood vessels. It is a process by which a primary vascular plexus is formed through differentiation of endothelial progenitor cells (48).

Vasculogenesis includes multiple steps: mesoderm formation, blood island differentiation, endothelial cell differentiation and primary capillary plexus formation (Figure I.2) (10). The embryonic mesoderm is the main source of vascular endothelial and hematopoietic progenitor cells; it also acts as the site of vasculogenesis. Induction of mesoderm during gastrulation results in the formation of blood islands on the yolk sac in mice on embryonic day (E) 6.5-7. Hematopoietic precursor cells are located internally in the blood islands and differentiate into common blood cells (11). Angioblasts, located peripherally within the blood islands, are the precursor cells that differentiate into endothelial cells at E8.5. Differentiated endothelial cells can

form a vascular lumen, deposit a basal lamina structure and prepare for the formation of a primitive vascular plexus near the site of their origin (10).

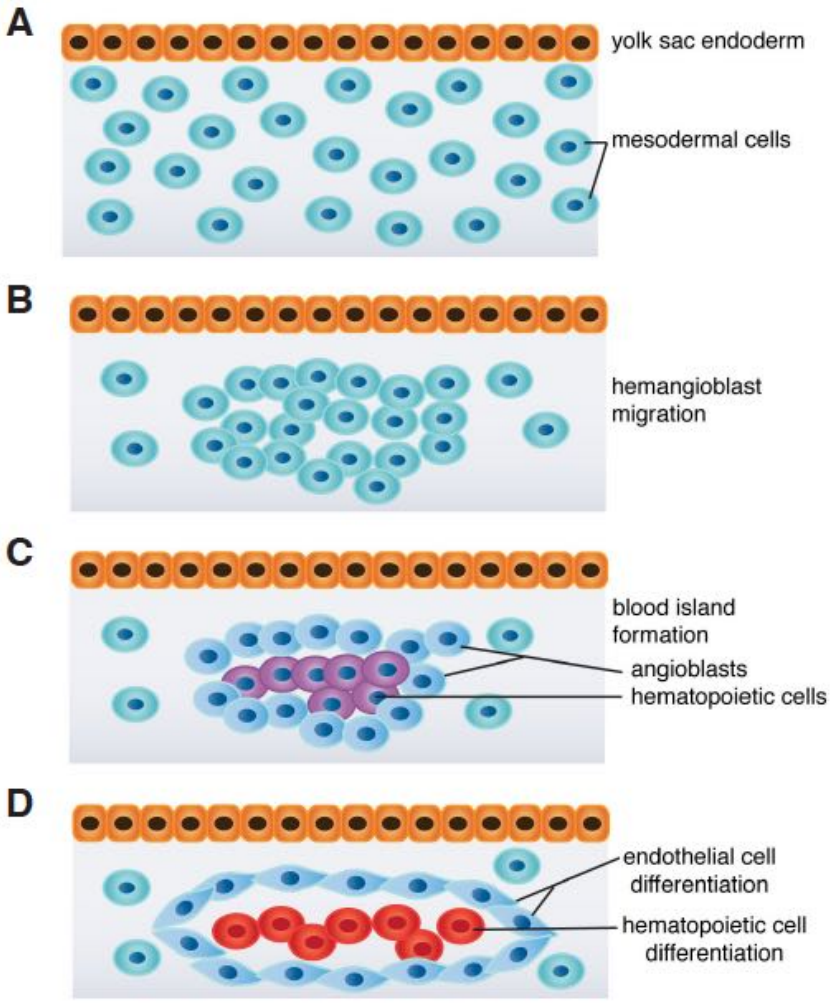

Figure I.2. Embryonic vasculogenesis and endothelial cell differentiation.

(A-B) During vasculogenesis, mesodermal cells differentiate into hemangioblasts, whose migration to the yolk sac leads to the formation of primitive blood islands. (C) In the blood islands, the peripheral hemangioblasts differentiate into endothelial cell precursor angioblasts, whereas the internal hemangioblasts differentiate into hematopoietic cells. (D) When activated, endothelial cells migrate and fuse together to become blood islands. These islands then remodel into tubular structures to generate the primary vascular plexus. Adapted from reference (11).

I.1.3.2 Angiogenesis

Angiogenesis, a process first described in tumor growth by Folkman *et al*., defines the formation of new blood vessels from pre-existing ones (12, 13). Angiogenesis takes place throughout development and adulthood, whereas vasculogenesis is generally thought to occur during a limited period early in embryonic development.

I.1.3.2.1 Types of angiogenesis

There are two main types of angiogenesis: sprouting and non-sprouting angiogenesis (also called intussusceptive angiogenesis). Both types contribute to the vascularization of organs and tissues during development (14, 15). Sprouting angiogenesis is defined as a general mechanism of vessel growth that coordinates migrating endothelial tip cells and proliferative endothelial stalk cells (Figure I.3A). Intussusceptive angiogenesis is defined as the process in which vessels are split from preexisting ones via the formation of transvascular tissue pillars (Figure I.3B) (16-18).

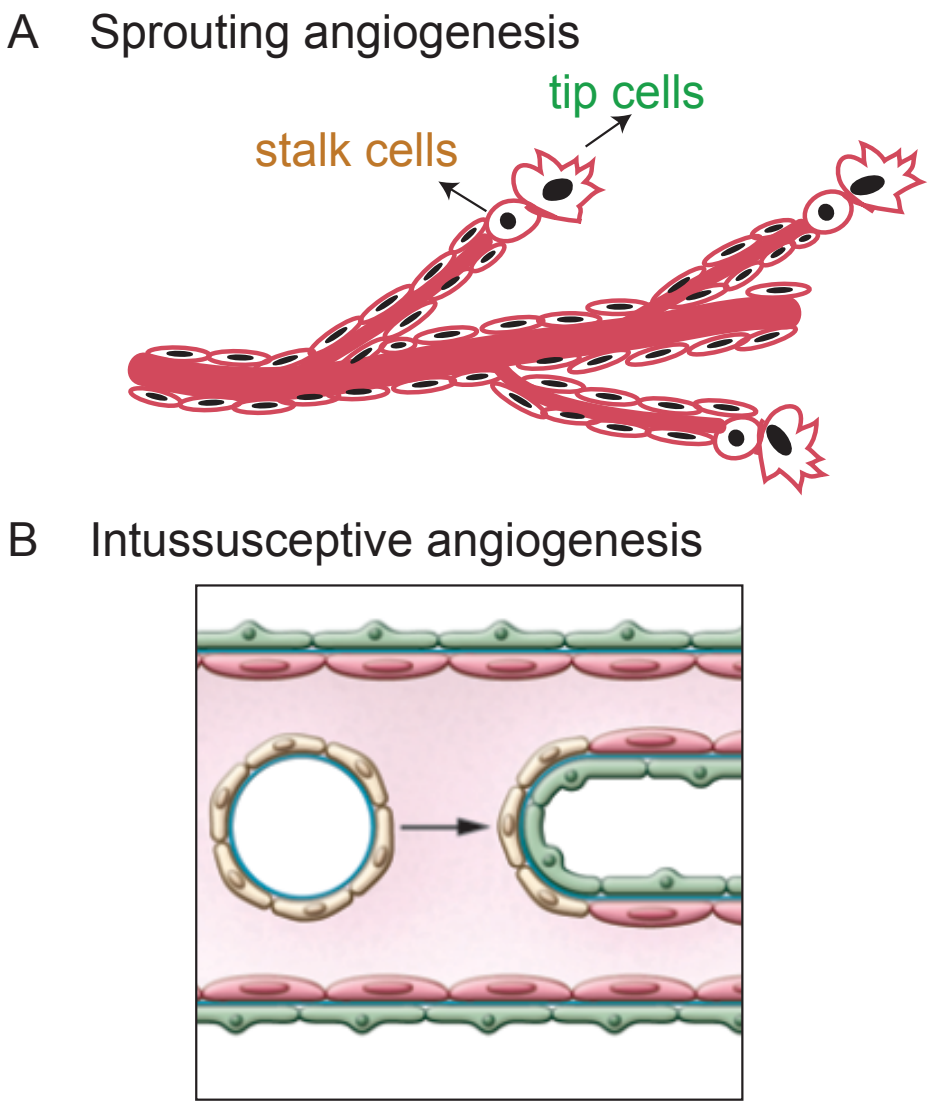

Figure I.3. Sprouting angiogenesis and intrussusceptive angiogenesis.

(A) During sprouting angiogenesis, tip cells produce filopodia in response to angiogenic growth factors, whereas stalk cells produce less filopodia but proliferate to form vascular lumen in response to stimuli. (B) During intussusceptive angiogenesis, pre-existing vessels split into two daughter vessels via the insertion of a tissue pillar. Adapted from reference (19).

I.1.3.2.1.1 Sprouting angiogenesis

Sprouting angiogenesis is a fundamental mechanism of vessel growth that takes place in several steps. In the presence of angiogenic growth factors, endothelial cells are activated and secrete proteases such as extracellular matrix metalloproteinases (MMPs); this leads to ECM degradation which allows endothelial cells to escape from the vessel wall and move towards the connective tissues. The endothelial cells that migrate at the vascular front are called tip cells. Tip cells contain long filopodia, membrane projections that assembled from parallel actin filaments in tight bundles that probe the environment for directional cues such as growth factors. Stalk cells are the endothelial cells that follow tip cells; they produce less filopodia but proliferate more in response to growth factors (20, 21). Activated endothelial cells can migrate into the surrounding matrix to form sprouts to connect with neighbouring vessels. The sprouts are able to establish lumens in which endothelial cells are differentiated to accommodate the local environment. Finally they mature by remodeling into a complex vascular network to co-ordinate with the existing surrounding vascular network that surrounds them. In summary, sprouting angiogenesis includes endothelial cell migration, proliferation, proteolytic degradation of ECM and the subsequent formation of capillary tubes (22).

One of the main characteristics of sprouting angiogenesis is the specification of endothelial cells into tip cells and stalk cells (23). Tip cells are migratory and polarized, whereas stalk cells are more involved in the formation of tubes, branches and vascular lumens, as well as the establishment of junctions with neighbor cells and the synthesis of basement membrane components (24, 25). Specialization of these two types of endothelial cells is transient and reversible. Once a vessel branch is formed, the active sprouting endothelial cells including both tip cells and stalk cells can ultimately transit to phalanx cells, the most quiescent endothelial cells

which are luminized and non-proliferating. This last process further promotes vessel integrity and vasculature stabilization through an increase in cell adhesion and the dampening response produced by stimulatory factors (26, 27).

I.1.3.2.2.2 Non-sprouting (Intussusceptive) angiogenesis

Intussusceptive angiogenesis was first described in a study that observed the appearance in the capillary network of tiny pillars on rat lung microvasculature in casts by scanning electron microscopy (18). It may take place as the result of proliferation of endothelial cells inside the vessel lumen and the subsequent fusion and splitting of the capillaries. In fact, both sprouting and intussusceptive angiogenesis can concur in the vascularization of organs or tissues—including lung, heart and yolk sac–during development, depending on the number of vessels already present when the organ starts to grow quickly. For example, intussusceptive angiogenesis predominates in the lung which contains endothelial precursors and is already vascularized by vasculogenesis, whereas sprouting angiogenesis happens in the brain anlage where there are no angioblasts (15).

During intussusceptive angiogenesis, four phases are necessary for the capillary wall to extend into the lumen and split a single vessel in two. First, a contact between the two facing capillary walls is established. Second, the endothelial cell junctions are permeabilized to allow growth factors and inflammatory cells to migrate into the lumen. Third, a core, filled with cells such as pericytes and myofibroblasts, is formed between the two new vessels at the contact region. During this phase, collagen fibers in the core provide ECM for the growth of the vessel lumen. Finally, the core is removed and a new vessel is formed. Intussusceptive angiogenesis is important due to its role in reorganizing pre-existing cells, which allows an increase in the number of capillaries without a corresponding increase in the number of endothelial cells. Furthermore, it is

of importance during development, especially when there is lack of resources to create a rich microvasculature with new cells which will eventually develop into a new vessel (17).

I.1.3.2.2 Proliferation and migration of endothelial cells during angiogenesis

During angiogenesis, growth factors and chemokines can stimulate endothelial cells to break out of their stable position in the vessel wall and coordinate sprouting, branching and new lumenized network formation (28-30). These processes require the coordination of endothelial cell proliferation, migration and polarization in response to a set of molecular cues that are integrated in both space and time.

Endothelial cell proliferation is important during angiogenesis to enable sprouts to expand in length and diameter, and form new vessels. A useful tool to assess endothelial cell proliferation is BrdU labeling, a technique commonly used in the detection of proliferating cells in living tissues. BrdU, a marker of S phase entry, can be incorporated into newly synthesized DNA during cell replication and be detected by specific antibody (31).

Endothelial cell migration can be driven by growth factors such as VEGF and basic fibroblast growth factor (bFGF), a process called chemotaxis or directional migration. Organogenesis, wound healing and immune responses are orchestrated of direction cell migration toward specific locations. During directional migration, endothelial cells adopt a front-rear polarized morphology where their leading edge form membrane protrusions and attach to the underlying substrate though new contacts. It involves the following main events (Figure I.4). First, at the cell front, actin assembly drives the protrusion of lamellipodia and filopodia to induce the extension of cell shape. Filopodia are membrane projections filled with actin filaments whereas

lamellipodia are cytoplasmic protrusions formed at the leading edge of migrating cells (32). Second, the protrusion is attached to the focal adhesion, a multimeric protein complex that provides an adhesive link between the intracellular actin and the ECM. Third, the cell body is contracted by stress fibers to allow its forward progress. Stress fibers are actin microfilaments located at the rear of the cell, which contract and pull the trailing edge forward to keep up with the rest of the cell. Finally, adhesions are dissembled and the rear of the cell is released (29, 33).

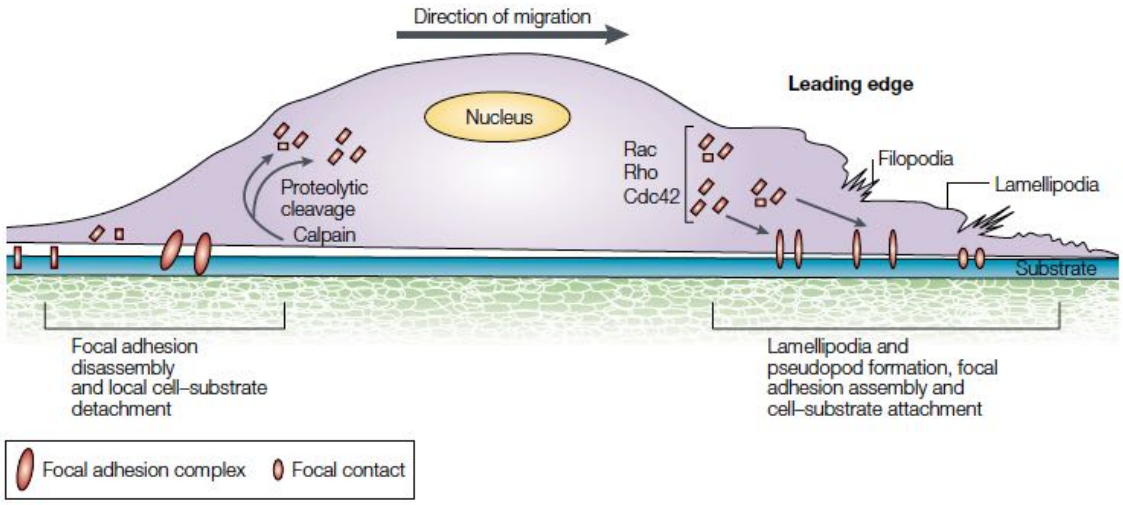

Figure I.4. Directed migration process of a cell.

For a cell to migrate, it first responds to stimuli by developing filopodia and lamellipodia structures at the leading edge. Cell morphology is modified under the control of a protein family of small GTPases (Rac1, RhoA and Cdc42) and this process is referred to as cell polarization. The lamellipodia formed at the leading edge of cells are stabilized and anchored to the underlying substrates through the de novo formation of focal adhesion attachments, which provides the necessary contraction required for the cell body to move forward. Forward cell movement is achieved through disassembly of focal adhesion and detachment at the rear of the cell, which allows retraction of the tail during active migration. Calpains are a family of calcium-activated proteases, which are able to promote disassembly of focal adhesion to facilitate cell migration. Adapted from reference (34).

As mentioned, when cells are migratory, they develop a front-rear axis of polarity. The front-rear polarity is essential for directional cell migration and is mediated by the localized activation of Rho GTPases and partition defective proteins (PARs). The Rho GTPases are composed of Cdc42, Rac and RhoA (35, 36). Both Cdc42 and Rac are at the front edge of the cell and play a role in the regulation of microtubule rearrangements. Cdc42 is involved in the formation of dynamic filopodia that sense the guidance cues at the beginning of migration while Rac affects actin polymerization associated with the formation of lamellipodia (37). RhoA, on the other hand, is active at the rear of the cells and controls the rear-end retraction, which allows forward mobilization by inducing actomyosin contractility (38). The activation of these proteins must be tightly controlled for the precision of cell orientation. PAR proteins including PAR3 and PAR6 are also widely recoganized as being involved in cell polarity (Figure I.5). The crosstalk between PAR proteins and the Rho family GTPases is critical for cell migration. Atypical protein kinase C (aPKC), another important regulator of polarity, can form a polarity complex with PAR6 at the leading edge of the cell, downstream of Cdc42. Cdc42 promotes aPKC activation when bound to PAR6. The PAR6/aPKC complex in turn phosphorylates PAR3. The abovementioned polarity complexes have been demonstrated to be involved in the initial polarization steps of endothelial cells to modulate migration (39, 40). In addition, PKCζ and β-catenin are known to function in a complex at adherens junctions and to localize at the leading edge together with PAR3 and PAR6 to promote directional and collective endothelial cell migration induced by the growth factor Ang-1 (40).

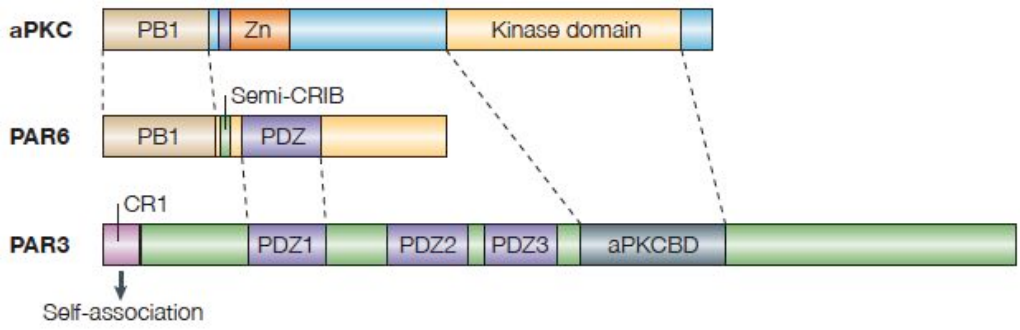

Figure I.5. Schematic representation showing the structures of polarity proteins aPKC, PAR3, and PAR6.

αPKC comprises a Ser/Thr kinase domain, a PB1 domain and a zinc-finger (Zn) motif. PAR6 is composed of several domains including a PB1 domain, semi-CRIB site and a PDZ domain. PAR3 contains three PDZ domains, a CR1 domain and a αPKC binding domain (aPKCBD). The PB1 (Phox and Bem1) domain, composed of approximately 80 amino acid residues, is conserved among animals, fungi, amoebas, and plants. The PDZ domain is a modular protein-interaction domain specialized for binding to short peptide motifs at the extreme carboxy termini of other proteins. The CRIB domain defines a domain containing the Cdc42/Rac interactive binding, and Semi-CRIB in PAR6 refers to a domain that is about half the usual length of a usual domain. The dotted lines show regions of the proteins that interact with each other. Adapted from reference (39).

I.1.3.2.3 Regulators of angiogenesis

The development of a proper vascular network is critical not only for development and differentiation during embryogenesis, but also for repair of wounded tissues and functional regeneration in the adult organism. Deregulation of angiogenesis is observed in numerous malignant, inflammatory, ischemic, infectious and immune disorders. It is implicated in several pathogeneses such as atherosclerosis, tumor development and rheumatoid arthritis (2, 41, 42).

Angiogenesis is important in tumorigenesis because the formation of new blood vessel is required for oxygen and nutrient exchange between the tumor periphery and the hypoxic core (43). Therefore, it is important to maintain a balanced angiogenesis. A considerable number of studies have revealed a variety of angiogenic factors in vertebrates including VEGF, Notch, Ang, Wnt, fibroblast growth factor (FGF) and Transforming growth factor-β (TGF-β).

VEGF is the central regulator of angiogenesis and participates in inducing proliferation, migration, permeability and survival of endothelial cells (44, 45). In physiological angiogenesis, VEGF induces angiogenic sprouting during early postnatal retinal development by controlling tip cell migration and stalk cell proliferation, depending on its spatial distribution (46). Also, *in situ* hybridization of healing wounds has shown increased expression of VEGF by epidermal keratinocytes and expression of VEGFR1 was elevated in vessels in the wound (47). In tumor angiogenesis, expression of VEGF is upregulated and the growth of tumors in mice can be inhibited by antibodies against VEGF (48).

The Notch pathway is an evolutionarily conserved signaling pathway that is required for normal embryonic development, regulation of tissue homeostasis and maintenance of stem cells in adults (49). Notch signaling controls multiple aspects of angiogenesis including cell specification (tip versus stalk cell), proliferation, motility and filopodia protrusion. Migratory tip cells express relatively high levels of the Notch ligand DLL4, whereas proliferative stalk cells express Jagged-1, a ligand which is able to activate Notch receptors (50, 51). In proliferating endothelial cells, Notch signaling is a negative regulator since evidence has shown that endothelial cell proliferation is increased when Notch signaling is suppressed (23, 52-54). Overactivation of Notch signaling reduces the migratory behavior of endothelial cells. For instance, overexpression

of the Notch intracellular domain in zebrafish inhibited the migration and generation of the filopodia of endothelial cells (53).

Angiopoietins belong to a family of vascular growth factors that are important in embryonic and postnatal angiogenesis. The most well studied angiopoietins are Ang-1 and Ang-2. Ang-1 is important for endothelial cell migration, adhesion and survival, whereas Ang-2 can disrupt the connections between the endothelium and perivascular cells, promote cell death and induce vascular regression (55). Tie-1 and Tie-2 are receptors for angiopoietins in angiogenesis and both are essential for vascular maturation (55). In Tie-1 null embryos, angiogenesis is not perturbed, however, vessels lose their integrity, leading to hemorrhage and death between E13.5 and birth (56). Tie-2 null embryos die between E9.5 and E12.5, and the vasculature remains poorly organized with only few numbers of endothelial cells and vessel branches (57). Upon Ang-1 binding, Tie-2 receptor molecules cluster to bring their kinase domains in close proximity and to allow trans-phosporylation and the initiation of downstream signal transduction (55).

Additionally, Wnts, a family of secreted growth factors, have been shown to function in angiogenesis and Wnt signaling can promote angiogenesis (58). Inactivation of Norrin, a Wnt ligand, results in Norrie disease which is characterized by ocular vascular defects (59). Abnormality in Frizzled 4, a Wnt receptor, leads to defects in retinal vascular development (60).

I.1.3.3 Neovascularization by endothelial progenitor cells

The dogma that endothelial cells are exclusively differentiated from angioblast was generally accepted. However, the identification of circulating endothelial progenitor cells (EPCs) in human peripheral blood has challenged this dogma (61). The progenitor cells are EPCs, which

express various markers including endothelial markers such as VE-Cadherin, CD31/PECAM-1, KDR, TIE-2, and Flk-1, as well as stem cell markers CD133 and CD45.

Homing and differentiation of EPCs require coordinated multistep signaling events including chemoattraction, adhesion, transmigration and ultimately differentiation into endothelial cells (62). Chemokine Stromal-cell-derived factor-1 and VEGF are known factors that initiate the mobilization of EPCs. EPCs reside within a stem cell niche in the bone marrow. MMP-9, a matrix metalloproteinase involved in the degradation of the ECM, causes the release of soluble Kit ligand from EPCs in the bone marrow, allowing EPCs to mobilize to the peripheral circulation (63). Once at the site, adhesion to activated endothelial cells leads to the transmigration of EPCs through the endothelial cell monolayer, which is an important step for homing of progenitor cells (64, 65). EPCs can then invade and migrate to the same site, where they mature into functional endothelial cells (66).

EPCs are capable of mobilizing into active sites of angiogenesis and function in neovascularization in animal models under ischemia. In mice, EPCs were demonstrated to promote corneal neovascularization by mobilizing in bone marrow in response to ischemia and granulocyte macrophage-colony stimulating factor (GM-CSF) (67). In pathological conditions, such as acute myocardial infarction, the mobilization of EPCs from bone marrow increases in order to induce re-endothelialization and neovascularization. It is believed this could potentially become a promising clinical therapy for vascular repair in coronary artery diseases (61).

I.1.4 Endothelial cell-cell junctions and cell permeability

Cell junctions are composed of protein complexes that provide contact and communication between neighbouring cells. For example, endothelial cells are linked by adhesive proteins that are organized in a complex network of junction structures, mostly composed of adherens, tight and gap junctional complexes (68-70) (Figure I.6). All three types of endothelial cell junctions are required for the maintenance of vascular integrity, which is important for tissue homeostasis.

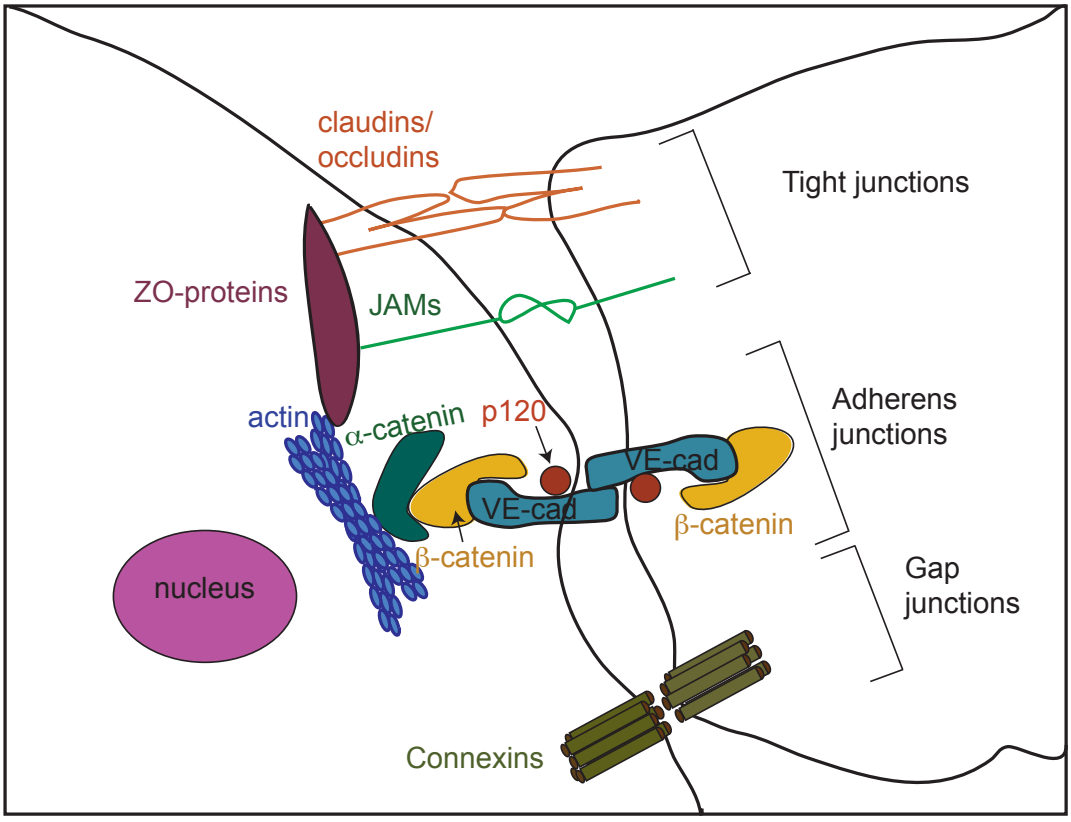

Figure I.6. Schematic representation of endothelial cell junctions.

Tight junctions are composed of claudins, occludins, JAMs, and ZO proteins. In adherens junctions, VE-cadherin mediates the adhesion between adjacent endothelial cells. The cytoplasmic tail of VE-cadherin binds to β-catenin and p120; this complex in turn binds α-catenin and then mediates the formation of actin bundles. Connexins in gap junctions mediate the transportation of small molecules between cells.

I.1.4.1 Adherens junctions

In endothelial cells, adherens junctions consist of the transmembrane protein cadherins including VE-, N- and M-cadherin and intracellular components such as p120-catenin, β-catenin and α-catenin that link this protein to the actin cytoskeleton (71) (Figure I.6). Adherens junctions perform multiple functions in the establishment of cell–cell adhesion and signal transduction, and are important for cell adhesion, cell survival, cell growth, and paracellular permeability to circulating leukocytes and solutes (68, 72-74).

I.1.4.1.1 VE-cadherin

The cadherin family includes the calcium-dependent adhesion molecules expressed in different tissues. E-cadherin is mostly present in epithelial cells, whereas N-cadherin can be found in the nervous system, smooth muscle cells, fibroblasts and endothelial cells. In endothelial cells, N-cadherin has been found on the cell membrane and clustered at cell-cell junctions (75, 76). VE-cadherin is expressed only in the endothelial cells as an exclusive signature and is one of the first markers expressed in endothelial cell progenitors when they start to be committed to the endothelial lineage (77).

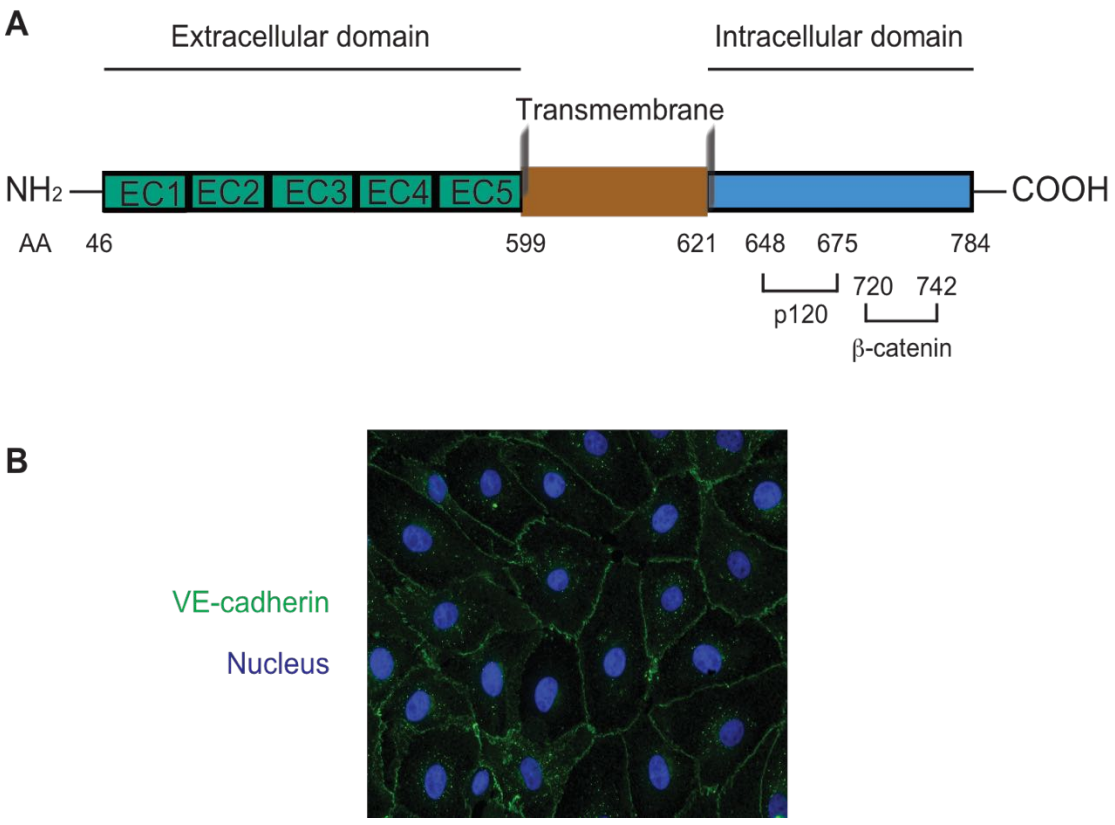

Figure I.7. Schematic structure and cellular localization of VE-cadherin.

(A) Primary structure of mouse VE-cadherin. β-catenin binds to the amino acid sites 720-742 whereas p120-catenin interacts with the juxta-membrane domain amino acid sites 648-675. AA: amimo acide. EC (1, 2, 3…5): extracellular domain (1, 2, 3…5). (B) Immunofluorescence microscopy image of VE-cadherin localization in confluent bovine artery endothelial cells. VE-cadherin is recognized by Goat anti-VE-cadherin (C19) and shown in green and DAPI labeled nucleus in blue. DAPI: 4',6-diamidino-2-phenylindole.

VE-cadherin contains five extracellular repeats (EC1-5), a transmembrane domain and a well-conserved cytoplasmic tail (Figure I.7A). VE-cadherin is localized at cell-cell contacts in confluent endothelial cells (Figure I.7B), where it forms calcium-dependent hemophilic adhesions with adjacent cells in the extracellular domain. The cytoplasmic tail of VE-cadherin can be divided into two regions: a juxtamembrane domain that binds to p120-catenin in order to regulate adherens junction stability and a catenin-binding domain that binds to β-catenin to link cadherins to the actin filament (78, 79). Phosphorylation of Tyr658 in the juxtamembrane domain was sufficient to prevent the binding of p120-catenin, and phosphorylation of Tyr731 at the catenin-binding domain caused dissociation of β-catenin (80).

During embryogenesis, VE-cadherin contributes to the remodeling and maturation of the vasculature (81-84). Deletion of the gene *Cdh5*, which encodes for VE-cadherin, results in early embryonic lethality due to defects in vascular remodeling and integrity (85). *In vivo*, the administration of blocking antibodies against VE-cadherin results in a dramatic increase in vascular permeability and hemorrhages during angiogenesis (86, 87). *In vitro*, VE-cadherin mediates cell contact inhibition of cell growth in endothelial cells and the presence of the last 82 amino acids at the C-terminus of the protein is required for this inhibitory effect (88).

I.1.4.1.2 β-catenin

β-catenin protein was initially identified as a ~90 kDa protein that associates with the cytoplasmic tail of E-cadherin (89, 90). β-catenin is expressed in almost all human tissues (91). In the adherens junctions of endothelial cells, β-catenin acts as a linker between the transmembrane protein VE-cadherin and the cytoplasmic protein α-catenin (72, 92-105).

The primary structure of the human β-catenin protein is composed of an NH2-terminal portion of approximately 150 amino acid, a COOH-terminal region of 115 amino acids and a central structural core of 12 repeats of 42 amino acids called armadillo repeats (ARMs) (106) (Figure I.8A). Each ARM consists of 3 helices (H1, H2 and H3), all together composed of 12 repeats stacked to form a superhelix. The superhelix contains a positively charged groove that is composed of the H3 helices and the side chains with the flanking loop regions (107) (Figure I.8B). The structure is very straight for the first 8 repeats and then curves between repeats 8 and 9 to form a pronounced cleft.

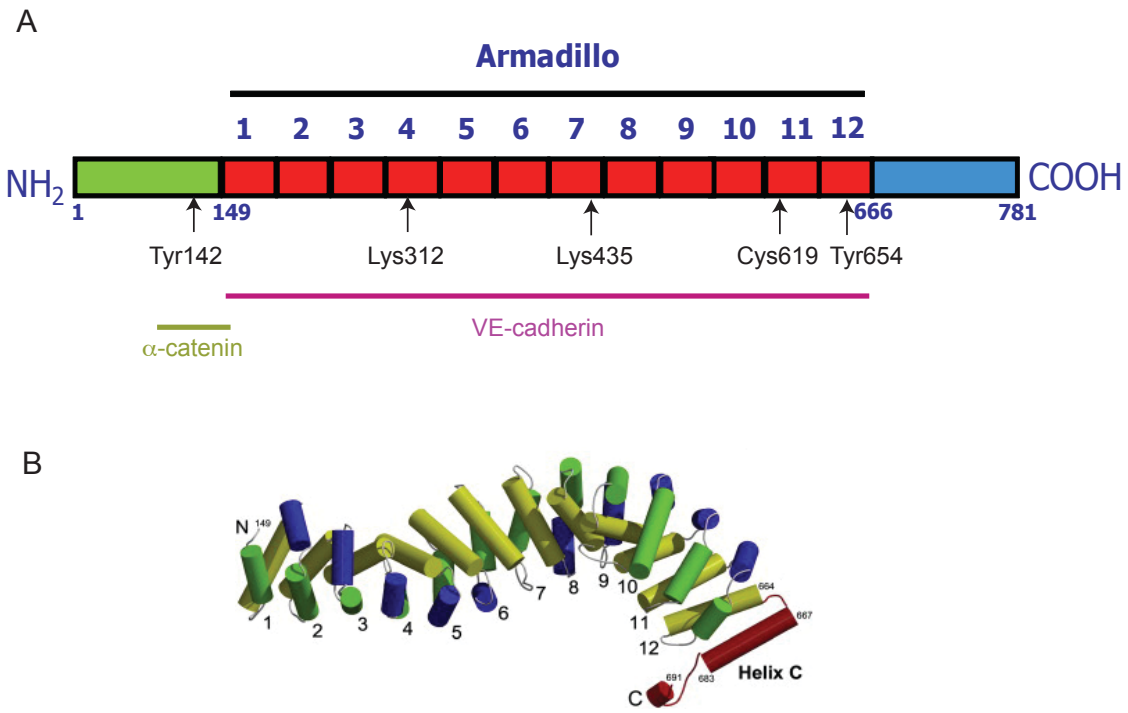

Figure I.8. Schematic representation of the primary and secondary structures of β-catenin.

(A) β-catenin is composed of an N-terminal, C-terminal and a central region that contains 12 armadillo repeats. Tyr654, Lys312, Lys435 and Cys619 of β-catenin are important amino acid sites for VE-cadherin/β-catenin association. β-catenin interacts with α-catenin via its N-terminal region, where the phosphorylation site Tyr142 is located. (B) The β-catenin crystal structure (residues 138–781) shows that each armadillo repeat is composed of three helices that are shown as blue (H1), green (H2) and yellow (H3) cylinders, and the C-terminal domain is shown in red. The C-terminal domain α-helix is called helix C. (B) is adapted from reference (108).

The β-catenin–cadherin interaction surface spans the entire length of the 12 ARM repeat domain of β-catenin (109). The crystal structure of E-cadherin/β-catenin has been well characterized and it shows that the β-catenin Tyr654 site forms a hydrogen bond with the E-cadherin Asp665 site. Phosphorylation of Tyr654 on β-catenin causes E-cadherin to dissociate from β-catenin probably because the phosphate group is too large to be accommodated in the interface and the negatively charged residue electrostatically repels E-cadherin Asp665 (109). Likewise, in endothelial cells, phosphorylation of β-catenin dramatically decreases its binding to VE-cadherin and disassembles the junctions, indicating that tyrosine phosphorylation of β-catenin can modify junctional stability (110). In addition, the Asp674 and Glu682 sites of E-cadherin form salt bridges with the β-catenin Lys435 and Lys312 residues, respectively, contributing to the formation of a complex (109, 111).

β-catenin binds α-catenin with its distal parts of the N-terminal and the adjacent first ARM repeat (112). Tyrosine kinases Fyn, Fer, or c-Met can phosphorylate Tyr142 site of β-catenin that is located in the binding domain and significantly decreasing α-catenin binding to β-catenin (113-115).

β-catenin has been implicated in the development of the vascular system. β-catenin null-mutant embryos show a loss of formation of the mesoderm, suggesting that β-catenin plays a vital role in mouse development during the gastrulation stage (116). Endothelial-specific deletion of the β-catenin gene in mice is embryonic lethal due to defects in vascularization and the development of the heart (117, 118). Moreover, β-catenin is a dual functional protein: in addition to its role in the regulation of the coordination of the cell-cell junction, β-catenin is an important component in

Wnt/β-catenin signaling pathway where it plays a role in the regulation of gene transcription. The role of β-catenin in the Wnt signaling pathway will be discussed in detail in section I.2.3.3.2.

I.1.4.1.3 α-catenin

α-catenin is an actin-binding and -bundling protein that contains a β-catenin binding domain in the N-terminus and actin-binding sites in the C-terminus (112, 119-121). It links the cadherin-β-catenin complex to the actin-based cytoskeleton by binding directly to filamentous actin (F-actin) or by interacting with the actin-binding proteins (93, 95, 96). Actin is a major cytoskeletal component of endothelial cells, which exists as globular actin (G-actin) and as a filamentous polymer called F-actin, a linear chain of G-actin subunits (122, 123).

α-catenin plays an important role in intercellular adhesion during development and differentiation. In *Drosophila*, cell adhesion is disrupted when α-catenin contains a mutation in the binding site for Armadillo, the homologue of β-catenin in *Drosophila* (124).

I.1.4.2 Tight junctions

Tight junctions are formed after adherens junctions during intracellular contact formation (125). Tight junctions create a series of contacts between the adjacent cells to close the extracellular space and form a tight intercellular barrier. For example, tight junctions play a central role in establishing the blood-brain barrier (BBB), a highly selective permeable barrier formed by brain endothelial cells, which is important for neuronal function (126). In fact, the complexity in the composition of tight junctions differs across various vasculatures. Endothelial cells in large

arteries generally possess more developed tight junctions compared to those in capillaries and venules, due to exposure to high rates of blood flow (127). In addition, the barrier function of tight junctions is to transport between cells and help to maintain cell polarity between the luminal and the abluminal side of the endothelial cells by separating their apical and basolateral compartments (128).

Tight junctions are composed of claudins, occludins, junctional adhesion molecules (JAMs) and zonula occludens (ZOs) (Figure I.6). Claudin family members are the major transmembrane components in the endothelial cell tight junctions that are located at the most apical part of the lateral surface (129). Claudins exhibit hemophilic or heterophilic adhesive properties through their extracellular domains and directly regulate the barrier function of tight junctions (130). Deletion of the claudin-5 gene in mice results in post-natal death due to a defective BBB (131). Occludin is another transmembrane protein in tight junctions containing two extracellular loops (132). Occludin is associated with increased tight junction function and is highly expressed in the BBB. Downregulation of occludin is associated with several diseases including stroke, diabetes and hypoxia (70). Molecules in the JAMs family are type I transmembrane glycoproteins composed of a single transmembrane domain. The extracellular domain of JAMs folds into two immunoglobulin-like domains. JAMs play important roles in the assembly of cell-cell junctions, establishment of cell polarity and regulation of leukocyte migration through endothelial cells (133-135). ZO proteins (ZO-1, ZO-2, and ZO-3) are cytoplasmic scaffold proteins that link transmembrane proteins to the cortical actin cytoskeleton. The localization of ZO-1 in the cell periphery is positively correlated with the confluence of endothelial cells, where tight junctions are formed (136). In endothelial cells, ZO-1 has been implicated in the regulation of adherens junctions, cell migration, and barrier formation (137).

I.1.4.3 Gap junctions

Gap junctions are intercellular channels that directly connect the cytoplasm of adjacent cells in order to transport water, ions and other small molecules between cells. Thus, gap junctions play an important role in cell communication (72). Connexins (Cx), such as Cx43, Cx40 and Cx37 are the main components of gap junctions. Six Connexins combined together to form one connexon; connexons act as channels between adjacent endothelial cells (Figure I.6). Studies show that vascular endothelial cell-specific ablation of Cx43 causes hypotension associated with significant induction of plasma nitric oxide (NO), indicating a role for Cx43 in vascular homeostasis (138). Another report showed that simultaneous deletion of Cx37 and Cx40 in mice resulted in severe vascular abnormalities and eventually lethality on postnatal day 1, underscoring the importance of endothelial cell gap junctions in the development of the vasculature (139).

I.1.4.4 Endothelial cell permeability

Endothelial cells separate blood from the different tissue compartments and modulate the passage of cells and solutes between blood and tissue. Vascular permeability increases when quiescent endothelium is exposed to stimuli such as growth factors and cytokines (140, 141). The passage of macromolecules, fluids and cells through the endothelial barrier can occur either through (transcellular) or between (paracellular) cells (Figure I.9) (142).

Small molecules are generally transported by transcytosis, which requires a trafficking vesicle system that involves caveolae and their coat proteins, caveolins (143). Caveolae are invaginations of the plasma membrane, which are also a special type of lipid raft. One of the first functions attributed to caveolae in endothelial cells is the ability to transfer molecules from the lumen of blood vessels to the sub-endothelial space by transcytosis. Caveolae can be released from the plasma membrane into cytoplasm during transcytosis, with different sizes between 50 and 100 nm. Caveolae-mediated transcytosis is a critical mechanism used for the transport of albumin, insulin, low-density lipoproteins and hormones across the endothelial barrier (144, 145).

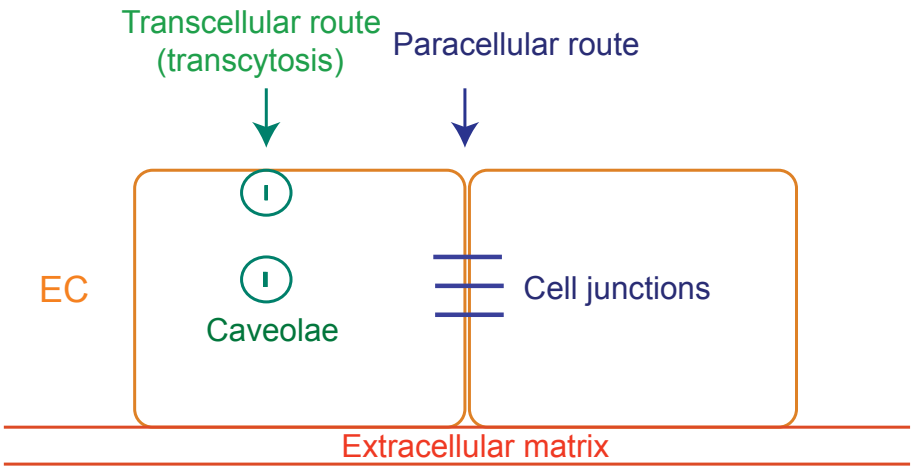

Figure I.9. Transport of molecules in endothelial cells.

The passage of macromolecules, fluids and cells through endothelial cells can take place through transcellular (through caveolae) or paracellular (through cell junctions). Adapted and modified from reference (142).

The passage of cells and large macromolecules is mostly done via the paracellular pathway and is finely controlled by the opening and closing of endothelial cell-cell junctions (142). Several factors, including VEGF, can increase endothelial cell permeability and leukocyte diapedesis through modulation of the VE-cadherin-catenin complex in the plasma membrane (146-149). The initial step of induction of the endothelial cell permeability involves the destabilization of adherens junctions (71, 150). VEGF stimulation can activate the Src family kinase (SFK) to induce tyrosine phosphorylation of VE-cadherin and β-catenin respectively, resulting in the subsequent disassembly of the adherens junctions (Figure I.10A) (151, 152). Another important mechanism in VEGF-mediated endothelial cell permeability is through VE-cadherin internalization, which also requires Src activation. In this scenario, Src kinase-induced phosphorylation of VE-cadherin provokes the internalization of VE-cadherin through clathrin-mediated endocytosis, a process in which cells absorb proteins through the inward budding of the plasma membrane vesicles containing the proteins being absorbed (80, 153-155) (Figure I.10B).

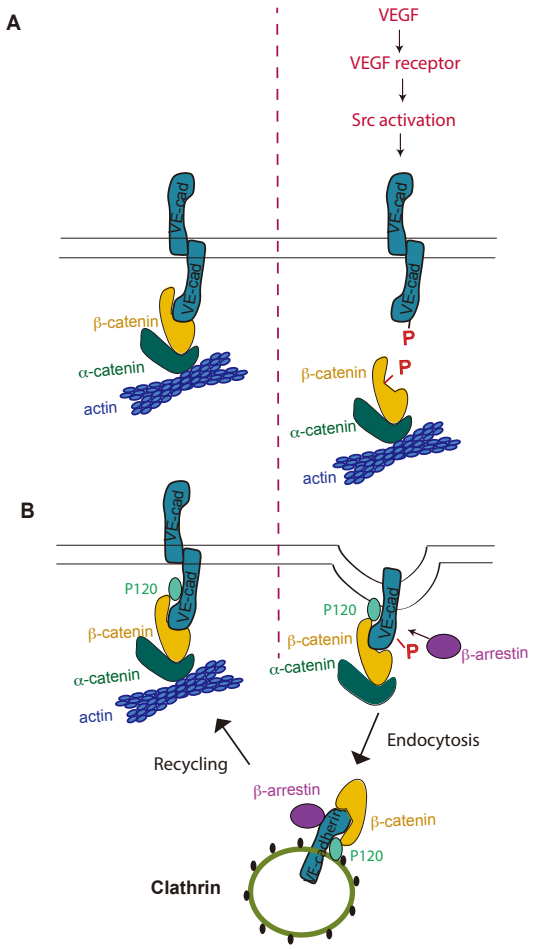

Figure I.10. The role of VE-cadherin in VEGF-induced permeability.

Homophilic interactions of VE-cadherin are expressed in adjacent endothelial cells to maintain the integrity of adherens junctions via the interaction of VE-cadherin with actin cytoskeleton using catenins. VEGF binds to its receptor and induces Src activation, resulting in the phosphorylation of VE-cadherin. (A) Src can induce not only VE-cadherin phosphorylation but also β-catenin phosphorylation, dissociating the cadherin-catenin complex in parallel. (B) Phosphorylation of VE-cadherin leads to its internalization into clathrin-coated pits. The process can cause dissociation of VE-cadherin from catenins and disassembly of cell adherens junctions, ultimately leading to increased permeability. Endosomal VE-cadherin may be recycled to the cell surface to participate in reorganization of adherens junctions in vascular remodeling (153, 156).

Endothelial cell permeability is important since the disruption of intercellular junctions between endothelial cells would increase the passage of macromolecules through the endothelium, thereby providing a pro-angiogenic microenvironment (157, 158). In summary, it has been found that endothelial cell junctions are crucial for cell permeability and angiogenesis in the literature review in this section. In the next section, I am going to discuss the importance of several signaling pathways in endothelial cells that are responsible for their functions, especially those involved in angiogenesis.

I.2. Signaling pathways in endothelial cells

I.2.1 VEGF/eNOS signaling pathway

VEGF binds to its receptors on the surface of endothelial cells and activates intracellular tyrosine kinases, triggering multiple downstream events, such as Phosphatidylinositol 3-kinases (PI3K)/Akt, phospholipase Cγ (PLCγ) and endothelial nitric oxide synthase (eNOS) signaling pathways, that regulate vascular development and function in both healthy organisms and those with diseases such as cancer.

I.2.1.1 VEGF ligands

VEGF was first identified by Senger *et al.* in 1983 as a functional factor in vascular permeability and vascular development (159). It is well regarded as an endothelial cell-specific mitogen and chemotactic agent that is part of the cysteine-knot growth-factor superfamily.

VEGF is expressed in various types of cells including endothelial cells, vascular smooth muscle cells, macrophages and tumor cells (160). VEGF can be found in mammals as part of a family of structurally homologous secreted glycoproteins: VEGF-A, VEGF-B, VEGF-C, VEGF-D and placental growth factor (P1GF) (161). All the five VEGFs can be alternatively spliced to form multiple isoforms. VEGF-A is generated by different types of cells including endothelial cells under stress conditions, and is highly expressed in tissues undergoing growth (162). The VEGF-A gene is differentially spliced into various isoforms: $VEGF_{121}$, $VEGF_{145}$, $VEGF_{165}$, $VEGF_{189}$ and $VEGF_{206}$ (Figure I.11). $VEGF_{165}$ is the most predominant and biologically active isoform and thus will be the form referred to for the remainder of the thesis.

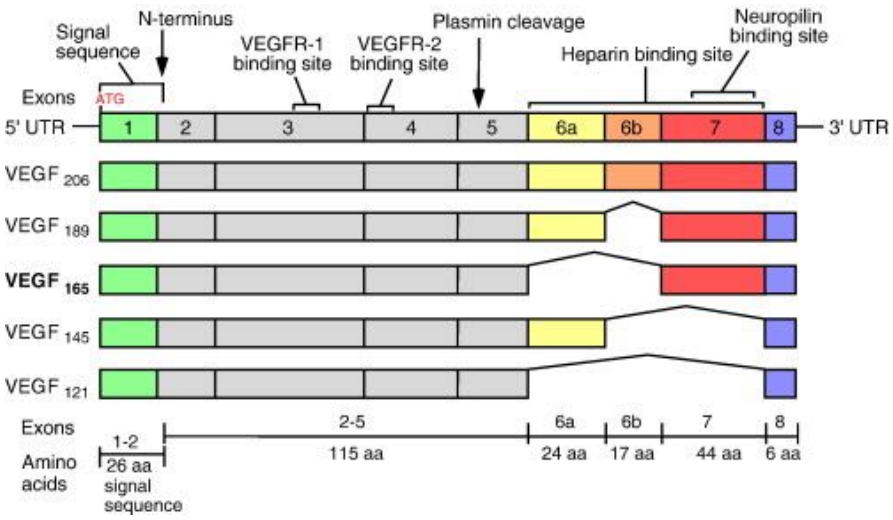

Figure I.11. Splice variants of the human VEGF-A mRNA.

There are eight exons in the VEGF-A gene and alternative splicing results in several isoforms of different sizes: $VEGF_{121}$, $VEGF_{145}$, $VEGF_{165}$, $VEGF_{189}$ and $VEGF_{206}$. Adapted from reference (163).

In humans, VEGF is usually produced as a dimeric glycoprotein: a 46 kDa homodimer composed of two 23 kDa subunits (163). Stress conditions such as hypoxia induce VEGF mRNA expression, either through the direct binding of the transcription factor hypoxia inducible factor (HIF) to the regulatory *cis*-acting enhancer elements located in the VEGF gene, or through the increased stabilization of its mRNA (164, 165). Additionally, a variety of cytokines and growth factors, including epidermal growth factor (EGF), TGF-β and interleukin 6 (IL-6), have been demonstrated to promote VEGF expression *in vitro* (166, 167). More recent studies have shown that the activation of the Wnt signaling pathway in colonic neoplasia strongly upregulates VEGF expression by modulating the T cell factor 4 binding element found in the VEGF promoter (168).

VEGF plays an important angiogenic role in inducing a directional, chemoattractive cue and is critical for endothelial cell permeability, proliferation and migration (169, 170). Deletion of the VEGF gene on one of the alleles would ultimately cause embryonic lethality due to a variety of defects in angiogenesis, suggesting that expression of VEGF on both alleles is crucial to the formation of a closed circulation system (171, 172). Overexpression of VEGF also leads to early embryonic lethality due to vascular defects, implying that embryonic survival requires the precise control of VEGF-A expression (171, 173).

Besides the activities in the vascular system, VEGF also plays a role in physiological functions such as wound healing, bone formation, hematopoiesis and development (174, 175).

I.2.1.2 VEGF receptors

VEGF factors regulate vascular development, angiogenesis and lymphangiogenesis by binding to a number of homologous receptor tyrosine kinases (RTKs) that are VEGF receptors (VEGFR), including VEGFR1/Flt-1, VEGFR2/Flk-1/KDR, and VEGFR3/Flt4, which are expressed mainly in endothelial cells in blood vessels and in the lymphatic system (176-181). VEGFRs are composed of an extracellular region of seven Ig-like domains, a short transmembrane domain, a juxtamembrane domain, an intracellular region containing a tyrosine kinase domain and a C-terminal tail (Figure I.12). VEGFRs are all essential for VEGF-induced angiogenesis since knockout mice for either isoform can lead to embryonic lethality due to vascular defects (182-185).

VEGFR1 is expressed in hematopoietic stem cells, monocytes, macrophages and vascular endothelial cells. Ablation of the VEGFR1 gene *Flt-1* in mice causes impairment of the vasculature and embryonic lethality on E9.0 (182). In the *Flt-1*-null mutant, abnormal blood island and vascular assembly was observed, suggesting that *Flt-1* is important for organization of embryonic vasculature.

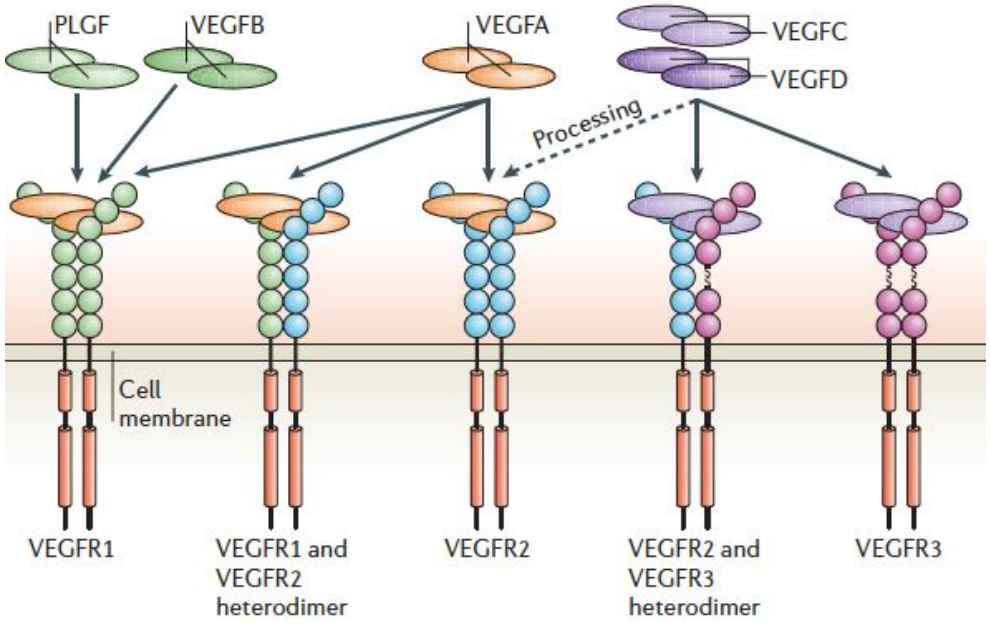

Figure I.12. The binding properties of VEGFRs (VEGFR1, 2 and 3) with different VEGF ligands.

Various VEGF ligands bind to the three VEGFRs, resulting in the formation of different VEGFR homo- or heterodimers. VEGF binds the extracellular part of the receptors that contains seven immunoglobulin-like subunits. VEGFA binds VEGFR1, VEGFR2, or the dimers of the two. The dimers formed by both VEGFB and PLGF bind VEGFR1. Proteolysis processing of VEGF-C and -D enables binding to VEGFR2. Adapted from reference (181).

VEGFR2, which is expressed in vascular and lymphatic endothelial cells, is responsible for most of the mitogenic and chemotactic effects of VEGF. VEGFR2-null mice die in utero between E8.5 and E9.5 as a result of impaired development of hematopoietic and endothelial cells, suggesting its indispensable role in the establishment of the vascular system (185). The phenotypes of VEGFR2-null mice resemble to a great extent those in VEGF-null mice. In addition, VEGFR2 expression is found in the embryonic precursors of endothelial cells, with its highest levels observed during embryonic vasculogenesis and angiogenesis, and during pathological processes such as neovascularization during tumor angiogenesis, confirming the essential role of VEGFR2 in vascular assembly (186, 187).

VEGFR3 is only found in lymphatic endothelial cells and binds both VEGF-C and VEGF-D (181). *Vegfr3-/-* mice die due to the defective vascular remodeling before the emergence of lymphatics, indicating that VEGFR3 is essential for blood vascular development (181).

In addition, neuropilins (Nrps) are a family of cell surface receptors involved in VEGF intracellular signaling transduction as co-receptors for VEGFRs (188). There are two NRP homologues including NRP1 and NRP2, which are transmembrane proteins (189, 190). NRP1 may either homomultimerize or form heteromultimers with NRP2 (188). NRP1 is able to bind and form complex with VEGFR2, whereas NRP2 is associated with VEGFR3 in a VEGFC- or VEGFD-dependent manner (191, 192).

I.2.1.3 VEGF signaling pathways

Even though the affinity of VEGFR1 for VEGF is much higher than that of VEGFR2, the effect of VEGF is mainly transduced by VEGFR2 on endothelial cells. VEGF has been shown to activate a number of different intracellular signaling pathways through VEGFR2, including protein kinase C (PKC), PI3K/Akt/PLCγ, mitogen-activated protein kinase (MAPK)/extracellular signal-regulated kinase (ERK), and p38 MAPK (Figure I.13). These signaling pathways play important roles in mediating endothelial cell survival, proliferation, migration, and permeability. Here, we focus on PI3K/Akt and PLCγ signaling because these pathways are involved in the activation of eNOS that results in the generation of NO from endothelial cells (193).

There are several phosphorylation sites which are important for the transduction of signals of VEGF (Figure I.13). For example, Tyr951 of VEGFR2, a binding site for the T-cell-specific adaptor, participates in the regulation of endothelial cell migration (194). Phosphorylation of Tyr1175 on VEGFR2 is essential for VEGF-induced PI3K and PLCγ activation and the downstream events (181). Additionally, Tyr801 is also an autophosphorylation site of VEGFR2 and is essential for activation of PI3K/eNOS signaling and generation of NO (193).

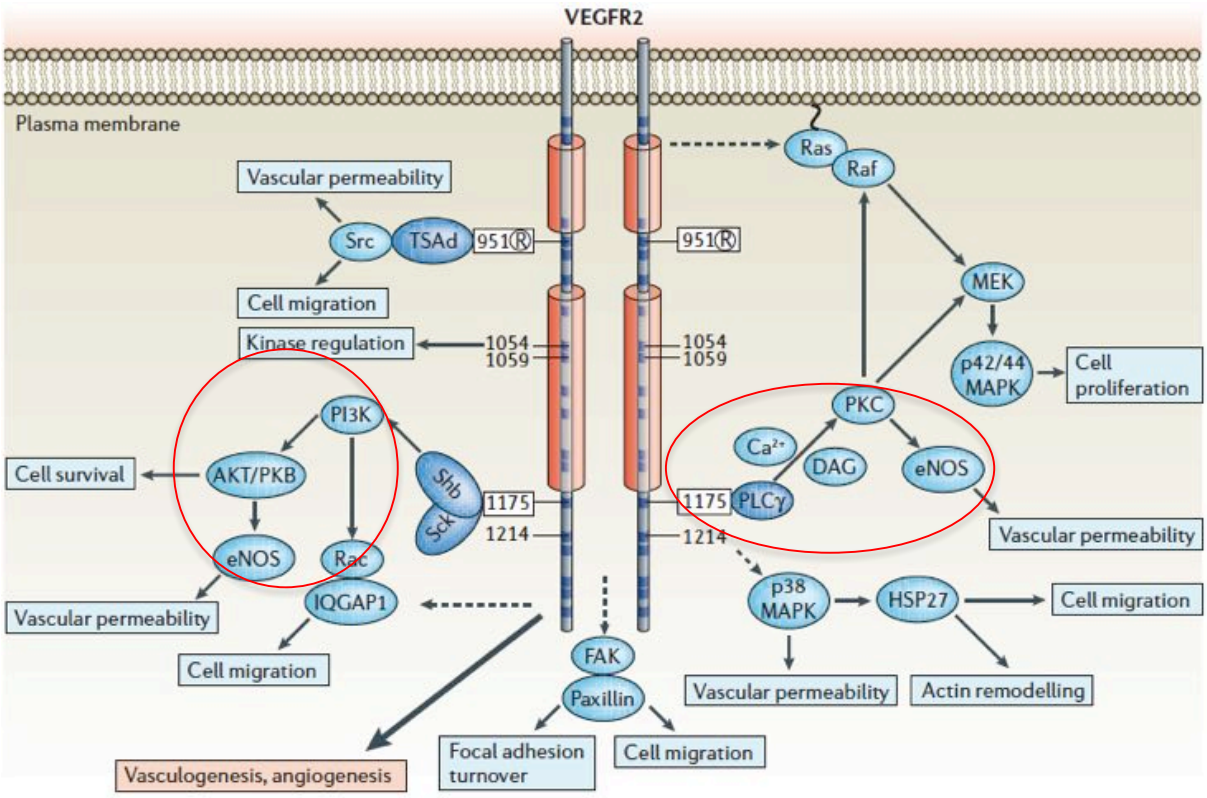

Figure I.13. Regulation of cell viability, cell migration, cell proliferation and vascular permeability via VEGFR2 signaling pathways.

Upon binding of VEGF, VEGFR2 is phosphorylated on several sites including Tyr951, Tyr1054, Tyr1059, Tyr1175 and Tyr1214. Phosphorylation of these sites on VEGFR2 is required to trigger signaling pathways such as PI3K, PLCγ, Src, Ras and p38; these pathways are important in cell survival, cell migration, cell proliferation, vascular permeability, focal adhesion turnover and actin remodeling. Adapted from reference (181).

I.2.1.3.1 PI3K/Akt signaling

Phosphatidylinositol 3-kinases (PI3K) are lipid kinase enzymes that function as a master regulator in a variety of signaling pathways. VEGF can activate PI3K, via several pathways including tyrosine kinases Src and FAK, adaptor proteins Gab1, Shb (SH2 and β-cells) and IQGAP1, or by direct binding of PI3K to phosphorylated Y1175 in VEGFR2 (195-202).

Akt (also known as protein kinase B, or PKB) is a serine/threonine kinase that resides in the cytosol in an active form and translocates to the plasma membrane when the cell is stimulated. Akt is a signaling intermediate protein downstream of PI3K that is important in VEGF-regulated endothelial cell biology. Three isoforms of Akt (Akt1-3) are expressed in endothelial cells and are activated by either PI3K through phosphoinositide-dependent kinase 1 or by inhibition of the phosphatase PTEN (194). In angiogenesis, activated Akt functions in angiogenesis in regulating endothelial cell survival, proliferation, and vascular permeability (203).

I.2.1.3.2 PLCγ signaling

VEGF induces phosphorylation of Y1175 at the cytoplasmic tail of VEGFR2, which further leads to binding of PLCγ. Phosphorylation of PLCγ leads to an increase of its enzymatic activity. PLCγ generates diacylglycerol (DAG) and inositol 1, 4, 5-trisphosphate (IP_3) by hydrolyzation of phospholipid phosphatidylinositol (4, 5)-bisphosphate (PIP_2). The generated IP_3 regulates the intracellular levels of Ca^{2+}. This in turn promotes activation of PKCγ followed by downstream activation of eNOS or ERK. PLCγ is a key mediator of VEGFR2-dependent proliferation. Indeed, PLCγ-deficient mice embryos die at approximately E9.0 with remarkably diminished vasculogenesis (204, 205).

I.2.1.3.3 eNOS activation

NO is a lipophilic, highly diffusible, and short-lived physiological messenger that has been defined as a classic relaxing factor (206, 207). NO is synthesized by nitric oxide synthase (NOS) from L-arginine and molecular oxygen. Oxygen first binds to the heme group of NOS, where it is reduced and incorporated into L-arginine to form NO and L-citrulline (208-210). There are three NOS isoforms including neuronal NOS (nNOS), inducible NOS (iNOS) and eNOS. nNOS is expressed in specific neurons of the brain and iNOS can be found in inflammatory diseases (211). eNOS is an abundant enzyme in the endothelium that produces endothelial-derived NO to maintain systemic blood pressure, modulate vascular remodeling and regulate angiogenesis (212-215). eNOS knockout mice display a mild hypertensive phenotype (213).

eNOS is a dimer composed of two identical monomers of 134 kD. It contains a reductase domain and an oxidase domain, separated by a calmodulin-binding sequence (216). The reductase domain displays binding sites for nicotinamide adenine dinucleotide phosphate (NADPH), flavin mononucleotide (FMN), and flavin adenine dinucleotide (FAD). The oxidase domain comprises binding sites for the heme group, zinc, the cofactor tetrahydrobiopterin (BH4), and the substrate L-arginine (211). Six phosphorylation sites important for eNOS enzyme activity have been identified so far: Ser116, Ser617, Ser635, Ser1179, Thr83 and Thr497.

Activation of eNOS, induced either by PLCγ-dependent Ca^{2+} influx or PI3K/Akt, can induce phosphorylation of eNOS on Ser1179 (Figure I.13). When PLCγ is activated, Ca^{2+} binds calmodulin (CaM) and promotes enzymatic activity of eNOS, resulting in the production of NO (217). Alternatively, Akt/PKB can directly phosphorylate eNOS on serine 1179 and activate the enzyme to enhance the production of NO (218).

Extensive research has shown that the regulation of eNOS activity is modulated by a range of proteins via their direct or indirect interactions with eNOS. In endothelial cells, eNOS is bound to caveolin-1, a negative regulator of eNOS enzyme activity, causing the release of NO through a consensus site in caveolae (219). The activation of eNOS involves dissociation from caveolin-1, association with heat shock protein 90 (Hsp90) and CaM binding. CaM is able to remarkably displace eNOS from its inhibitor caveolin-1. Hsp90 has been demonstrated to facilitate this displacement, too (220). In addition, AHA1, the activator of Hsp90 ATPase increases the association of eNOS and Hsp90, which contributes to increased eNOS activity, NO production and endothelial cell migration (221). Subcellular distribution of eNOS affects its enzymatic activity. For instance, localization of eNOS to either the intracellular membrane or the Golgi is necessary for VEGF-induced NO production (222).

I.2.2. NO signaling: the roles of cGMP and S-nitrosylation

NO has drawn a lot of attention due to its regulatory roles in many pathophysiological conditions such as cell viability, proliferation, migration, differentiation, leukocyte endothelial interactions, platelet aggregation and angiogenesis (223, 224). NO signals and transduces its biological activities through both cyclic guanosine monophosphate (cGMP)-dependent and – independent mechanisms.

I.2.2.1 NO/cGMP

The receptor for the signaling molecule NO is the soluble guanylate cyclase (sGC), an enzyme composed of two homologous hemes. NO can lead to at least a 200-fold increase in sGC activity. However, ODQ (1H-[1,2,4]Oxadiazolo[4,3-a]quinoxalin-1-one), a highly selective and irreversible heme-site inhibitor of sGC (225, 226), is able to bind sGC competitively with NO.

The classic NO signaling pathway is activated when NOS-derived NO diffuses and binds to sGC, inducing a conformational change resulting in the activation of the enzyme and generation of the second messenger, cGMP (227, 228). cGMP then activates protein kinase G (PKG), which phosphorylates many downstream cellular proteins such as phospholipase C, G-proteins, Ca^{2+}-ATPase and myosin phosphatase (229).

Most of the effects of NO occur through NO-sGC signaling including its effect on physiological functions related to the cardiovascular, neuronal and gastrointestinal systems (230). For example, in the vascular system, NO-induced relaxation of vascular smooth muscle occurs predominantly through sGC and PKG (231). This signaling has also been described in terms of its role in regulating angiogenesis through its effects on endothelial cell migration (232).

I.2.2.2 S-nitrosylation, a cGMP-independent mechanism for NO signaling

I.2.2.2.1 S-nitrosylation reaction

In addition to the binding of NO to its receptor sGC, physiological NO-modification of protein may be caused by S-nitrosylation (SNO), a posttranslational modification involving covalent adduction of an NO moiety (NO•) to a thiol side chain of reactive cysteines to form an S-nitrosothiol (233, 234).

SNO can occur downstream from cellular NOS activity. For instance, activation of eNOS leads to several downstream SNO-derived proteins including eNOS itself. SNO can also be caused by NO donors such as S-Nitrosoglutathione (GSNO), S-Nitroso-N-acetyl-DL-penicillamine (SNAP) and 3,3-Bis(aminoethyl)-1-hydroxy-2-oxo-1-triazene (DETA NONOate). Among these NO donors, GSNO is thought to be the main non-protein SNO source in cells and in extracellular fluids (235). SNO of G protein-coupled receptor kinase 2 by either eNOS or GSNO is reported to attenuate the desensitization and internalization of the G protein-coupled receptors (236).

Cysteine is a unique amino acid because it contains a thiol side chain. A proposed mechanism for generating of protein or cysteine nitrosothiols in vivo is as follows:

$2RSH + O_2 + 4NO \rightarrow 2RSNO + 2NO_2 + 2H^+$ or,

$RS^{\bullet} + NO \rightarrow RSNO$

SNO is reversible, and the stability of cellular SNO proteins varies due to denitrosylation, which can be carried out by the oxidoreductase thioredoxin (Trx) and S-nitrosoglutathione reductase (GSNOR) (237-240). Therefore, SNO can finely modulate the function of the target

proteins in the intracellular environment by dynamically regulating the balance between SNO and denitrosylation of the proteins.

SNO has been implicated in the regulation of protein activity, localization and stability. SNO of proteins exerts significant influence of NO on cell signal transduction including transcription regulation, DNA repair and apoptosis (241-245). For example, SNO of caspase-3 on the catalytic site cysteine 163 causes inactivation of proteins, leading to inhibition of apoptosis (246).

I.2.2.2.2 SNO detection strategies

The use of mass spectrometry (MS) enables the detection of a 29 Da weight increase due to the replacement of hydrogen on S-H with -NO group, showing direct evidence of an S-nitrosylated cysteine on peptides. Kaneko *et al.* reported the first observation of the predominant S-NO bond cleavage using MS (247). However, the stability of SNO in each peptide is dependent on the sequence of the peptide. Therefore, the optimal voltage of ionization for different peptides should be determined separately and extensive adjustment of experimental parameters is demanded in the direct MS-based detection of SNO group.

Using MS, our group has identified several cysteine residues in β-catenin as potential SNO targets by analyzing and comparing peptides from control and GSNO-treated GST-β-catenin. During the analysis, the SNO targets were identified in the treated samples, according to the calculation of neutral loss of 29 Da (Table I.1) (156).

Peptides	GSNO treatment	Peak height (counts)	Area	Observed m/z	Experimental mass (Da)	Calculated mass (Da)
1a-^{293}FLAITTDCLQILAYGNQESK	-	1340	663	ND		2227.1194
1b-^{293}FLAITTDC_{NO}LQILAYGNQESK	+	74	27	753.0432	2256.1078	2256.1096
2a-^{377}LVQNCLWTLR	-	7780	6426	623.3286	1244.6426	1244.67
2b-^{377}LVQNC_{NO}LWTLR	+	1510	592	637.8422	1273.6698	1273.660
3a-^{436}MMVCQVGGIEALVR	-	6910	5657	753.3761	1504.7376	1504.7563
3b-^{436}MMVC_{NO}QVGGIEALVR	+	1230	452	767.8832	1533.7518	1533.7466
4a-^{454}AGDREDITEPAICALR	-	7320	3346	577.2706	1728.79	1728.8464
4b-^{454}AGDREDITEPAIC_{NO}ALR	+	1950	761	586.9572	1757.8498	1757.8366
5a-^{516}NLALCPANHAPLR	-	5810	3499	695.3645	1388.7144	1388.7346
5b-^{516}NLALC_{NO}PANHAPLR	+	2920	760	709.8724	1417.7302	1417.7248
6a-^{613}VAAGVLCELAQDK	-	8970	6001	658.8423	1315.67	1315.6806
6b-^{613}VAAGVLC_{NO}ELAQDK	+	**11000**	**3076**	673.3365	1344.6584	1344.6707

Table I.1. Analysis of nitrosylated peptides from β-catenin treated with GSNO.

Mass spectrometry analysis of control and GSNO-treated GST-β-catenin samples. Non-modified peptides from control sample and S-nitrosylated peptides from GSNO treated sample are shown. Area and peak height, statistical data of the detected S-nitrosylated peptides and unmodified counterparts are presented. The analysis shows that residues C300, C381, C439, C466, C520 and C619 of β-catenin are potential targets for SNO modification. Adapted from reference (156).

The biotin switch method, initially described by Jaffrey *et al.* in 2001, is a widely used strategy for the detection and identification of SNO proteins (303). Biotin switch is an indirect SNO detection strategy that involves substitution of S-nitrosylated cysteine residues on proteins using several main steps. First, the free cysteine residues on proteins are blocked by thiol-specific methanethiolation reagents, such as methyl methanethiosulfonate (MMTS); second, nitrosothiol bonds are selectively decomposed with ascorbate, which results in the reduction of nitrosothiols to thiols; and last, the nascent thiol groups from reducing nitrosylated cysteines are biotinylated by labeling with biotin-HPDP (N-[6-(Biotinamido)hexyl]-3′- (2′-pyridyldithio)-propionamide. The biotinylated proteins are probed with antibodies to recognize the targeted SNO proteins using immunoblotting (303).

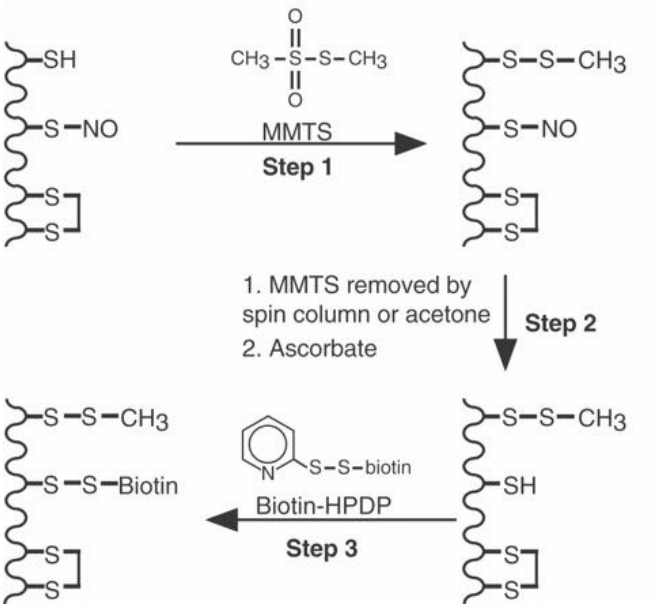

Figure I.14. Schematic diagram of the biotin-switch assay.

Adapted from reference (248).

I.2.2.2.3 SNO motif

The first suggested SNO motif is the acid-base motif comprised of acidic (Asp, Glu) and basic (Arg, His, Lys) residues; it has been established from previous observation that the thiol of Cys93 is linked to the β-submit of hemoglobin (His-Cys93-Asp). Cys93 is located between an acidic and a basic amino acid. In fact, the cysteine sulfur would be apposed to basic or acidic side chains due to the configuration of the hemoglobin tetramer. Consequently, acid-base catalysis alternatively promotes nitrosylation and denitrosylation of Cys93 in a conformation-dependent manner (249). However, the acid-base motif may reach another level of complexity. More specifically, the target cysteine may not be positioned with acidic and/or basic residues according to the primary sequence, but may emerge into a three-dimensional protein structure. For example, the acid-base motif in aquaporin-1 is composed of Asp128, Cys189 and His180, which are closer in the tertiary protein configuration than in the primary structure. The 'hydrophobic compartment' motifs for S-nitrosylation may be of particular importance.

I.2.2.2.4 Biological effects induced by protein SNO

Protein SNO has been shown to exert a wide range of biological effects including inhibiting enzyme activity, promoting cell migration and inhibiting cell proliferation.

SNO of proteins can affect their enzyme activity. Indeed, cysteine residues are present at the active site and are involved in the catalysis of numerous enzymes including cathepsin B and caspases (234, 250). In addition, de-nitrosylation of caspase-3 not only unblocks the cysteine active site, but also relieves the inhibition of protein-protein interactions regulated by the enzyme (251).

SNO has been proposed to have an inhibitory role in cancer cell proliferation. JS-K, a NO-donating prodrug, inhibited Jurkat T cell growth and β-catenin/TCF4 transcriptional activity through SNO of nuclear β-catenin (252). NO has also been shown to exert an inhibitory effect on cell proliferation and early studies suggested that this effect of NO on mitogenesis could be cGMP dependent (253-256). However, studies with sGC inhibitor ODQ have provided evidence that some of the anti-mitogenic effects of NO donors are cGMP-independent (257, 258).

There is evidence showing that SNO of proteins affects angiogenesis. SNO of the small actin-binding protein cofilin-1 by eNOS-derived NO contributes to actin cytoskeleton remodeling and VEGF-stimulated endothelial cell migration (259).

Furthermore, SNO plays an essential role in the control of gene transcription by affecting either enzyme activity or stability of transcription factors. One of the mechanisms related to the regulatory role of NO on transcriptional activity is due to SNO of critical cysteine residues in the DNA-binding region of transcriptional factors. For example, SNO of NF-κB by iNOS-derived NO can regulate gene transcription by inhibiting NF-κB-dependent DNA binding, promoter activity and transcription of target genes (260-262). Alternatively, NO can also affect transcription activity by controlling the stability of transcriptional factors. For instance, the activity of transcriptional factor Hypoxia-inducible factor 1 (HIF-1) can be upregulated by NO-mediated SNO due to the modulation of protein stabilization (263).

I.2.3 Wnt/β-catenin signaling

I.2.3.1 Wnt family proteins

Wnts are secreted cysteine-rich glycoproteins that play important roles as growth factors in both development and disease. In humans, 19 Wnt genes have been identified (Table I.2). Wnt1 is the first protein discovered and was initially named *Int1 proto-oncogene* since its locus was linked to the mouse tumor virus-driven tumorigenesis (264). *In vivo*, Wnt2 is expressed in the fetal vessels of the placenta in mice embryos at the age 7.5-8.5 days post coitum (265), and Wnt5a and Wnt10b are expressed in the blood vessels of the mouse embryonic yolk sac (266). *In vitro*, Wnt5a, Wnt7a and Wnt10b are expressed in primary endothelial cells, and Wnt5a is expressed in vascular smooth muscle cells (267).

Wnts are generally characterized as hydrophobic due to the posttranslational addition of palmitate and/or palmitoleic acid to one or two residues, such as Cys77 and/or Ser209 in Wnt3a (268, 269). The first identified biologically active Wnt proteins were wingless protein (Drosophila) and Wnt3a (270, 271). Isolation of the active form of Wnt proteins has provided insight into the function of these molecules, as demonstrated in the induction of self-renewal in hematopoietic stem cells (269).

Wnts are composed of approximately 350 amino acids. The first crystal structure of the Wnt-Frizzled receptor complex has only recently been revealed. Janda *et al.* showed that the Xenopus Wnt8 (XWnt8) contains a two-domain structure that extends and binds to the cysteine-rich domain of Frizzled-8, a Wnt receptor (272). They revealed for the first time that a protein fold to an unusual two-domain structure with the amino-terminal (NTD) and the carboxy-terminal domains (CTD) (Figure I.14). The NTD is made up of a series of α-helices and five disulfide

bridges formed by cysteine residues, whereas the CTD is mainly composed of two β-sheets that are linked by six disulfide bridges.

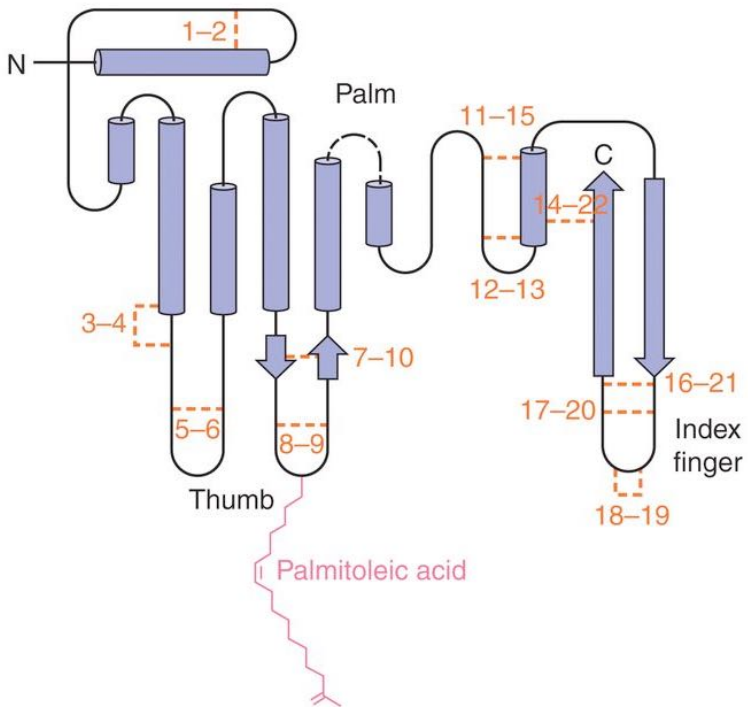

Figure I.15. Diagram of the secondary structure of XWnt8.

Disulfides formed between cysteine residues are shown as orange lines. The palmitoleic acid site is shown as the red zigzag line. The Wnt structure resembles a hand with a "thumb" and "index fingers" extended to interact with Frizzled 8 at two distinct binding sites. Adapted from reference (272).

The presence of Wnt family proteins and their signaling spans the beginning of embryonic development all the way through to adulthood, where it plays a role in tissue homeostasis (273). In addition, Wnts have been implicated in the control of stem cell self-renewal, differentiation, apoptosis, cell proliferation and cell motility (274). Deregulation of Wnts can lead to a number of multiple disorders including cancer, inflammatory diseases and neurological disorders (275).

I.2.3.2 Wnt receptors

More than 15 receptors and co-receptors that form a complex network have been identified for Wnts. Among these receptors, Frizzled (Fz) and LDL receptor-related proteins (LRPs) are the best characterized.

The ten members of the Fz family (Fz1-10) are the most widespread, high-affinity Wnt receptors. Fzs are principle receptors for Wnts, and are known to bind Wnt5A, -B, -6, -7A, -7B, -8B, -9B and -10B (275). The Fz protein is a seven-transmembrane protein that contains an extracellular region called a cysteine-rich domain (CRD). Wnts can bind the CRD of Fz to transduce signals and this process is facilitated by co-receptors such as LRPs. The structural complex of Wnt and Fz (XWnt8/Fz8-CRD) shows that there are two main sites in XWnt8 that are important for its binding with Fz8-CRD. One of those sites is a palmitoleic acid lipid group at the top of Wnt's "thumb" structure extended into a deep groove in the Fz8-CRD. The other binding site, at the top of Wnt "index finger", links with a different side of the Fz8-CRD (Figure I.14) (272).

LRPs, composed of LRP5, LRP6 and Arrow in Drosophila, are long single-pass transmembrane proteins that can act as co-receptors in Wnt/β-catenin signaling. LRP6 is the best studied LRP and its extracellular domain mediates the interaction between Wnt and Fz, resulting

in the formation of a ternary complex (276). The principle mechanism of regulation of LRP6 function is through phosphorylation of its intracellular domain, which contains five Pro-Pro-Pro-Ser/Thr-Pro motifs. Phosphorylation of these motifs is required for Wnt/β-catenin signaling (277, 278).

Besides the well-known receptors Fz and LRP, there are several other transmembrane proteins that act as specific receptors for Wnt proteins and/or Wnt agonists. Receptor tyrosine kinase-like orphan receptors such as Ror2, can bind to Wnt5A using a CRD motif similar to that of the Fz (279, 280). R-spondins, a family of cysteine-rich secreted proteins, can interact with members of the LGR5 family on the cell surface to promote Wnt signaling (281). The Derailed/Ryk receptors are single-pass transmembrane tyrosine kinases, which act as co-receptors with the Fz proteins for Wnt signaling and play important roles in the central nervous system, associated with axon guidance and neural tube formation (282). In *Drosophila*, WNT5 can signal through the Derailed/Ryk receptor and mediate signaling in the embryonic central nervous system (283).

I.2.3.3 Wnt signaling pathways

Wnt proteins have been implicated in both canonical and non-canonical pathways. However, β-catenin is only involved in the canonical Wnt signaling pathway (Figure I.15). Wnt1, Wnt3a, Wnt7b, Wnt8 and Wnt10b are generally implicated in the canonical pathway, whereas Wnt5A and Wnt11 are commonly linked to the non-canonical pathways (284).

I.2.3.3.1 Non-canonical Wnt pathways

The non-canonical Wnt pathways include the planar cell polarity (PCP) pathway and the Wnt/Ca^{2+} pathway (Figure I.15, a and c). PCP signaling activates the small GTPases RHOA and RAC1, which in turn induce activation of RHO kinase (ROCK) and JUN-N-terminal kinase (JNK), leading to actin polymerization and microtubule stabilization (285). This pathway was found to play important roles in the regulation of cell polarity and motility (286). The Wnt-Ca^{2+} pathway activates calmodulin-dependent kinase II (CAMKII), protein kinase C (PKC) and calcineurin (287). Calcineurin further activates Nuclear Factor of Activated T cells (NFAT) to induce the transcription of genes involved in both cell fate and migration. PCP and Wnt/Ca^{2+} pathways are capable of antagonizing β-catenin signaling (285).

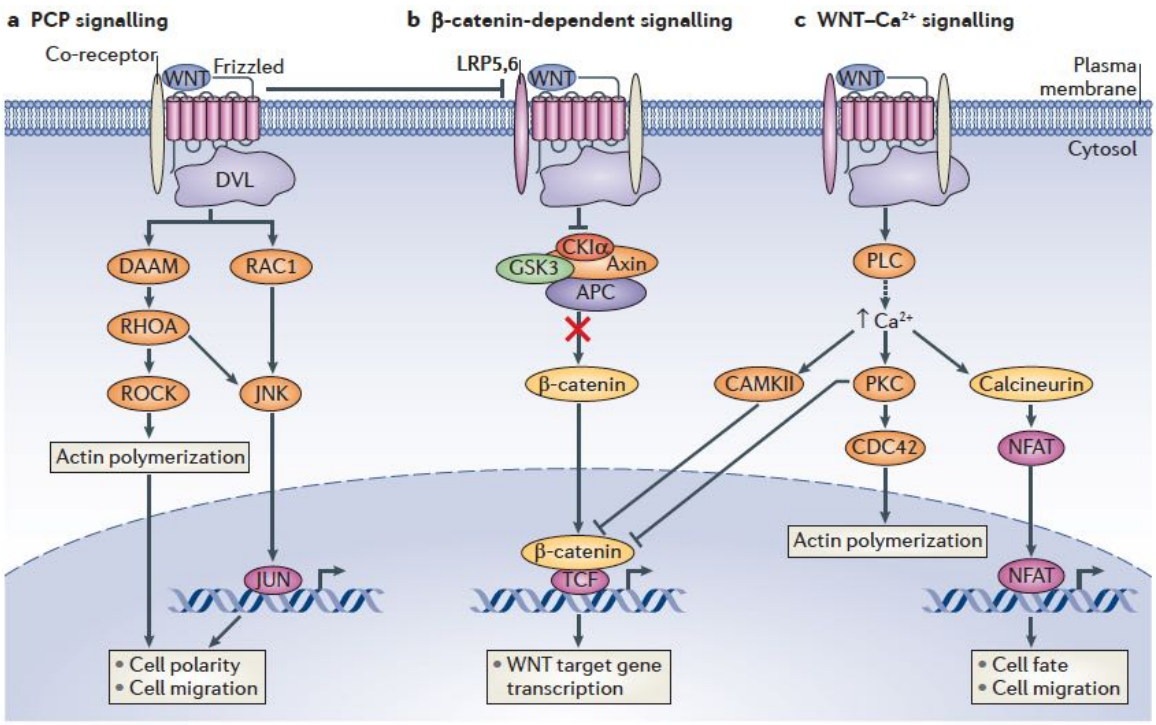

Figure I.16. Canonical and non-canonical Wnt signaling pathways.

(a) Non-canonical Wnt/PCP signaling. (b) Canonical Wnt/β-catenin signaling. (c) The non-canonical WNT-Ca2+ pathway. PCP, planar cell polarity; ROCK, RHO kinase; GSK3: glycogen synthase kinase 3; CKIα: casein kinase Iα; APC: adenomatosis polyposis coli; NFAT: nuclear factor of activated T cells. Adapted from reference (285).

I.2.3.3.2 Wnt/β-catenin pathway (Canonical Wnt pathways)

Wnt proteins such as Wnt1, Wnt3, Wnt3a, Wnt7a, Wnt7b and Wnt10b have been demonstrated to activate Wnt/β-catenin signaling, a β-catenin-dependent pathway. β-catenin has been shown to play an important role in structural and signaling functions. Alternatively, β-catenin also acts as a linker between cadherin and cytoskeleton through its binding with α-catenin. In addition, as a crucial member of the Wnt signaling pathway, when it enters into the nucleus, β-catenin binds transcriptional factors (T cell factor/Lymphoid enhancer factor) and modulates the transcription of target genes (288). But how does β-catenin switch between its roles in adhesion and transcription? We will explain this next by describing the status of β-catenin in the absence (Wnt-off state) or in the presence (Wnt-on state) of the Wnt signal.

I.2.3.3.2.1 Wnt-off state

Wnt-off state characterizes the constitutive degradation of cytosolic β-catenin in the absence of Wnt activity. When Wnt/β-catenin is not activated, β-catenin levels are regulated by a destruction complex composed of the Ser/Thr kinases Casein-Kinase 1 α (CKIα), and Glycogen Synthase Kinase-3β (GSK3β), tumor suppressor proteins adenomatous polyposis coli (APC) and Axin (289, 290). The "priming" kinase CK1α phosphorylates β-catenin on Ser45 and GSK-3β successively phosphorylates β-catenin in the degradation complex on the residues Ser33, Ser37 and Thr41 (291). APC and Axin serve as scaffold protein in sequestering β-catenin. GSK-3β directly phosphorylates APC, and the phosphorylation of APC enhances its binding to β-catenin *in vitro* (292). Axin can be phosphorylated by GSK-3β and significantly enhance the phosphorylation of β-catenin by GSK-3β by bring the two proteins into close proximity (293). Phosphorylated β-catenin can be recognized by the β-transducin repeats-containing protein (β-

TrCP1), which leads to the ubiquitination of β-catenin by an E2 ligase (289, 291, 292, 294) (Figure I.16a).

Dickkopf (Dkk) and Kremen (Krm) are antagonists in the Wnt pathway due to their role in blocking the access to the LRP co-receptor (295, 296). In addition, the Wnt-inhibitory factor (WIF-1) can directly interact with Wnts, resulting in negatively regulated Wnt signaling (297).

I.2.3.3.2.2 Wnt-on state

Recent studies show that Wnt induces plasma membrane-associated LRP6 aggregates in vertebrate cells. Activation of the canonical Wnt pathway is initiated when the Wnt ligand binds to Fz-LRP5/6, a co-receptor complex, resulting in the phosphorylation of LRP6 by CK1 (298-302) (Figure I.16b). This leads to the subsequent recruitment of dishevelled (Dvl), a phosphoprotein that acts downstream of Fz receptors and blocks the destruction complex (303-305). Dvl has been suggested to cause sequestration of the rate-limiting component Axin from the destruction complex. As a consequence, the activity of cytosolic GSK3β is inhibited and protein phosphatase 2A (PP2A) dephophsphorylates Axin and APC. Unphosporylated Axin and APC can no longer recruit β-catenin to the destruction complex for its phosphorylation (306). Failure of phosphorylation of β-catenin at Ser33/Ser37/Thr41 sites prevents its degradation by proteasome, allowing it to be stabilized in the cytoplasm (277, 307-309).

Additionally, in pathological conditions, β-catenin can also escape degradation when the N-terminal amino residues Ser33, Ser37, Thr41 and Ser45 are mutated, or when APC is mutated in colon carcinoma (310).

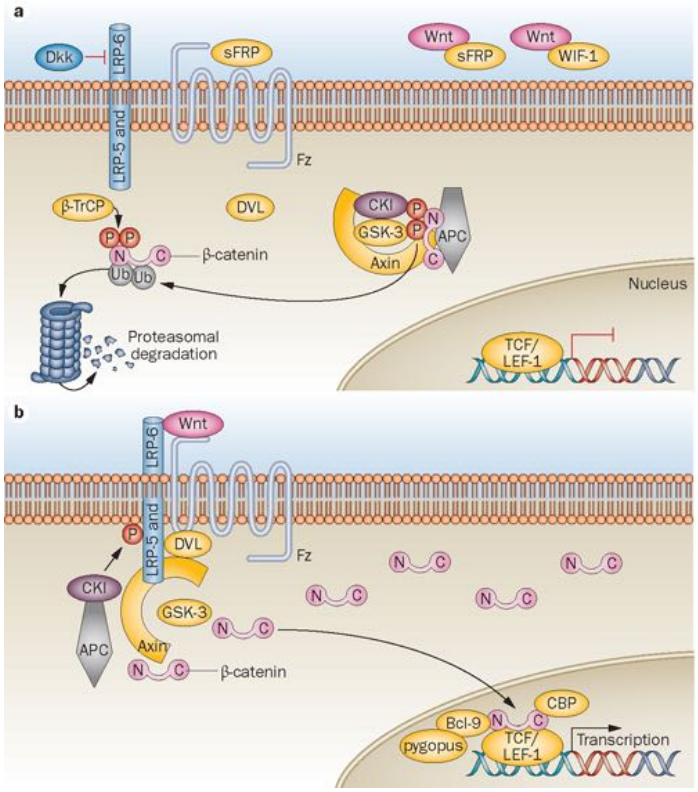

Figure I.17. The Wnt signaling cascade.

(a) In the Wnt-off state, Wnt proteins are bound to sFRP or WiF-1. Wnt receptors LRP-5 and LRP-6 are blocked by DKK proteins. In addition, noncanonical Wnts and sFRPs can bind to Fz receptors and compete with canonical Wnts. Cytoplasmic β-catenin is phosphorylated by the destruction protein complex composed of CK1, GSK-3, APC and Axin. The phosphorylation of β-catenin leads to further ubiquitination and degradation by proteasome. Wnt target genes are repressed because the transcription factors are bound to repressors. (b) In the Wnt-on state, Wnt ligands induce LRP-5 and LRP-6 phosphorylation and recruitment of Axin and DVL proteins to the plasma membrane, which disrupts the β-catenin destruction complex, leading to stabilization and translocation of β-catenin to the nucleus. Nuclear β-catenin interacts with transcriptional co-activators including Bcl-9, the pygopus homologs and CBP, to activate TCF/LEF1 transcription factors and induce expression of target genes. Abbreviations: APC, adenomatous polyposis coli protein; Bcl-9, B-cell lymphoma 9 protein; β-TrCP, F-box/WD repeat-containing protein 1A (formerly termed β-transducin repeat-containing E3 ubiquitin protein ligase); CKI, casein kinase I; CBP, cAMP response element binding protein (CREB)-binding protein; Dkk, Dickkopf protein;

DVL, dishevelled homologs; Fz, Frizzled family receptors; GSK-3, glycogen synthase kinase-3; LRP, low-density lipoprotein receptor- related proteins; TCF/LEF-1, T-cell-specific transcription factor/lymphoid enhancer- binding factor 1; sFRP, secreted frizzled-related protein; Ub, ubiquitin. Adapted from reference (311).

I.2.3.3.2.3 β-catenin enters the nucleus and binds TCF/LEF

β-catenin is stabilized by the activation of the Wnt/β-catenin signaling and can shuttle between the cytoplasm and the nucleus. Given that β-catenin does not contain a nuclear localization signal (NLS) or a nuclear export signal (NES) within its sequence, much of the research has focused on understanding how β-catenin translocates into the nucleus. β-catenin has been shown to directly interact with different nuclear pore complex components (NPCs) and pass through the nuclear pores (312). Another molecular mechanism proposed for nuclear translocation of β-catenin is through its association with the Forkhead-box transcription factor FoxM1, which directly interacts with ARM11-12 and thereby induces β-catenin nuclear import in mammalian cells (313). It is plausible that FoxM1 provides its NLS to β-catenin and induces the formation of the β-catenin-FoxM1 complex, which can be imported to the nucleus. Inside the nucleus, β-catenin-FoxM1 would then bind together with TCF on the promoter region of Wnt target genes. Hence, FoxM1 may act as a direct modulator of β-catenin-mediated transcription.

Once shuttled into the nucleus, β-catenin can activate the transcription of Wnt/β-catenin target genes but requires DNA binding partners to bind the promoters since it does not contain a DNA binding domain (108). TCF transcription factors (TCF1, TCF3, TCF4, and LEF1) are the main nuclear partners of β-catenin for its guidance to specific DNA loci. TCF transcriptional

factors can bind to a specific motif in the minor groove of the target DNA (CCTTTGAT(G/C)) via their high mobility group (HMG) domain (314). When β-catenin is not present, TCF transcriptional factors act as repressors by forming complexes with a transcriptional repressor named Groucho/TLE. However, in the presence of β-catenin, the binding of β-catenin physically displaces Groucho/TLE and converts TCF transcriptional factors into activators, transducing the Wnt signal to activate target genes (315, 316).

TCF/LEF proteins can bind the armadillo repeats 4-10 and anchor β-catenin in specific promoters of transcription (107). The salt bridges formed between Asp16 of TCF and Lys435 of β-catenin have been shown to be important both structurally and biochemically in the binding of β-catenin to the TCF4 and LEF-1 (108, 111, 317, 318). Likewise, Lys312 is also critical for the β-catenin-TCF4 interaction (Figure I.17A). In addition, the N-terminal regions, especially the Leu12 - Phe21 region of TCF4, serve as binding sites for β-catenin. In fact, Ile19 and Phe21 regroup into the hydrophobic pocket formed by residues including Cys466 at Arm8 of β-catenin (Figure I.17B).

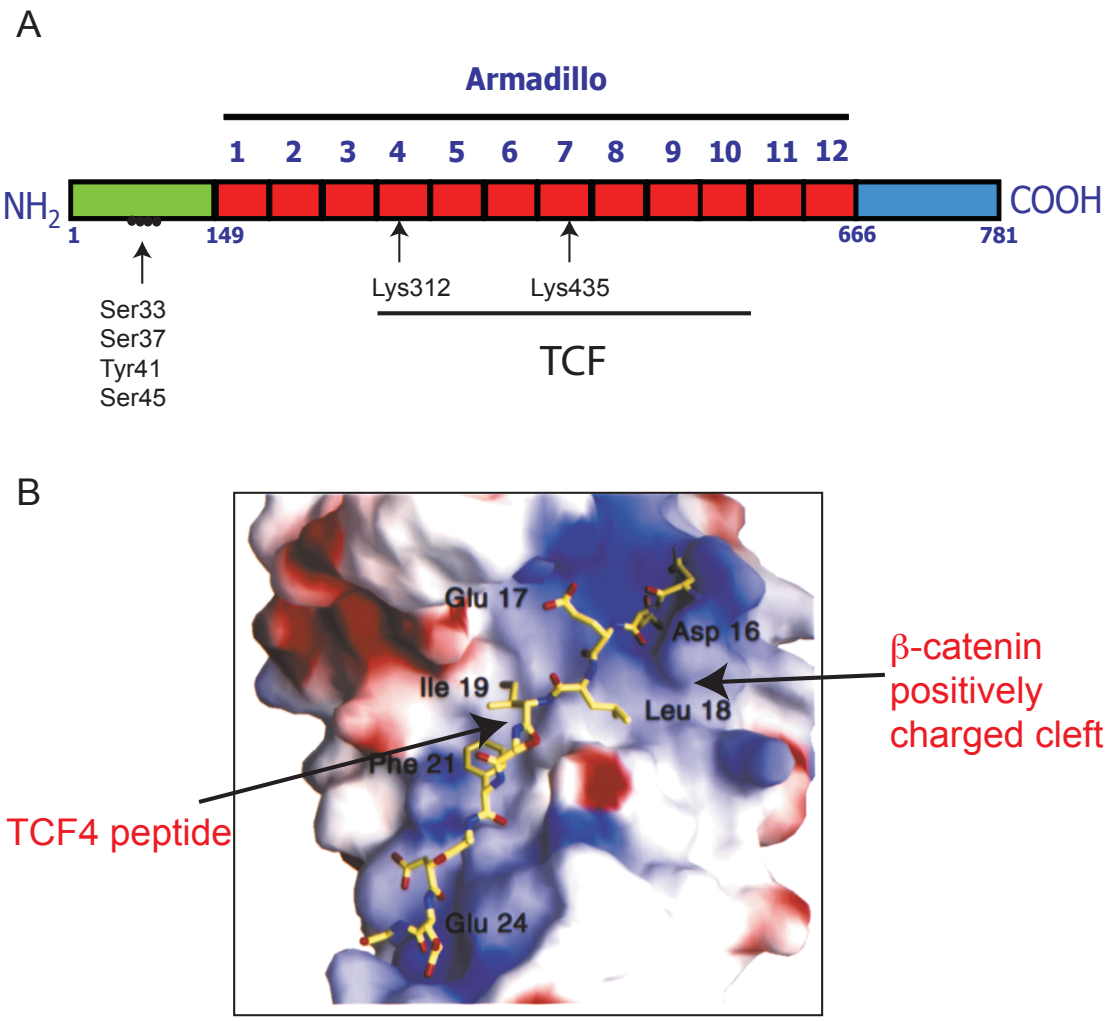

Figure I.18. β-catenin interacts with transcription factor TCF4.

(A) Schematic representation of the primary structure of β-catenin showing that TCF interacting with ARM 4-10. Ser33, Ser37, Tyr41 and Ser45 are N-terminal residues of β-catenin important for its proteasome degradation. Lys312 and Lys435 are known sites located in the ARM of β-catenin crucial for interaction between β-catenin and TCFs. (B) The TCF4's extended region, peptide 12-24, binds a positively charged groove formed by the ARM8 region of β-catenin. The color of β-catenin is shown according to its electrostatic potential (blue indicates positive charge; and red shows negative charge). Leu18, Ile19 and Phe21 residues of TCF4 form hydrophobic interactions with the center of the cleft of β-catenin. (B) is adapted from reference (317).

Moreover, β-catenin also interacts with B-Cell CLL/Lymphoma 9 (BCL9) to recruit Pygopus, a critical transcriptional co-activator in Wnt signaling (319). Many other transcriptional co-activators such as Brg1 and CBP/p300 as well as transcriptional inhibitors such as ICAT and Chibby, have been demonstrated to be partners of β-catenin in different stages of transcription (320-326).

I.2.3.3.2.4 Wnt/β-catenin target genes

In response to canonical Wnt, β-catenin is stabilized in the cytoplasm, translocated into the nucleus, and partnered with TCF/Lef family transcription factors to modulate expression of numerous target genes including *c-Myc* (327), *cyclin D1* (328, 329), *Connexin 43*, *uPAR*, *c-jun* (330), *fibronectin* and *PPAR* (331). These genes further contribute to the growth-promoting activities mediated by β-catenin signaling. Table I.3 shows a representative list of target genes of Wnt/β-catenin signaling that exist during embryonic development and in pathological conditions. *AXIN2* gene is regarded as the universal transcriptional target and a general indicator of Wnt pathway activity and Axin2 protein is expressed in almost all the tissues, especially in a number of murine and human tumors (332-334). Interestingly, Axin2 attenuates Wnt signaling due to its role in regards to β-catenin degradation, acting in a negative feedback loop (335, 336). Another well-recognized Wnt target gene is *naked* (Nkd), which has also been recently identified as an antagonist for Wnt signaling. Nkd binds to Dvl and blocks the β-catenin pathway. Moreover, VEGF has been described recently as a β-catenin target gene in HeLa cells and colon cancer cells, and may have influence on angiogenesis due to its role as an angiogenic factor (168). A comprehensive, list of Wnt/ β-catenin targets can be found at "the Wnt Homepage" http://web.stanford.edu/group/nusselab/cgi-bin/wnt/target_genes (Table I.3).

Gene	Organism/system	Direct/Indirect	up/down	Ref.
C-MYC	human colon cancer	yes	up	He 1998
CYCLIN D	human colon cancer	yes	up	Tetsu 1999; Shtutman 1999 Disputed by Sansom, 2005
Connexin43	rat cardiomyocytes	?	up	Ai 2000
C-JUN	human colon cancer	yes	up	Mann B 1999
uPAR	human colon cancer	?	up	Mann B 1999
TCF-1	human colon cancer	yes	up	Roose 1999
LEF1	human colon cancer	yes	up	Hovanes, 2001; Filali 2002
MMP-7	human colon cancer	yes	up	Brabletz 1999; Crawford 1999
AXIN-2	human colon cancer	yes	up	Yan 2001; Lustig 2002; Jho 2002
Fibronectin	Mouse lung		up	De Langhe 2005
CD44	human colon cancer	?	up	Wielenga 1999
EPHB/EPHRIN-B	human colon cancer	?	up/down	Batlle, 2002
BMP4	human colon cancer	?	up	Kim 2002
CLAUDIN-1	human colon cancer	yes	up	Miwa 2002
VEGF	human colon cancer	yes	up	Zhang, 2001
ENDOTHELIN-1	human colon cancer		up	Kim 2004
JAGGED	human colon cancer		up	Rodilla, 2009
DICKKOPF	Various cells, tumors		up	Niida. 2004; Gonzalez-Sancho 2004 Chamorro 2004
SOX9	Intestine		up	Blache 2004
SOX17	gastrointestinal tumors		up	Du, 2009
SOX2	Xenopus retina		up	Van Raay, 2005
DELTA-LIKE 1	somites			Galceran, 2004; Hofmann 2004

Gene	Organism/system	Direct/Indirect	up/down	Ref.
MMP-26	Human			Marchenko 2002
NANOG	ES			Pereira, 2006; Cole 2008
OCT 4	ES		up	Cole 2008
SNAIL	ES/EB		up	Ten Berge 2008
FIBRONECTIN	ES/EB		up	Ten Berge 2008
FRIZZLED 7	EC cells	yes	up	Willert 2002
WNT3A	EC cells			Zhang 2009
MMP2, MMP9	T cells			Wu 2007
BMP4	Xenopus	?	down	Baker 1999
TWIST	Wnt1 induced mammary cancer		up	Howe, 2003
E-CADHERIN	ES/EB		down	Ten Berge 2008
Jagged1	Mouse hair follicle		up	Estrach, 2006
Interleukin 8	Endothelial cells			Masckauchan 2005
WINGLESS	Drosophila	?	up or down	Yu 1998

Table I.2. List of Wnt target genes identified in cancer and development in a variety of species. Information in this table is adapted and selected from 'The Wnt Homepage' (http://web.stanford.edu/group/nusselab/cgi-bin/wnt/target_genes).

I.2.3.3.2.5 Wnt/β-catenin signaling and angiogenesis

The Wnt/β-catenin pathway has been well characterized in development-related angiogenesis processes such as embryogenesis and organogenesis during gastrulation, pattern formation and stem cell differentiation (265, 337, 338). Using TOP-Gal Wnt reporter mice, it was demonstrated that inhibition of Wnt/β-catenin signaling by delivering a Wnt inhibitor to developing embryos leads to severe angiogenic defects (339). Wnt/β-catenin is also implicated in disorders such as cancer and inflammatory diseases (340-343). Accumulation of β-catenin in the cytoplasm and nucleus of the endothelium is found in endothelial cells of neovessels of human glioblastoma multiforme tumors, medulloblastomas and other types of tumors (344-347).

Extensive data demonstrate that Wnt/β-catenin signaling is important to promote endothelial cell proliferation in angiogenesis (2, 348-351). In cell culture, ectopic expression of Wnt1 was shown to activate the Wnt/β-catenin canonical signaling pathway, stabilize cytosolic β-catenin in endothelial cells and promote endothelial cell proliferation (349). During embryonic development, when vessels proliferate, β-catenin can be detected in the endothelial cell nuclei and cytoplasm of capillaries, arteries and veins (343, 345, 352, 353). Also, β-catenin is often found in the nucleus and cytoplasm of endothelial cells under pathological angiogenesis or vascular remodeling. In a rat model of myocardial infarction, where coronary artery ligation leads to cardiac ischemia, β-catenin accumulates in the cytoplasm of endothelial cells in both newly formed small vessels and in activated infarct areas of pre-existing large arteries in the infarct area. These observations link the presence of intracellular β-catenin to a proliferative state of endothelial cells (70, 344). One very recent study revealed that canonical Wnt10B could enhance proliferation of human corneal endothelial cells through Wnt/β-catenin activation and upregulation of *CYCLIN D1* (350).

In addition to its role in proliferation, β-catenin is also important in other aspects in angiogenesis. In adherens junctions, phosphorylation or SNO of β-catenin promotes endothelial cell permeability, which is a critical step for the initiation of angiogenesis (156). MMPs mediated degradation and remodeling of the ECM is also a critical event in angiogenesis and MMP-7 is upregulated in colon cancer cells under Wnt/β-catenin activation (354). *Cox-2*, a gene upregulated in response to Wnt/β-catenin signaling in mouse epithelial cells, stimulates endothelial cell migration and the inhibition of cox-2 reduces angiogenesis and tumor growth (355).

In summary, β-catenin accumulation takes place in both developmental and pathological angiogenesis and could therefore be used as a therapeutical target for many diseases.

I.3. Role of eNOS-derived NO in transcriptional regulation in endothelial cells: transcriptomic studies

NO has been shown to influence transcription through regulation of transcription factors and modulation of gene expression. NO can induce the binding of transcription factor cAMP-response-element-binding protein (CREB) to its promoters and the expression of target genes to promote survival and differentiation of neural cells, by both activating cGMP signaling and promoting SNO of nuclear proteins (356). NO downregulates expression of transcription factor N-Myc through cGMP signaling, resulting in negative regulation of proliferation in neuronal precursors. Other oncogenic transcription factors, including c-fos, c-jun, and NF-κB, are also regulated by NO signaling, either in a cGMP-dependent way or through nitrosative conformational changes. Moreover, NO can regulate transcription by SNO of transcriptional factors to affect their DNA binding activity or protein stabilization. For example, SNO of NF-κB by iNOS-derived NO regulates gene transcription by inhibiting NF-κB-dependent DNA binding activity, and SNO of HIF-1 promotes its transcriptional activity due to the modulation of the protein stabilization (261-263).

For all living cells, regulation of gene expression by extracellular and intracellular signals is a fundamental mechanism of development and hemeostasis. Indeed, the ultimate step in many signal transduction pathways is the regulation of transcription factors that can modulate the expression of specific genes. The transcriptional regulation by growth factors and cell endogenous proteins in endothelial cells play important roles in endothelial function and angiogenesis.

Transcriptomic approaches allow for broad comparisons of global gene expression profiles and can shed light on novel pathways that are relevant to development and pathogenesis (357,

358). In recent years, transcriptomic studies have provided valuable information about the networks of molecular signals that regulate the pathophysiology of blood vessel walls. Here, the main focus of the review is on studies examining the effect of VEGF, eNOS and eNOS-derived NO on the transcriptional profiling of endothelial cells.

I.3.1 Effect of VEGF on the regulation of gene expression in endothelial cells

VEGF is the major trigger of vasculogenesis and physiological angiogenesis (359). Also, regulation of endothelial cells by VEGF has been widely studied during development and in pathologic disorders such as inflammatory conditions, cancer, diabetes, and coronary heart disease (360-362).

Transcriptomic studies contributing to the identification of novel genes produced in response to VEGF treatment would further help researchers to understand the effect of VEGF on endothelial cell transcriptional profiling of endothelial cells. There are solid data showing that VEGF can strongly induce the expression of transcription factors and genes involved in inflammation and angiogenesis (363). Using microarray analysis of endothelial cells treated with VEGF at different time points, one study showed that the earliest and most highly VEGF-upregulated genes are the transcription factors *NR4A2* (Nuclear Receptor Subfamily 4, Group A, Member 2, also known as *Nurr1*) and *EGR3* (Early Growth Response 3), which are responsible for inducing secondary genes important for angiogenesis (363). The study revealed the regulation of other genes with a more delayed induction kinetics such as transcription factors *HLX1* (H2.0-like homeobox 1), *MEF2C* (Myocyte Enhancer Factor 2C), *PER1* (Period Circadian Clock 1), the

secreted *IGFBP3* (Insulin-like growth factor-binding protein 3) and *CCRL1* (Chemokine CC Receptor-like-1).

In another study, using a human cDNA chip containing more than 7,000 genes, Abe *et al*. observed that in HUVEC, genes known to be involved in angiogenesis—such as *COX2* (cyclooxygenase-2), *HB-EGF* (heparin-binding epidermal growth factor-like growth factor), *EGR1* (Early growth response protein 1), *CYR61* (Cysteine-rich angiogenic inducer 61), and *ANG2* (Angiopoietin-2), were upregulated after 24 hours of VEGF treatment (364). Moreover, VEGF also induced a novel angiogenic inhibitor, bactericidal/permeability-increasing protein encoded by *BPI*.

VEGF-regulated gene expression was also analyzed using Affymetrix oligonucleotide arrays. Liu *et al*. found that the transcription factors of the *NR4A* nuclear receptor family members *NUR77*, *NURR1*, and *NOR2* and zinc-finger transcription factor *EGR3* were strongly induced in HUVECs-treated with VEGF (365). The up-regulation of NR4A nuclear receptor genes by VEGF partially explains how VEGF can modulate inflammation due to the important role of NR4A genes in some inflammatory diseases and cancer. In addition, VEGF-induced genes are enriched in the following families of molecular factors: cytokine receptors, phosphorylases and growth factors.

To summarize, these studies pinpoints genes involved in different signaling pathways and functions, providing insights to better understand the mechanisms implicating VEGF in angiogenesis and in inflammatory diseases.

I.3.2 Effect of eNOS and NO in the regulation of gene expression in endothelial cells

Genes regulated by eNOS depletion in mice were analyzed by comparing the changes in gene expression between eNOS-KO and WT mice (366). Using the microarray method, this study showed that in heart tissues of eNOS-KD and WT mice, a total of 480 genes were different including 39,000 transcripts and variants. The differentially regulated genes include *Ramp2* (Receptor (G Protein-Coupled) Activity Modifying Protein 2), *Ctgf* (Connective Tissue Growth Factor), *Wisp2* (WNT1 Inducible Signaling Pathway Protein 2) and *Peg3* (paternally expressed gene 3) in cell signaling, *Fbn1* (Fibrillin 1), *Frzb* (Frizzled-Related Protein) and *Ddx1* (DEAD (Asp-Glu-Ala-Asp) Box Helicase 1) in development, *Cxcl13* (Chemokine (C-X-C Motif) Ligand 13) and *Gpx3* (Glutathione Peroxidase 3) in immune response, *Lox*, *Egr1* (Early growth response protein 1), *Nars* (Asparaginyl-TRNA Synthetase) in protein expression and *Nrap (*Nebulin-Related Anchoring Protein) and *Acta1* (Actin, Alpha 1, Skeletal Muscle) in cell structure. The differentially regulated genes were grouped into several functional categories on the basis of their gene ontology biological process annotations. These categories are: cell signaling, communication, transport, cell structure, motility, metabolism, development, immune response, apoptosis, and protein expression. Almost all the differentially expressed genes within the cell structure/motility subcategory showed increased expression in eNOS-KO mice, such as *Acta1* (α-skeletin actin), *Comp* (cartilage oligomeric matrix protein) and *Myh7* (myosin heavy polypeptide 7).

Further studies to identify new genes affected by eNOS and a more comprehensive gene ontology analysis would allow a better understanding of the effect of eNOS on transcriptional profiling in endothelial cells.

I.3.3 A transcriptomic study technology: RNA sequencing analysis

RNA sequencing (RNA-seq) is a very powerful tool for transcriptomic studies that accurately records short sequences of nucleotides sampled from millions of mRNA molecules in the transcriptome with a high level of reproducibility (333-336).

I.3.3.1 RNA sequencing technology

Initially, transcriptomics studies largely relied on hybridization-based microarray technologies and offered were limited in their ability to fully catalogue and quantify the diverse RNA molecules that are expressed in the genome (337, 338). RNA-seq technology is more widely used by researchers these days because it offers benefits that are not common in arrays. First, it provides unbiased detection of novel transcripts: instead of depending on species- or transcript-specific probes like arrays, sequences are mapped to libraries of known transcripts in RNA-seq experiments, making it possible to discover of novel genes and isoforms (339-341). Second, RNA-seq has a broader dynamic range: when using the array hybridization technology, gene expression measurement is limited by background at the low end and signal saturation at the high end, whereas RNA-seq allows for quantification of discrete, digital sequencing read counts, offering a broader dynamic range. Third, RNA-seq has higher specificity and sensitivity compared to microarrays. Finally, RNA-seq can more easily detect rare and low-abundance transcripts. Sequencing coverage depth can easily be increased to detect rare transcripts, single transcripts per cell, or weakly expressed genes. These benefits have been validated in a study comparing both techniques (342). Therefore, the introduction of high-throughput next-generation sequencing technologies such as RNA sequencing revolutionized transcriptomics by allowing RNA analysis through cDNA

sequencing at a massive scale and providing a progressively fuller knowledge of quantitative aspects of transcript biology (333, 335, 343, 344).

In a typical RNA-seq experiment, mRNA is first synthesized into cDNA, followed by a process of random fragmentation that cuts the full-length mRNA transcripts into shorter fragments (335, 345). A size selection process is then performed, allowing fragments with the proper size (ranging from 25bp to 300bp) to be selected for sequencing on a high-throughput platform, such as IlluminaGA/HiSeq, SOliD or Roche 454. The direct output of an RNA-seq experiment is tens or hundreds of millions of short reads known as 'paired-ends', whose sequences are reads from the transcript fragments in the prepared cDNA library. The procedures are described in Figure I.18.

Dealing with the large volume of RNA-seq data is time-consuming and challenging. For example, Illumina Hiseq2000 can generate up to 200 million 100-nt reads (approximately 50 GB of data) in one lane in one sequencer run. These data must be processed for assembly into transcripts, quantitated and analyzed with bioinformatic tools before any insights can be discovered with respect to their biological meaning.

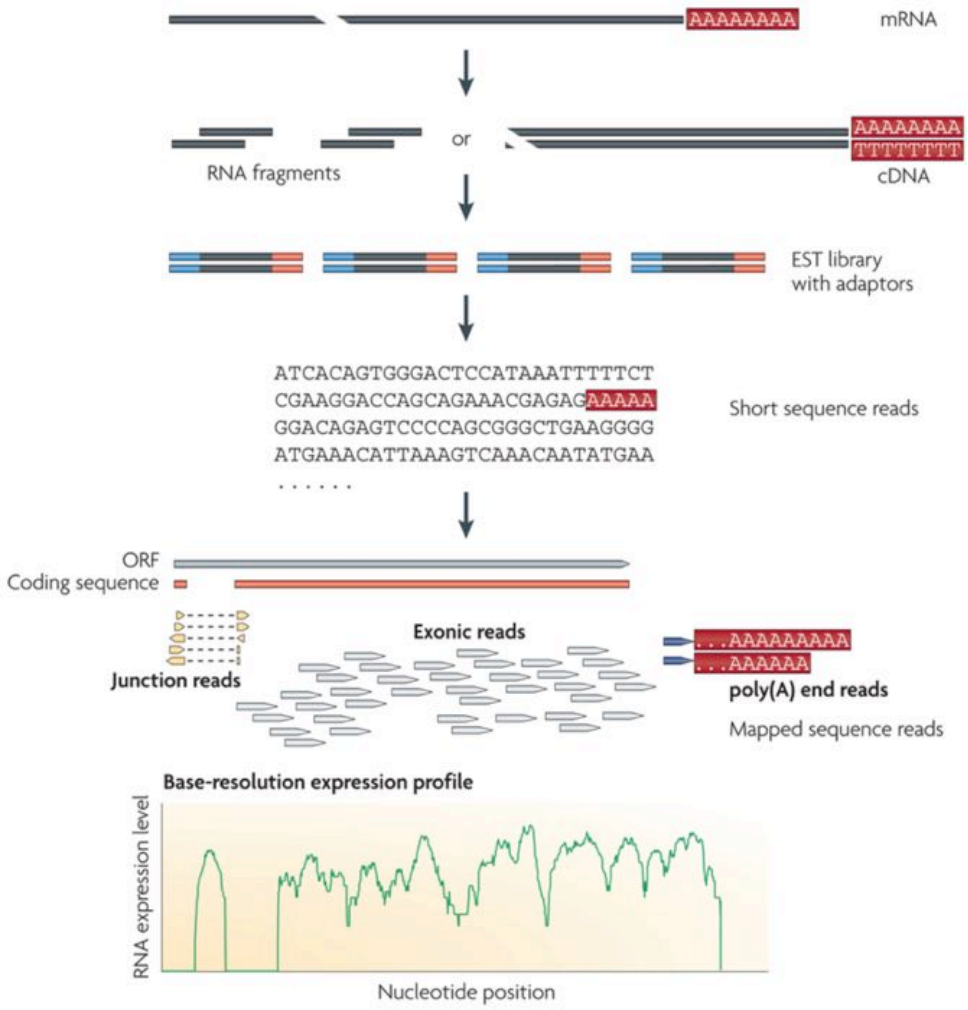

Figure I.19. A typical RNA sequencing experiment.

Long RNAs are first converted into a library of cDNA and a short sequence is obtained from each cDNA using high-throughput sequencing technology. The sequencing reads are aligned with the reference genome/transcriptome to be classified as exonic reads, junction reads and poly(A) end-reads. These three different types of reads are then used to generate a base-resolution expression profile for each gene. The bottom illustration shows the gene expression profile using a yeast ORF with one intron as an example. Adapted from reference (367).

I.3.3.2 Bioinformatic analysis tools

Further bioinformatic analysis include Gene Ontology (GO) enrichment and Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment that were conducted to identify the related biological modules and pathologic pathways. GO is a language for annotation and also an essential tool for the unification of biology. There are three main domains of GO: 1) biological process, which describes a biological objective to which the gene or gene product contributes; 2) molecular function, referring to the biochemical activity (including specific binding to ligands or structures) of a gene product; and 3) cellular component, which defines the specific region in the cell where a gene product is active. KEGG is a database resource for understanding the high-level functions and utilities of the biological system, such as the cell, the organism and the ecosystem; knowledge of these entities and processes is obtained from molecular-level information, especially from large-scale molecular datasets generated by genome sequencing and other high-throughput experimental technologies (http://www.genome.jp/kegg/).

For comparison of more than two experimental groups, gene cluster analysis is one of the most useful techniques for analyzing gene expression data. By grouping genes together based on the similarities of their expression profiles and functional categories, cluster analysis can help to reveal the effect of certain condition on the transcription. Hierarchical (pairwise single-, average-, maximum- and centroid-linkage) clustering is one of the most popular types of clustering. Similarity between gene expression data can be assessed using Pearson and uncentered correlation analysis, and the Euclidean distance. For my doctoral project, I used a popular open source program for gene clustering that is known as Gene Cluster 3.0.

I.4 Objectives and hypothesis

The proliferation, migration and polarization of endothelial cells are critical for angiogenesis. VEGF can activate eNOS to produce NO in endothelial cells and eNOS derived-NO is capable of inducing SNO of β-catenin at cell-cell junctions and modulating endothelial permeability. Additionally, NO has been implicated in the transcriptional control of gene expression. Therefore, the overall goals of my thesis work were to investigate the regulatory roles of NO on transcription in endothelial cells, particularly with respect to the modulation of the transcriptional activity of β-catenin.

In the first part of the study, we hypothesize that SNO modulates the transcriptional activity of β-catenin and affects Wnt signaling in endothelial cells. The specific goals of this first study were to address the following questions:

(1) What is the regulatory role of eNOS-derived NO on the transcriptional activity of β-catenin?

(2) What is the mechanism responsible for the effect of NO on the transcriptional activity of β-catenin and on endothelial cell proliferation?

(3) Does VEGF-stimulated eNOS activation and NO production affect Wnt signaling?

Given the known effects of NO on gene transcription derived from the first part of my study and other studies showing that NO directly regulates transcriptional activity, we performed whole transcriptome sequencing to investigate the effects of eNOS expression on transcription in endothelial cells. Therefore the goals of the second part of my study are as follows:

(1) To investigate the direct effect of eNOS on endothelial function, especially on endothelial cell polarization by comparing the gene differential expression in control cells and eNOS-knockdown cells;

(2) To determine the role of eNOS in VEGF-regulated endothelial cell transcriptional profiling by comparing the list of differentially expressed genes in VEGF-treated control cells and VEGF-treated eNOS-depleted cells.

In summary, through investigation of the role of eNOS on the endothelial cell transcriptome, especially the effect of eNOS-derived NO on the transcriptional activity of β-catenin, the studies present in this thesis contribute to a better understanding of the roles of eNOS and eNOS-derived NO on transcription in endothelial cells.

Chapter II. eNOS-dependent S-nitrosylation of β-catenin prevents its association with TCF4 and inhibits proliferation of endothelial cells by Wnt3a (Article submitted to MCB)

eNOS-dependent S-nitrosylation of β-catenin prevents its association with TCF4 and inhibits proliferation of endothelial cells by Wnt3a

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ABSTRACT

Nitric oxide (NO) produced by endothelial NO synthase (eNOS) modulates many functions in endothelial cells. S-nitrosylation (SNO) of cysteine residues on β-catenin by eNOS-derived NO has been shown to influence intercellular contacts between endothelial cells. However, the implication of SNO in the regulation of β-catenin transcriptional activity is ill-defined. Here we report that NO inhibits the transcriptional activity of β-catenin and endothelial cell proliferation induced by activation of Wnt/β-catenin signaling. Interestingly, induction by Wnt3a of β-catenin target genes, such as Axin2, is repressed in an eNOS-dependent manner by VEGF. We identify Cys466 of β-catenin as the critical residue for the repressive effects of NO on β-catenin transcriptional activity. Furthermore, we observed that Cys466 of β-catenin, located at the binding interface of the β-catenin/TCF4 transcriptional complex, is essential for disruption of this complex by NO. Importantly, Cys466 of β-catenin is necessary for the inhibitory effects of NO on Wnt3a-stimulated proliferation of endothelial cells. Thus our data define the mechanism responsible for the repressive effects of NO on the transcriptional activity of β-catenin and link eNOS-derived NO to the modulation by VEGF of Wnt/β-catenin-induced endothelial cell proliferation.

INTRODUCTION

Wnt/β-catenin signaling pathway is involved in physiological and pathological angiogenesis, the process of blood vessel formation from preexisting vasculature, through its transcriptional regulation (2, 353, 368). Canonical Wnt proteins, namely Wnt1, Wnt3a, Wnt7a, Wnt7b and Wnt10b, have been demonstrated to activate β-catenin signaling in endothelial cells (ECs) and regulate target genes that affect the angiogenic process (348, 350, 353, 369). In addition to its essential structural role in cadherin-based adhesions, β-catenin is a regulator of Wnt-mediated gene expression through its association in the nucleus to the T-cell factor/lymphoid enhancing factor (TCF/LEF) (370-372). In the absence of Wnt stimulation, free cytoplasmic β-catenin levels are kept low by a degradation complex that includes glycogen synthase kinase 3β (GSK3β), adenomatous polyposis coli (APC) and Axin (291, 336, 373). Upon Wnt stimulation, β-catenin is stabilized in the cytoplasm and subsequently translocalized to the nucleus, interacts with TCF/LEF and enhances expression of genes involved in cell proliferation, differentiation, cell fate and survival (273, 350, 374, 375).

In ECs, growth factors such as VEGF regulate β-catenin activity to promote angiogenesis. It is well established that VEGF must induce signaling at adherens junctions of ECs in order to induce blood vessel formation (181, 376). VEGF activates the phosphatidylinositol 3-kinase (PI3K)/Akt/endothelial nitric oxide synthase (eNOS) signaling pathway and stimulates nitric oxide (NO) production from ECs, which is essential for VEGF-regulated angiogenesis (218, 377, 378). NO exerts many biological functions in ECs by binding to its receptor guanylate cyclase and inducing cyclic guanosine monophosphate (cGMP) (227, 379). In addition, NO is capable of regulating protein function and cellular signal transduction by modifying thiol groups on cysteine residues by means of S-nitrosylation (SNO) (233, 234, 380). We previously showed that VEGF-

stimulated NO production in ECs induces SNO of β-catenin at Cys619 and promotes its dissociation from the adherens junction protein VE-cadherin. This results in the opening of intercellular contacts between ECs and in increased permeability to macromolecules in response to VEGF (156). We also identified other Cys residues on β-catenin that could be targets for SNO, namely Cys300, 381, 439, 466 and 520 (156). Furthermore, Cys213 and 520 were identified as potential SNO sites in large-scale proteomics assays (381, 382). In addition to its effects at cell-cell junctions, it has been shown that NO can inhibit the transcriptional activity of β-catenin in the nucleus and reduce proliferation of cancer cells (252, 383). However, it is still unclear whether SNO of β-catenin is responsible for both effects of NO, at cell junctions and in the nucleus, and what are the mechanisms responsible for these effects.

Herein, we demonstrate that eNOS-derived NO attenuates Wnt/β-catenin signaling in ECs by decreasing the transcriptional activity of β-catenin. This inhibitory effect of NO on β-catenin activity is caused by SNO of β-catenin on Cys466. We show that SNO of the Cys466 residue of β-catenin causes its dissociation from the transcriptional factor TCF4 that results in a reduction of Wnt3a-stimulated transcription of target genes. In addition, we show that SNO of β-catenin on Cys466 inhibits proliferation of ECs stimulated by Wnt3a and that VEGF-stimulated eNOS activation and NO production inhibits Wnt3a signaling, possibly forming an inhibitory feedback loop on Wnt/β-catenin signaling. These findings define SNO as a modulator of β-catenin transcriptional activity, which is important for Wnt/β-catenin signaling and in the control of EC proliferation.

MATERIALS AND METHODS

Cell culture and reagents

Bovine aortic endothelial cells (BAECs), were obtained from VEC Technologies (Rensselaer, NY) and grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10 % (v/v) fetal bovine serum (FBS; HyClone, Logan, UT), 2 mM L-glutamine, 100 U/ml penicillin and 100 mg/ml streptomycin. COS-7 and HEK293 cells were grown in DMEM supplemented with 10 % FBS (Invitrogen), 2 mM L-glutamine, 100 U/ml penicillin and 100 mg/ml streptomycin. HEK293T stable Wnt-reporter cells that express the β-catenin (Firefly) luciferase reporter TOPFlash and Renilla luciferase were provided by Dr. Stéphane Angers (University of Toronto, Canada) (384). The following primary antibodies were used: anti-myc-Tag (9B11), anti-β-actin (8H10D10) and anti-BrdU (Bu20a) mAb from Cell Signaling; anti-β-catenin, anti-eNOS and anti-Annexin II mAb from BD Transduction Laboratories; anti-TCF-4 (6H5-3) mAb from upstate; anti-Flag (M2) mAb from Sigma. Recombinant Human VEGF-A (VEGF herein) obtained from R&D System was used for cell stimulation throughout this study. S-nitrosoglutathione (GSNO) was from Sigma-Aldrich. NOC-18 was from Santa Cruz.

Plasmids, siRNA and cell transfections

Bovine S1179D-eNOS and S1179A-eNOS (in pcDNA3) were provided by Dr. William C. Sessa (Yale University School of Medicine, New Haven, CT). Human Flag-TCF-4 was provided by Dr. Hans Clevers (University Medical Centre Utrecht, The Netherlands). M50 Super 8xTOPFlash and M51 Super 8x FOPFlash vectors were purchased from addgene. Renilla luciferase reporter vector pRL-TK was from Promega. Full-length human β-catenin was purchased from Open Biosystems (Huntsville, AL) and subcloned in pCMV-3Myc (Stratagene). Single point mutations of myc-β-

catenin plasmid causing codon changes from cysteine to serine for residues Cys213, Cys300, Cys381, Cys439, Cys466, Cys520 and Cys619 were generated by using QuickChange site-directed mutagenesis kit (Stratagene). The sequences of mutagenic sense primers can be found in supplemental information (Table S1). All the mutations were verified by DNA sequencing.

Small interfering RNA (siRNA) control and siRNA against eNOS were generated by Thermo Scientific Dharmacon. The sense sequences are as follows: 5'-CCAGGAAGAAGACCUUUAAUU-3' and 5'-CCAACAUGCUGCUGGAAAUUU-3' for eNOS-siRNA, as well as 5'-AUGAACGUGAAUUGCUCAAUU-3' for control siRNA (CT-siRNA). Cell transfections were performed using Lipofectamine 2000 according to manufacturer's protocol (Invitrogen).

Wnt3a-conditioned medium

Wnt3a conditioned medium was collected by culturing Wnt3a stable transfected Mouse L cells (CRL-2647, ATCC) according to manufacture's instructions. Conditioned medium from parental mouse L cells not producing Wnt3a (CRL-2648) was also collected to use as control (CT). The activity of Wnt3a-conditioned medium was monitored by luciferase assay in TOPFlash stable HEK293 cell line. Wnt3a-conditioned medium that induced at least 50 fold of luciferase activity comparing to CT medium was used all experiments.

Luciferase assay

COS-7 cells were transfected with the β-catenin-dependent luciferase (Firefly) reporter plasmid TOPFlash or the negative control FOPFlash and Renilla luciferase (pRL-TK) as an internal control. In some instances, HEK293T stable Wnt-reporter cells expressing TOPFlash and Renilla

were used. Luciferase activity was determined using the Dual-luciferase reporter assay system (Promega, Madison, WI) 48h after transfection of the indicated plasmid constructs.

BrdU proliferation assay

Cells were incubated with 0.03 mg/ml BrdU at 37°C for 30 minutes, fixed with 70% ethanol for 5 minutes and then denaturated with 1.5M HCl for 30 minutes at room temperature. After incubating with PBS-1%BSA-0.3%Triton-X100 to block non-specific staining for 1h, cells were incubated with BrdU antibody overnight at 4°C. After three washes with PBS, cells were then incubated with Alexa-Fluor568 conjugated goat anti-mouse antibody (Invitrogen) for another two hours. The samples were then counterstained with DAPI (4,6-diamidino-2-phenylindole) to stain the nuclei and analyzed with Zeiss Axio Observer.Z1 microscope.

RNA extraction and qRT-PCR

Total RNA was extracted with an RNeasy Mini kit (Qiagen). After DNase I treatment, cDNA was synthesized using the SuperScript II Reverse Transcriptase kit for RT-PCR (Invitrogen) from 1ug total RNA according manufacturer's instructions. Real-time PCR was performed with the SYBR Select Master Mix (Applied Biosystems) and Eco™ Real-Time PCR System (Illumina) or ViiA™ 7 Real-Time PCR System (Life Technologies). Gene expression analysis was performed by using the comparative cycle threshold (ΔCT) method, normalized using reference genes β-actin and GAPDH expression and presented as the mean fold change (±SEM) compared with control. The sequences of primers can be found in Table S2.

Immunoprecipation (IP) and immunoblotting

For IP from whole cell lysate, cells were solubilized in lysis buffer containing 1% Triton X-100, 50 mM Tris-HCl, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 20 mM sodium fluoride, 1 mM sodium pyrophosphate, 1 mM orthovanadate and protease inhibitor cocktail (Roche Diagnostics). For TCF4 IP from ECs nuclei lysates, nuclei were first separated as described (385). Soluble proteins were incubated with primary antibodies (2 μg) at 4°C overnight. Protein A-Sepharose (Sigma; 50 ml of a 50 % slurry) or anti-Flag M2 affinity gel (for Flag IP) was added and incubated for an additional hour. The immune complexes were precipitated, separated by SDS-PAGE, transferred onto a nitrocellulose membrane (Hybond-ECL, GE Healthcare) and western blotted. Detection and quantification were performed by a LI-COR Odyssey infrared imaging system (LI-COR Biosciences) using the appropriate Alexa 680 or Alexa 800 labeled secondary antibodies (Invitrogen) or by a chemiluminescence-based detection system Image Quant (ECL; GE healthcare).

NO release analysis

Cell culture medium from COS-7 cells expressing S1179D- or S1179A-eNOS and myc-tagged β-catenin was collected and processed for the measurement of nitrite (NO_2^-), the stable breakdown product of NO in aqueous solution. NO-specific chemiluminescence was measured using a NO analyzer (NOA 280i; GE Ionics Instruments) (386).

Protein degradation assay

COS-7 cells overexpressing myc-WT- or myc-C466S-β-catenin were treated with 100 μg/ml cycloheximide (CHX) in serum free media for 24 hours. Cells were washed twice with cold PBS and lysed in RIPA buffer (50 mM NaCl, 50 mM Tris, 0,1 mM EDTA, 0,1 mM EGTA, 0.1% SDS,

0.1 % deoxycholic acid, 1% NP-40) completed with 20 mM sodium fluoride, 1 mM sodium pyrophosphate, 1 mM sodium orthovanadate, and complete EDTA free protease inhibitor (Roche). Protein was separated by SDS-PAGE, transferred onto a nitrocellulose membrane and western blotted. The detection of the signal was performed with Image Quant and the densitometric analysis was performed using Image J. The results are expressed as a ratio of the levels of Myc in treated cells versus the non-treated cells (t=0). The half-lives of proteins were calculated with linear regression equation.

Statistics

Values are reported as mean ± SEM. Statistical comparisons were performed by ANOVA followed by Bonferroni's *post hoc* test using GraphPad Prism 5.0. Student's t-test was used to compare two datasets (Figure 5*B*). A probability value $P < 0.05$ was considered as statistically significant.

RESULTS

eNOS-derived NO inhibits the transcriptional activity of β-catenin

To determine if eNOS-derived NO inhibits the transcriptional activity of β-catenin, we expressed in COS-7 cells increasing amounts of myc-β-catenin and a constitutively active mutant of eNOS, S1179D-eNOS. Then, we examined the transcriptional activity of β-catenin using TOPFlash, a luciferase reporter construct that contains TCF/LEF binding sites upstream of luciferase (387). We found that expression of S1179D-eNOS significantly attenuated TOPFlash activity induced by β-catenin (Figure 1A). To confirm that the inhibitory effect of eNOS on β-catenin is due to its enzymatic activity and NO production, we compared the effect of inactive eNOS, S1179A-eNOS, with S1179D-eNOS on the transcriptional activity of β-catenin. In contrast to S1179D-eNOS, expression of S1179A-eNOS did not reduce the activation of the β-catenin reporter TOPFlash (Figure 1B). We validated that S1179D-eNOS expression produced significant amounts of NO compared to S1179A-eNOS when co-transfected with β-catenin (Figure S1). In addition, treatment with the NOS inhibitor L-NMMA prevented the inhibitory effect of S1179D-eNOS on β-catenin transcriptional activity and treatment with the soluble guanylate cyclase inhibitor ODQ failed to interfere with the capability of eNOS to inhibit β-catenin transcriptional activity (Figure 1C). Finally, the constitutively activated chimeric VP16-LEF1 protein, a fusion protein between the activation domain of VP16 and LEF1 (384) known to activate TOPFlash independently of β-catenin transactivation properties, was insensitive to S1179D-eNOS expression (Figure S2A). Collectively, these results suggest that eNOS-derived NO directly inhibits the transcriptional activity of β-catenin and this is done independently of cGMP generation.

NO inhibits Wnt/β-catenin signaling in endothelial cells

Next, we investigated whether NO affects β-catenin transcriptional activity in ECs. We found that the NO donor GSNO inhibited TOPFlash activity induced by β-catenin overexpression (Figure 2A). Similarly, GSNO treatment of BAECs inhibited Wnt3a-induced expression of Axin2, a known β-catenin target gene (Figure 2B). Moreover, we examined if NO could inhibit EC proliferation stimulated by Wnt/β-catenin signaling. BAECs were exposed to Wnt3a in absence or presence of GSNO in the culture media. BrdU incorporation assays revealed that treatment of cells with GSNO or NOC-18, a more physiological NO donor, inhibited EC proliferation induced by Wnt3a (Figure 2C). In addition, GSNO also inhibited proliferation of BAECs that was stimulated by transient expression of myc-tagged β-catenin (Figure 2D and S2B). Finally, similar to the β-catenin transcriptional activity assays (Figure S2A), proliferation of BAECs induced by expression of the constitutively active chimeric VP16-LEF1 protein was not affected by GSNO treatment (Figure S2B). Taken together, these results show that NO inhibits EC proliferation promoted by activation of Wnt/β-catenin signaling and this could be mediated through decreased transcriptional activity of β-catenin by NO.

VEGF-stimulated NO production inhibits Wnt3a-mediated activation of β-catenin

Since eNOS-dependent NO production is central for the effects of VEGF in ECs, we investigated whether eNOS activation by VEGF affects Wnt/β-catenin signaling. BAECs were transfected with siRNA against eNOS or with control (CT) siRNA. First, in CT-siRNA transfected BAECs, treatment with Wnt3a, and to a lesser extent with VEGF, increased mRNA levels of the β-catenin target gene Axin2 (Figure 3A). Interestingly, when BAECs were treated with both VEGF and Wnt3a this resulted in a reduction in Axin2 mRNA when compared to Wnt3a treatment alone

(Figure 3A). Remarkably, the inhibitory effect of VEGF on Wnt3a-stimulated induction of Axin2 mRNA was completely abolished in eNOS-depleted BAECs (Figure 3A). VEGF and Wnt3a are both known to promote proliferation of ECs, thus we examined the effect of VEGF treatment on Wnt3a-stimulated BAEC proliferation and on cyclin D1 mRNA levels, a β-catenin target gene involved in cell cycle progression. We observed that treatment with VEGF or Wnt3a alone increased BrdU incoporation in BAECs. In contrast, proliferation of BAECs induced by co-treatment with Wnt3a and VEGF was reduced when compared to Wnt3a treatment alone (Figure 3B). Similarly, induction of cyclin D1 mRNA levels by Wnt3a was reduced by VEGF co-treatment (Figure 3C). Taken together, these results suggest that VEGF-stimulated eNOS activation and NO production negatively regulate transcription of β-catenin target genes and cell proliferation induced by Wnt3a.

SNO of β-catenin decreases its transcriptional activity

We hypothesized that SNO is responsible for the inhibition of transcriptional activity of β-catenin by NO. We have previously shown by LC-MS/MS analyses that cysteine residues 300, 381, 439, 466, 520 and 619 of β-catenin are possible substrates for NO and sites of SNO (156). In addition, Cys213 was identified as a potential SNO sites in another proteomics study (382). To determine which cysteine residue of β-catenin is attributed to the inhibitory effects of NO on the transcriptional activity of β-catenin, we generated β-catenin mutants where cysteine residues were replaced by non-nitrosylable serine. Hence, the following β-catenin mutants were generated: C213S-, C300S-, C381S-, C439S-, C466S-, C520S- and C619S-β-catenin. We tested the sensitivity of these mutants to eNOS-mediated inhibition of β-catenin transcriptional activity (Figure 4A). Wild-type (WT) and the β-catenin mutants were co-expressed with S1179D-eNOS

and the transcriptional activity of β-catenin was monitored using the TOPFlash reporter. Our results show that, among all the non-nitrosylable mutants tested; only C466S-β-catenin was insensitive to the inhibitory effects of NO (Figure 4A). This suggests that NO inhibits the transcriptional activity of β-catenin through SNO of Cys466.

Next, we examined if Cys466 is important for mediating the inhibitory effects of VEGF on β-catenin signaling in ECs. Myc-tagged WT, C466S- or C619S-β-catenin were expressed in BAECs and the effects of VEGF treatment on the induction of Axin2 mRNA by β-catenin overexpression were determined (Figure 4B). Similar to Figure 2A, overexpression of WT-β-catenin did induce transcription of Axin2 which was inhibited by VEGF-treatment. In contrast, the induction of Axin2 transcription by C466S-β-catenin expression in BAECs was resistant to VEGF treatment. Similar to the effect of VEGF on WT-β-catenin, induction of Axin2 mRNA levels by the C619S-β-catenin mutant was inhibited by VEGF treatment. These results obtained in BAECs suggest that residue Cys466 of β-catenin is a substrate for SNO and is responsible for the repression of β-catenin transcriptional activity by VEGF-stimulated NO production.

SNO of Cys466 disrupts the interaction between β-catenin and TCF4

Previous crystallographic studies of the TCF4/β-catenin complex revealed that the N-terminal portion of TCF4 (residues 13 - 25) binds a positively charged groove created by the armadillo repeats 4 to 9 of β-catenin. Interestingly, the side chains of residues Ile19 and Phe21 of TCF4 are thought to form hydrophobic contacts with residues Cys466, Pro463 and the aliphatic portion of Arg386 on β-catenin (317). Figure 5*A* highlights the proximal position of Cys466 of β-catenin relatively to Ile19 of TCF4. Therefore, it is reasonable to hypothesize that addition of an

NO group on Cys466 of β-catenin could affect the stability of the interaction with TCF4. To investigate this, TCF4 was immunoprecipitated from nuclear extracts of ECs stimulated with Wnt3a in absence or in presence of GSNO and the interaction with β-catenin was determined. Our results show that Wnt3a stimulation of BAECs induced the association of β-catenin with TCF4 (Figure 5B). Interestingly, treatment of cells with GSNO diminished the interaction between β-catenin and TCF4 promoted by Wnt3a stimulation (Figure 5B). Next, to determine if Cys466 of β-catenin is important for the effects of NO on disruption of the interaction between β-catenin and TCF4, we transfected HEK293T cells with WT- or C466S-β-catenin together with Flag-tagged TCF4 in presence or in absence of constitutively active S1179D-eNOS. Cell lysates were immunoprecipitated with an anti-Flag antibody and the TCF4/β-catenin complex was resolved. In the absence of eNOS, basal association of C466S-β-catenin to TCF4 was similar to that of WT-β-catenin (Figure 5C). Co-expression of S1179D-eNOS decreased the interaction between WT-β-catenin and TCF4. In contrast, the association between C466S-β-catenin and TCF4 was not affected by the expression of S1179D-eNOS (Figure 5C). Finally, the protein stability of β-catenin is also a determinant of its transcriptional activity; the half-lives of WT- and C466S-β-catenin proteins expressed in COS7 in presence of S1179D-eNOS cells were not different (15.7 ± 2.7 hr and 14.8 ± 2.4 hr, respectively). Together, these results suggest that SNO at residue Cys466 of β-catenin results in dissociation from TCF4 and this may be responsible for the decrease of β-catenin transcriptional activity mediated by NO.

Cys466 of β-catenin is necessary for inhibition of EC proliferation by NO

Next, we examined if SNO of Cys466 of β-catenin could be responsible for the inhibitory effects of NO on Wnt/β-catenin-induced EC proliferation. To test this, we expressed WT- or C466S-β-catenin in BAECs and determined by BrdU incorporation assays the inhibitory capacity of GSNO on β-catenin-dependent or on Wnt3a-stimulated cell proliferation. As showed previously, GSNO decreased proliferation of BAECs that overexpress WT-β-catenin. In contrast, BAECs that express C466S-β-catenin were completely resistant to the inhibitory effects of GSNO (Figure 6A). Furthermore, Wnt3a-induced induced proliferation of cells expressing WT-β-catenin was decreased by GSNO treatment (Figure 6B). Importantly, Wnt3a-stimulated proliferation of BAECs expressing C466S-β-catenin was not affected by GSNO. These results confirm that SNO of Cys466 of β-catenin is responsible for the inhibitory effects of NO on Wnt/β-catenin-stimulated proliferation of ECs.

DISCUSSION

Herein, we investigated the influence of NO on β-catenin transcriptional activity and its consequences on Wnt/β-catenin-mediated EC proliferation. We elucidate a new mechanism that explains how eNOS-derived NO modifies Wnt/β-catenin signaling to regulate proliferation of ECs. The main findings are 1) that eNOS-derived NO inhibits β-catenin transcriptional activity and EC proliferation stimulated by Wnt3a; 2) that VEGF may act as a feedback inhibitor for Wnt/β-catenin signaling through the generation of NO by eNOS; 3) that SNO of Cys466 of β-catenin is a central modifier of the interaction with TCF4; 4) that disruption of the β-catenin/TCF4 interaction by SNO of Cys466 may be responsible for the inhibition of the transcriptional activity of β-catenin by NO and 5) for the repression of Wnt/β-catenin-stimulated EC proliferation by NO. Taken together, our findings have uncovered a mechanism used by NO to modulate Wnt/β-catenin signaling which may explain some of the important effects produced by eNOS in ECs and during angiogenesis.

We have previously shown that SNO of Cys619 of β-catenin at cell junctions promotes its dissociation from VE-cadherin and is important for VEGF-induced EC permeability (156). It is also well established that once release from cell junctions β-catenin may act in concert with TCF/LEF transcription factors in the canonical Wnt signaling pathway to modulate gene expression in many cell types (117, 288, 301, 348, 349) This study now reveals that NO can affect both functions of β-catenin in ECs through SNO of another cysteine residue, Cys466. Previous studies have shown that NO donors, including NO-donating aspirin or other NO-releasing chemical entities, could repress the transcriptional activity of β-catenin and some have suggested that SNO of β-catenin could be responsible for this effect (252, 383, 388-391). Chemical NO-donating agents have shown promise on cancer cells where they attenuate Wnt/β-catenin signaling,

reduce proliferation and induce apoptosis (252, 390). However, the direct targets of NO, responsible for attenuating Wnt/β-catenin, were not identified. To our knowledge, this study provides the first demonstration that modification of Cys466 of β-catenin by NO is responsible for disruption of the β-catenin/TCF4 association, which results in inhibition of the transcriptional activity of the complex. As stated above, previous structural studies have defined residues 13-31 of TCF4 to form a minimal binding unit for the armadillo repeats 4 to 9 of β-catenin (392). Cys466 is located in the 8th armadillo repeat of β-catenin and interacts with Ile19 of TCF4 (317). Further analysis of the β-catenin/TCF4 complex (PDB file: 1JPW) by molecular modeling reveals that modification of the thiol group of Cys466 by SNO causes a shift of Ile19 of TCF4, which results in decreased binding energy (-7.4 kcal/mol) between β-catenin and TCF4, calculated using the Molecular Mechanics-Generalized Born Surface Area (MM-GBSA) method (393). In addition, Cys466 is close to Lys435 of β-catenin that has also been suggested to play an important role in the interaction with TCF4 via a salt bridge hydrogen bond with Asp16 of TCF4 (317, 392). Hence, it is likely that SNO of Cys466 disrupts the interaction between β-catenin and TCF4 by inducing conformational changes that affect binding to TCF4 and result in decreased transcriptional activation.

Cross-regulation between NO and Wnt/β-catenin signaling has been shown in other systems. Interestingly, it appears that the Wnt/β-catenin activation state determines the modulatory effects of NO. This study and others show that NO is inhibitory on β-catenin transcriptional activity when Wnt/β-catenin signaling is activated (252, 383, 388-391). Consistent with these results, we reveal that VEGF-mediated eNOS activation in ECs acts as an inhibitor Wnt3a-induced β-catenin activation and cell proliferation (Figure 3) (168, 394). Inversely, it has been shown that NO may act as a positive modulator of β-catenin activity under low basal Wnt activation. For

instance, expression of iNOS (NOS2) in embryonic stem cells influences cell differentiation and early lineage commitment through activation of β-catenin (395). Another study showed that β-catenin associates with eNOS in quiescent EC and that eNOS activation causes cGMP-dependent nuclear translocation of β-catenin and influences angiogenesis (396). In agreement with these studies, our results show that, in resting ECs, VEGF activates β-catenin signaling and this is prevented by knockdown of eNOS (Figure 3A). In contrast to the inhibitory role of NO on β-catenin, the involvement of SNO in the positive effects of NO on β-catenin nuclear signaling remains to be defined. Perhaps when β-catenin is associated with VE-cadherin at cell-cell contacts, VEGF-induced NO dissolves adherens junctions through SNO of Cys619 and this could promote the transcriptional activity of β-catenin by allowing the translocation from the plasma membrane to the nucleus (156).

VEGF was previously identified as one of the target genes of Wnt/β-catenin signaling (168, 394). Our results now reveal that VEGF, through the production of NO, may engage in a negative feedback loop regulating Wnt signaling in ECs. We show that the inhibitory effect of VEGF on Wnt signaling depends on eNOS activation and NO-mediated dissociation of β-catenin from the transcription factor TCF4. Both VEGF and Wnt exert angiogenic properties, however, distinct outcomes of VEGF and Wnt in the regulation of EC specification and vascular remodeling have been reported (353, 397). During angiogenic sprouting, VEGF signaling is implicated in endothelial tip cell specification, maintenance and migration whereas Wnt-dependent intracellular signals are involved in the promotion of stalk cells (397, 398). Our results allow us to speculate that inhibition of Wnt3a-induced proliferation of ECs by VEGF-mediated eNOS activation may be relevant during angiogenesis in order for VEGF to suppress proliferative signals in tip cells by restraining Wnt/β-catenin signaling. This could contribute to maintaining the migratory phenotype

of endothelial tip cells during sprouting. Therefore, it would make sense that Wnt-induced VEGF may somehow affect the outcomes of Wnt in the remodeling due to its inhibitory effect for Wnt/β-catenin in certain context.

In summary, our results demonstrate that NO directly modulates Wnt/β-catenin nuclear signaling resulting in decreased β-catenin transcriptional activity and in reduced EC proliferation stimulated by Wnt3a. Our results shed light on a mechanism that explains the inhibitory effects of NO on β-catenin transcriptional activity. This mechanism involves VEGF-induced SNO of β-catenin at Cys466 by eNOS-derived NO, which disrupts the β-catenin/TCF4 complex and results in decreased transcription of β-catenin target genes and proliferation of ECs. Thus, NO affects the function of β-catenin at the plasma membrane through SNO of Cys619 and in the nucleus by modifying Cys466. This study further highlights the roles of eNOS-derived NO in EC biology and angiogenesis.

AUTHOR CONTRIBUTIONS

Y.Z. designed and performed the experiments, analyzed the data, prepared the figures, and wrote the manuscript; R.C. and C.D. performed the experiments and analyzed the data; J.-P.G. designed and supervised the experiments, analyzed the data, prepared the figures, and wrote the manuscript.

Conflict of interest: The authors declare that they have no conflict of interest.

ACKNOWLEDGEMENTS

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II.7. Figures and Legends

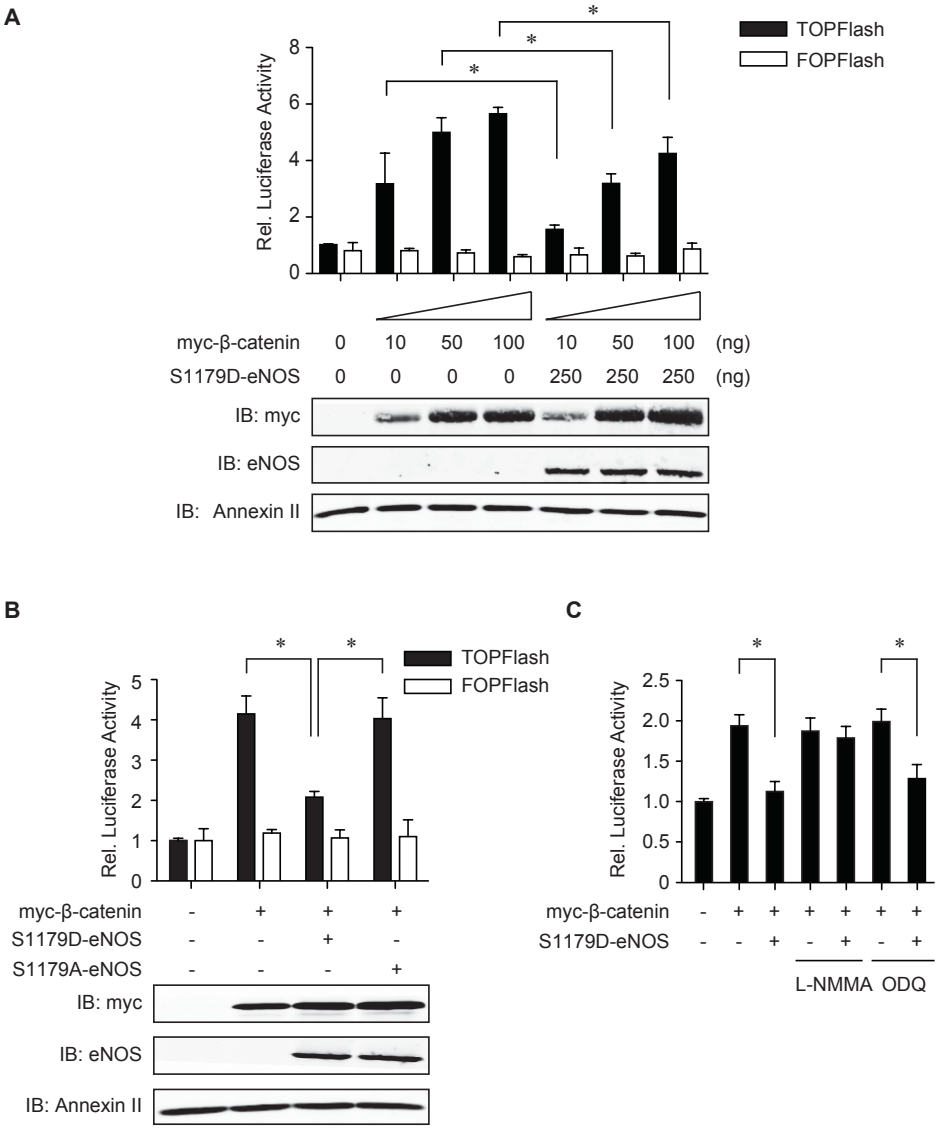

Figure II.1. eNOS-derived NO inhibits transcriptional activity of β-catenin.

(*A*) β-catenin luciferase reporter assay of COS-7 cells expressing TOPFlash and transfected with the indicated amounts of myc-tagged β-catenin and S1179D-eNOS. The FOPFlash luciferase reporter serves as negative control (n=4). Transfection levels of myc-tagged β-catenin and eNOS were monitored by immunoblot (IB) and Annexin II was used as a loading control. (*B*) β-catenin luciferase reporter assay of COS-7 cells expressing TOPFlash or FOPFlash and transfected with myc-tagged β-catenin and active (S1179D) or inactive (S1179A) eNOS (n=3). (*C*) β-catenin luciferase reporter assay of HEK293T stably expressing the TOPFlash reporter and transfected as

indicated with myc-tagged β-catenin and/or S1179D-eNOS. Cells were treated with the NOS inhibitor L-NMMA (0.1mM) or with the soluble guanylate cyclase inhibitor ODQ (10 μ M) for 8h when indicated (n=3). Data are represented as mean ± SEM. *$P<0.05$.

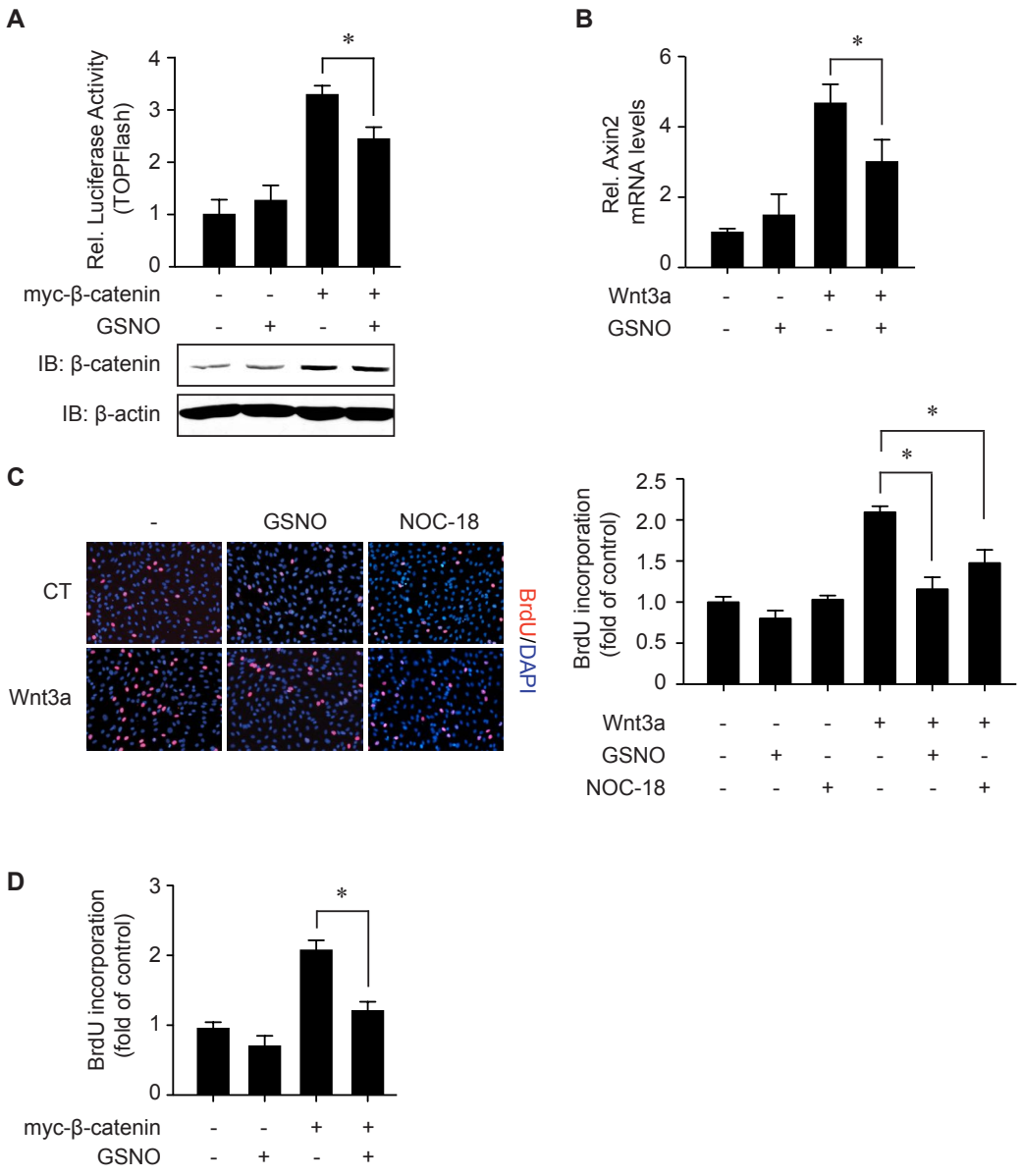

Figure II.2. NO inhibits β-catenin transcriptional activity and EC proliferation induced by Wnt3a.

(*A*) β-catenin luciferase reporter assay of BAECs expressing myc-tagged β-catenin in presence or absence of GSNO (0.1mM; 18h) (n=3). Transfection levels of myc-tagged β-catenin were monitored by immunoblot (IB) and β-actin was used as a loading control. (*B*) qRT-PCR analysis of *AXIN2* mRNA levels in BAECs treated with Wnt3a-conditioned medium and in presence or absence of GSNO (0.1mM; 24h) (n=4). (*C*) BrdU incorporation assay in BAECs treated with Wnt3a and in presence or absence of GSNO (0.1mM; 24h). Representative immunofluorescence

images of BrdU incorporation in BAECs (left panels). Cell nuclei were stained with DAPI. The percentage of BrdU positive cells for each treatment was normalized to non-treated (right panel; n=3). (*D*) BrdU incorporation assay in BAECs expressing myc-tagged β-catenin in presence or absence of GSNO (0.1mM; 18h) (n=3). Data are represented as mean ± SEM.*$P<0.05$.

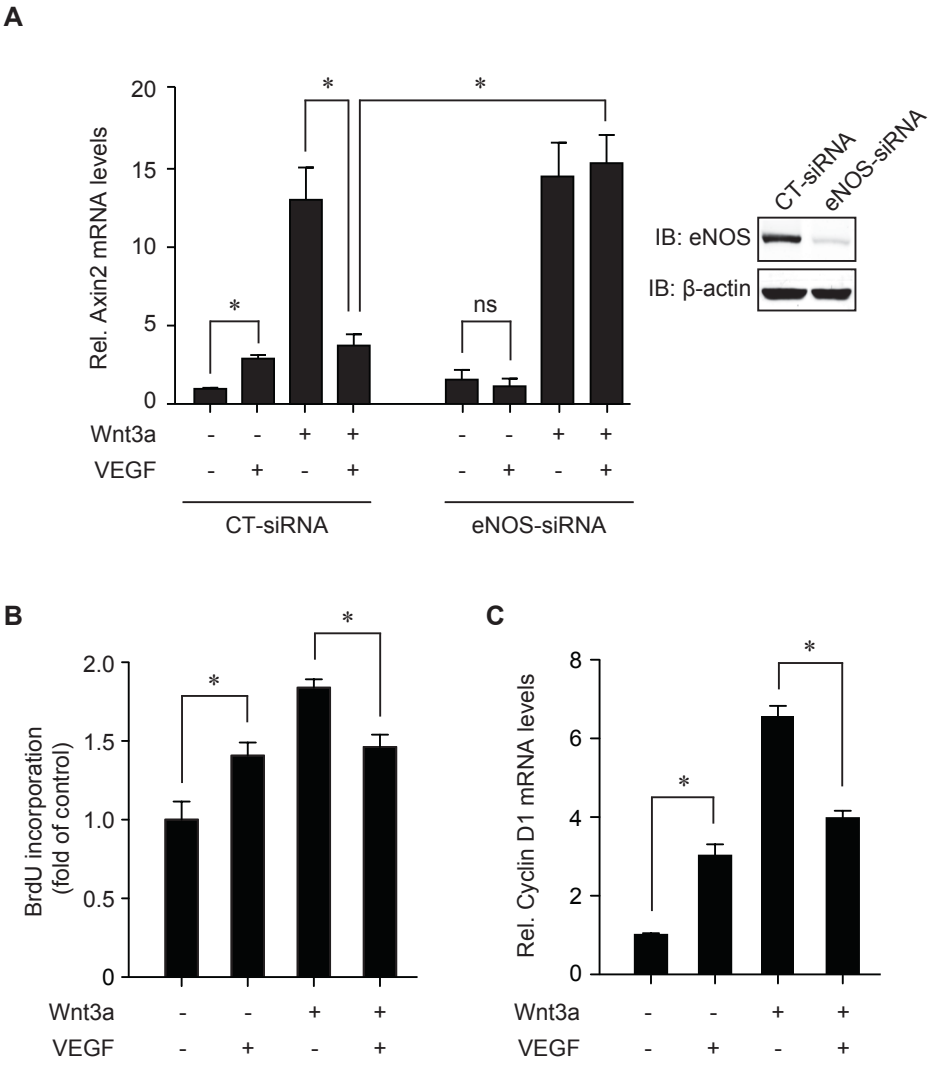

Figure II.3. VEGF inhibits Wnt/β-catenin signaling in an eNOS-dependent manner.

(A) qRT-PCR analysis of Axin2 mRNA levels in control or eNOS-depleted BAECs treated with Wnt3a-conditioned medium and/or VEGF (40ng/mL; 24h) as indicated (n=3). eNOS was depleted in BAECs by transfection of siRNA against eNOS (eNOS-siRNA) and control (CT) siRNA was used for comparison. Depletion of eNOS was monitored by immunoblot (IB) and β-actin was used as a loading control. *(B)* BrdU incorporation assay in BAECs treated with Wnt3a-conditioned medium and/or VEGF (40ng/mL; 24h) as indicated. The percentage of BrdU positive cells for each treatment was normalized to non-treated (n=3). *(C)* qRT-PCR analysis of cyclin D1 mRNA levels in BAECs treated with Wnt3a-conditioned medium and/or VEGF (40ng/mL; 6h) as indicated (n=3). Data are represented as mean ± SEM.**P*<0.05; ns, not statistically significant.

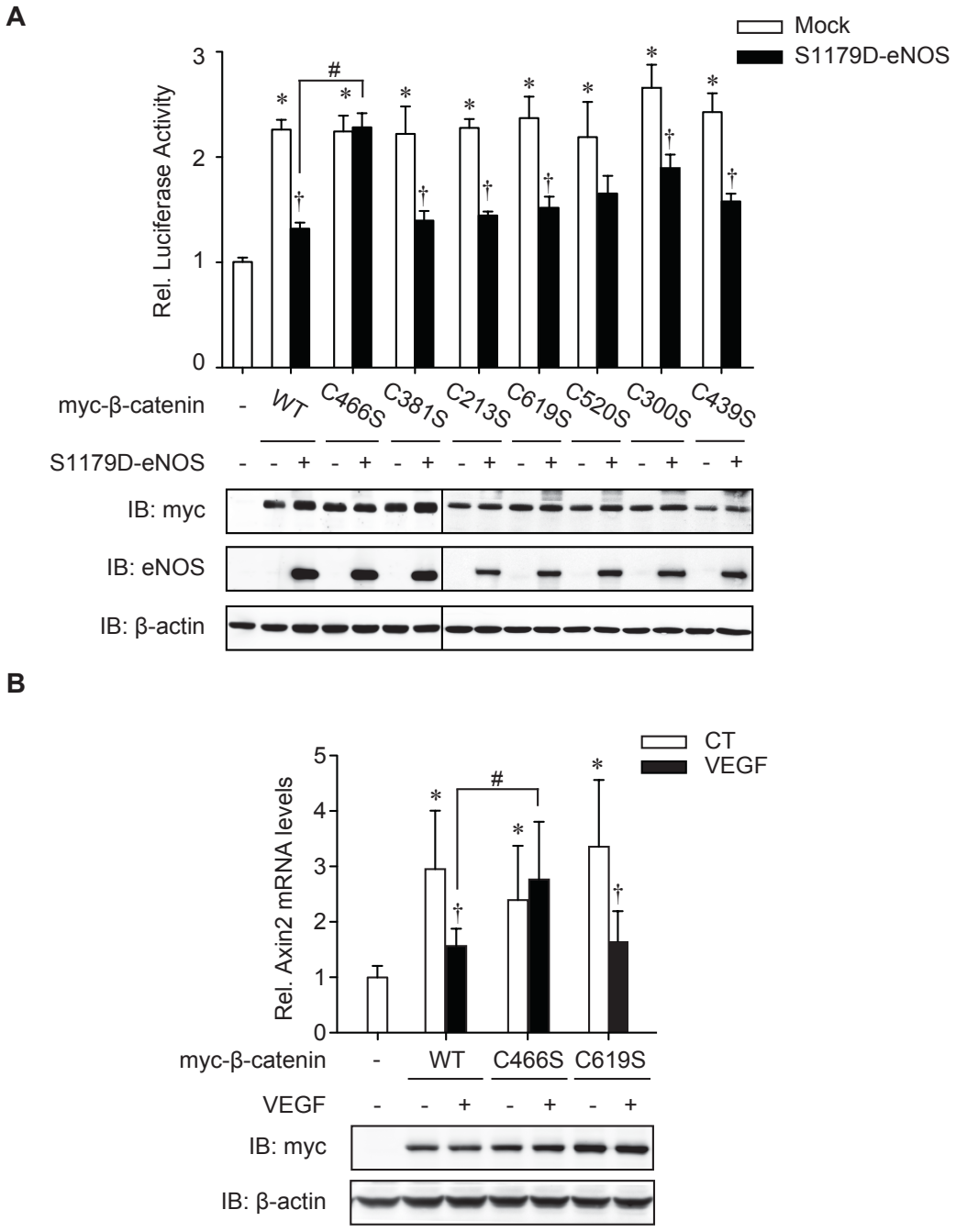

Figure II.4. SNO of Cys466 is responsible for the inhibitory effect of NO on β-catenin transcriptional activity.

(*A*) β-catenin luciferase reporter assay in HEK293T stably expressing the TOPFlash reporter and transfected with myc-tagged wild-type (WT) β-catenin or the indicated non-nitrosylable cysteine to serine mutated β-catenin constructs. Where indicated, cells were co-transfected with S1179D-

eNOS or empty vector pcDNA3 (mock) (n=3). Data are represented as mean ± SEM. *$P<0.05$ *vs.* mock transfected; † $P<0.05$ *vs.* corresponding mutant without S1179D-eNOS; # $P<0.05$ *vs.* cells transfected with WT-β-catenin and S1179D-eNOS. Transfection levels of myc-β-catenin and eNOS were validated by immunoblot (IB) and β-actin was used as a loading control. (*B*) qRT-PCR analysis of *AXIN2* mRNA levels in BAECs expressing myc-tagged WT-, C466S- or C619-β-catenin treated or not with VEGF (40ng/mL; 6h) (n=4). Transfection levels of myc-tagged β-catenin were validated by immunoblot (IB) and β-actin was used as a loading control. Data are represented as mean ± SEM. *$P<0.05$ *vs.* non-transfected and non-stimulated cells; † $P<0.05$ *vs.* cells expressing myc-WT-β-catenin without stimulation; # $P<0.05$ *vs.* cells expressing myc-WT-β-catenin and VEGF-treated.

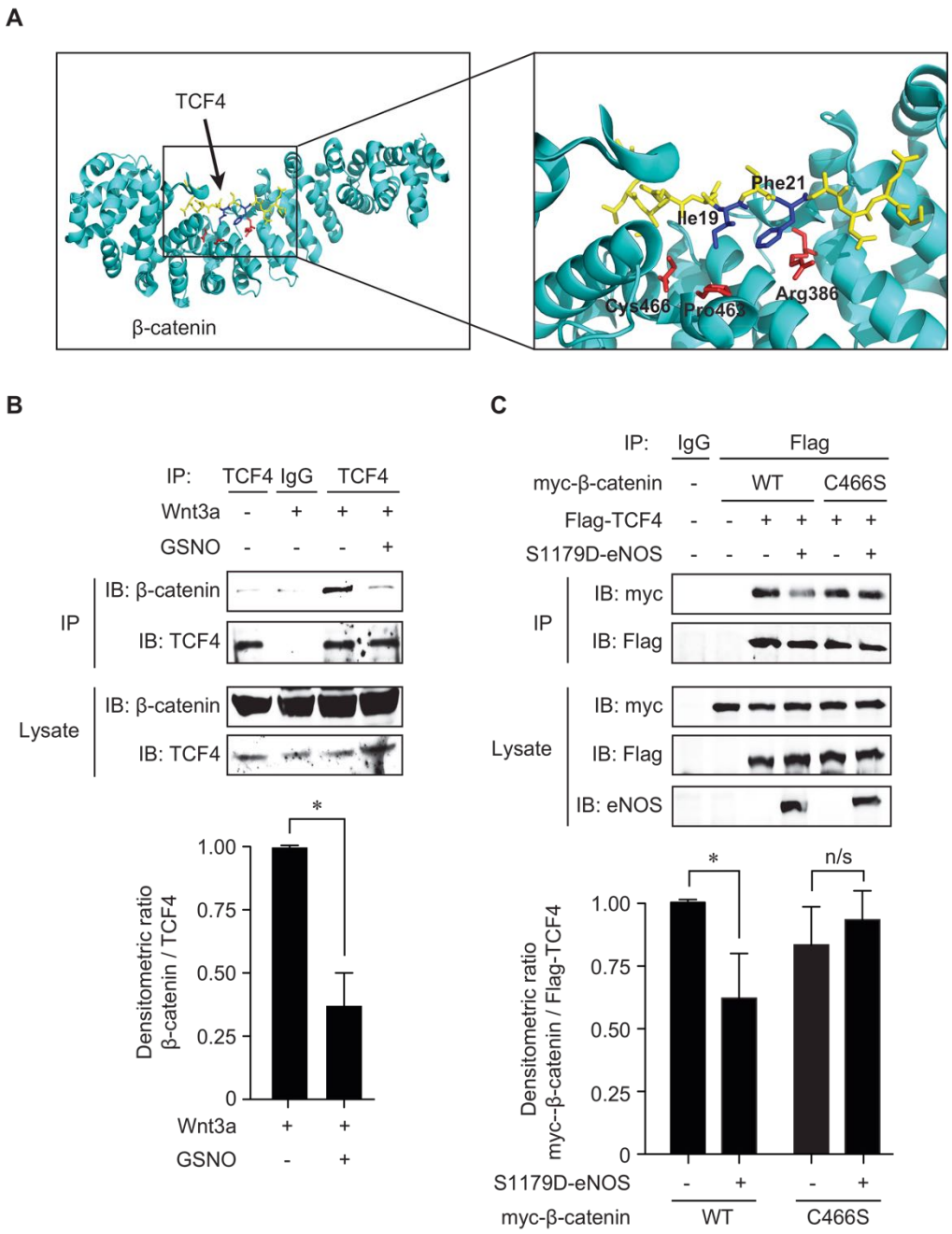

Figure II.5. Cys466 of β-catenin is necessary for NO-mediated disruption of the β-catenin/TCF4 complex.

(*A*) Three-dimensional representation of human β-catenin (cyan) in complex with a TCF4 peptide (residues 13-25; yellow). The side chains of residues Cys466, Pro463 and Arg386 of β-catenin are shown in red and residues Ile19 and Phe21 of TCF4 are highlighted in purple. The image was

generated based on analysis of PDB 1JPW protein structure (317) with the PyMol Molecular Graphics system (Version 1.7.4). (*B*) Co-immunoprecipitation (IP) of β-catenin and TCF4 from the nuclear fraction of BAECs treated with Wnt3a conditioned medium overnight and in presence or absence of GSNO (0.1mM; 30min). Non-immune IgG was used for control IP. Bar graph shows the normalized densitometric ratio, quantified by Image J, of β-catenin to TCF4 levels (n=3). Data are represented as mean ± SEM. *$p<0.05$ (*C*) Co-IP of myc-WT- or myc-C466S-β-catenin with Flag-TCF4 from HEK293 cell lysates transfected with and S1179D-eNOS where indicated. Levels of β-catenin and TCF4 constructs in the IP were monitored using anti-myc and anti-Flag. Bar graph shows the normalized densitometric ratio, quantified by Image J, of myc-β-catenin to Flag-TCF4 levels (n=3). Data are represented as mean ± SEM. *$p<0.05$.

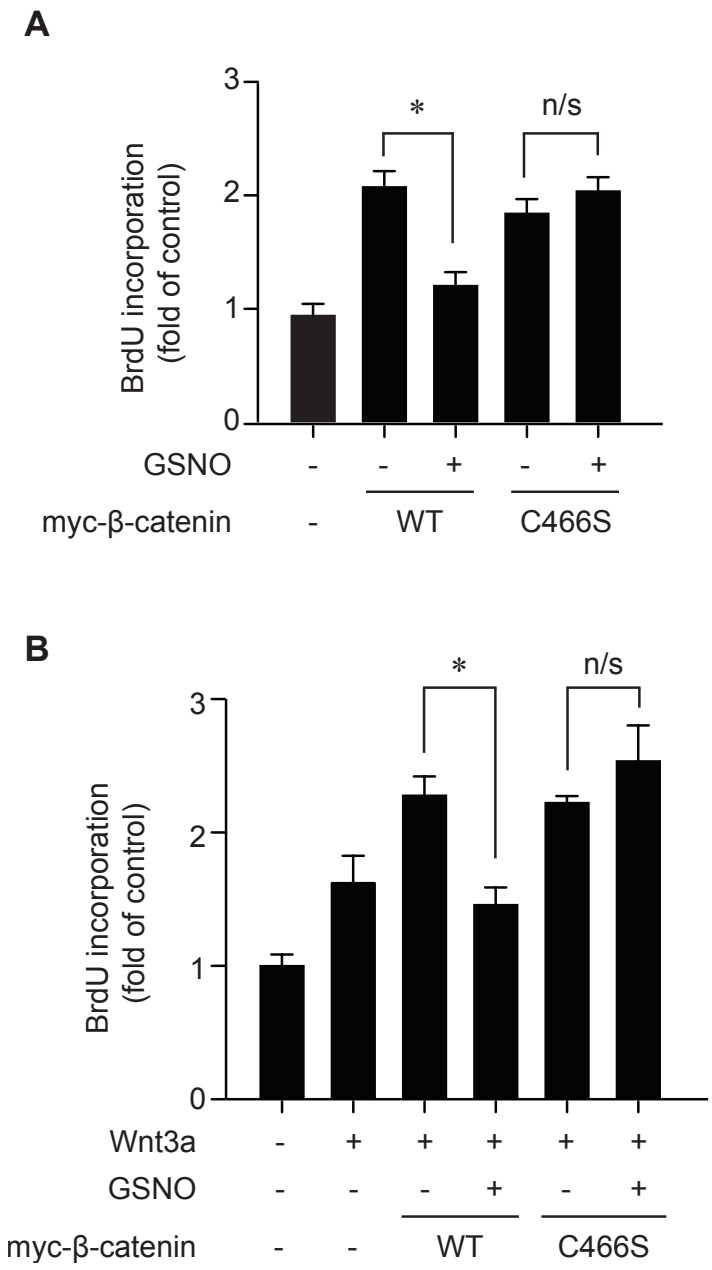

Figure II.6. SNO of Cys466 is critical for the inhibitory effects of NO on EC proliferation.

(*A*) BrdU incorporation assay in BAECs expressing myc-WT- or C466S-β-catenin in presence or absence of GSNO (0.1mM; 18h) (n=3). *(B)* BrdU incorporation assay in BAECs expressing myc-tagged WT-, or C466S-β-catenin and treated with Wnt3a conditioned medium in presence or absence of GSNO (0.1mM; 18h) (n=3). The percentage of BrdU positive cells for each treatment was normalized to non-treated. Data are represented as mean ± SEM. *$p<0.05$.

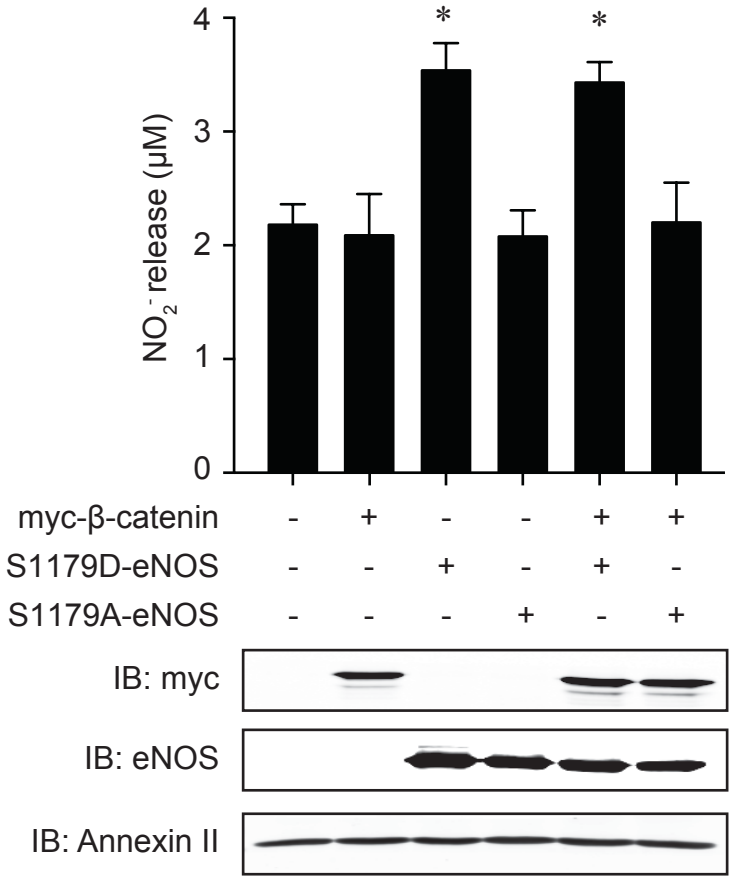

Figure II.S1. S1179D-eNOS expression produces significant amounts of NO compared to S1179A-eNOS when co-transfected with β-catenin.

NO levels were monitored 48 hours post-transfection in culture medium of COS-7 cells expressing myc-β-catenin together with S1179D-eNOS or S1179A-eNOS. Transfection levels of myc-β-catenin and eNOS were monitored by immunoblot (IB) and β-actin was used as a loading control. Nitrite levels were normalized to protein amounts (n=3). Data are represented as mean ± SEM. *$p<0.05$ *vs.* non-transfected cells.

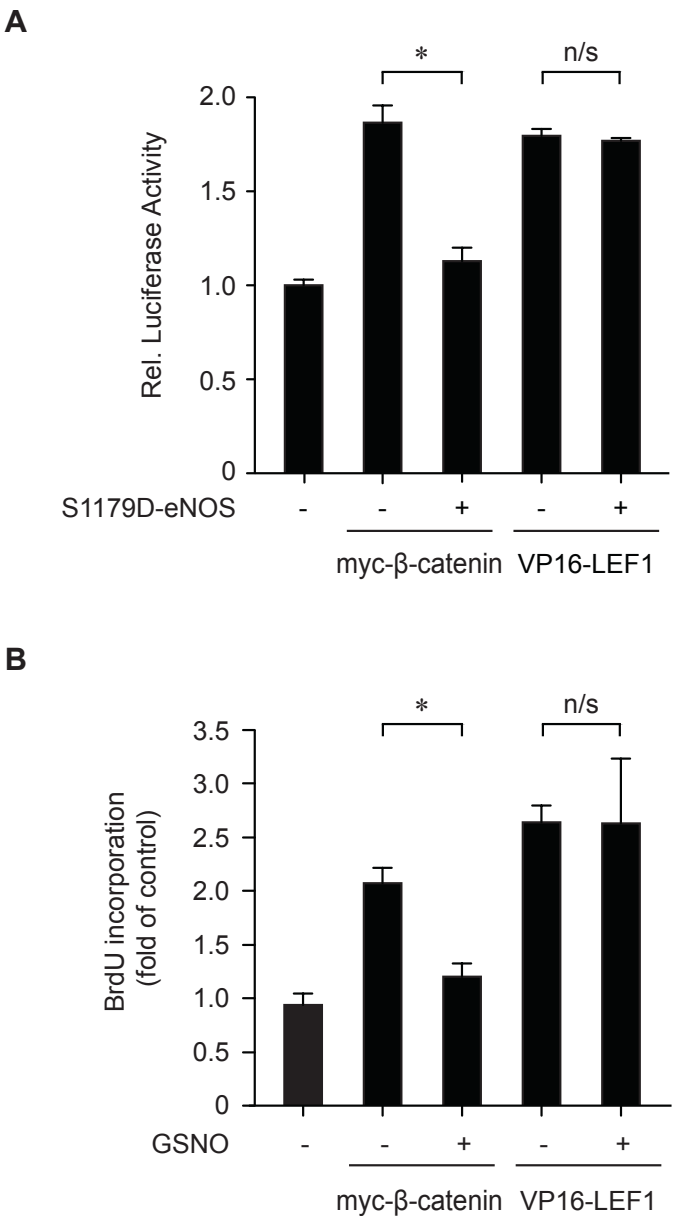

Figure II.S2. Transcriptional activity and cell proliferation induced by VP16-LEF1 are insensitive to the effects of NO.

(A) β-catenin luciferase reporter assay of HEK293T stably expressing the TOPFlash reporter and transfected with myc-tagged β-catenin or VP16-LEF1 in presence or absence of S1179D-eNOS (n=3). *(B)* BrdU proliferation assay of BAECs expressing myc-tagged β-catenin or VP16-LEF1 as indicated and in absence or presence of GSNO (0.1mM; 18h) (n=3). Data are represented as mean ± SEM. **P*<0.05; ns, not statistically significant.

Primer Name	Sequences
Bcat_ForC213S	GTA GAA ACA GCT CGT AGT ACC GCT GGG ACC
Bcat_ForC300S	TTC TTG GCT ATT ACG ACA GAC AGC CTT CAA ATT TTA GCT TAT G
Bcat_ForC381S	AAC GTC TTG TTC AGA ACA GTC TTT GGA CTC TCA GG
Bcat_ForC439S	AGA ACA AGA TGA TGG TCA GCC AAG TGG GTG GTA TA
Bcat_ForC466S	CAC TGA GCC TGC CAT CAG TGC TCT TCG TCA TCT G
Bcat_ForC520S	GGA TTG ATT CGA AAT CTT GCC CTT AGT CCC GCA AAT CA
Bcat_ForC619S	CTG CAG GGG TCC TCA GTG AAC TTG CTC AGG

Table II.S1.Mutagenesis sense primers (bovine)

Primer Name	Sequences
*AXIN2*_FOR	GGGAGAAATGCGTGGATACTT
*AXIN2*_REV	TTGTAGATCGCTTTGGCTACTC
β-actin_FOR	GACAGGATGCAGAAAGAGATCA
β-actin_REV	AATCCACACGGAGTACTTGC
GAPDH_FOR	CAACGTGTCTGTTGTGGATCTG
GAPDH_REV	TGTAGCCTAGAATGCCCTTGAG

Table II.S2. qRT-PCR primers (bovine)

Chapter III. Investigation of the regulatory role of eNOS on transcription in endothelial cells by RNA sequencing analysis (in preparation)

RNA sequencing analysis reveal the regulatory role of eNOS on transcription in endothelial cells in cell polarization, cell integrity and immune responses

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AUTHOR CONTRIBUTIONS

Y.Z. designed and performed the experiments, analyzed the data, prepared the figures, and wrote the manuscript; R.C analyzed the data and prepared figures III.3 and III.5; J.-P.G. designed and supervised the experiments, analyzed the data, prepared the figures, and wrote the manuscript.

Conflict of interest: The authors declare that they have no conflict of interest.

III.1. Abstract

eNOS activity and NO production regulate the expression of multiple genes in endothelial cells. However, the regulatory role of eNOS at the whole transcriptome level in endothelial cells remains to be explored. Using RNA sequencing analysis, we show that genes regulated by expression of eNOS are involved in cell integrity, immune response and cell polarization in endothelial cells. Furthermore, KEGG signaling pathway analysis shows that genes regulated by eNOS are enriched in many signaling pathways including Ras signaling and Rap1 signaling, which are important for endothelial cell migration. Moreover, clustering analysis of differentially regulated genes in VEGF-treated cells and VEGF-treated eNOS-depleted cells reveal that eNOS activity may influence expression of genes in angiogenesis in response to VEGF, including those involved in chemotaxis. Furthermore, results from real-time quantitative PCR analysis confirm that genes involved in polarization such as *PARD3A*, *PARD3B*, *PKCZ*, *CRB1* and *TJ3* are up-regulated by eNOS knockdown. Our studies show that levels of eNOS and VEGF-regulated eNOS activity modulate transcription in endothelial cells.

III.2. Introduction

Expression of eNOS and the biological product NO affect many signaling pathways including cell differentiation, cell proliferation, cell polarity and cell apoptosis, the production of cytokines, the expression of adhesion molecules, and the synthesis and deposition of extracellular matrix components (399-401). NO has been implicated in the regulation of transcription factors and the modulation of gene expression to modulate many functions in endothelial cells. For example, NO downregulates the expression of the transcription factor N-Myc through cGMP signaling, resulting in negative regulation of proliferation of neuronal precursors (356). Also, NO signaling has been shown to regulate other transcription factors such as c-fos, c-jun and NF-κB in a cGMP-dependent way (356). Alternatively, NO can regulate transcription through SNO of transcription factors by affecting their DNA binding activity or protein stabilization. For instance, SNO of NF-κB with exogenous iNOS-derived NO regulates gene transcription by inhibiting NF-κB-dependent DNA binding activity and SNO of HIF-1 promotes its transcriptional activity due to the modulation of protein stabilization (261-263).

Modulation of eNOS activity may be an attractive strategy for altering transcription in endothelial cells. Growth factors such as VEGF activate eNOS signaling pathway by the induction of calcium flux and the phosphorylation of NOS via PI3K-Akt pathway to stimulate NO production, which is essential for VEGF-regulated angiogenesis (218, 377, 378). A microarray study shows that genes differentially expressed in the heart of eNOS knockout mice are observed in the cellular processes including apoptosis, proliferation, inflammation, motility, constriction and regeneration (366). VEGF has been shown to regulate expression of genes in endothelial cells, such as *COX2*, *EGR1* and *angiogpoietin 2* (364); however, the correlation between VEGF-regulated genes and eNOS activity is still unknown.

RNA sequencing technique has provided researchers with a powerful tool for characterization of the transcriptome using properties for quantification and transcript discovery. RNA sequencing analysis reveals that thrombin treatment of human pulmonary microvascular endothelial cells upregulates genes enriched in the network of inflammatory response (402). Differentially expressed genes in APC mutated cells and wildtype cells obtained from RNA sequencing shows that genes regulated by loss of function of APC are associate with cell polarity (403).

In this study, we sought to investigate the effect of eNOS on the transcriptome-wide regulation of gene expression. Therefore, we carried on RNA sequencing analyses to study the effect of loss of eNOS and VEGF-induced eNOS activity on transcription in endothelial cells.

III.3. Materials and Methods

Cell culture

BAECs, obtained from VEC Technologies, were cultured in Dulbecco modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum (HyClone Laboratories), 2.0 mM L-glutamine, 100 U/mL penicillin, and 100 μg/mL streptomycin.

Antibodies and reagents

The following primary antibodies were used: anti-β-actin (8H10D10) from Cell Signaling Technology; anti-eNOS from BD Transduction Laboratories. Recombinant Human VEGF-A (VEGF herein) was obtained from the BRB Preclinical Repository of the NCI-Frederick Cancer Research and Development Center.

siRNA design and cell transfections

Small interfering RNA (siRNA) control and siRNA against eNOS were generated by Thermo Scientific Dharmacon. Two different siRNA sequences were designed for bovine eNOS, and the sense sequences are as follows: 5'-CCAGGAAGAAGACCUUUAAUU-3' and 5'-CCAACAUGCUGCUGGAAAUUU-3' for eNOS. The sequence of control siRNA is 5'-AUGAACGUGAAUUGCUCAAUU-3'. BAECs were transfected with siRNA using Lipofectamine 2000 according to the manufacturer's protocol (Invitrogen). BAECs were transfected with 50mM of control or eNOS siRNA at 80-90% confluency for 48h. Cells were then serum-starved for 6h before treated with VEGF (40ng/mL) for an additional 6h.

Immunoblot

BAECs were solubilized with a lysis buffer containing 1% Nonidet P-40, 0.1% sodium dodecyl sulfate (SDS), 0.1% deoxycholic acid, 50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 0.1mM ethylenediaminetetraacetic acid (EDTA), 0.1mM ethyleneglycoltetraacetic acid (EGTA), 20mM sodium fluoride, 1mM sodium pyrophosphate, and 1mM sodium orthovanadate and protease inhibitor cocktail (Roche Diagnostics). Cells were washed with cold PBS and centrifuged at 14,000 rpm for 10min. Soluble proteins were separated by SDS-PAGE, transferred onto a nitrocellulose membrane (Hybond-ECL, GE Healthcare) and western blotted. Detection and quantification were performed by a LI-COR Odyssey infrared imaging system (LI-COR Biosciences) using the appropriate Alexa-Fluro 680 labeled secondary antibodies (Invitrogen).

RNA extraction, electropherogram and Quantitative RT-PCR (qRT-PCR) analysis

Total RNA was extracted and suspended in commercial RNase-free water with an RNeasy Mini kit (Qiagen). After DNase I treatment, cDNA was synthesized from 1ug total RNA using the SuperScript II Reverse Transcriptase kit for RT-PCR (Invitrogen) according manufacturer's instructions. cDNA (5ng) was amplified in triplicate in a reaction volume of 10uL with 5uL SYBR Select Master Mix (Applied Biosystems).

The quantity and quality of the RNA were evaluated using RNA electropherograms (Agilent 2100 Bioanalyzer) and the RNA integrity number (404).

Real-time quantitative PCR was performed with ViiATM 7 Real-Time PCR System (Applied Biosystems). Gene expression analysis was performed by using the comparative cycle threshold (ΔCT) method, normalized with the expression of reference genes β-actin and GAPDH and

presented as the mean fold change (±SEM) compared with control sample. Bovine Egr3 forward primer is GGTGACCATGAGCAGTTTGC, and its reverse primer is AAGGCGAACTTTCCCAAGT. Bovine β-actin forward primer is GACAGGATGCAGAAAGAGATCA, and its reverse primer is AATCCACACGGAGTACTTGC.

RNA sequencing

The RNA samples (~100 ng/μL in 10 μL) were submitted to IRCM (Institut de Recherches Clininiques de Montreal) molecular biology core facility to capture mRNA using poly(T)-coated magnetic beads, followed by reverse transcription to generate cDNA library. The whole-transcriptome sequencing was done at Genome Quebec using an Illumina sequencer Hiseq2000.

Alignment of reads

The reads from RNA-seq were aligned to the reference genome UMD 3.1, a Bos Taurus genome, with TopHat software by the bioinformatics core facility of IRCM, using the Ensembl annotation provided with the Illumina iGenomes. Htseq-count script was used to count the number of reads aligned to each gene. These counts were then normalized relative to the sequencing depth with DESeq, an R package to analyze count data from RNA-Seq and test for differential expression. The levels of gene expression were calculated with Cufflinks, a software estimates the abundances of transcripts, and tests for differential expression in RNA-Seq samples (405).

Gene differential expression analysis

Gene differential expression was calculated by using the fold changes of number of reads by algorithms plugged in Microsoft Excel. In each experimental condition, genes up-/down-regulated more than 2 fold (>2 or <0.5 fold; >1 or <-1 log fold) in treatment condition comparing to the control condition were defined as regulated genes. The lists of regulated genes were further submitted for bioinformatics analysis.

Bioinformatics analysis

Bioinfomatics analysis was performed using DAVID (the Database for Annotation, Visualization and Integrated Discovery), an online resource that provides bioinformatics tools for analysis of large lists of genes derived from genomic studies. The lists of regulated genes were submitted to DAVID Bioinformatics Resources 6.7, and the enrichment of gene ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway was analyzed. The submitted gene IDs were converted from bovine Ensemble ID to human Ensemble ID since human database contains a larger list of characterized genes. The conversion was performed using the online bioinformatics tool 'dbOrtho' from the biological database network.

Each identifier was mapped to its corresponding objects provided by DAVID database. In the analyses of biological process, cellular components and molecular function, the GO terms 'GOTERM_BP_FAT', 'GOTERM_CC_FAT' and 'GOTERM_MF_FAT' were applied. EASE score, a conservative adjustment to the Fisher exact probability, was used to calculate Modified Fisher Exact P-value. The enrichment of GO terms was considered significant when the P-value is <0.05. In the analysis of pathway enrichment, the term KEGG_ pathway was applied and pathways with a Modified Fisher Exact P-Values <0.05 were considered enriched.

KEGG_pathway analysis was performed using resources from Search Tool for the Retrieval of Interacting Genes/Proteins (STRING), a database that contains information of known and predicted protein-protein interactions.

For gene cluster analysis, the regulated genes were submitted for clustering analysis using the software Gene Cluster 3.0. First, submitted genes were filtered by applying the available selection ‘log fold MaxVal – MinVal ⩾1’ to remove all genes that do not have more than two fold changes (equals one log fold) between two conditions. In other words, only genes whose expression level was different of at least two fold were selected. The filtered genes were clustered with hierarchical clustering methods by selecting ‘average linkage clustering’. The hierarchical clusters were visualized by TreeView software as a heat map with hierarchical cluster.

III. 4. Results

Quality analysis of RNA-Seq data

To study the effect of eNOS on transcriptome-wide regulation of gene expression, we have designed an RNA-seq experiment using mRNA from BAECs (Figure III.1). To carry out the RNA-seq experiments, BAECs were transfected with either control siRNA or siRNA against eNOS. The transfected BAECs were then either treated with VEGF or left untreated. Total RNA was isolated from four types of cells:

(a) BAECs transfected with control siRNA (CT siRNA);

(b) BAECs transfected with CT siRNA followed by VEGF treatment;

(c) BAECs transfected with eNOS siRNA;

(d) BAECs transfected with eNOS siRNA followed by VEGF treatment.

Before submitting the RNA samples for sequencing, we verified the experimental conditions using immunoblot and qRT-PCR analysis. First, results from the immunoblot analysis showed that eNOS levels were knocked down by ~80% in the samples transfected with siRNA against eNOS (Figure III.2A). Second, the results from the RT-PCR analysis demonstrated that the expression of *Egr3*, a well-known VEGF-responsive gene, was markedly increased in VEGF-treated cells (Figure III.2B).

In addition, we validated the quality of the RNA by determining the RNA integration number (RIN) for each sample. In our experiment, RIN values were above 9 in all the samples, which is greater than the recommended level of ~8 for this type of experiment, indicating excellent RNA integrity (Figure III.2C).

The sequencing yielded more than 40 million reads from each sample and ~14,000 genes were mapped from these reads (Table III.1). The number of reads assigned for each gene were used to calculate the gene differential expression.

RNA-seq analysis results showed that there was a 0.14-fold change in the expression of *NOS3*, the gene that encodes eNOS, in the eNOS knocked down cells when compared to the control cells. This is consistent with the results obtained from the immunoblot, which showed an 80% knockdown of eNOS at the protein level (Figure III.2 A). In addition, the expression of *Egr3*, was markedly increased in VEGF-treated cells in the RNA-seq analysis; a similar result was observed in the RT-PCR analysis (Figure III.2 B).

The distribution patterns of the gene expression in the different analyses are shown in a histogram (Figure III.3). As previously mentioned, the analyses were performed in endothelial cells to study (i) the effect of VEGF on the regulation of gene expression, (ii) the effect of eNOS knockdown on the regulation of gene expression and (iii) the effect of eNOS knockdown on the regulation of gene expression in the presence of VEGF treatment. The threshold (>1 log2 fold and < -1 log2 fold) was set to determine the regulated genes in each analysis. VEGF treatment of BAECs regulated the expression of 1,093 genes, including 467 upregulated and 626 downregulated genes. Comparison of control siRNA and eNOS siRNA transfected BAECs yielded 1,542 regulated genes, including 808 upregulated and 734 downregulated that were affected by eNOS knockdown. When compared the genes differentially expressed in VEGF-treated cells and VEGF-treated eNOS-depleted cells, expression of 1,635 genes was regulated, including 816 upregulated and 819 downregulated genes.

We started the analysis by comparing untreated cells and VEGF-treated cells in order to investigate the transcriptional profiling of endothelial cells in response to VEGF treatment. Since

a number of previous studies have examined the effect of VEGF on transcription using arrays, we were able to validate our results by comparing them with published data (365, 366, 370, 372). Our analysis identified 1,093 genes regulated by VEGF and a list of profoundly regulated genes is shown in Table III.S1. Comparing these findings to the results of published microarray studies, we were able to validate a few differentially expressed genes discovered in our analysis. Our results showed that the NR4A nuclear receptor family genes including *NR4A-1, -2* and *-3* were upregulated 71-, 26- and 69-fold, respectively. As shown by Liu *et al*, VEGF markedly induced expression of all these genes encoding the NR4A nuclear receptor family genes 17-fold (365). This more marked increase of the gene expression observed in our analysis is probably due to the higher concentration of VEGF used to treat the samples in our study. Liu's study also showed that expression of early response genes *Egr1, and Egr3* (Early Growth Response 1 and 3) was upregulated to ~10 fold by VEGF. Similarly, our data showed that in VEGF-treated cells, expression of the *Egr1* gene was upregulated to 3-fold, while expression of the *Egr3* gene upregulated ~16-fold. In addition, our analysis also showed evidence of other VEGF-regulated genes, such as *DSCR1* (Down syndrome critical region gene 1, *RCAN1* in the bovine genome), *CXCR7* (C-X-C chemokine receptor type 7), *RND1* (Rho-related GTP-binding protein Rho6), *CLDN5* (Claudin-5) and *ANGPT2* (Angiopoietin-2), which have been identified as VEGF-responsive genes in other studies (364, 406, 407). GO annotations and pathway enrichment were also analyzed for the VEGF-regulated genes (Figure III.S1 and Table III. S1). Similarly, we obtained a list of differential expressed genes in the VEGF-treated eNOS-depleted cells by comparing to untreated cells. The expression of these genes was further compared with the VEGF-regulated genes.

Differential expressed genes in eNOS knockdown endothelial cells

To study the effect of eNOS knockdown on endothelial cells, we compared expression of genes in cells transfected with control and eNOS siRNA. A list of genes which were most differentially expressed in cells transfected with CT and eNOS siRNA was generated (Table III.4). The genes most markedly upregulated by eNOS depletion include *SLC16A9* (proton-linked monocarboxylate transporter, MCT), *PKP2* (plakophilin 2), *GJA -9* and *-1* (gap junction proteins -9 and -1), *CRB1* (crumbs family member 1), *MMP2* (matrix metallopeptidase 2), and *FAS* (Fas cell surface death receptor). We found that the expression of *MMP2* is upregulated when endogenous eNOS is knocked down. This is in agreement with previous reports showing that NO synthase inhibition in coronary vessels resulted in increased MMP2 activity and expression of eNOS in smooth muscle cells caused inhibition of MMP2 activities (408, 409). Crumbs (CRB) have been shown to be crucial for polarization in epithelial cells, and CRB1, expressed in the neural retina and adult brain in drosophila, may control proper development of polarity in the eye (410, 411). Therefore, the upregulation of *CRB1* by eNOS knockdown found in our analysis may suggest a potential link between cell polarity and the expression of eNOS. The genes most profoundly downregulated by eNOS knockdown include *PMCH* (pro-MCH precursor), *RBM20* (probable RNA-binding protein 20), and *TRIM9* (Tripartite Motif Containing 9). *TRIM9* encodes a brain-specific E3 ubiquitin ligase, which is repressed in the brains of Parkinson's disease patients (412). Expression of genes such as *ANGPT2* (angiopoietin-2) and *CLDN 5* (claudin 5), which encode proteins important for the regulation of vascular integrity in endothelial cells, was found to be significantly decreased by eNOS knockdown. Interestingly, genes involved in the Notch pathway, such as *HES1* (hairy and enhancer of split-1), *DLL4* (Delta-Like 4 (Drosophila)) and *NOTCH4* (Notch 4), were significantly downregulated in eNOS-depleted endothelial cells, indicating potential crosstalk between eNOS and Notch signaling.

GO annotations of genes regulated by eNOS knockdown

To investigate the function of the genes regulated by eNOS, 1,542 genes regulated by eNOS knockdown were submitted to determine the GO annotations including **biological process**, **cellular component**, and **molecular function** (Figure III.4). In the analysis of **biological process** ontology, we identified that the most enriched GO terms for the genes regulated by eNOS knockdown include inflammatory response (GO: 0006954), response to wounding (GO: 0009611), regulation of response to external stimulus (GO: 0032101), defense response (GO: 0006952), MAPKKK cascade (GO: 0000165), cell adhesion (GO: 0007155) and biological adhesion (GO: 0022610). In the analysis of **cellular component** ontology, the most enriched GO terms for the list of genes regulated by eNOS knockdown were plasma membrane part (GO: 00044459), plasma membrane (GO: 0006886), extracellular **cellular components** region part (GO: 0005576), intrinsic to plasma membrane (GO: 0031226), integral to plasma membrane (GO: 0005887), and apical junction complex (GO: 0043296). Furthermore, we found that the regulated genes were enriched in the **molecular function** ontology including components involved in pattern binding (GO: 0001871), polysaccharide binding (GO: 0030247), carbohydrate binding (GO: 0030246), and growth factor binding (GO: 0019838).

Pathway analysis of genes regulated by eNOS knockdown in endothelial cells

The KEGG pathway enrichment analysis showed that many pathways including leukocyte transendothelial migration (hsa04670), cell adhesion molecules (hsa04514), ECM-receptor interaction (hsa04512), PI3K-Atk signaling pathway (hsa04151), pathways in cancer (hsa05200), axon guidance (hsa04360), regulation of actin cytoskeleton (hsa04810), focal adhesion (hsa04510), Ras signaling pathway (hsa04014) and VEGF signaling pathway (hsa04370) were enriched for genes regulated by eNOS knockdown (Table III.3).

In our analysis, the most significantly enriched pathway in eNOS knockdown cells is leukocyte transendothelial migration (has04670), which involves genes including *CXCL12* (Chemokine (C-X-C Motif) Ligand 12), *CLDN5* (Claudin 5), *ESAM* (endothelial cell adhesion molecule), *MMP2* (Matrix Metallopeptidase 2), *MAPK11* (mitogen-activated protein kinase 11) and *PIK3CB* (Phosphatidylinositol-4,5-Bisphosphate 3-Kinase, Catalytic Subunit Beta).

In addition, since Ras signaling activation has been implicated in cell migration (413, 414), thus the finding that the Ras signaling pathway is enriched for the genes regulated by eNOS depletion in our analysis may provide more evidence for the involvement of Ras signaling in the migration of endothelial cells at the transcriptional level. The genes enriched in the Ras signaling pathway (hsa04014) include *PAK7* (P21-activated kinase 7), *MRAS* (Ras-related protein M-Ras), *FGFR3* (fibroblast growth factor receptor 3), *RASA4* (Ras P21 protein activator 4), *CSF1* (macrophage colony-stimulating factor 1), *NGFR* (nerve growth factor receptor), *KSR1* (kinase suppressor of Ras 1), and *SHC3* (Src homology 2 domain-containing-transforming protein C3). Interestingly, MRAS has been shown to play a key role in polarized migration during tumorigenic growth through its ability to recruit polarity proteins and modulate Ras-ERK pathway (415), therefore, the modulation of expression of *MRAS* by eNOS knockdown may suggest a link between eNOS and Ras signaling.

Genes regulated by VEGF-induced eNOS activation

Due to our interest in studying the effect of eNOS-derived NO on transcription, we sought to investigate the expression of genes regulated by VEGF-induced eNOS activation. Therefore, we compared two lists of genes: the first list contains those genes identified in VEGF-treated cells; the second list includes genes identified in VEGF-treated eNOS-depleted cells. In this part of the

analysis, we chose genes whose expression showed more than a 2-fold difference between the first list and the second list in order to investigate the influence of eNOS knockdown in VEGF-treated cells (only the genes differentially expressed in either the first list or the second list were chosen for this comparison) (Table III.4). Expression of *IL11*, which encodes a cytokine promotes cancer cells (416), was upregulated by VEGF significantly in cells transfected in control siRNA and eNOS siRNA but the upregulation was more than 2-fold different. These results suggest that eNOS plays a positive role in VEGF-regulated expression of *IL11*. Additionally, VEGF stimulation increased level of ADAM12 in endothelial cells and ADAM12 overexpression resulted in increase of cell migration, therefore, it would be interesting to reveal the link among eNOS, VEGF-regulated expression of *ADAM12* and ADAM12-mediated endothelial cell migration (417).

GO analysis of genes regulated by VEGF-induced eNOS activation

To determine the biological functions of genes regulated by VEGF-induced eNOS activation, we performed GO enrichment analysis for the genes differentially expressed in VEGF-treated cells and VEGF-treated eNOS-depleted cells (Figure III.5). We found that the most enriched **biological processes** include cell-cell signaling (GO: 0007267), behavior (GO: 0007610), cell adhesion (GO: 0007155), biological adhesion (GO: 0022610), immune response (GO: 0006955), neuron development (GO: 0048666), neuron differentiation (GO: 0030182), chemotaxis (GO: 0006935), and angiogenesis (GO: 0001525). Moreover, we investigated the **cellular component** GO enrichment and we identified that plasma membrane part (GO: 0044459), extracellular region part (GO: 0044421), extracellular region (GO: 0005576), plasma membrane (GO: 0005886), intrinsic to plasma membrane (GO: 0031226), extracellular space (GO: 0005615), integral to plasma membrane (GO: 0005887), actin cytoskeleton (GO: 0015629), and apical part of cell (GO: 0045177) were found to be significantly enriched. Furthermore, the significantly

enriched **molecular functions** consist of growth factor binding (GO: 0019838), calcium ion binding (GO: 0005509), thrombin receptor activity (GO: 0015057), cytoskeletal protein binding (GO: 0008092), integrin binding (GO: 0005178), ion channel activity (GO: 0005216), growth factor activity (GO: 0008083), and chemokine activity (GO: 0008009). VEGF and activation of eNOS have been implicated in the reciprocal regulation of angiogenesis (418), the enrichment of angiogenesis as a biological process from our RNA-seq analysis may provide another piece of evidence to for further exploration of VEGF-regulated angiogenesis at genomic level.

Pathway analysis of genes regulated by VEGF-induced eNOS activation

The KEGG pathway analysis of genes regulated by VEGF-induced eNOS activation showed that the following pathways were enriched: Rap1 signaling pathway (hsa04015), pathways in cancer (hsa05200), PI3K-Akt signaling pathway (hsa04151), calcium signaling pathway (hsa04020), cytokine-cytokine receptor interaction (hsa04060), cGMP-PKG signaling pathway (hsa04022), Ras signaling pathway (hsa04014), axon guidance (hsa04360), TNF signaling pathway (hsa04668), Wnt signaling pathway (hsa04310) and the hedgehog signaling pathway (hsa04310) (Table III.5). It is known that the Rap1 and Ras signaling pathways are involved in VEGF-stimulated cell migration and NO stimulates the Ras-ERK1/2 MAP kinases signaling pathway (419, 420). Therefore, the differential expression of these enriched genes may offer novel insights into the mechanisms involved in these signaling pathways. Moreover, pathway enrichment analysis showed that eNOS knockdown affects the regulation of Wnt signaling by VEGF, in consistent with our previous findings that eNOS knockdown prevents the regulation of the Wnt target genes *AXIN2* and *CYCLIN D1* by VEGF (Zhang et al., unpublished).

Clustering analysis of genes regulated by VEGF-induced eNOS activation

Hierarchical clustering, in which genes with similar expression patterns are grouped together, is the most popular method for gene expression data analysis. Here, we generated a heat map to show the hierarchical clustering of differential gene expression (Figure III.6). In the clustering analysis, we obtained five clusters. From these, two clusters which contain genes with most profoundly difference were chosen for further analysis: Cluster 1 contains 90 genes whose expression was significantly higher in VEGF-treated cells compared to VEGF-treated eNOS-depleted cells, whereas Cluster 2 is composed of 86 genes whose expression was significantly higher in VEGF-treated eNOS-depleted cells compared to VEGF-treated cells. In other words, in Cluster 1, knockdown of eNOS upregulated the expression of VEGF-regulated genes, suggesting eNOS-derived NO may repress their expression. In contrast, in Cluster 2, depletion of eNOS downregulated the expression of VEGF-regulated genes *CCL8*, *CCL19*, *CCL21* and *CCL24*, suggesting that eNOS-derived NO promoted the expression of these genes. Chemokines *CCL19*, *CCL21*, *CCL24*, *CCL25*, *CXCL8* and *CXCL10* were reported to induce chemotaxis of M1 macrophages (421).

To further explore the functions of genes involved in the two clusters, we analyzed the genes in Cluster 1 and Cluster 2 using DAVID Gene Functional Classification tool, an online resource capable of organizing and condensing large gene lists into biologically meaningful modules (422). With this tool, the differentially regulated genes in Cluster1 and Cluster2 were grouped into different functional categories. The terms for the function categories found in Cluster 1 include glycoprotein, polymorphism, ion transport, transmembrane and potassium transport; in Cluster 2 there are glycoprotein, signaling, chemotaxis, actin-binding, calmodulin-binding, metalloprotease, cytokine, inflammatory response and G protein-coupled receptor.

Since we are interested in the functional categories that are important for angiogenesis and endothelial cell biology, we focused on the genes classified in the Cluster 2 categories: **signaling, chemotaxis, inflammatory response** and **metalloprotease**. Genes that encode for receptors were classified in the **signaling** function category, such as *FZD7* (frizzled 7) in Wnt signaling, *IL1RL1* (interleukin 1 receptor-like 1) in inflammation, *NGFR* (nerve growth factor receptor) in fibroblast growth factor signaling, and *PDGFB* (Beta-Type Platelet-Derived Growth Factor Receptor) in PI3K pathway. Genes enriched in the **chemotaxis** and **inflammatory response** functions include genes that encode for chemokines, such as *CCL8* (chemokine (C-C motif) ligand 8), *CCL19* (chemokine (C-C motif) ligand 19), *CCL22* (chemokine (C-C motif) ligand 22), and *CCL24* (chemokine (C-C motif) ligand 24). Genes classified in the **metalloprotease** category include *ADAM12* (ADAM metallopeptidase domain 12), *ADAMTS3* (ADAM metallopeptidase with thrombospondin type 1 motif, 3), *DPEP2* (dipeptidase 2) and *LMLN* (leishmanolysin-like peptidase).

Validation of eNOS-regulated polarization genes by qRT-PCR analysis

Some evidence showed that eNOS downregulation increased the polarization of endothelial cells, suggesting that eNOS plays an important role in directed endothelial cell migration via the regulation of cell polarity (Oubaha *et. al.*, not published). Therefore, we were interested in studying the genes involved in cell polarization that were modulated by the downregulation of eNOS. Notably, in our RNA sequencing analysis, genes which were differentially expressed in cells transfected with eNOS siRNA, were enriched in a GO annotation, the apical junction complex (GO:0043296), which categories genes important for cell polarization. A heat map was generated to show all the differentially expressed genes found in this annotation in eNOS-depleted cells (Figure III.5). The genes include *PARD6A* (partitioning defective 6, PAR6), *PARD6B* (partitioning

defective 6 homolog beta), *PKCZ* (protein kinase C, zeta), *TJP3* (ZO-3), *CRB1 (*crumbs homolog 1), *PARD3B* (partitioning defective 3 homolog beta), and *PARD3* (partitioning defective 3, PAR3); these genes are known to play an important role in cell polarization. Next, we validated the levels of mRNA in these genes by qRT-PCR (Figure III.7A), and the results from qRT-PCR analysis were in agreement with those obtained in our RNA-seq analysis (Figure III.7B).

III.5 Discussion

In the present study, we performed a comprehensive transcriptome analysis of BAECs using the Illumina RNA sequencing technique. Using eNOS knockdown endothelial cells as a model, we revealed that the expression of ~1,500 genes was increased or decreased at least 2-fold when eNOS was knocked down, indicating that eNOS influences transcription in endothelial cells to a great extent. Particularly, eNOS knockdown has been shown to regulate the expression of genes involved in immune response, cell integrity and cell polarization. In addition, KEGG signaling pathway analysis showed that genes regulated by eNOS knockdown were enriched in the Ras signaling pathway, which is crucial for the polarity and directed cell movements of endothelial cells (423), implying a potential link among eNOS, Ras signaling and endothelial cell polarity. Furthermore, the analysis of gene differential expression in VEGF-activated cells versus VEGF-activated eNOS-depleted cells yielded ~1,600 genes whose expression differed by at least 2-fold in the two samples, suggesting that eNOS contributes to VEGF-regulated transcription in endothelial cells. Gene clustering analysis and functional classification of these genes suggests that eNOS activity modulates the expression of genes involved in chemotaxis in VEGF-treated endothelial cells.

In eNOS-/- mice, characterization by changes in gene expression by microarray analysis revealed the expression of genes related to heart failure and immune response was regulated by eNOS knockout (366). Our study, on the other hand, using transcriptome-wide RNA sequencing analysis, demonstrates an integral role of eNOS in endothelial cell culture at the transcriptomic level. Our analysis shows that eNOS knockdown significantly decreases expression of *ANGPT-2,* which encodes Ang-2, a protein involved in the regulation of vascular integrity in endothelial cells of the interior tumor vessels. NO has been implicated in the regulation of endothelial integrity and

activation of PI3K/Akt/eNOS signaling positively regulated Ang-2 release, suggesting that NO regulates the secretion of Ang-2 from endothelial cells (424, 425). Here, our results show a positive correlation between eNOS and the total Ang-2 synthesized by endothelial cells, indicating that increased *Ang-2* expression by eNOS may contribute to the effect of NO on the Ang-2-induced destabilization of vessels. Moreover, eNOS knockdown downregulates the expression of *CLDN 5*, gene encoding for claudin 5, another endothelial cell-specific protein indispensable for the organization of tight junctions. Decreases in claudin 5 can be destructive to the organization of tight junctions and may lead to increased endothelial cell permeability (131), therefore, our results may imply a link between eNOS and cell integrity in genomic level.

Moreover, our results show that genes important in the Notch signaling pathway such as *DLL4*, *DLL1*, *Notch4*, *Hes1* and *Hey1* are downregulated in endothelial cells where the eNOS levels are knocked down, indicating a potential cross-regulation between eNOS and Notch. Notch signaling promotes the distinction between the leading tip endothelial cell and the growing stalk cell in the formation of vascular sprouts (426), thus eNOS may regulate the tip/stalk cell specification through modulation of the important players in Notch signaling.

Furthermore, results herein from both RNA sequencing and qPCR analysis show an inverse correlation between expression of eNOS and polarization genes. PAR3, PAR6 and aPKC are well-known proteins involved in cell polarization in many different contexts including in endothelial cells (72, 427). To our knowledge, for the first time, we observe that eNOS knockdown increases the expression *PARD6A*, *PARD6B*, *PKCZ*, *TJP3*, *CRB1*, *PARD3B* and *PARD3,* suggesting a role of eNOS in the modulation of expression of genes involved in polarization in endothelial cells (Figure III.7). Recently, the molecular control of endothelial cell polarity has been a subject of investigation, similar to the already well-studied topic of epithelial polarity (428). It is known that

NOS inhibitor L-NAME significantly inhibits endothelial cell migration and cell polarization is a critical step during directed cell migration (429, 430). Therefore, the results might provide novel mechanistic insights for the influence of eNOS or the production of NO on the polarization of endothelial cells. In the analysis of signaling pathways enriched for genes regulated by eNOS downregulation, we show that Ras signaling pathway is among the most affected signaling pathways. K-Ras has been observed together with some well-known polarity regulators including Cdc42, Rac1 and Rap1b, which are important for the development of an endothelial cell apical membrane surface during tubulogenesis (423). In T cells, eNOS selectively activates N-Ras on the Golgi through S-nitrosylation of cysteine 118 and modulates polarization of Golgi/cytoplasm (431). Thus, further investigation of crosstalk between eNOS and Ras signaling may provide more comprehensive mechanistic insight into the effect of eNOS on cell polarity.

Additionally, genes whose expression was regulated by eNOS knockdown were enriched in the biological processes of inflammatory response (GO: 0006954) and immune response (GO: 0006955). NO also plays a critical role in the immunity by inhibiting the adhesion of platelets and leukocytes to endothelium (401). Using eNOS (-/-) mice, a study has shown that NO is critical for reducing leukocyte adhesion (432). In agreement with these results, in the RNA sequencing analysis, we found that in endothelial cells, eNOS knockdown upregulated expression of *SELE*, which encodes for E-selectin, a cell adhesion molecule expressed only on endothelial cells (433); this suggests a negative role of NO in regulation of leukocyte-endothelial cell adhesion. Therefore, investigation of the expression of genes involved in the two processes may provide new knowledge concerning the role of NO in the immune response. These genes include *TLR2* (toll-like receptor 2), *TLR6* (toll-like receptor 6), *TLR7* (toll-like receptor 7), *TNFRSF4* (tumor necrosis factor ligand superfamily member 4), *CD14* (CD14 molecule), *NOX1* (NADPH oxidase 1), *NOX4* (NADPH

oxidase 4), *CFI* (complement factor I), *C5* (complement component 5) and *C7* (complement component 7).

VEGF is one of the most potent angiogenic factors in the regulation of endothelial cell-directed migration and eNOS plays an important role in VEGF-regulated endothelial cell migration (429). However, the effect of eNOS on VEGF-regulated gene expression is still unknown. In this study, comparison of expression of the genes in VEGF-treated cells and VEGF-treated eNOS-depleted cells shows that genes that encode for chemokines, including *CCL8*, *CCL19*, *CCL22* and *CCL24,* were clustered together in a hierarchical clustering analysis. Since VEGF is known to activate eNOS to generate NO, these results suggest that eNOS-derived NO in endothelial cells stimulated by VEGF treatment can increase the expression of chemokines such as CCL8, CCL19, CCL22 and CCL24. Chemokines are a large group of small cytokines known for their chemotactic ability to regulate the recruitment of leukocytes to sites of inflammation. In addition to the regulatory role in leukocyte function, chemokines can affect angiogenesis through their binding to the chemokine receptors expressed on endothelial cells, leading to increased cell migration in concert with other angiogenic factors (434). Therefore, the modulation of eNOS in VEGF-regulated expression of chemokines may indicate a novel role of eNOS in VEGF-regulated angiogenesis.

In summary, our results show the expression of more than a 1,500 genes which are involved in inflammatory responses, cell integrity, cell polarization, chemotaxis and other cellular functions in endothelial cells regulated by eNOS expression and activity. Our findings also indicate that eNOS may regulate Ras signaling, Notch signaling and other signaling pathways, which are critical for endothelial cell functions.

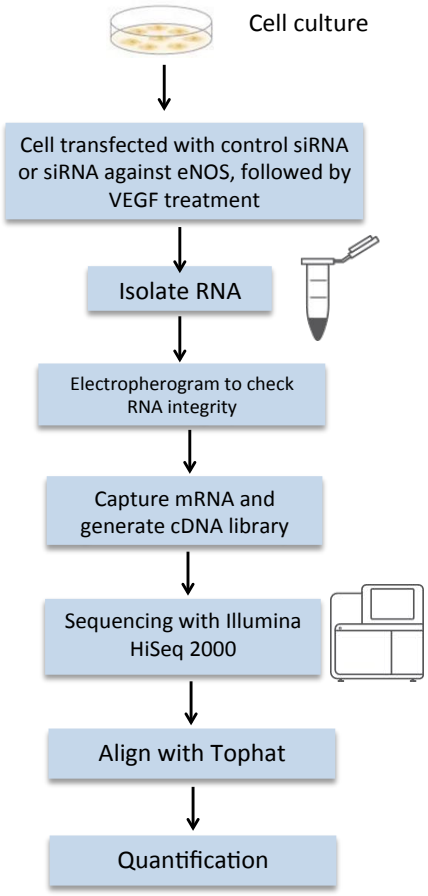


Figure III.1. RNA-seq experimental design and workflow.

BAECs were transfected with control (CT) siRNA or eNOS siRNA for 48h. Cells were then starved in serum-free medium followed by VEGF treatment (40ng/mL) for 6h. Total RNA was isolated and electropherogram was performed to ensure the integrity of RNA. cDNA library was generated, followed by RNA sequencing using an Illumina sequencer. The generated reads from the sequencing were submitted and aligned to UMD3.1 bovine genome with TopHat software. Gene expression was quantified with Cufflinks and Microsoft Excel software.

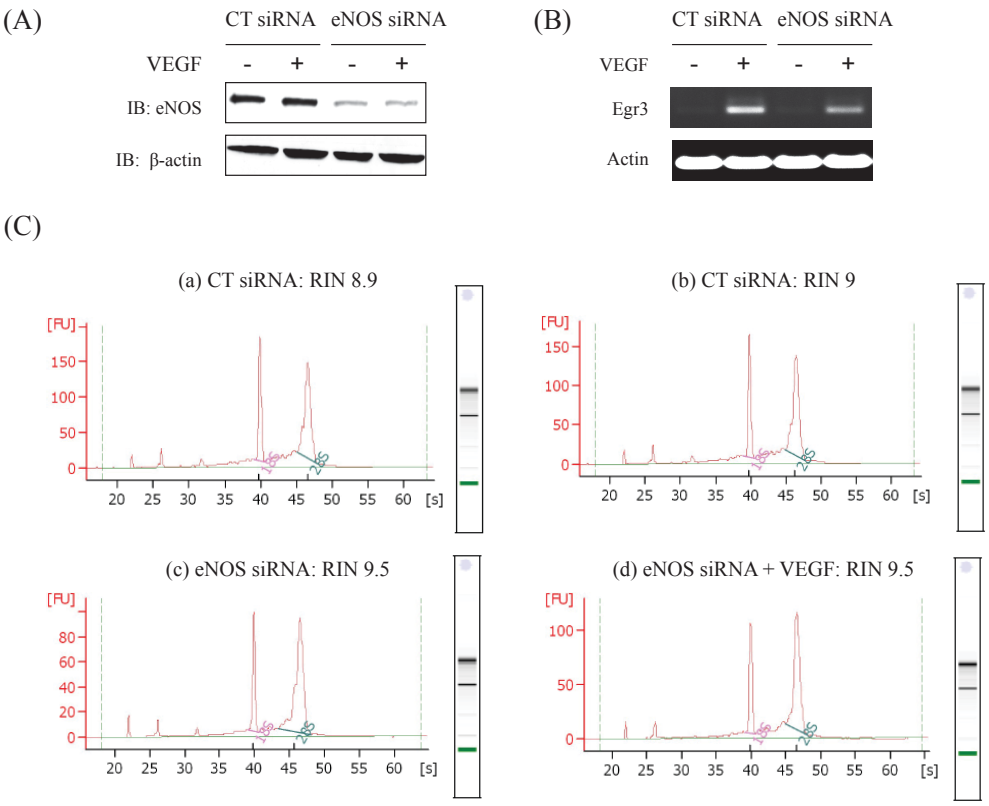


Figure III.2. Validation of sample preparation before sequencing.

BAECs were transfected with control (CT) siRNA or eNOS siRNA, then subjected to VEGF treatment (40ng/mL) for 6h. (A) Depletion of eNOS was monitored by immunoblot (IB) and β-actin was used as a loading control. (B) RT-PCR analysis of *EGR3* mRNA levels in BAECs treated with VEGF and actin was used as a control. (C) Total RNA was isolated and an electropherogram was performed to ensure the integrity of RNA. RIN values obtained from the electropherogram and the peaks correspond to 28S and 16S rRNAs.

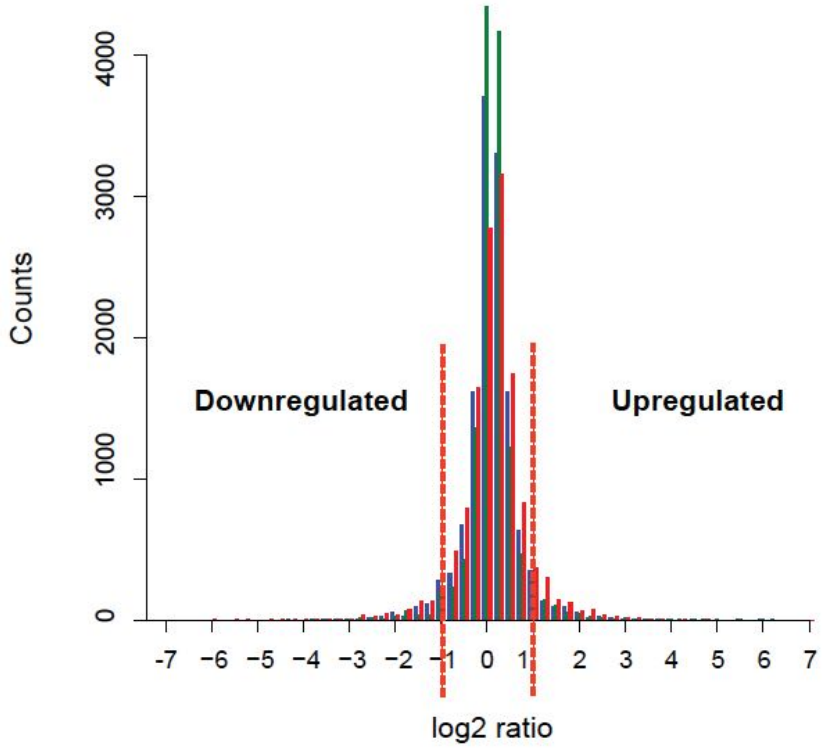


Figure III.3. Distribution patterns of total identified genes and regulated genes

A histogram shows the distribution pattern of the expression of all genes identified in the analyses. Blue bars represent the number of genes in the analysis which compares control untreated cells and VEGF-treated cells. Red bars show the number of genes in the analysis in which cells transfected with control siRNA and eNOS siRNA were compared. Green indicates genes in the analysis in which VEGF-treated cells and VEGF-treated eNOS-depleted cells were compared. X axis indicates the expression level of genes. Y axis shows the number of genes identified in the similar level. In the analyses, genes whose expression levels were more than 2 fold different between two conditions were defined as regulated genes. The graph was generated with Graphical Proteomics Data Explorer (GProx) software using the values of log2 ratio. Dotted lines indicate our threshold (>1 log2 fold and < -1 log2 fold) used to define the up- and downregulated genes.

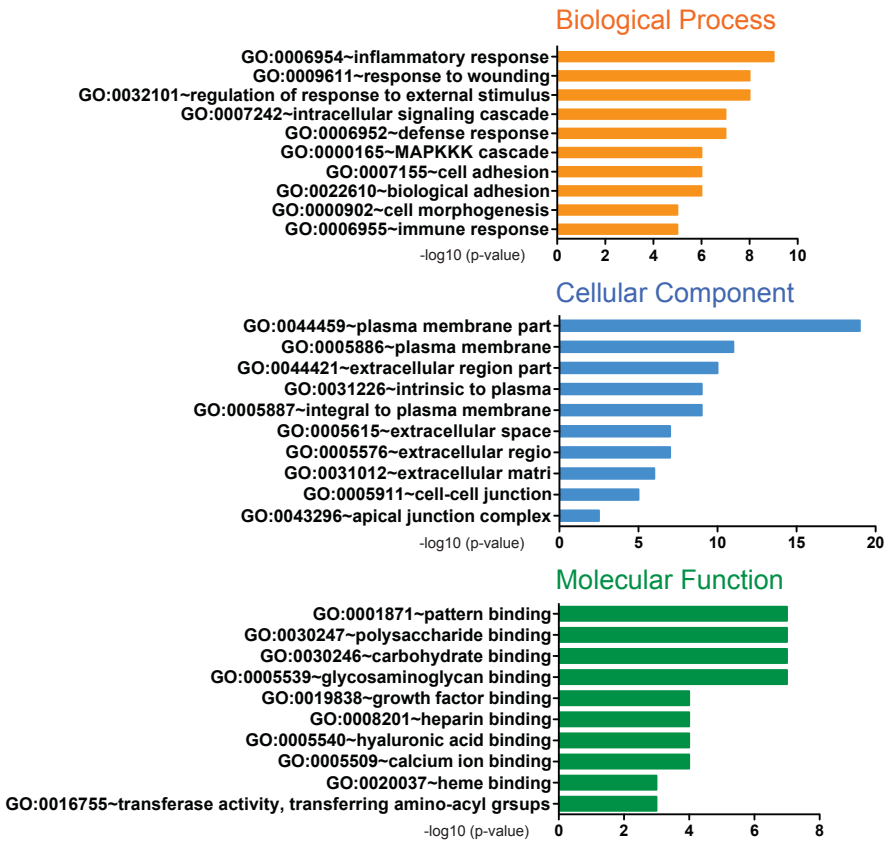


Figure III.4. GO enrichment analysis of genes regulated by eNOS knockdown and a heat map showing fold changes of selected genes.

The list of differentially expressed genes in eNOS-depleted cells was submitted to determine the enrichment of **biological processes**, **cellular component**, and **molecular function**. The Fisher Exact p-value was used to define the significantly enriched GO terms. X-axis shows a log10 transformation of the p-value of the enrichment. The GO terms with values –log10 (p-value) > 1.3 are considered significantly enriched (p-value < 0.05). Y-axis shows the names and identification numbers of the GO annotation terms.

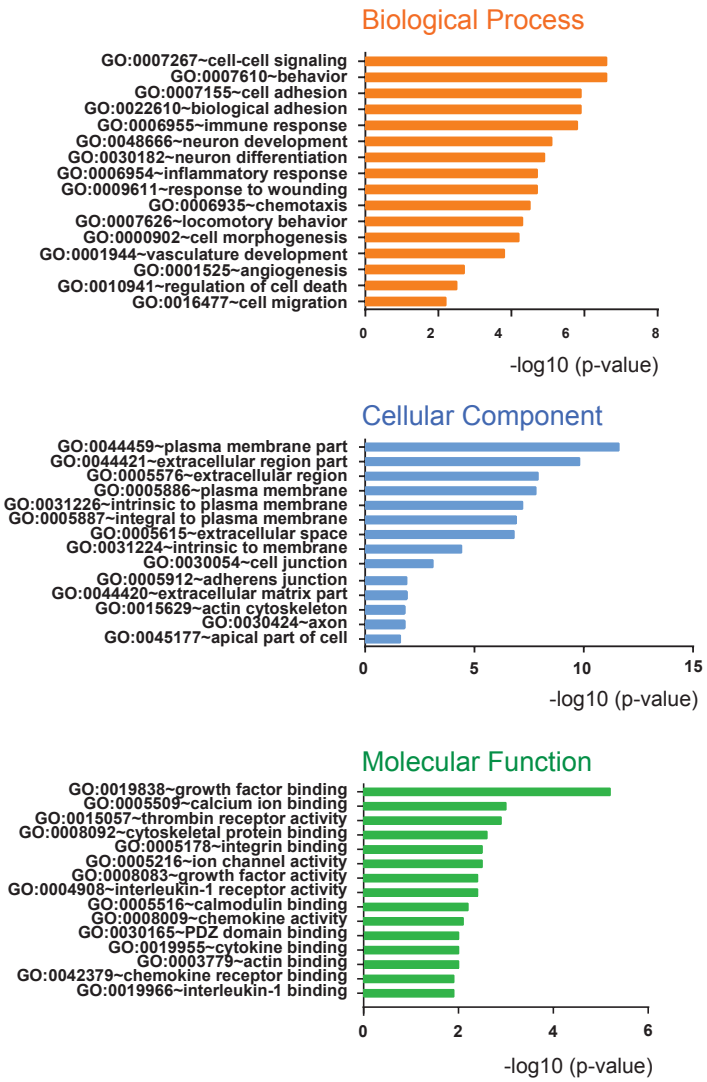


Figure III.5. GO enrichment analysis of genes differentially expressed in VEGF-treated cells and VEGF-treated eNOS-depleted cells.

BAECs were transfected with with eNOS siRNA or control siRNA, then treated with VEGF for 6h or left untreated. The differentially expressed genes (> 2-fold) in VEGF-treated cells and VEGF-treated eNOS-depleted cells were submitted to the DAVID database and the enrichment of the biological processes, cellular components, and molecular function was characterized. The Fisher Exact p-value was used to define the significantly enriched GO terms. The x-axis shows the log10 transformation of the p-value of the enrichment. The GO terms with values –log10 (p-value) > 1.3 are considered significantly enriched (p-value < 0.05). The y-axis lists the names and identification numbers of the GO annotation terms. In the heat map, red indicates upregulation of genes and green indicates downregulation of genes. The numbers show the log fold change in gene expression.

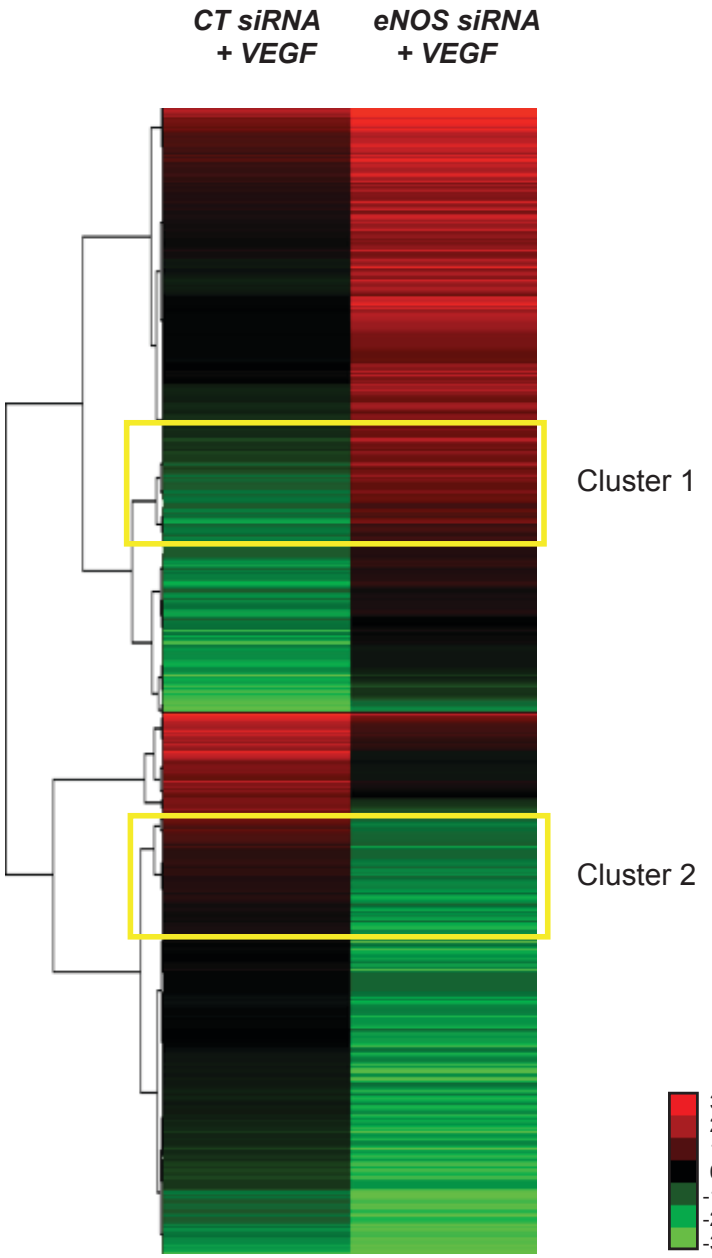
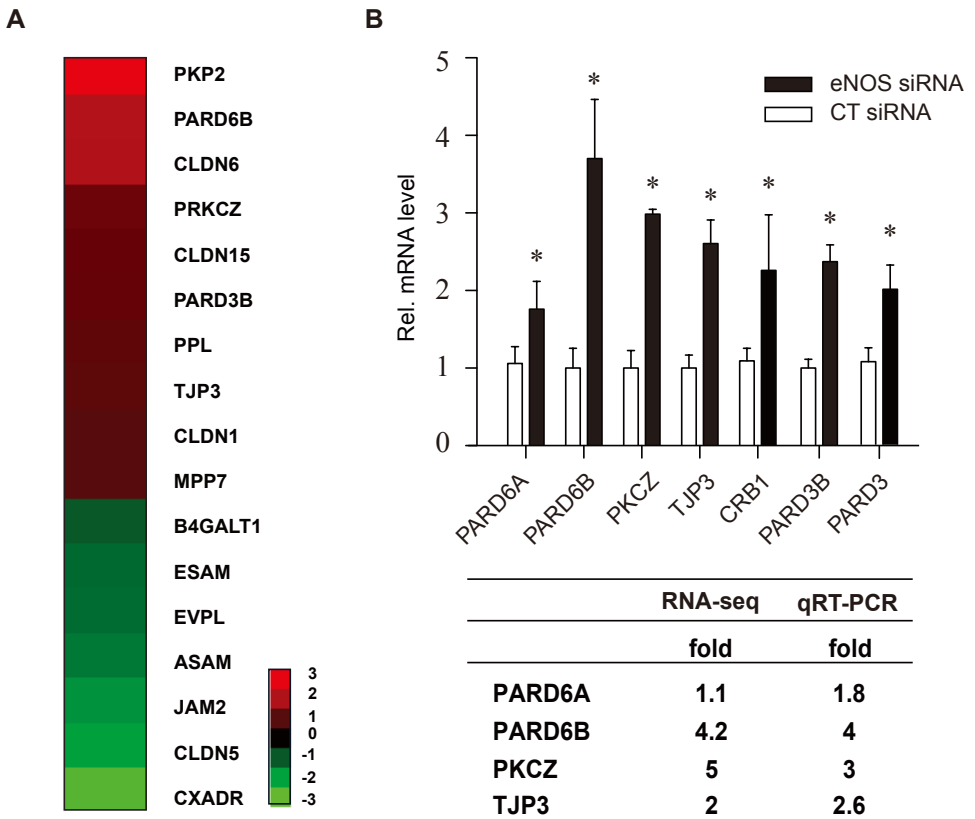


Figure III.6. Hierarchical clustering of genes in VEGF-treated cells and VEGF-treated eNOS-depleted cells.

BAECs were transfected with with eNOS siRNA or control siRNA, then treated with VEGF for 6h or left untreated. The differentially expressed genes (> 2-fold) in VEGF-treated cells and VEGF-treated eNOS-depleted cells were submitted to Gene Clustering analysis. Red indicates the upregulation of gene expression and green indicates the downregulation of gene expression. Numbers beside the color bar show the log fold changes.



	RNA-seq	qRT-PCR
	fold	fold
PARD6A	1.1	1.8
PARD6B	4.2	4
PKCZ	5	3
TJP3	2	2.6
CRB1	3.9	2.6
PARD3B	2.2	2.4
PARD3	0.9	2

Figure III.7. Expression of selected eNOS-knockdown-regulated genes was validated by qRT-PCR analysis.

(A) A heat map showing expression of genes regulated by knockdown of eNOS and enriched in apical junction complex (GO:0043296). Red color indicates upregulation and green represents the downregulation of genes. The numbers at the side of the color chart show log fold changes of the gene expression. (B) qPCR analysis of BAECs were transfected with 50mM CT siRNA or eNOS siRNA for 48h. N=3, error bars represent the SEM. *P<0.05; one-way ANOVA. The table in the bottom shows the results of fold change of gene expression obtained from RNA-seq and qRT-PCR.

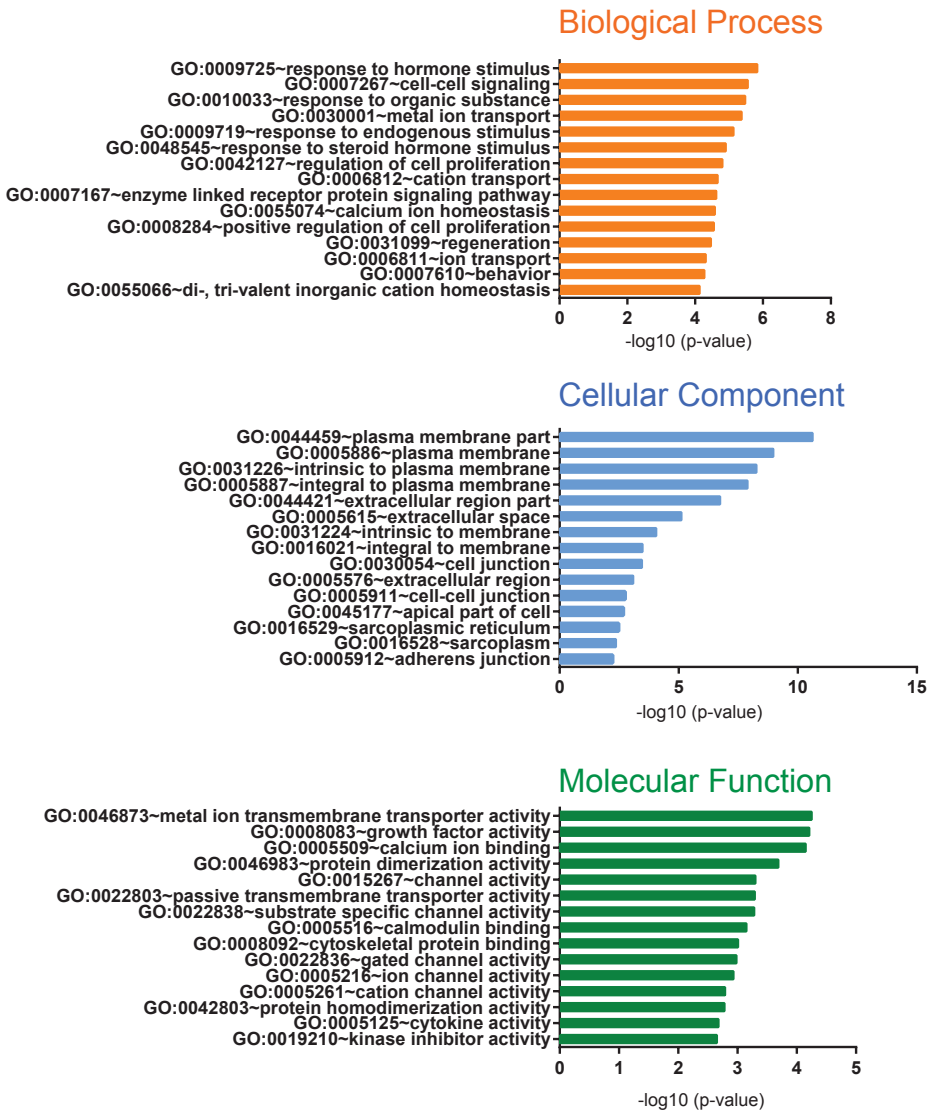


Figure III.S1. GO enrichment analysis of VEGF-regulated genes in endothelial cells.

BAECs were treated with VEGF (40ng/mL) for 6h or left untreated. The genes which are > 2-fold differentially expressed in VEGF-treated BAECs *vs* those in control cells were considered as VEGF-regulated genes. The VEGF-regulated genes were submitted to the DAVID database and the enrichment of the **biological processes**, **cellular components**, and **molecular functions** were characterized. The Fisher Exact p-value was used to define the significance of enriched GO terms. X-axis shows a log10 transformation of the p-value of the enrichment. The GO terms with values –log10 (p-value) > 1.3 are considered significantly enriched (p-value < 0.05). Y-axis shows the names and identification numbers of the GO annotation terms.

Sample Name	Number of Reads	Number of Genes
(a) CT siRNA	43,329,070	13,869
(b) CT siRNA + VEGF	46,273,531	13,864
(c) eNOS siRNA	41,960,838	14,004
(d) eNOS siRNA + VEGF	48,664,733	14,051

Table III.1. Number of reads obtained and identified genes in RNA-seq samples.

Ensembl Gene ID	Gene Name	Description	Log Fold	Fold
ENSBTAG00000019792	SLC16A9	Solute Carrier Family 16, Member 9	4.7	25
ENSBTAG00000002651	PKP2	plakophilin-2	4.2	18
ENSBTAG00000017561	HHIPL2	Hedgehog Interacting Protein-Like 2	4.2	18
ENSBTAG00000017312	SST	somatostatin precursor	4.2	18
ENSBTAG00000007175	AVPR1A	vasopressin V1a receptor	4.0	16
ENSBTAG00000030338	GJA9	Gap junction protein	4.0	16
ENSBTAG00000010179	COL5A3	Collagen, Type V, Alpha 3	3.7	13
ENSBTAG00000009331	CPAMD8	C3 And PZP-Like Alpha-2-Macroglobulin Domain-Containing 8	3.6	12
ENSBTAG00000009180	NRSN1	neurensin-1	3.6	12
ENSBTAG00000008944	CRB1	crumbs homolog 1 precursor	1.9	4
ENSBTAG00000000977	CADM1	cell adhesion molecule 1 precursor	1.5	3
ENSBTAG00000001835	GJA1	Gap junction alpha-1 protein	1.5	3
ENSBTAG00000019267	MMP2	matrix metalloproteinase-2	1.4	3
ENSBTAG00000010785	FAS	Tumor necrosis factor receptor superfamily member 6	1.3	3
ENSBTAG00000007307	SELE	E-selectin	1.2	2
ENSBTAG00000013008	PMCH	pro-MCH precursor	-6.2	0.01
ENSBTAG00000023891	RBM20	probable RNA-binding protein 20	-5.6	0.02
ENSBTAG00000010103	TRIM9	E3 ubiquitin-protein ligase TRIM9	-5.4	0.02
ENSBTAG00000014270	UNC5B	netrin receptor UNC5B precursor	-4.8	0.03
ENSBTAG00000009255	DNHD1	dynein heavy chain domain 1	-4.8	0.04
ENSBTAG00000014113	CCL8	C-C motif chemokine 8	-4.7	0.04
ENSBTAG00000018398	GPR4	G-protein coupled receptor 4	-4.4	0.05
ENSBTAG00000020814	C2CD4A	C2 calcium-dependent domain-containing protein 4A	-4.3	0.05
ENSBTAG00000016398	DNAJC22	DnaJ homolog subfamily C member 22	-4.2	0.05
ENSBTAG00000021842	FCGR2	Low affinity immunoglobulin gamma Fc region receptor II	-4.2	0.06
ENSBTAG00000037811	CCL2	C-C motif chemokine 2	-2.5	0.17
ENSBTAG00000000569	HES1	Transcription factor HES-1	-2.6	0.17
ENSBTAG00000010361	DLL4	delta-like protein 4 precursor	-2.5	0.18
ENSBTAG00000025424	NOTCH4	neurogenic locus notch homolog protein 4 precursor	-2.3	0.20
ENSBTAG00000011034	ANGPT2	Angiopoietin-2	-2.3	0.21
ENSBTAG00000022020	CLDN5	Claudin-5	-2.0	0.25
ENSBTAG00000015717	HEY1	Hairy/enhancer-of-split related with YRPW motif protein 1	-1.8	0.30
ENSBTAG00000020661	ABCA1	ATP-binding cassette sub-family A member 1	-1.5	0.35

Table III.2. A list of genes whose expression was profoundly regulated by eNOS knockdown in endothelial cells.

BAECs were transfected with eNOS siRNA or control siRNA for 48h. Differentially expressed genes in cells where eNOS was knocked down *vs* control cells are are shown based on their fold changes.

GO_Id	Term	Gene Number	-log10 (p-value)
hsa04670	Leukocyte transendothelial migration	22	5.1
hsa04514	Cell adhesion molecules (CAMs)	23	4.3
hsa04512	ECM-receptor interaction	16	3.7
hsa04151	PI3K-Akt signaling pathway	40	3.3
hsa05200	Pathways in cancer	38	3.1
hsa04360	Axon guidance	19	3.1
hsa04810	Regulation of actin cytoskeleton	27	3
hsa04510	Focal adhesion	26	2.8
hsa04014	Ras signaling pathway	27	2.6
hsa04022	cGMP-PKG signaling pathway	21	2.6
hsa02010	ABC transporters	8	2.3
hsa04370	VEGF signaling pathway	10	2.2
hsa04020	Calcium signaling pathway	21	2.1
hsa04620	Toll-like receptor signaling pathway	14	2
hsa04115	p53 signaling pathway	10	1.8
hsa04270	Vascular smooth muscle contraction	14	1.6
hsa04010	MAPK signaling pathway	25	1.4

Table III.3. KEGG pathway enrichment analysis of genes regulated by eNOS knockdown.

BAECs were transfected with eNOS siRNA or control siRNA for 48h. Differentially expressed genes in cells where eNOS was knocked down *vs* control cells were considered as genes regulated by eNOS knockdown. The regulated genes were submitted for KEGG signaling pathway analysis. The GO terms with values –log10 (p-value) > 1.3 are considered significantly enriched (p-value < 0.05).

Ensembl_gene_id	Gene name	VEGF	eNOS siRNA+ VEGF	Ratio
ENSBTAG00000014560	*HLX*	41.6	14.0	3.0
ENSBTAG00000047400	*IL11*	9.4	3.1	3.0
ENSBTAG00000004555	*LRP2*	7.7	2.7	2.9
ENSBTAG00000006894	*NOS2*	4.6	2.2	2.1
ENSBTAG00000013451	*RHOH*	4.5	1.5	3.0
ENSBTAG00000020737	*SOX8*	3.9	0.9	4.3
ENSBTAG00000001002	*TCF7*	2.5	0.9	2.8
ENSBTAG00000046409	*EGR2*	2.3	0.7	3.0
ENSBTAG00000014113	*CCL8*	2.9	0.4	6.8
ENSBTAG00000012444	*ADAM12*	2.0	0.4	4.3
ENSBTAG00000026275	*CCL24*	1.4	0.5	3.0
ENSBTAG00000015483	*CCR8*	1.4	0.5	3.2
ENSBTAG00000017718	*CCL22*	1.4	0.2	6.5
ENSBTAG00000020957	*FZD5*	0.9	0.2	4.1
ENSBTAG00000000569	*HES1*	0.9	0.4	3.1
ENSBTAG00000012295	*NKD1*	0.5	0.1	4.0
ENSBTAG00000015717	*HEY1*	0.5	0.2	2.3
ENSBTAG00000025424	*NOTCH4*	0.4	0.1	2.7
ENSBTAG00000010963	*IL16*	0.3	0.1	2.9

Table III.4. A list of genes whose expression was differentially regulated in eNOS-depleted and VEGF-regulated eNOS-depleted cells.

BAECs were transfected with eNOS siRNA or control siRNA for 48h, followed by VEGF stimulation. Differentially expressed genes in treated cells *vs* control cells (control siRNA) are are shown based on their fold changes.

GO_Id	Term	Gene Number	-log10 (p-value)
hsa04015	Rap1 signaling pathway	23	3.8
hsa05200	Pathways in cancer	31	3.6
hsa04151	PI3K-Akt signaling pathway	31	3.3
hsa04020	Calcium signaling pathway	19	3.0
hsa04060	Cytokine-cytokine receptor interaction	25	3.0
hsa04022	cGMP-PKG signaling pathway	17	2.7
hsa04014	Ras signaling pathway	21	2.6
hsa04514	Cell adhesion molecules (CAMs)	15	2.6
hsa04810	Regulation of actin cytoskeleton	20	2.5
hsa04611	Platelet activation	14	2.5
hsa04064	NF-kappa B signaling pathway	11	2.4
hsa04510	Focal adhesion	19	2.3
hsa04360	Axon guidance	13	2.1
hsa04512	ECM-receptor interaction	10	2.1
hsa04640	Hematopoietic cell lineage	10	2.1
hsa04670	Leukocyte transendothelial migration	12	2.0
hsa04062	Chemokine signaling pathway	16	1.9
hsa04668	TNF signaling pathway	11	1.8
hsa04310	Wnt signaling pathway	12	1.5
hsa04340	Hedgehog signaling pathway	6	1.5
hsa04370	VEGF signaling pathway	6	1.3

Table III.5. KEGG signaling pathways analysis of genes differentially expressed in VEGF-treated cells and VEGF-treated eNOS-depleted cells.

BAECs were transfected with with eNOS siRNA or control siRNA, then treated with VEGF for 6h or left untreated. The differentially expressed genes (> 2-fold) in VEGF-treated cells and VEGF-treated eNOS-depleted cells were submitted for KEGG signaling pathway analysis. The GO terms with values –log10 (p-value) > 1.3 are considered significantly enriched (p-value < 0.05).

Ensembl_gene_id	Gene name	Description	Log Fold	Fold
ENSBTAG00000005260	SPP1	osteopontin precursor	6.2	71
ENSBTAG00000000507	NR4A1	Nuclear receptor subfamily 4 group A member 1	6.1	69
ENSBTAG00000014560	HLX	H2.0-like homeobox protein	5.4	42
ENSBTAG00000003650	NR4A2	Nuclear receptor subfamily 4 group A member 2	4.7	26
ENSBTAG00000025246	ZIC2	zinc finger protein ZIC 2	4.3	19
ENSBTAG00000004399	LRRN3	Uncharacterized protein	4.1	18
ENSBTAG00000000128	FGF18	fibroblast growth factor 18 precursor	3.9	15
ENSBTAG00000018232	STOML3	stomatin-like protein 3	3.7	13
ENSBTAG00000004305	RGS16	Regulator of G-protein signaling 16	3.3	11
ENSBTAG00000018773	RND1	Rho-related GTP-binding protein Rho6	3.3	10
ENSBTAG00000020035	RCAN1	Calcipressin-1	2.6	6
ENSBTAG00000018424	CXCR7	C-X-C chemokine receptor type 7	1.8	4
ENSBTAG00000010069	EGR1	early growth response protein 1	1.6	3
ENSBTAG00000016344	PIK3R6	phosphoinositide 3-kinase regulatory subunit 6	-4.4	0.0
ENSBTAG00000003054	INSRR	insulin receptor-related protein precursor	-3.9	0.1
ENSBTAG00000047116	TPPP	Tubulin polymerization-promoting protein	-3.6	0.1
ENSBTAG00000001305	ATP2B2	plasma membrane calcium-transporting ATPase 2	-3.6	0.1
ENSBTAG00000011010	PRND	prion-like protein doppel precursor	-3.4	0.1
ENSBTAG00000006720	GJA5	gap junction alpha-5 protein	-3.2	0.1
ENSBTAG00000006811	EPHA3	ephrin type-A receptor 3 precursor	-3.1	0.1
ENSBTAG00000016291	RHOV	Rho-related GTP-binding protein RhoV	-2.9	0.1
ENSBTAG00000019864	MAPK15	mitogen-activated protein kinase 15	-2.8	0.1
ENSBTAG00000011598	SOX2	transcription factor SOX-2	-2.3	0.2
ENSBTAG00000022020	CLDN5	Claudin-5	-1.6	0.3
ENSBTAG00000011810	ANGPTL2	angiopoietin-related protein 2 precursor	-1.2	0.4
ENSBTAG00000012295	NKD1	protein naked cuticle homolog 1	-1.1	0.5

Table III.S1. Profoundly regulated genes in VEGF-treated endothelial cells.

BAECs were treated with VEGF (40ng/mL) for 6h or left untreated. The differentially expressed genes in VEGF-treated BAECs *vs* those in control cells on their fold changes.

Chapter IV. CONCLUSION

NO has been shown to affect transcription through either SNO of proteins or cGMP-dependent signaling (356, 435). The work presented here is the first to explicitly describe that SNO of β-catenin on Cys466 residue is responsible for the effect of eNOS-derived NO on the transcriptional activity of β-catenin, dissociation of β-catenin from transcription factor TCF4, and the proliferation of endothelial cells. Also, this study demonstrates that VEGF may act as a feedback inhibitor for Wnt/β-catenin signaling through the induction of eNOS-derived NO. Moreover, the transcriptomic-wide analysis shows the influence of endogenous eNOS expression and VEGF-induced eNOS activation on transcription and on many endothelial cell functions such as cell polarization, endothelial cell integrity, immune response and chemotaxis. Practically, the results from RNA sequencing analysis show that the expression of genes involved in cell polarization, such as *PARD3A*, *PARD3B, PARD6A, PARD6B, PKCZ, TJP3* and *CRB1,* is upregulated in eNOS-depleted endothelial cells and the upregulation is further confirmed by qRT-PCR analysis. These findings may provide novel mechanistic insights for the study of influence of eNOS on cell polarization. In summary, the work presented in this thesis shows that eNOS activation and its bioactive product NO play a multitude of roles in the regulation of endothelial transcription including the β-catenin transcriptional activity. Several findings highlighted in this thesis will be discussed in more details in the discussion that follows.

SNO of Cys466 on β-catenin is responsible for the modulation of the transcriptional activity of β-catenin by NO

β-catenin has a dual function: it links cadherin to the cytoskeleton and it acts as a transcriptional regulator of Wnt signaling. Our laboratory has previously reported that SNO of Cys619 residue of β-catenin at cell junctions promotes its dissociation from VE-cadherin and is important for VEGF-induced endothelial cell permeability and these results indicate that SNO regulates the function of β-catenin in cell-cell junction (156). In cancer cells, β-catenin has been demonstrated as an important regulator of cell proliferation and NO-donating agents show attenuation of Wnt/β-catenin signaling (252). Our data here shows that NO causes SNO of β-catenin on Cys466 residue to affect its function in Wnt/β-catenin signaling.

SNO of proteins has been demonstrated as a mechanism used by NO to affect transcription (261, 435). SNO of transcription factor AP-1 affects its specific DNA-binding activities in cultured cerebellar granule cells (436). NO utilizes several independent signaling pathways to induce gene expression, such as PI3K, PKC, NF-kB and p53 signaling (437). Also, NO-regulated genes are binned into different functional categories including signaling, metabolism, cell cycle, stress response and transcription (437). Here we demonstrate that NO modulates the transcriptional activity of β-catenin and expression of the target genes through SNO of β-catenin and the dissociation of TCF4/β-catenin transcriptional complex in endothelial cells. Our results are in agreement with the crystal structure study showing that Cys466 is located in a hydrophobic groove of β-catenin and at the binding interface of β-catenin/TCF4 (317). However, our data do not rule out the possibility that the decrease of the transcriptional activity of β-catenin can be also caused by its nuclear translocation. To our knowledge, despite the fact that β-catenin is a known substrate

for SNO, this is the first study to show that SNO of β-catenin on Cys466 is responsible for disruption of the β-catenin/TCF4 transcriptional complex and the effect on TCF-dependent transcription. Therefore, these findings uncover a novel mechanism used by NO to modulate Wnt/β-catenin signaling and endothelial cell proliferation.

VEGF regulates Wnt/β-catenin signaling

It is well established that VEGF regulates angiogenesis and this effect is at least partially due to its capacity to activate eNOS signaling and induce the production of NO (45, 194). The results presented in this thesis show that VEGF inhibits Wnt/β-catenin signaling through the cross regulation of β-catenin transcriptional activity by the production of NO, consistent with the findings from other studies showing that NO has an inhibitory effect on β-catenin transcriptional activity in cancer cells where Wnt/β-catenin signaling is activated (252, 383, 388-391).

Interestingly, *VEGF* is a target gene of Wnt/β-catenin signaling (394), thus VEGF may engage in a negative feedback loop regulating Wnt signaling. Similarly, *AXIN2*, another target gene of Wnt/β-catenin signaling, which encodes a component of the β-catenin degradation complex that controls the amount of β-catenin, also acts as a negative regulator for Wnt signaling (438). The negative feedback effect of VEGF on Wnt is in agreement with their distinct outcomes in the regulation of angiogenesis in certain context (23). During vascular remodeling, the Wnt/β-catenin pathway increases vascular stability by targeting claudin-3 and PDGF-B (439). By contrast, VEGF is important in the formation of unstable and highly permeable vessels by promoting the vascular permeability (353, 440-442). In the process of endothelial specification, analysis of the developing retinal vasculature in mice demonstrate that canonical Wnt-signaling is abundant in

stalk cells, whereas little signaling activity can be found in tip cells, in which VEGF is a potent inducer of sprouting (397). These findings often link Notch signaling with Wnt; however, our results allow us to speculate that Wnt-induced VEGF may somehow affect the outcomes of Wnt in remodeling and endothelial specification due to its inhibitory effect on Wnt/β-catenin.

Our results also show that VEGF induces Wnt/β-catenin in resting quiescent endothelial cells through the activation of eNOS, which supports the findings of a study showing that eNOS associates with β-catenin and induces its translocation into the nucleus (396). It appears that NO may act as a positive modulator of β-catenin activity under basal Wnt activation, even though its action is mostly inhibitory under active Wnt state. Moreover, it is generally accepted that the subcellular localization of β-catenin regulates its function: the membrane-bound pool of β-catenin mediates cell-cell adhesion, whereas the cytoplasmic and nuclear pools of the protein are components of the Wnt signaling pathway (443). In the quiescent endothelial cells, β-catenin is mostly localized at the cell membrane; it is believed that NO may increase Wnt signaling by translocating β-catenin into the nucleus (443, 444). During this process, despite the decrease in β-catenin-TCF4 binding due to SNO of β-catenin, the overall transcriptional activity of β-catenin may still be promoted. However, in Wnt-activated endothelial cells, where β-catenin is stabilized and dominated in the cytoplasm and nucleus, SNO of β-catenin may exhibit inhibitory properties and act to limit the Wnt signal in this scenario.

Roles of endogenous eNOS and VEGF-induced eNOS activation on transcription in endothelial cells by transcriptomic-wide analysis

In eNOS knockout mice, microarray analysis revealed that the expression of genes involved in immune response was regulated by eNOS (366). Here, using eNOS knocked down endothelial cells, our results from the RNA sequencing analysis show that eNOS knockdown affects the transcription of more than 1500 genes in endothelial cells, including those involved in immune response, cell integrity, cell polarization and migration. In our analysis, expression of *ANGPT-2* is significantly decreased by eNOS knockdown*,* indicating that eNOS may modulate vascular integrity through the regulation of the protein level of Ang-2. eNOS-derived NO has already been shown to regulate the secretion of Ang-2 from endothelial cells (424, 425), therefore our results may imply a correlation between eNOS and Ang-2-induced destabilization of vessels. Moreover, eNOS depletion downregulates the expression of the gene encoding for claudin 5. The decrease of claudin 5 leads to increased endothelial cell permeability (131), thus our results showing downregulation of claudin 5 by eNOS may reveal a link between eNOS and cell integrity at the transcriptional level. Furthermore, our data from both RNA sequencing and qPCR analysis show an inverse correlation between expression of eNOS and polarization genes *PARD6A*, *PARD6B*, *PKCZ*, *TJP3*, *CRB1*, *PARD3B* and *PARD3,* suggesting a role of eNOS in the polarization in endothelial cells. These genes encode for PAR3, PAR6, aPKC and crumbs, well-known polarization proteins in many different types of cells including endothelial cells (72, 411, 427). Therefore, the findings that eNOS regulates expression of polarization genes may contribute for the better understanding of the molecular control of endothelial cell polarization.

In addition, we show that Ras signaling pathway is enriched for genes regulated by eNOS knockdown. K-Ras has been linked with cell polarity due to the regulation of Cdc42, Rac1 and Rap1b, which are important proteins in the development of an endothelial cell apical membrane surface during tubulogenesis (423). N-Ras on the Golgi is activated by eNOS through S-

nitrosylation of cysteine 118 and involved in polarization (431). Thus, further investigation of crosstalk between eNOS and Ras signaling may provide mechanistic insight into the effect of eNOS on cell polarity.

VEGF is a potent angiogenic factor in the regulation of endothelial cell-directed migration and eNOS has been shown to be critical in VEGF-regulated endothelial cell migration (429). Our analysis of differential gene expression in VEGF-treated cells and VEGF-treated eNOS-depleted cells shows that genes encoding for chemokines *CCL8*, *CCL19*, *CCL22* and *CCL24* were differentially expressed in these two conditions. The differential expression indicates that eNOS activation in endothelial cells stimulated by VEGF treatment may increase the expression of the chemokines CCL8, CCL19, CCL22 and CCL24. Chemokines are known for their chemotactic ability to regulate the recruitment of leukocytes to sites of inflammation (434, 445). CXC chemokines has displayed pleiotropic effects in immunity, regulating angiogenesis and CXCR2 is activated on endothelium during angiogenesis (434). Therefore, the modulation of eNOS in VEGF-regulated expression of chemokines may indicate a novel role of eNOS in VEGF-regulated chemotaxis of cells.

In summary, our data demonstrate that NO can introduce profound changes in mRNA profiles of endothelial cells and employ a multitude of signaling pathways to control gene expression. These findings may serve as an important starting point for elucidating signaling cascades from NO to gene transcription.

Chapter V. PERSPECTIVES

In the present study, we demonstrate that SNO of β-catenin inhibits proliferation of endothelial cell. eNOS-drived NO has been demonstrated to play a role in the mobilization and differentiation of endothelial progenitor cells and Wnt/β-catenin signaling is well known to be important in the regulate endothelial differentiation during vascular development (375, 446). In the future, whether SNO of β-catenin on cysteine 466 affects differentiation of endothelial cells through modulating expression of genes involved in cell differentiation could be investigated.

Given that here we show eNOS knockdown increases expression of polarization genes in endothelial cells, it would be of interest to look for the polarization phenotypes related to angiogenesis in both eNOS-depleted cells and eNOS knocked out mice due to the modulation of polarization gene expression by eNOS. In addition, the expression of candidate genes such as *DLL4* in eNOS knockdown or overexpressed endothelial cells can be validated using qRT-PCR analysis. Functional studies such as the investigation of the effect of eNOS on tip/stalk endothelial cell specification can be examined by characterizing specific cell markers of tip cells in cultured cells or in the retinal cells of eNOS-/- mice.

Moreover, the expression of the genes encoding for the chemokines CCL8, CCL19, CCL22 and CCL24 could be validated by qRT-PCR analysis in VEGF-treated cells and VEGF-treated eNOS-depleted cells. Additionally, to confirm the effect of NO on the expression of genes of chemokines, NO donors or inhibitors can be used to treat cells and the expression of genes encoding for chemokines can be compared in control cells and treated cells.

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