

Mechanisms of Hypoxia-Induced Neurovascular Remodeling in PlGF Knockout Mice

By

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ABSTRACT

Due to the high metabolic demand and low capacity for energy storage of the brain, neurons are vitally reliant on a constant oxygen supply. Under chronic mild hypoxic conditions (10% oxygen), angiogenesis is induced in the brain in an attempt to restore tissue oxygen tension to normal levels. In brain hypoxia, vascular endothelial growth factor (VEGF) plays a critical role in angiogenesis; however, the role of its homolog placental growth factor (PlGF) is unknown. Using PlGF knockout (PlGF^{-/-}) mice exposed to whole body hypoxia (10% oxygen) for 7, 14 and 21-days, we show that PlGF^{-/-} animals exhibit a delay in the angiogenic response of the brain to hypoxia. PlGF^{-/-} microvessels had a significant increase in fibrinogen accumulation and extravasation, which correlated with disruption of the tight-junction protein claudin-5. These vessels displayed large lumens, were surrounded by reactive astrocytes, lacked mural cell coverage and endothelial VEGF expression, and regressed after 21 days of hypoxia. The lack of PlGF, in combination with reduced VEGF expression levels observed in the brain of PlGF^{-/-} animals during the first 5 days of hypoxia, is likely the cause of the delayed angiogenic response and the prothrombotic phenotype of these mice. *In vitro* studies conducted to analyze mechanisms involved in the impaired angiogenic phenotype and enhanced astrocytic reactivity to hypoxia of PlGF^{-/-} animals indicated that: i) PlGF^{-/-} mouse brain endothelial cells exhibit alterations in intracellular signaling pathways associated with sprouting (ERK1/2) and vessel branching morphogenesis (GSK-3 β) and ii) PlGF^{-/-} astrocytes overexpress VEGF receptor-2 (VEGFR-2) which through activation of the ERK1/2 signaling pathway leads to a more proliferative astrocytic phenotype. These astrocytes were more resistant to oxygen and glucose deprivation (OGD) than PlGF^{+/+} astrocytes, a characteristic that was shown to be independent of the classical

antiapoptotic VEGFR-2-dependent PI3K/Akt pathway. The findings presented in this thesis demonstrated a critical role of PlGF in vascular remodeling in the hypoxic brain.

DEDICATION

To my wife Melissa Vance, my two sons Jack Nuno Andrade, and Rio Thomas Andrade for their love and support.

ACKNOWLEDGMENT

To my advisors, Dr. Dana Stanimirovic and Dr. Maria Moreno: “No written word, no spoken plea, Can teach our youth what they should be, Nor all the books on all the shelves, It’s what the teachers are themselves.”-- Anonymous

I would like to thank Dr. Dana Stanimirovic for giving me the opportunity to embark on this journey, encouraging me and supporting me. She was a strong and calm constant, especially during difficult times.

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As a boy, Abin-Alsar overheard a conversation between his father and a dervish.

“Careful with your work”, said the dervish. “Think of what future generations will say about you.”

“So what?”, replied his father, “When I die, everything shall end, and it will not matter what they say.”

Abin-Alsar never forgot that conversation.

During his whole life, he made an effort to do good, to help people and go about his work with enthusiasm.

He became well-known for his concern for others.

When he died, he left behind a great number of things which improved the quality of life in his town.

On his tombstone, he had the following epitaph engraved:

“A life which ends with death, is a life not well spent.”-- Paulo Coelho

“A pessimist sees the difficulty in every opportunity; an optimist sees the opportunity in every difficulty.” -- Sir Winston Churchill

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LIST OF ABBREVIATIONS

ABC: ATP-binding cassette

β -actin: beta-actin

BBB: blood-brain barrier

Bcl-2: B-cell lymphoma-2

BEC: brain endothelial cells

BREC: bovine retinal endothelial cell

C: control

Ca^{2+} : calcium

$[\text{Ca}^{2+}]_i$: intracellular concentration of Ca^{2+} .

CBF: cerebral blood flow

CLN: capillary-like networks

CNS: central nervous system

CM: conditioned media

cPLA2: cytosolic phospholipase A2

CRT: control

DAG: sn-1,2-diacyl- glycerol

Da: Daltons

Dll4: Delta-like ligand-4

DMEM: Dulbecco's modified Eagle's medium

ECM: extracellular matrix

EDG1: endothelial differentiation sphingolipid G-protein-coupled receptor-1

eNOS: endothelial nitric oxide synthase

ER: endoplasmic reticulum

ERK1/2: extracellular regulated kinase

FAK: focal adhesion kinase

FGF: fibroblast growth factor

FiO₂: fractional inspired oxygen

Flk-1: Fetal liver kinase

Flt-1: fms-related tyrosine kinase 1

GFAP: glial fibrillary acidic protein

GLUT1: Glucose transporter 1

GSK-3 β : glycogen synthase kinase-3 β

H: hypoxia

h or hr: hours

HBSS: Hank's buffered salt solution

HIF: hypoxia-inducible factor

hMSCs human mesenchymal stem cells

H/R: reoxygenation

HS: high sensitivity

HSP27: heat- shock protein 27

HSPG: heparan sulfate proteoglycans

HUVEC: human umbilical vein endothelial cells

IgG: Immunoglobulin G

IP3: inositol (1,4,5)-trisphosphate

JAM: junctional adhesion molecule

KDR: kinase insert domain receptor

LCM: Laser-capture microdissection

MAGUK: membrane-associated guanylate kinase

MAPKAP 2/3: MAPK-activating protein kinase-2 and 3

MAPK: mitogen-activated protein kinase

MCAO: middle cerebral artery occlusion

MBEC: mouse brain endothelial cells

MCM: mouse melanoma conditioned media

mGluRs: metabotropic glutamate receptors

MMP: matrix metalloproteinase

MRPs: multidrug resistance-associated proteins

N: normoxia

NG2: nerve/glial antigen 2

NIHSS: National Institutes of Health Stroke Scale

NO: nitric oxide

NP: neuropilin

NRT: non-reverse transcriptase

OGD: oxygen and glucose deprivation

P8: postnatal day 8

p38 MAPK: p38 mitogen-activated protein kinase

PaO₂: arterial oxygen partial pressure

PaCO₂: arterial carbon dioxide partial pressure

PBS: phosphate-buffered saline

PDGF: platelet-derived growth factor

PDGFR- β : platelet-derived growth factor receptor- β

p-ERK1/2: phospho-extracellular regulated kinase

PECAM-1: platelet-endothelial cell adhesion molecule-1

P-gp: P-glycoprotein

p-GSK-3 β : phospho-glycogen synthase kinase-3 β

PGI₂: prostacyclin

PI: propidium iodide

PIP₂: phospholipid phosphatidylinositol (4,5)-bisphosphate,

PIP₃: phosphatidylinositol (3,4,5)-trisphosphate

PI3K: phosphoinositide 3-kinase

PKC: protein kinase C

PLC: phospholipase C

PLGF: placental growth factor

PLGF^{+/+}: PLGF wild-type

PLGF^{-/-}: PLGF knockout

PO₂: oxygen partial pressure

PtO₂: tissue oxygen partial pressure

PVDF: polyvinylidene difluoride

Q-PCR: Real-Time quantitative polymerase chain reaction

RGS-5: regulator of G protein signaling 5

RT: room temperature

RT-PCR: reverse transcriptase-polymerase chain reaction

Sck: Shc-like protein

SH2: Src homology-2

α -SMA: α -smooth muscle actin

SMC: smooth muscle cell

SPK: sphingosine kinase

SSeCKS: src-suppressed C-kinase substrate

sVEGFR-1: soluble vascular endothelial growth factor receptor-1

TAF: tumor-angiogenesis factor

TGF- α : transforming growth factor- α

TNF- α : tumor necrosis factor- α

VE-cadherins: vascular endothelial-cadherins

VEGF: vascular endothelial growth factor

VEGFR-1: vascular endothelial growth factor receptor-1

VEGFR-2: vascular endothelial growth factor receptor-2

VRAP: VEGFR-associated protein

VSMC: vascular smooth muscle cell

ZO: zonula occludens

CHAPTER 1: INTRODUCTION

The brain is a highly metabolic organ and is extremely sensitive to oxygen levels. Neurons require an uninterrupted supply of oxygen and nutrients for proper neuronal activity. The cerebral vasculature plays an important role in this homeostatic process. The neuronal and cerebral vascular system are intimately in contact, and continuously communicating to maintain the balance between local oxygen delivery and tissue oxygen consumption (Ndubuizu *et al* 2010). This functional interaction is referred to as neurovascular coupling (Hamel 2006). In conditions where the oxygen supply to the brain is interrupted, functional and structural changes occur in the cerebral vasculature in order to restore brain homeostasis. In mild hypoxic conditions, systemic compensatory mechanisms such as increased ventilation and cerebral blood flow (CBF) are initiated to maintain oxygen supply to the brain. However, if hypoxic conditions persist cerebral vascular remodeling occurs through a complex process known as angiogenesis, the growth of new capillaries from preexisting vessels.

1.1 Anatomy of the brain vasculature

The brain is irrigated by an intricate network of blood vessels that originate from the internal carotid and vertebral arteries. At the base of the brain the vertebral arteries merge to form the basilar artery, which in combination with the carotid arteries, form a circle of communicating vessels known as the circle of Willis. This is the source of the major cerebral arteries, which form a network of pial vessels on the surface of the brain. These vessels get progressively smaller and project into the brain parenchyma via invaginations of the pia mater called the Virchow-Robin space. As the vessels penetrate deeper into the brain,

this space disappears and the vasculature continues reducing in size originating a dense capillary mesh that supplies every neuron in the brain. The cerebral capillaries are heterogeneously distributed in the brain; areas such as gray matter with high metabolic demand contain higher capillary density than areas that require less metabolic demand such as in the white matter of the brain (Klein *et al* 1986).

1.1.1 Cellular anatomy of the neurovascular unit

Cerebral vessels share similar characteristics with other peripheral vascular systems, however, they also exhibit unique features, which allow a precise control of the brain homeostasis. Brain vessels present the following common vascular anatomical structures:

The *tunica adventitia*, the outer layer of the vessel, is composed primarily of collagen fibers, produced by neighboring fibroblast, that run parallel to the long axis of the vessel. This layer provides tensile strength and attachment of the vessel to the enveloping connective tissue of the organ it supplies. As the vessels get smaller, this layer shrinks and disappears at the level of arterioles and capillaries.

The *tunica media*, the middle layer, is composed of smooth muscle cells positioned concentrically around the vessel. It also contains collagen fibers, elastic fibers, and proteoglycans produced by the smooth muscle cells. The number of smooth muscle cell layers decreases as the caliber of the vessel becomes smaller (arteries to arterioles); the smooth muscle cells are substituted by pericytes at the capillary level.

The *tunica intima*, the innermost layer, is composed of the endothelium that lines the luminal side of the vascular wall and by the basement membrane on the abluminal side of the endothelium. In this layer, pericytes are located within the basement membrane and are in intimate contact with endothelial cells. At the capillary level, the basement membrane

separates the endothelium from other cells of the central nervous system (CNS). In the brain, the endothelial cells are tightly sealed and form a solute restrictive barrier called the blood brain barrier (BBB), which will be discussed in detail in the next section. The BBB is a distinctive feature of the endothelium of cerebral capillaries, and is important for the maintenance of brain homeostasis.

Capillary brain endothelial cells (BEC) and surrounding astrocytes, pericytes, neurons, and extracellular matrix (ECM) of the basement membrane or basal lamina, together form a functional unit referred to as the neurovascular unit (Zlokovic 2008). The spatial relationship of each of the different cells of the neurovascular unit (Figure 1.1) is an important anatomical feature that is critical to its function.

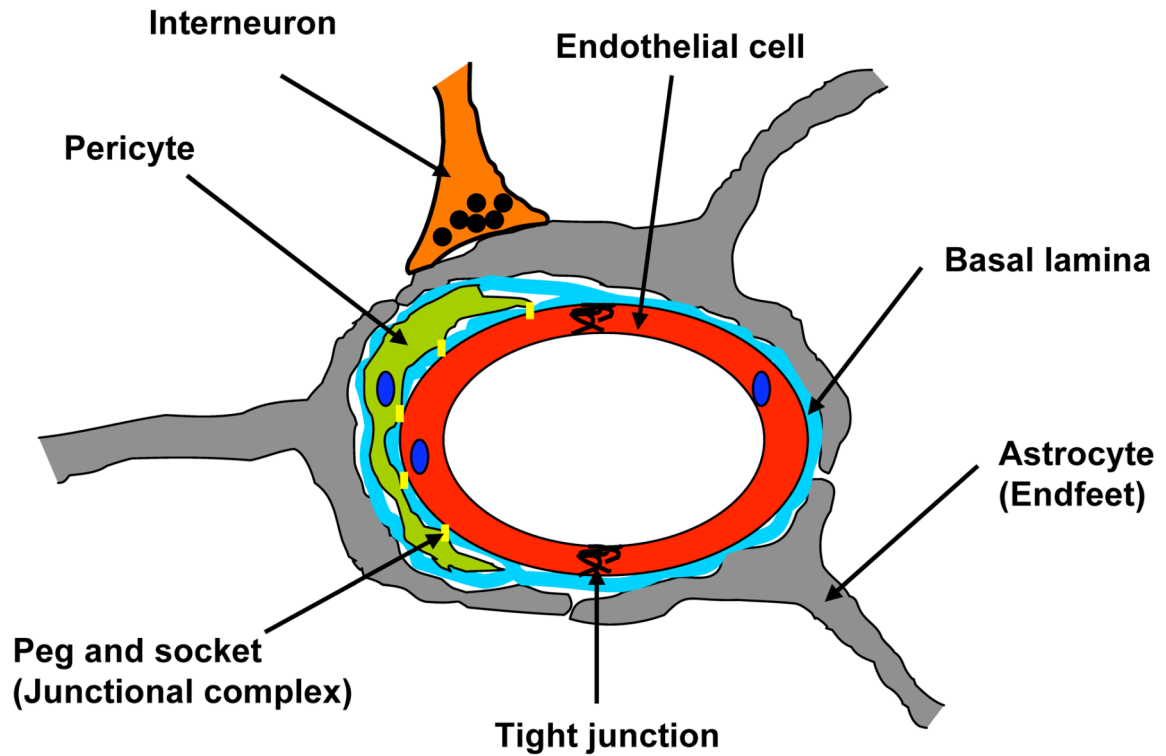


Figure 1.1 Schematic illustration of a cross-section of the neurovascular unit showing astrocytic perivascular endfeet processes. Peg and socket junctional complexes, are regions where BEC and pericytes form direct contact. Adapted from (Iadecola 2004).

The close interaction between the different cellular and acellular components of the neurovascular unit, enables efficient paracrine signaling, important for normal brain function and disease processes, such as stroke. The cellular constituents (BEC, pericytes, astrocytes and neurons) of the neurovascular unit will be highlighted in detail for the purpose of this thesis.

1.1.2 Brain endothelial cells

The BEC layer provides the CNS with an important physical, functional, and metabolic barrier, called the BBB. The BBB limits the access of circulating hydrophilic molecules, such as peptides, and proteins from entering the brain parenchyma. However, small gaseous molecules such as oxygen, and carbon dioxide, as well as small lipophilic molecules less than 500 Da such as ethanol, can diffuse freely through the BEC lipid bilayer. Unlike endothelium of the peripheral tissues, cerebral endothelium lacks fenestrae and exhibits low pinocytosis. BEC are attached to each other by transmembrane tight junctions consisting of occludin, claudin, junctional adhesion molecule (JAM) and the endothelial cell-selective adhesion molecule (Cardoso *et al* 2010). The tight junction proteins prevent paracellular permeability, and restrict the movement of ions such as Na⁺ and Cl⁻ into the brain therefore promoting the high electrical resistance ($\sim 1500\text{-}2000\ \Omega\cdot\text{cm}^2$) in the BBB (Bazzoni and Dejana 2004).

The tight junctions are formed by a complex set of molecules with distinct biological function. Occludin was the first tight junction protein discovered (Furuse *et al* 1993). It is involved in barrier function and increases the electrical resistance of the BBB (Bamforth *et al* 1999). Claudins are a multigene family with at least 24 member proteins identified so far (Hawkins and Davis 2005). Claudins-5, -3 and -12 are localized at the BBB (Nitta *et al*

2003); each of the claudin transmembrane proteins blocks the diffusion of a group of molecules of a certain size (Zlokovic 2008). For instance, claudin-5 knockout affects the ability of the BBB to restrict small molecules (<800 Da) but not larger molecules from entering the brain (Nitta *et al* 2003). The junctional adhesion molecule-A and the endothelial cell-selective adhesion molecule affect the junctional tightness, and are involved in transendothelial migration of leukocytes (Padden *et al* 2007). In addition, tight junction proteins have also been implicated in regulating gene expression, cell proliferation and differentiation (Gonzalez-Mariscal *et al* 2009). These cellular effects are mediated by intracellular multidomain adaptor proteins of the peripheral membrane-associated guanylate kinase (MAGUK) family such as zonula occludens (ZO) -1, -2, and -3, which link the tight junction to the cytoskeleton in BEC (Zlokovic 2008). The tight junctions form the physical barrier of the BBB.

The functional component of the BBB is represented by the efflux proteins and selective transporters expressed on both the luminal and abluminal surface of BEC. Active efflux proteins, primarily localized at the luminal side of the microvessels, including ATP-binding cassette (ABC) transporters, the multidrug resistance transporter P-glycoprotein (P-gp) and several multidrug resistance-associated proteins (MRPs), work together to reduce the penetration of many potential toxic lipophilic and anionic compounds into the brain (Zlokovic 2008). However, the high efficiency of these pumps is also one of the great challenges for the development of brain specific therapeutics, as most of these compounds cannot evade this system and fail to access the brain (Neuwelt *et al* 2011; Zhang *et al* 2003).

Due to the high constraints of the BBB, nutrients and other essential molecules enter the brain by various transport systems. These transporters ferry glucose, amino acids, nucleosides and other substance into the brain. Glucose transporter 1 (GLUT1) is one of the

most characterized transporters since it is involved in delivering the main energy source, glucose, into the brain (Zlokovic 2008). These transporters are regulated by the metabolic demand of the brain, and the concentration of substrates in the plasma.

Peptidases and nucleotidases located extracellularly, and monoamine oxidase, and cytochrome P450 found in intracellular compartments of the BEC modify and inactivate several neuroactive and toxic substances, forming a metabolic barrier (Abbott *et al* 2006; el-Bacha and Minn 1999).

Collectively, these studies emphasize the important function of the BBB in brain homeostasis. Breakdown of this barrier under certain pathological conditions such as stroke (Fischer *et al* 2002; Zhang *et al* 2000) leads to neuronal injury and death (Zlokovic 2008).

1.1.3 Pericytes

Pericytes are mural cells that belong to the same cell lineage of the vascular smooth muscle cells (Armulik *et al* 2005). They can be distinguished from smooth muscle cells by their location relative to the endothelium, morphology, and to a certain extent, the expression of various molecular markers (Armulik *et al* 2005; Krueger and Bechmann 2010). However, depending on the size of the vessel (intermediate size to small microvessels) transitional forms of pericytes and smooth muscle cells express common markers, such as α -smooth muscle actin (α -SMA), desmin and regulator of G protein signaling 5 (RGS-5), among others, that also vary depending on the organ, type of vessel, and developmental stages, which makes the identification of these cells very challenging (Armulik *et al* 2005; Diaz-Flores *et al* 2009; Krueger and Bechmann 2010).

During development, pericytes guide BEC in the organization of the growing vessel wall (Virgintino *et al* 2007). Pericytes are located within the basement membrane surrounding the cerebral microvessels and in intimate contact with BEC; both cell-types contribute to the formation of the basement membrane (Armulik *et al* 2005; Iadecola 2004). Direct BEC-pericyte connections occur at junctional complexes, regions called peg-socket contacts, where the basement membrane is absent (Figure 1.1) (Cuevas *et al* 1984). The close relationship between pericytes and BEC suggest that pericytes play an important role in modulating microvessel function in normal and pathological conditions (Al Ahmad *et al* 2010; Armulik *et al* 2010; Bell *et al* 2010).

In response to neuronal oxygen demands, pericytes, stimulated by secreted vasoactive agents from local active neurons, constrict or relax, therefore affecting capillary diameter and brain perfusion (Peppiatt *et al* 2006). Pericyte coverage promotes vessel stability (Bell *et al* 2010; Lindahl *et al* 1997; Nishioku *et al* 2009), by inhibiting endothelial proliferation (Orlidge and D'Amore 1987), reducing endothelial cell migration (Sato and Rifkin 1989), and participating in the formation of basement membrane (Virgintino *et al* 2007), which leads to BEC differentiation and quiescence (Al Ahmad *et al* 2009; Armulik *et al* 2005; von Tell *et al* 2006). In addition, secretion of angiopoietin-1 by pericytes contributes to endothelial cell survival and vessel maturation through the activation of Tie2 receptors on endothelial cells (Thomas and Augustin 2009) and downstream stimulation of the Akt/survivin signaling pathway (Papapetropoulos *et al* 2000).

Pericyte coverage is primarily promoted through the secretion of endothelial cell platelet-derived growth factor (PDGF)-B which binds to PDGF receptor beta (PDGFR β) on pericytes, promoting proliferation, migration, and recruitment of pericytes to the vascular wall (Armulik *et al* 2005; Lindahl *et al* 1997).

Under developmental and pathological conditions, activated endothelial cells secrete angiopoietin-2, which through an autocrine fashion acts as an antagonist to Tie2 receptors, and disrupts vessel stabilization by promoting pericyte detachment (Armulik *et al* 2005; Diaz-Flores *et al* 2009; Reiss *et al* 2007). Reduction in pericyte coverage in $\text{Pdgfr}\beta^{+/-}$ mice leads to age-dependent neuronal damage and neuroinflammation (Bell *et al* 2010). Deficient PDGFR β signaling induces pericyte degeneration, which leads to reduced levels of brain microcirculation and BBB breakdown. These changes precipitate neurodegeneration and neuronal inflammation (Bell *et al* 2010).

Recently, two independent studies have revealed the importance of pericytes in modulating BBB function in BEC (Armulik *et al* 2010; Daneman *et al* 2010). Daneman *et al.* (2010) demonstrated that pericyte coverage inversely correlates with vessel permeability. This study shows that pericytes do not induce BBB-specific genes on endothelial cells, but rather inhibit the expression of molecules that promote vessel permeability and immune cell infiltration. Interestingly, the highest pericyte coverage is found in the brain microvasculature (Armulik *et al* 2005). Pericytes have also been shown to affect functional aspects of the BBB, altering the structure of the tight junctions and vesicle trafficking (Daneman *et al* 2010). Armulik *et al.* (2010) showed that lack of pericytes increased BBB permeability to water and a range of tracers of different molecular weights (Armulik *et al* 2010). The permeability was found to be modulated by increased transcytosis in the endothelium (Armulik *et al* 2010). In addition, pericyte-deficient mice exhibited dysfunctional BEC-astrocyte contact (Armulik *et al* 2010). Taken together these findings demonstrate that pericytes are active participants in capillary function and maintenance. Pericyte-endothelial interaction is an area that is currently under intense investigation.

1.1.4 Astrocytes

Astrocytes, the most abundant cells in the brain, were initially thought to act as a ‘glue’ that held the molecular and cellular structures of the CNS together. However, astrocytes are now viewed as important players for normal brain function and repair. Astrocytes located between neurons and capillaries extend “endfeet processes” that physically link neighboring neurons with their surrounding capillaries (Figure 1.1). This allows astrocytes to sense changes in the neuronal microenvironment and adjust microvascular function accordingly. In addition to the homeostatic function of astrocytes, these cells are particularly critical to neuronal survival and brain repair after injury (Chen and Swanson 2003).

Astrocytes are broadly divided into three distinct populations based on morphological characteristics and spatial organization: radial astrocytes located around ventricles, protoplasmic astrocytes located in gray matter, and fibrous astrocytes located in white matter (Shehab *et al* 1990). However, increasing evidence indicates that astrocytes are a very heterogeneous population of cells that exhibit different structural, functional, chemical, and molecular characteristics and play distinct roles in different brain regions. For instance, they express different gap junction proteins (Wallraff *et al* 2004), receptors, transporters (Matthias *et al* 2003; Zhou and Kimelberg 2001), and ion channels (Verkhratsky and Steinhauser 2000). These specific molecular characteristics expressed in different subtypes of astrocytes, allow these cells to perform a range of homeostatic functions such as maintenance of pH balance (Deitmer and Rose 1996), delivery of glucose and metabolic substrates to neurons (Forsyth 1996), and clearance of neuronal waste such as metabolic byproducts and neurotransmitters released during synaptic transmission (Nedergaard *et al* 2003). Furthermore, astrocytes are closely associated with synapses and have been shown to

facilitate synaptic plasticity in the hippocampus (Nishiyama *et al* 2002; Yang *et al* 2003b). Astrocytes participate in delivering oxygen to active neurons (Anderson and Nedergaard 2003; Zonta *et al* 2003). For example, astrocyte processes expressing metabotropic glutamate receptors (mGluRs), sense synaptic activity through the release of glutamate from the synaptic cleft (Zonta *et al* 2003). Activation of mGluRs induces an increase in intracellular Ca^{2+} concentration that spreads to the astrocytic endfeet, plastered on the microvessel wall (Figure 1.1) (Zonta *et al* 2003). The elevation of Ca^{2+} concentration induces the release of vasoactive factors from the astrocytic endfeet, resulting in dilation or vasoconstriction of the surrounding microvessels during neuronal activity (Gordon *et al* 2008; Mulligan and MacVicar 2004; Zonta *et al* 2003). These contrasting effects of intracellular Ca^{2+} in astrocytes are dependent on the metabolic state of the neuronal microenvironment (Gordon *et al* 2008). Collectively, these studies highlight the important role that astrocytes play in sensing neuronal oxygen demands within the microenvironment.

Several lines of evidence have implicated astrocytes in promoting and modulating the BBB phenotype in BEC (Al Ahmad *et al* 2010; Janzer and Raff 1987; Yamagata *et al* 1997). Perivascular astrocytes increase the tightness of tight junctions in the BBB (Dehouck *et al* 1990), promote the expression and localization of transporters in the BBB of the endothelium (McAllister *et al* 2001; Schinkel 1999) and induce the expression of several enzymes associated with the metabolic barrier in the cerebral endothelium (Abbott *et al* 2006; Hayashi *et al* 1997; Sobue *et al* 1999). Expression of src-suppressed C-kinase substrate (SSeCKS) in astrocytes increases BBB by inducing ZO-1, ZO-2, claudin-1 and occludin in human BEC, and inhibits vessel permeability by increasing angiopoietin-1 production (Lee *et al* 2003).

Astrocytes are generally more resistant to hypoxic conditions than other cell-types in the CNS (Anderson *et al* 2003; Chen and Swanson 2003). However, they are highly

vulnerable to the coupling of acidosis and hypoxia during cerebral ischemia, a restriction of blood flow to brain tissue such as in stroke (Bondarenko and Chesler 2001; Swanson *et al* 1997). Differences in sensitivity to hypoxia have been reported between different populations of astrocytes in different parts of the brain (Lukaszevicz *et al* 2002; Xu *et al* 2001b; Zhao and Flavin 2000).

In pathological conditions, such as stroke, astrocyte survival has been correlated with neuronal survival (Chen and Swanson 2003). After brain injury astrocytes upregulate glial fibrillary acidic protein (GFAP) an intermediate filament involved in astrocyte activation (Li *et al* 2007). Reactive astrocytes minimize brain injury by clearing glutamate and ions released from injured neurons, and clearing metabolic byproducts (Chen and Swanson 2003). Astrocytes also secrete neurotrophic factors that promote neuronal survival, and minimize damage to neighboring cells by the formation of the glial scar (Krum *et al* 2008). However, the glial scar can also have a detrimental effect in functional recovery by acting as a barrier to neuronal regeneration (Beck *et al* 2008).

Under ischemic conditions, astrocytes secrete growth factors that promote the sprouting of new capillaries from neighboring vessels towards the infarcted tissue (Chow *et al* 2001) in order to restore blood circulation into the damaged area.

Taken together, these studies highlight the essential role that astrocytes play in brain homeostasis, neuroprotection and cerebral revascularization, and underline the importance of the survival of these cells after brain injury for brain repair.

1.1.5 Perivascular neurons

Anatomical studies have shown that cortical microvessels and/or associated astrocytic endfeet are innervated by noradrenergic, serotonergic, cholinergic and GABA-ergic neurons

originating from either subcortical pathways (locus coeruleus, raphe nucleus, basal forebrain), or local cortical interneurons (Hamel 2006; Hawkins and Davis 2005). These neurons release vasoactive neurotransmitters and neuropeptides including NO, vasoactive intestinal polypeptide, dopamine, substance P, serotonin, GABA, noradrenalin, and acetylcholine (Iadecola 2004). Chemical or electrical stimulation of these subcortical areas elicits increases or decreases in cortical CBF (Hamel 2006). This is achieved through the activation of receptors for vasoactive neurotransmitters and neuropeptides on microvascular endothelial and/or smooth muscle cells as well as surrounding astrocytes that can either dilate or constrict cortical microvessels (Hamel 2006).

Modulation of intraparenchymal CBF by active neuronal afferents is highly complex processes requiring the interplay of cellular networks and circuitry that together control hemodynamic responses (Kocharyan *et al* 2008). Studies have shown that stimulation of specific subcortical areas induces the activity of specific subsets of cortical interneurons, which activate neuronal, vascular, and/or astroglial targets that together promote the full expression of the hemodynamic responses (Kocharyan *et al* 2008).

Pathological conditions have demonstrated the critical role perivascular neurons have on cortical microvessel function. For instance, chemical lesions of the noradrenergic neurons of the locus coeruleus increases the vulnerability of the BBB to acute hypertension (Hawkins and Davis 2005) whereas loss of cholinergic innervation of cortical microvessels has been associated with the impaired cerebrovascular function observed in Alzheimer's disease (Tong and Hamel 1999).

1.2 Hypoxia

Hypoxia refers to conditions where there is an inadequate supply of oxygen to organs or tissues. Hypoxia occurs under pathological conditions, such as pulmonary fibrosis or scarring of the lungs, and anemic conditions referred to as anemic hypoxia. Under these situations, oxygen delivery is impaired due to either deficiency in gas exchange in the lung or reduced oxygen carrying capacity by the blood.

Hypoxia also occurs in high altitudes (18,000 feet or ~5,500 m) where a decrease in the normal atmospheric oxygen partial pressure (PO_2) at sea level (160 mmHg) results in a reduction of arterial hemoglobin saturation (hypoxemia) (LaManna *et al* 2004).

Hypoxia at 18,000 feet is considered mild hypoxia; it is equivalent to a sea level fractional inspired oxygen (FiO_2) of ~10%, which results in a drop in arterial oxygen partial pressure (PaO_2) from 100 mmHg to ~50 mmHg (Xu and Lamanna 2006). Under these conditions, acute compensatory mechanisms, such as increased blood flow, are initiated and maintain proper physiological function (Dore-Duffy and LaManna 2007). Moderate hypoxia resulting in a PaO_2 between ~30 and 50 mmHg leads to impaired thought processes, and severe hypoxia $PaO_2 < \sim 35$ mmHg leads to loss of consciousness (Xu and Lamanna 2006). While mild hypoxia is not associated with neuronal damage, moderate and severe hypoxia is correlated with neuronal death in a time and degree of hypoxia-dependent manner (Xu and Lamanna 2006).

Prolonged exposure to mild hypoxia (i.e. mild chronic hypoxia) occurs over weeks to months, and is followed by adaptive systemic changes. However, long-term adaptation to hypoxia occurs over generations, examples of this are demonstrated in populations living in high altitudes in Himalayas, and Andes, (Beall *et al* 1998).

Rats exposed to one week of mild chronic hypoxia react by systemic adaptation involving a decrease in metabolism, an increase in respiratory rate that leads to a drop in arterial carbon dioxide partial pressure (PaCO_2) below 22 mmHg (LaManna *et al* 1992). The alkalosis resulting from the drop in PaCO_2 is counteracted by bicarbonate produced in the kidney, therefore, maintaining proper acid-base balance (LaManna *et al* 2004). Blood flow almost doubles during mild hypoxia, despite the drop in PaCO_2 , in order to compensate for the hypoxemia that occurs during this period (Xu and Lamanna 2006). In addition, the hematocrit, packed red blood cell volume, also increases, restoring the oxygen content in the blood; therefore, oxygen delivery (in $\text{ml O}_2 \times 100 \text{ g}^{-1} \times \text{min}^{-1}$) is resorted to baseline levels resulting in renormalization of blood flow by three weeks of hypoxia (Xu and Lamanna 2006). Although oxygen delivery is restored to prehypoxic values further adaptive changes occur at the tissue level, particularly in the brain.

Neurons are highly metabolic cells that are extremely sensitive to oxygen levels and changes in their microenvironment. Although the brain represents about 2% of the total body weight it accounts for 20% of the total body oxygen consumption (Quaegebeur *et al* 2011). The systemic changes that occur during mild chronic hypoxia leads to almost normal oxygen delivery to the brain (LaManna *et al* 1992). However, due to the low PaO_2 and the near zero tissue oxygen partial pressure (Pto_2) the driving force for oxygen diffusion down its concentration gradient, from capillary to tissue, is low (LaManna *et al* 2004). To compensate for the low oxygen diffusion, the cerebral vasculature undergoes major structural changes through a remodeling process called angiogenesis. This results in the formation of new blood vessels and the reduction of the intercapillary and diffusion distance (LaManna *et al* 2004). This adaptive mechanism also occurs after cerebral ischemia where arterial

occlusion results in severe oxygen and glucose deprivation within the penumbra surrounding the infarcted tissue.

1.3 Angiogenesis

Angiogenesis, the sprouting of new capillaries from pre-existing vessels, is a highly complex multi-step process involving the spatial and temporal orchestration of several pro- and anti-angiogenic factors. Oxygen plays a critical role in this process. Hypoxia promotes, directly and indirectly, the expression of several angiogenic factors and receptors in the endothelium and the surrounding hypoxic tissue.

Angiogenesis is essential during embryonic development. Endothelial progenitors differentiate into mature endothelial cells that form a primitive vascular plexus through a process known as vasculogenesis. Angiogenesis is initiated from this plexus, driven by secreted angiogenic factors from the oxygen deprived expanding tissue (Carmeliet 2005a; Risau 1997). In the adult, angiogenesis is stimulated during the ovarian cycle, in the placenta during pregnancy, and during wound healing and repair. Angiogenesis has been associated with several pathologies involving hypoxia and inflammation, including obesity, asthma, cancer, diabetes, cirrhosis, multiple sclerosis, autoimmune diseases, ischemic heart disease, preeclampsia, and stroke (Carmeliet 2005a).

Under normal physiological conditions endothelial cells are in a quiescent state. However, in response to hypoxia, endothelial cells become activated by the upregulation of angiogenic factors. When intracellular oxygen levels drop below a critical threshold, hypoxia sensing proteins, such as hypoxia inducible factor (HIF) prolyl-4 hydroxylases, stabilizes HIF-1 α . Intracellular accumulation of HIF-1 α promotes heterodimerization with

its constitutively expressed partner HIF-1 β . This heterodimeric transcription factor translocates to the nucleus where it initiates a signaling cascade that leads to extensive changes in gene expression, including glucose uptake, increased expression of glycolytic enzymes and increased expression of angiogenic factors. All this allows cells to adapt to low oxygen levels (Forsythe *et al* 1996; Ratan *et al* 2007).

Angiogenesis is a complex and coordinated processes that requires the interaction of a large number of ligands and receptors and the fine balance between multiple stimulating and inhibitory signals. The process includes the disruption of inter-endothelial tight junctions, retraction of pericytes from the abluminal surface of the capillary, release of proteases from the activated endothelial cells, breakdown of the basal lamina, degradation of the ECM surrounding vessels, endothelial cell migration toward angiogenic/chemotactic stimuli and their proliferation, formation of tube-like structures, fusion of the newly formed vessels, and initiation of blood flow.

Several factors have been identified as inducers of angiogenesis, including acidic fibroblast growth factor (FGF), basic FGF, transforming growth factor- α (TGF- α), tumor necrosis factor- α (TNF- α) and angiopoietins (Yancopoulos *et al* 2000). However, due to its potency in inducing angiogenesis and its involvement in several biological systems, VEGF and its signaling pathways, have been the most extensively studied (for reviews see (Ahmed *et al* 2000; Carmeliet 1999; Carmeliet 2005b; Dai and Rabie 2007; Gerber and Ferrara 2003; Lambrechts and Carmeliet 2006; LeCouter *et al* 2002; Sanchez *et al* 2010; Zachary 2005).

VEGF is secreted as a homodimeric glycoprotein by hypoxic tissues and exerts its pro-angiogenic effects through tyrosine kinases VEGF receptor-1 (VEGFR-1), also known as Flt-1, (de Vries *et al* 1992) and VEGFR-2, also known as Flk-1/KDR (Quinn *et al* 1993).

VEGF also interacts with coreceptors, neuropilin-1 (NP-1) and NP-2. These receptors were initially described as receptors for axon guidance factors belonging to the class-3 semaphorin sub-family (Neufeld *et al* 2002). Among the variety of angiogenic factors, VEGF and its receptors VEGFR-1 and VEGFR-2 play a central role in initiating angiogenesis, stimulating endothelial cell survival, proliferation, and migration (Ferrara *et al* 2003).

Endothelial VEGFR-2 activation promotes vascular permeability through the induction of phosphoinositide 3-kinase (PI3K)/Akt pathway, stimulating endothelial nitric oxide synthase (eNOS) expression and NO release (Fulton *et al* 1999). VEGFR-2 stimulates phosphorylation of endothelial cell-cell junctional adhesive molecules including: VE-cadherins, beta-catenin, plakoglobin, p120, and platelet/endothelial cell adhesion molecule 1 (PECAM-1)/CD31. Phosphorylation of these proteins promotes the loosening of cell-cell contacts and increases vessel permeability (Esser *et al* 1998). VEGFR-2 also stimulates endothelial cell survival through the PI3K/Akt signaling, promoting antiapoptotic pathways such as B-cell lymphoma-2 (Bcl-2) (Gerber *et al* 1998a).

Angiopoietin-1 produced by pericytes activates endothelial Tie2 receptors that induce proliferation, migration, and production of basement membrane proteinases (Augustin *et al* 2009). Detachment of pericytes from the microvessels increases vessel destabilization (Diaz-Flores *et al* 2009). These activated pericytes can be identified by the increased expression of the nerve/glia antigen 2 (NG2) marker (Ozerdem and Stallcup 2003; Virgintino *et al* 2007).

Matrix metalloproteinases (MMPs) produced by activated endothelial cells and pericytes degrade the basement membrane (Virgintino *et al* 2007). Other proteinases, including plasminogen activators, heparinases, and cathepsins also contribute to the degradation of the ECM (Carmeliet 2003). The proteolytic breakdown of ECM proteins

such as laminin-5 (Giannelli *et al* 1997) or collagen IV (Xu *et al* 2001a) exposes cryptic epitopes that promote endothelial cell and pericyte migration (Hangai *et al* 2002). Furthermore, basement membrane breakdown allows VEGF activated endothelial cells to sprout; these cells are called tip cells, and extend processes called filopodia (De Smet *et al* 2009; Gerhardt *et al* 2003). The filopodia interacts with the exposed pro-angiogenic matrix-bound proteins, such as basic FGF, and follow a VEGF gradient produced by the surrounding hypoxic cells and matrix-bound VEGF in the microenvironment (Basilico and Moscatelli 1992; De Smet *et al* 2009; Gerhardt *et al* 2003; Park *et al* 1993). NP-1, VEGFR-1, and VEGFR-2 expressed at the tip cells are involved in this complex process (Gerhardt *et al* 2003; Jones *et al* 2009; Kearney *et al* 2004). VEGFR-2-dependent actin reorganization contributes to the migration of tip cells towards the hypoxic stimulus (Rousseau *et al* 1997) while VEGFR-1 promotes vessel branching morphogenesis (Jones *et al* 2009). NP-1 is believed to be involved in lateral extension, however, its role in this process is still unclear (De Smet *et al* 2009). Other receptors such as integrins, composed of α and β subunits, interact with the ECM components (fibronectin, laminin, vitronectin and collagen). The interaction between integrins and the ECM is bidirectional and contributes to cytoskeletal rearrangements and the amplification of the angiogenic signals (De Smet *et al* 2009; Eliceiri *et al* 1998).

Endothelial tip cells activated by VEGF produce Delta-like ligand-4 (Dll4) which binds to Notch on neighboring endothelial cells and suppresses the tip cell phenotype in these cells through the reduction of VEGFR-2 expression. This decreases migration, inhibits filopodia extension, and vessel branching (Hellstrom *et al* 2007), these endothelial cells become stalk cells in the growing endothelial sprout (De Smet *et al* 2009; Hellstrom *et al*

2007). The Dll4-Notch signaling between endothelial cells contributes to the correct sprouting and branching patterns of the growing vasculature (Hellstrom *et al* 2007).

Elongation of the sprout is achieved by the proliferation of endothelial stalk cells (Gerhardt *et al* 2003). VEGF activation of VEGFR-2 in stalk cells promotes proliferation through the classical extracellular regulated kinase (Erk) pathway (p42/44 mitogen-activated protein kinase (MAPK)) (Takahashi *et al* 1999). Stalk cells form junctions, lay down ECM, and are involved in lumen formation.

Underneath the stalk cells reside a group of endothelial cells called the phalanx cells which exhibit reduced mitogenic activity to VEGF (De Smet *et al* 2009). These cells form close contact with pericytes, which keep them in a quiescent state (Augustin *et al* 2009). However, little is known about the molecular characteristics of phalanx cells (De Smet *et al* 2009).

In the final stages of angiogenesis endothelial cells secrete PDGF-B, which stimulates PDGFR β in pericytes and promote recruitment of these cells (Gerhardt *et al* 2003; Lindahl *et al* 1997). Pericytes and smooth muscle cells also express VEGFR-1; activation of this receptor has been shown to stimulate pericyte and smooth muscle cell recruitment by VEGF and its homolog placental growth factor (PlGF) (Cao *et al* 2010; Ishida *et al* 2001; Lutun *et al* 2002b). Other pathways also contribute to pericyte recruitment, including sphingosin-1-phosphate/endothelial differentiation sphingolipid G-protein-coupled receptor-1 (EDG1), TGF- β /TGF-beta receptor-I and Angiopoietin/Tie2 (Diaz-Flores *et al* 2009). Angiopoietin-1 secreted by pericytes, activates endothelial cell Tie2 receptors, promoting vessel stability and inhibiting vascular permeability (Papadimitrakopoulos *et al* 2000; Pizurki *et al* 2003; Thomas and Augustin 2009).

At this stage, basement membrane is formed around the new vessel by both pericytes and endothelial cells. Angiogenesis is inhibited by the exposure of matrix-bound anti-angiogenic factors such as endostatin, and angiostatin (O'Reilly *et al* 1994; O'Reilly *et al* 1997), as well as soluble factors thrombospondin-1, -2 (Zhou *et al* 2010), canstatin, and tumstatin (Carmeliet 2003).

In the resolution of angiogenesis, endothelial cells return to a quiescent state by the perfusion of the new vessels; blood flow is an important factor in promoting this process (Shiu *et al* 2004). In addition, oxygen-sensing molecules in the endothelial cells such as prolyl-hydroxylase domain-2 also contribute to the normalization of the endothelium (De Smet *et al* 2009; Mazzone *et al* 2009).

This general overview of angiogenesis outlines some of the main steps and factors in this process. However, tissue dependent differences have been observed (Carmeliet 2003). Distinct mechanisms modulating pathological and physiological angiogenesis have also been identified. For example, differences in vessel morphology and function, interaction of secreted factors and receptors during angiogenesis, such as the synergistic effect between VEGF and its homolog PlGF have been investigated (Autiero *et al* 2003b; Carmeliet 2000; Greenberg *et al* 2008). PlGF, while not crucial for developmental angiogenesis, plays a critical role in inducing VEGF-driven angiogenesis under pathological conditions (Autiero *et al* 2003b; Carmeliet 2000).

The coordinated interaction of many signaling events among the different angiogenic factors is still not completely known, and is an area of intense research. For the purpose of this thesis VEGF, and more specifically PlGF, will be explored in more detail in the following sections, particularly in the context of brain hypoxia and ischemia.

1.4 VEGF family members and receptors

VEGF, also known as VEGF-A, is structurally related to PDGF, and belongs to a gene family that includes PlGF, VEGF-B, VEGF-C, and VEGF-D. These proteins share a common structure of eight characteristically spaced cysteine residues in a VEGF homology domain (Roy *et al* 2006). VEGF-C and VEGF-D have been reported to play an important role in lymphangiogenesis (Tammela *et al* 2005). In humans, VEGF is produced as six different dimeric isoforms from alternative splicing of 8 exons (VEGF₁₂₁, VEGF₁₄₅, VEGF₁₆₅, VEGF₁₈₃, VEGF₁₈₉ and VEGF₂₀₆) (Dore-Duffy *et al* 2007). In mice, 5 isoforms similar to human VEGF have been identified, each one of them exhibiting one amino acid less than their human counterparts (VEGF₁₂₀, VEGF₁₄₄, VEGF₁₆₄, VEGF₁₈₈ and VEGF₂₀₅). Each of the VEGF variants have distinct and overlapping angiogenic properties, and differ in their ability to bind to heparan sulfate proteoglycans (HSPG) on the surface of cells and ECM (Tammela *et al* 2005). The diverse signaling potential of the VEGF family members are further increased by the capability of VEGF-A to form heterodimers with either PlGF or VEGF-B (Cao 2009).

Knockout studies in mice have shown that VEGF₁₆₄ is the principle isoform responsible of the VEGF-driven angiogenic activity (Roy *et al* 2006). Mice lacking even a single *Vegf* allele die at E11-E12, indicating the importance of this factor in development (Ferrara *et al* 1996).

Despite the high affinity of VEGF to VEGFR-1 (Cross *et al* 2003) VEGFR-2, however, is the primary transducer of the VEGF-dependent angiogenic signals in physiological and pathological conditions (Figure 1.2) (Ferrara *et al* 2003; Millauer *et al* 1993). VEGFR-2 is a 200-230 kDa homodimer with high affinity for VEGF, VEGF-C, and VEGF-D. VEGFR-2 is critical for proper vascular development and survival in mice

(Shalaby *et al* 1995). VEGF activation of VEGFR-2 induces proliferation in endothelial cells through the classical Erk1/2 signaling pathway (Keyt *et al* 1996; Takahashi *et al* 1999), cell survival through the activation of the PI3K/Akt signal transduction pathway (Gerber *et al* 1998b), and activation of eNOS, stimulating the production of the vascular permeability molecule NO (Dimmeler *et al* 1999; Fulton *et al* 1999). VEGFR-2 modulates endothelial cell junctions that affect vessel permeability (Esser *et al* 1998). In concert with cytoskeletal regulation, through p38-MAPK pathway, VEGFR-2 promotes endothelial cell migration (Rousseau *et al* 1997).

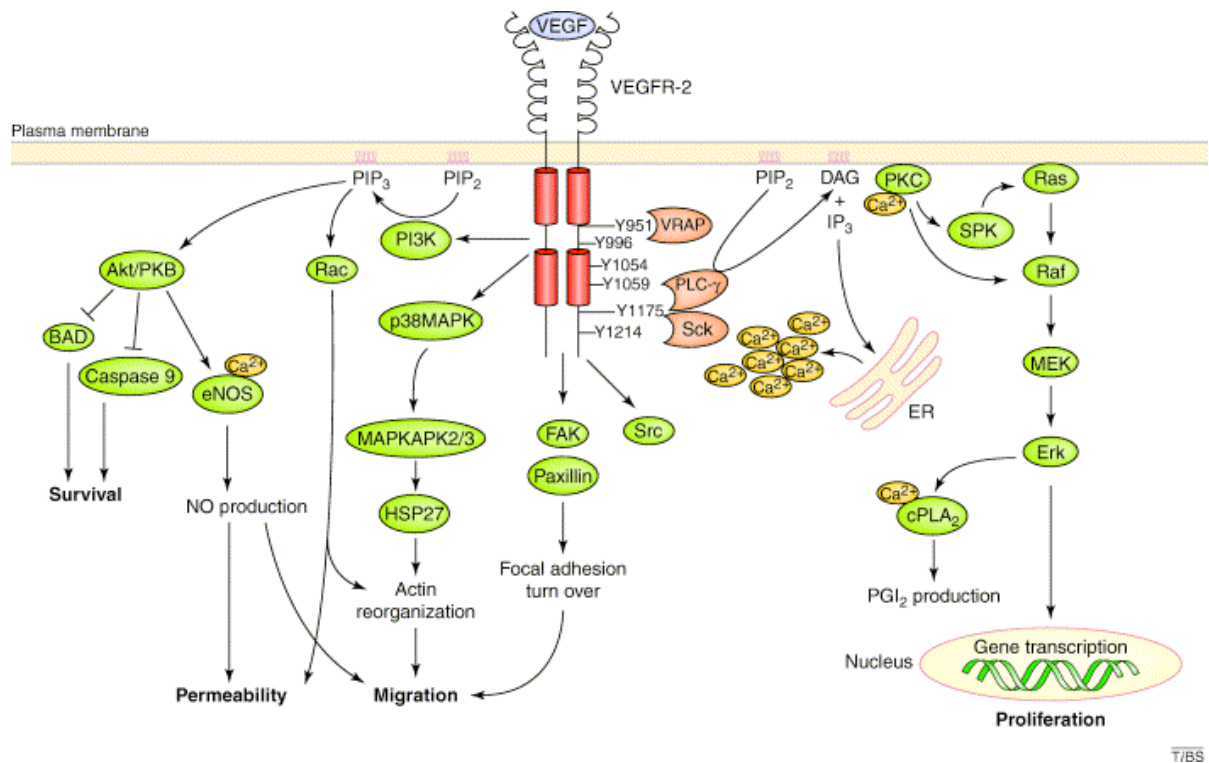


Figure 1.2 Schematic illustration of VEGFR-2 intracellular signaling. VEGF binding to VEGFR-2 induces autophosphorylation of specific intracellular tyrosine residues. Several intracellular adaptor proteins such as VEGFR-associated protein (VRAP), Sck and phospholipase C (PLC)-γ bind to tyrosine residues via their Src homology-2 (SH2) domains, leading to activation of these proteins. This induces signal transduction pathways such as Erk signaling pathway, which promotes proliferation, the Akt/PKB pathway promoting survival, permeability, and migration. VEGFR-2 activation also stimulates cytoskeletal regulation and cell migration through the p38 MAPK and focal adhesion kinase signal transduction pathway. Used with permission from (Cross *et al* 2003).

VEGFR-2 signaling is modulated by its ability to form complexes with other co-receptors, and heterodimerize with VEGFR-1. Complexes containing VEGFR-2 and NP-1 enhance the binding capacity of VEGF, specifically the VEGF₁₆₅ isoform, and increases endothelial cell chemotaxis (Soker *et al* 1998; Soker *et al* 2002). It has been hypothesized that NP-1 presents VEGF to VEGFR-2 in a manner that enhances the effectiveness of the receptor-mediated signal transduction (Ferrara *et al* 2003). Converging evidence indicates that NP-1 and NP-2 are involved in modulating VEGFR-2-dependent signaling in angiogenesis, but the signal transduction pathways, and the definitive role of these receptors are not fully characterized (Staton *et al* 2007).

1.5 PlGF and its receptors

In contrast to VEGF, very little is known about the molecular mechanism(s) of other VEGF homologs such as PlGF. Although both PlGF and VEGF were characterized in the early 90s, a literature search in February 2011 for PlGF resulted in 623 published papers while for VEGF resulted in 33928 published works. Increasingly, however, several reports have started implicating PlGF in different pathological conditions involving angiogenesis, including preeclampsia (Schmidt *et al* 2009), cardiovascular function (Iyer and Acharya 2002), cancer (Luttun *et al* 2004), and diabetic retinopathy (Praidou *et al* 2010). PlGF has also been detected in the brain under pathological conditions (Beck *et al* 2002; Donnini *et al* 1999; Nomura *et al* 1998), however, the role it plays in this context is not fully defined. Recent studies indicate potential roles of PlGF in neuroprotection and angiogenesis in the ischemic brain (Beck *et al* 2002; Du *et al* 2010; Liu *et al* 2006).

In humans, alternative splicing of PlGF RNA produces at least four polypeptides with different dimerization properties, heparin and receptor binding affinity (Figure 1.3A). PlGF-

1 was initially isolated from a term placenta cDNA library; it is a 149-amino acid glycosylated dimeric secreted protein, which binds to VEGFR-1 (Maglione *et al* 1991). Subsequently, a second isoform, PlGF-2 was discovered and found to contain a highly basic 21-amino acids insertion near the carboxyl end of the protein, which was not present in the originally described PlGF-1 protein (Hauser and Weich 1993). This insertion increased PlGF-2 affinity to heparin (Hauser and Weich 1993). Maglione *et al.* (1993) further characterized the human PlGF gene and determined its location on chromosome 14, and found that the two different mRNAs are produced from this single-copy gene in different cell lines and tissues (Maglione *et al* 1993). Human PlGF cDNA hybridized to sequences present in the genomic DNA of *Drosophila*, *Xenopus*, chicken and mouse, which indicates that the PlGF gene is highly conserved during evolution (Maglione *et al* 1993). Functional studies showed that both PlGF isoforms have a similar mitogenic potency on bovine aortic endothelial cells (Hauser and Weich 1993).

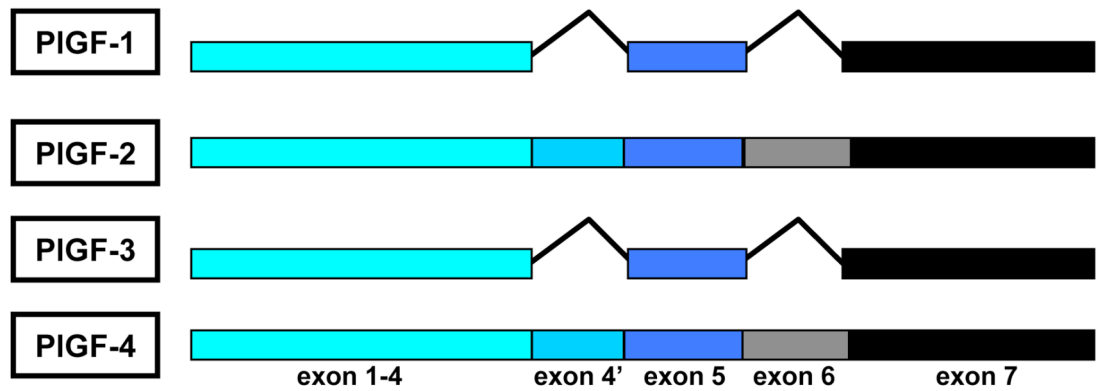
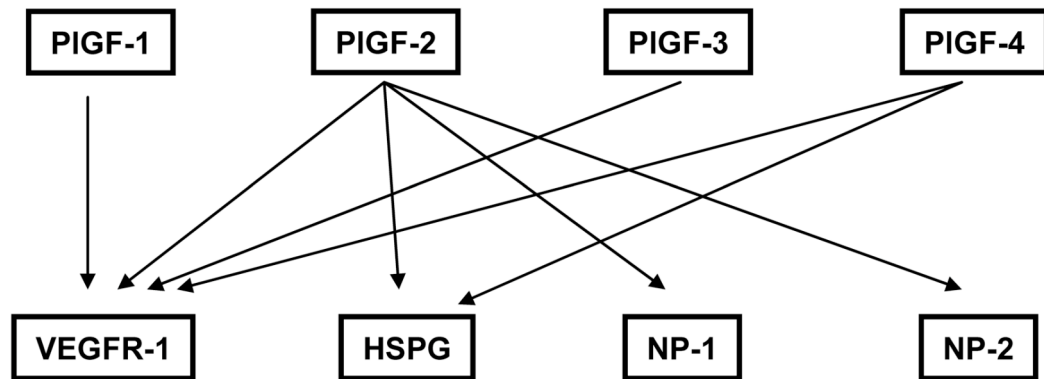
A**B**

Figure 1.3 (A) Illustration demonstrating the four spliced variants of the PIGF gene. PIGF gene has seven exons. PIGF-1 and PIGF-3 mRNAs lack exons 4' and 6. PIGF-3 mRNA has a 216 base insert between exons 4 and 5. PIGF-4 contains the same sequence as PIGF-3 in addition to a heparin-binding domain present in PIGF-2 **(B)** Receptor-binding of PIGF isoforms to VEGFR-1, heparan sulfate proteoglycans (HSPG), NP-1 and NP-2. Used with permission from (Ribatti 2008).

The third isoform, PlGF-3 variant, is similar to PlGF-1 except for an in-frame insertion of 72-amino acids near the C-terminal portion; PlGF-3 lacks a heparin-binding domain (Cao *et al* 1997). In 2003, a fourth variant, PlGF-4, was identified in normal human trophoblast and human umbilical vein endothelial cells (HUVEC). This isoform is composed of the same sequence as PlGF-3 but containing the heparin-binding domain previously thought to be present only in PlGF-2 (Yang *et al* 2003a). In contrast to PlGF-2, very little is known about the biological functions of PlGF-3 and -4.

PlGF-1 has been demonstrated to act as an inhibitor of angiogenesis by the formation of inactive PlGF-1/VEGF heterodimers in tumors (Eriksson *et al* 2002; Schomber *et al* 2007). It has been associated with promoting arteriogenesis in the mouse ischemic hindlimb (Li *et al* 2006), and it has been shown to be a marker in breast cancer associated with poor prognosis (Escudero-Esparza *et al* 2010).

In the mouse, only one PlGF isoform, with 78% similarity (65% identity) to the human PlGF-2, has been identified (DiPalma *et al* 1996). Mouse PlGF-2 is a secreted homodimer 158-amino-acid-long glycoprotein, whose gene is located on chromosome 12 of the mouse genome (DiPalma *et al* 1996).

PlGF-2 binds to three receptors VEGFR-1, NP-1 and -2 (Figure 3B) (Ribatti 2008). VEGFR-1 is a 180-kDa homodimer receptor tyrosine kinase with high-affinity for PlGF, and VEGF. It is expressed in vascular endothelial cells (Quinn *et al* 1993), pericytes, smooth muscle cells (Bellik *et al* 2005; Luttun *et al* 2002c), astrocytes (Choi *et al* 2007; Krum *et al* 2008), and hippocampal neurons (Choi *et al* 2007). VEGFR-1 knockout in mice results in death at E8.5-9 due to obstruction of vessels by an overgrowth of endothelial cells (Fong *et al* 1995). This receptor has been shown to have weak intracellular tyrosine kinase activity; deletion of its kinase domain resulted in normal development in mice (Hiratsuka *et al* 1998).

A secreted soluble form (sVEGFR-1), which lacks the transmembrane and intracellular parts of the receptor, was discovered and shown to function as an inhibitory sink for VEGF (Carmeliet *et al* 2001; Kendall *et al* 1996). This finding, along with the fact that VEGFR-1 knockout resulted in death due to an over-growth of endothelial cells, led to the hypothesis that VEGFR-1 may act as an inert negative regulator by sequestering VEGF (Carmeliet *et al* 2001).

However, subsequent studies have directly implicated VEGFR-1 in inducing signaling pathways associated with endothelial cell-cell or cell-matrix interactions during vascular development (Fong *et al* 1995); induction of transcription factors FosB and c-Fos (Holmes and Zachary 2004), cell motility (Taylor *et al* 2010), vessel permeability (Vogel *et al* 2007), endothelial morphogenesis (Funahashi *et al* 2010; Jones *et al* 2009; Mavria *et al* 2006) and survival (Cai *et al* 2003; Mavria *et al* 2006). Many of these effects involve PlGF activation of VEGFR-1.

PlGF-driven intracellular signaling pathways are not characterized as extensively as those triggered by VEGF. PlGF promotes survival in tumor endothelial cells and macrophages (Adini *et al* 2002). This effect is mediated through VEGFR-1 and is associated with an increase in the antiapoptotic gene survivin (Adini *et al* 2002). PlGF-VEGFR-1 signaling induces vessel branching, and formation of complex vascular networks by stimulating integrin recycling from intracellular stores to the endothelial cells membrane (Jones *et al* 2009). In agreement with these findings, anatomical analysis of VEGFR-1 knockout mice, which die at ~E8.5, revealed the presence of a highly disorganized vascular network (Fong *et al* 1995). Although VEGF can activate VEGFR-1 signaling, PlGF specifically promotes VEGFR-1-dependent increase in capillary-like networks (CLN) networks *in vitro* (Cai *et al* 2003). Furthermore, PlGF-stimulated CLN networks are

sustained for significantly longer periods than those induced by VEGF (Cai *et al* 2003). The mechanism involves VEGFR-1-activated PI3K/Akt pathway with the subsequent increase in the antiapoptotic protein Bcl-2 in endothelial cells (Cai *et al* 2003). Recently, PlGF was shown to induce endothelial cell sprouting through VEGFR-1, a mechanism involving notch-1 signaling (Funahashi *et al* 2010). Taken together, these studies show that PlGF induces distinct signaling pathways through VEGFR-1, and plays a critical role in endothelial cell sprouting and branching during angiogenesis.

Interestingly, monocytes, macrophages and hematopoietic stem cells exclusively express VEGFR-1 (Clauss *et al* 1996; Hattori *et al* 2002). PlGF has been reported to induce responses associated with recruitment, differentiation and activation of these cells during inflammation and arteriogenesis (Luttun *et al* 2002b).

PlGF binding to NP-1 is heparin dependent (Mamluk *et al* 2002; Migdal *et al* 1998) and plays a role in endothelial cell migration (Migdal *et al* 1998). In several pathologies, the simultaneous expression of PlGF and NPs have been observed (Beck *et al* 2002; Escudero-Esparza *et al* 2010; Henno *et al* 2010; Lacal *et al* 2000). However, the effects of PlGF on NP-1 and -2 signaling remains largely uncharacterized.

1.5.1 Synergistic effect of PlGF on VEGF-driven angiogenesis

Evidence of VEGFR-1/VEGFR-2 heterodimerization was reported by Kendall *et al.* (1996), demonstrating that sVEGFR-1 formed a VEGF-stabilized complex with the extracellular domain of VEGFR-2 in vitro, suggesting that these two receptors are capable of forming a heterodimeric receptor complex (Kendall *et al* 1996). In support of this hypothesis, an earlier study revealed the existence of endogenous PlGF and VEGF heterodimers with biochemical activity (DiSalvo *et al* 1995). In addition, PlGF was shown

to potentiate the action of low, marginally efficacious concentration of VEGF on endothelial cells, implicating PlGF as a potential modulator of VEGF (Park *et al* 1994). Subsequently, Autiero *et al.* (2003) using PlGF knockout (PlGF^{-/-}) and wild-type (PlGF^{+/+}) mice reported that VEGFR-1 and -2 heterodimerization occurs under pathological angiogenesis, and is a key mechanism in PlGF amplification of VEGF-mediated angiogenesis (Autiero *et al* 2003b).

In contrast to VEGF, loss of PlGF does not affect normal development in mice (Luttun *et al* 2002a). However, under pathological angiogenesis, PlGF is upregulated and enhances VEGF-dependent angiogenesis in the peripheral vascular system (Autiero *et al* 2003b; Carmeliet *et al* 2001; Luttun *et al* 2002a). It was postulated that PlGF stimulates angiogenesis by competing with VEGF for VEGFR-1 (Carmeliet *et al* 2001; Mac Gabhann and Popel 2004), resulting in an increase in the amount of available VEGF to activate its primary receptor VEGFR-2 (Carmeliet *et al* 2001). In addition, PlGF activation of VEGFR-1 was shown to synergize with VEGF activation of VEGFR-2 promoting a stronger angiogenic response (Carmeliet *et al* 2001).

Autiero *et al.* (2003) proposed a more complete model to describe the mechanisms involved in the PlGF synergistic enhancement of VEGF-driven angiogenesis (Figure 1.4). First, PlGF/VEGF heterodimers promote the heterodimerization of VEGFR-1 and -2, resulting in the stimulation of unique intracellular signaling pathways that amplify angiogenic responses (Autiero *et al* 2003b). Secondly, VEGFR-1 activation by PlGF promotes intermolecular cross talk that, either directly or indirectly, transactivates VEGFR-2 and primes the receptor for VEGF. Thirdly, PlGF and VEGF induce distinct biological responses through VEGFR-1 signaling (Autiero *et al* 2003b). The net result of these complex interactions between VEGF and its homolog PlGF, and their receptors, increases

the angiogenic response of the tissue to hypoxia. These findings single out PlGF as an important modulator of VEGF in pathological angiogenesis in the peripheral vascular system.

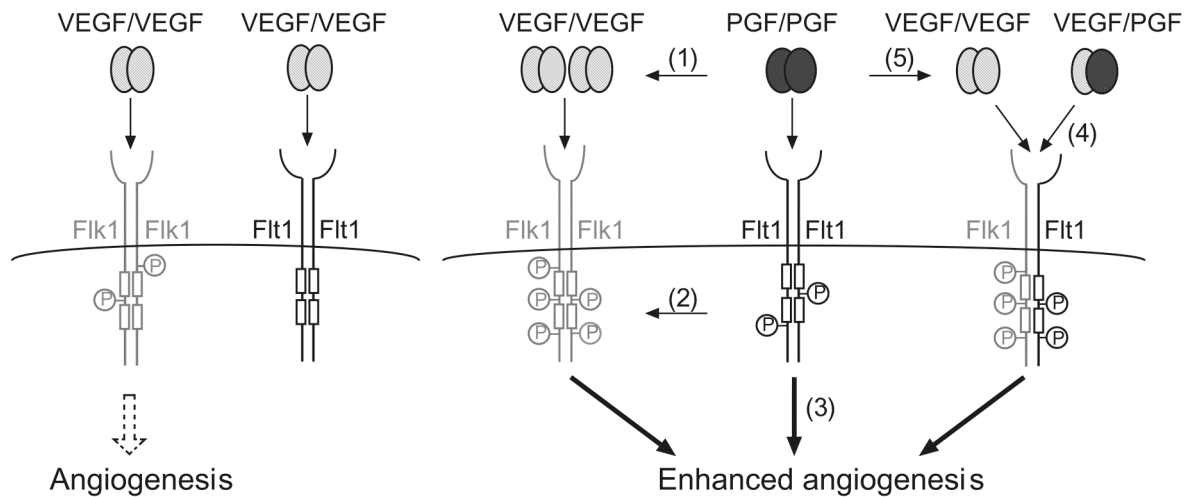


Figure 1.4 Schematic model of the synergism between VEGF and PlGF. In the absence of PlGF, VEGF activation of VEGFR-2 (Flk-1) induces tyrosine phosphorylation of VEGFR-2, leading to angiogenic signaling ('P' phosphotyrosine site). When PlGF levels are absent or low VEGFR-1 (Flt1) seems to be a reservoir to VEGF, with minimal signaling activity. In pathological conditions PlGF expression is increased and enhance angiogenesis by several mechanisms. (1) PlGF displaces VEGF from VEGFR-1 and makes more VEGF available to activate VEGFR-2. (2) PlGF activates VEGFR-1, leading to transphosphorylation of VEGFR-2 and increased VEGFR-2 phosphorylation. (3) PlGF transmits signals through VEGFR-1, inducing its own signaling pathways. (4) PlGF forms a heterodimer with VEGF (VEGF/PlGF), which can activate and transmit angiogenic signals through the VEGFR-2/VEGFR-1 heterodimer receptor complex. (5) PlGF displaces VEGF homodimers, which stimulate VEGFR-2/VEGFR-1 heterodimerization, from VEGFR-1. Used with permission from (Autiero *et al* 2003b)

1.6 Brain angiogenesis induced by chronic mild hypoxic conditions

Mild hypoxic conditions, such as those encountered in high altitudes, elicit various systemic cardiovascular and respiratory adaptations, such as increased CBF and ventilation to maintain oxygen delivery to the brain (LaManna *et al* 2004). Prolonged or chronic exposure to mild hypoxia (10% oxygen), equivalent to 18000 feet (~5500 m), results in a more dramatic mechanism of adaptation in the brain, angiogenesis (Dore-Duffy and LaManna 2007). This model of systemic (whole body) chronic mild hypoxia has been used to unravel the mechanism(s) through which hypoxia stimulates angiogenesis in the CNS (Dore-Duffy and LaManna 2007; Ratan *et al* 2007).

Experimental hypoxia in animals can be induced by two methods: i) by decreasing the partial pressure of oxygen in a normobaric chamber, producing “hypoxic hypoxia” (Xu and Lamanna 2006); ii) decreasing the overall barometric pressure in “Wright chambers,” which are chambers for housing small animals that withstand a partial vacuum, resulting in “hypobaric hypoxia” (Xu and Lamanna 2006). No important physiological adaptive differences in animals subjected to hypoxia using either method have been observed (Xu and Lamanna 2006).

Studies by LaManna *et al.* (1992) showed that rats exposed to 21 days (3 weeks) of hypoxia failed to gain weight, exhibited a significant increase in hematocrit (to a level of 71%) and CBF, and a strong stimulation of angiogenesis (microvessel density), compared to normoxic controls (LaManna *et al* 1992). The systemic adaptation led to an attenuation of the CBF to prehypoxic baseline levels, while the angiogenic response stimulated by tissue hypoxia, restored tissue oxygen levels to normoxic levels by 21 days of hypoxia (Xu and Lamanna 2006).

Under chronic hypoxia, the brain initiates a robust angiogenic response that extends over a period of 14-21 days (LaManna *et al* 1994; Li *et al* 2010; Mironov *et al* 1994; Pichiule and LaManna 2002; Xu and Lamanna 2006). In this animal model the angiogenic activity is maximal during the first 14 days of hypoxia, during which several angiogenic factors were identified.

After 6 hr of hypoxia vessel destabilization occurs by the upregulation of angiopoietin-2 in pericytes and endothelial cells (Pichiule and LaManna 2002; Thomas and Augustin 2009). Angiopoietin-2 induction continues for at least 14 days. However, between 14 and 21 days, angiopoietin-2 protein expression returns to control levels despite continued hypoxia (Pichiule and LaManna 2002). Rats that were subjected to 21 days of hypoxia followed by a recovery period of 21 days of normoxia, showed vessel regression due to BEC apoptosis during the first 14 days of recovery. During this period, angiopoietin-2 protein levels significantly increased again before returning to basal levels after 21 days of normoxic recovery (Pichiule and LaManna 2002). These results indicate that angiopoietin-2 plays an important role in vascular remodeling associated with both angiogenesis and vessel regression. Furthermore, since angiopoietin-2 is involved in microvascular plasticity during adaptation and deadaptation to hypoxia, the fate of BEC, whether to undergo angiogenesis or apoptosis, appears to be mediated by the presence of other angiogenic factors (Pichiule and LaManna 2002).

Under hypoxic conditions VEGF is the primary inducer of angiogenesis. Marti *et al.* (1998) showed that mice exposed to 6 hr of systemic hypoxia (6% oxygen) were sufficient to induce VEGF expression in glial cells adjacent to BEC but not in cortical microvessels (Marti and Risau 1998). Consistent with these findings, VEGF expression was demonstrated to be upregulated in capillary pericytes, and pericapillary astrocytes in hypoxic

mice (Dore-Duffy and LaManna 2007; Li *et al* 2010). VEGF expression in this model is biphasic, increasing slightly after 12 h of hypoxia, and reaching a maximum after 2 days before transiently decreasing after 4 days of hypoxia (Kuo *et al* 1999). VEGF protein expression then increased again after 7 days of hypoxia before returning to normoxic levels after 21 days of exposure to hypoxia (Kuo *et al* 1999). The first peak of VEGF expression was detected in activated capillary pericytes, while the second peak of VEGF expression was detected in pericapillary astrocytes (Dore-Duffy and LaManna 2007; Li *et al* 2010).

VEGF expression correlated with a significant increase in microvessel density during the first week of hypoxia (Kuo *et al* 1999). However, the expression of VEGF returned back to normal levels after this first week, despite continuous exposure to hypoxia (Kuo *et al* 1999). This implies that VEGF regulation in the brain is under tissue metabolic balance rather than to PaO₂, which remains low under hypoxic conditions (Kuo *et al* 1999). This study supports the hypothesis that VEGF is important for initiation of angiogenesis but not required to maintain increased brain vascularity during prolonged hypoxia (Kuo *et al* 1999).

Factors known to play a role in angiogenesis during early development have been identified under pathological conditions involving hypoxia. In the developing brain high levels of the ECM protein fibronectin and its receptor $\alpha 5\beta 1$ integrin in BEC, is associated with developmental angiogenesis (Milner and Campbell 2002). Fibronectin has been shown to elicit BEC survival and proliferation (Wang and Milner 2006). In contrast, vessel maturation is associated with the loss of fibronectin and $\alpha 5\beta 1$ integrin (Milner *et al* 2008). In the adult brain, under hypoxic conditions, the developmental mechanisms responsible of activating angiogenesis are recapitulated, suggesting that the fibronectin- $\alpha 5\beta 1$ interaction represents a fundamental pro-angiogenic pathway in the CNS (Milner *et al* 2008). Similar to

VEGF, fibronectin and $\alpha 5\beta 1$ integrin expression increased within the first week of hypoxia, and decreased thereafter, while the overall capillary density continued to increase over the 14-day time course (Milner *et al* 2008). In a subsequent study, BEC fibronectin- $\alpha 5\beta 1$ expression and proliferation were shown to peak after 4 days of hypoxia (Li *et al* 2010), which suggests that the strongest stimulus of BEC proliferation might occur within the first 4 days of hypoxia when VEGF expression are also high (Li *et al* 2010).

In agreement with the described proliferative phase of BEC during hypoxia, astrocyte activation and proliferation was reported to increase and reach a maximum after 4 days and continue to increase up to the 14-day time-point (Li *et al* 2010). Astrocytes are functionally and physically associated with cerebral vessels and quickly respond to changes occurring at the endothelial level (Nedergaard *et al* 2003). A direct correlation was found between the density of vessels that stained positive for laminin, an ECM protein found in vascular basement membrane, and $\alpha 6\beta 4$ integrin and dystroglycan, two proteins that are expressed in astrocytic endfeet, which are associated with anchoring astrocyte endfeet to the vascular basement membrane (Li *et al* 2010). This implies that during the first 2 weeks of hypoxia when angiogenesis in the brain is still ongoing, the new capillaries are closely matched with the presence of new reactive astrocyte endfeet (Li *et al* 2010).

In summary, hypoxia induces the accumulation of HIF-1, leading to an increase in VEGF expression and secretion, first from pericytes and then from astrocytes, within the first week; this results in the activation and proliferation of BEC. Both pericytes and BEC, secrete angiopoietin-2 that activates Tie2 receptors on BEC, and promote vessel destabilization and pericyte detachment (Pichiule and LaManna 2002; Thomas and Augustin 2009). Within the first week, integrin and fibronectin expression is upregulated, and in conjunction with VEGF,

promote BEC proliferation. During this time MMPs are being secreted by pericytes and astrocytes (Dore-Duffy *et al* 2007) that breakdown the vessel basement membrane, allowing sprouting of new capillaries. Astrocytes closely follow the growing microvasculature and attach themselves to the new capillaries (Li *et al* 2010). Once new vessels are established and functional, and tissue hypoxic signal is reduced, capillaries return to a quiescent state (Dore-Duffy *et al* 2007).

Focal ischemia in the brain results from an occlusion of a brain-supplying artery and is accompanied with a sudden reduction in oxygen (hypoxia) and nutrient supply to the tissue downstream of the occluded vessel (del Zoppo 2009). Hypoxia is an important driving force of angiogenesis during cerebral ischemia and is mediated by induced gene expression of VEGF and its two receptors VEGFR-1 and VEGFR-2 (Marti *et al* 2000).

Various mechanisms and factors activated during chronic hypoxia appear to be recapitulated in ischemic conditions (Ratan *et al* 2007). The transcription factor HIF-1 induces expression of several genes, including VEGF, in both surviving neurons and BEC after stroke (Ratan *et al* 2007), similar to what was observed in hypoxic conditions. Furthermore, glucose deprivation has also been shown to increase VEGF-A expression via HIF-1–related signaling (Maltepe *et al* 1997). Activation of the endoplasmic reticulum stress sensor IRE1 is a common pathway linking hypoxia- and hypoglycemia-dependent responses to the up-regulation of VEGF (Drogat *et al* 2007).

PlGF has also been reported to be upregulated in the brain under ischemic conditions (Beck *et al* 2002; Du *et al* 2010; Hayashi *et al* 2003; Liu *et al* 2006), however, the molecular regulation and function of PlGF during ischemia has not been fully understood.

1.7 Research hypothesis and objectives

Prolonged exposure to low oxygen environment leads to functional metabolic changes followed by structural adaptations through vascular remodeling (LaManna et al., 2004). In rats, doubling of the brain capillary density occurs at 1 week of hypoxic exposure, reaching the maximum level at 3 weeks (Lauro and LaManna, 1977). Several angiogenic factors have been associated with this remodeling and VEGF in particular has been shown to be an active participant. In the peripheral vascular system PlGF acts synergistically with VEGF to enhance angiogenesis, under pathological conditions (Carmeliet *et al* 2001). Yet the role of PlGF in hypoxia-induced brain angiogenesis remains unexplored.

Chronic mild hypoxia triggers angiogenesis in the brain in the attempt to restore proper tissue oxygen tension. The immediate angiogenic response is vital to neuronal survival. *We hypothesized that brain angiogenesis will be impaired in PlGF knockout mice subjected to mild chronic hypoxia, due to the lack of the synergistic effect between PlGF and VEGF in these mice.*

Angiogenesis in the brain is accompanied by increased BBB permeability (Valable et al., 2005). In contrast to VEGF that is known as a very potent permeability factor, the role of PlGF in modulating BBB opening is still not clear. During angiogenesis, PlGF is associated with vessel maturation and stability by participating in the recruitment of pericytes and smooth muscle cells to the newly formed vessels (Autiero *et al* 2003a; Du *et al* 2010; Liu *et al* 2006; Luttun *et al* 2002b; Luttun *et al* 2002c; Roncal *et al* 2008). Therefore, *we hypothesized that if PlGF knockout animals show a reduced angiogenic response, decreased BBB leakiness can be expected, at least during the proliferative phase of angiogenesis. However, hypoxic animals lacking PlGF may display greater BBB leakiness than wild-type mice in the later phases of hypoxia when vascular morphogenesis takes place.*

PlGF vascular effects have been associated with the selective stimulation of VEGFR-1 on endothelial cells, and activation of VEGFR-1-dependent ERK1/2 and GSK-3 β intracellular signaling pathways known to be involved in sprouting and branching morphogenesis (Funahashi *et al* 2010; Jones *et al* 2009; Mavria *et al* 2006). *We hypothesized that lack of the VEGFR-1 ligand PlGF in PlGF^{-/-} BEC will result in alterations in the intracellular signaling pathways associated with sprouting and branching morphogenesis in BEC, leading to impaired angiogenic response to hypoxia.*

It has been shown that VEGF not only stimulates the growth and survival of endothelial cells but also of neurons and astrocytes (Silverman *et al* 1999) and that these effects are mostly mediated by VEGFR-2 (Jin *et al* 2000). Survival signals upon VEGF binding to VEGFR-2 are triggered through the activation of the PI3K/Akt signal transduction system. Proliferative effects mediated by VEGFR-2 are dependent on activation of Erk signal transduction pathway (Takahashi *et al* 1999; Xiao *et al* 2007). Under pathological conditions astrocytes become activated, and VEGF has been shown to induce astrocyte reactivity through VEGFR-1 (Krum *et al* 2008; Mani *et al* 2005; Schmid-Brunclik *et al* 2008), however, the synergistic effect of PlGF on VEGF-dependent astrocyte activity is not known. *We hypothesized that lack of PlGF may affect astrocyte proliferation, and survival under ischemic/hypoxic conditions.*

The goal of this thesis is to investigate the potential role of PlGF in the process of vascular remodeling in the brain during chronic hypoxic conditions (7, 14, and 21 days), using PlGF knockout (PlGF^{-/-}) mice. These mice present in the PlGF gene a partial deletion of exon 3 and exon 4-6, which contain the essential cysteine residues necessary for the proper dimerization, and biological activity of PlGF (Luttun *et al* 2002a).

This thesis combines *in vitro* and *in vivo* experiments to evaluate the consequence of PlGF knockout on brain angiogenesis and cell survival during hypoxia. *In vivo* studies provide a global view of the role of PlGF in hypoxia-induced neurovascular remodeling. In this part of the study, the outcome of the complex interaction between the different components of the neurovascular unit were evaluated during the course of hypoxia. The experiments *in vitro* dissect the effect of PlGF knockout on both the ability of endothelial cells to respond to angiogenic stimuli and the capacity of astrocytes to react and survive hypoxic/ischemic conditions. Experiments are designed to delineate the impact that the lack of PlGF may have on the biological responses of astrocytes and endothelial cells individually as well as on their mutual interaction during the process of angiogenesis. Information extracted from *in vitro* and *in vivo* studies were compared and mutually validated.

The specific aims of this thesis are:

- 1) To determine the effect of PlGF knockout on cerebrovascular remodeling *in vivo*, specifically on angiogenesis, vessel maturation, BBB permeability and astrocyte reactivity. This study is described in Chapter 2.
- 2) To investigate the effect of PlGF knockout on signaling pathways involved in vascular branching and sprouting in brain endothelial cells submitted to hypoxia. This study is described in Chapter 3.
- 3) To determine the effect of PlGF knockout on astrocyte reactivity to oxygen and glucose deprivation and investigate the molecular mechanisms involved. This study is described in Chapter 4.

An overview of all major findings and their significance to neurovascular remodeling during hypoxia is presented in Chapter 5.

CHAPTER 2

PlGF Knockout Delays Brain Vessel Growth and Maturation upon Systemic Hypoxic Challenge

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Contribution of the co-authors:

- Dr. Danica Stanimirovic and Dr. Maria Moreno provided scientific guidance and supervised project. Manuscript was written by Moises Freitas-Andrade and edited and revised by Dr. Maria Moreno and Dr. Danica Stanimirovic.
- Claudie Charlebois assisted with mouse brain sectioning, immunofluorescence and Western blots as well as PCR experiments.
- Dr. Peter Carmeliet provided transgenic PIGF knockout and wild-type mice, and provided critical review of the manuscript.

2.1 ABSTRACT

In this study, we have investigated the potential role of PlGF in hypoxia-induced brain angiogenesis. To this end, PlGF wild-type (PlGF^{+/+}) and PlGF knockout (PlGF^{-/-}) mice were exposed to whole body hypoxia (10% oxygen) for 7, 14 and 21 days. PlGF^{+/+} animals exhibited a significant ~40% increase in angiogenesis after 7 days of hypoxia compared to controls, while in PlGF^{-/-} this effect only occurred after 14 days of hypoxia. No differences in pericyte/smooth muscle cell (SMC) coverage between the two genotypes were observed. After 14 days of hypoxia, PlGF^{-/-} microvessels had a significant increase in fibrinogen accumulation and extravasation compared to those of PlGF^{+/+}, which correlated with endothelial cell disruption of the tight junction protein claudin-5. These vessels displayed large lumens, were surrounded by reactive astrocytes, lacked both pericyte/SMC coverage and endothelial VEGF expression, and regressed after 21 days of hypoxia. VEGF expression levels were found to be significantly lower in the frontal cortex of PlGF^{-/-} compared to those in PlGF^{+/+} animals during the first 5 days of hypoxia, which in combination with the lack of PlGF may have contributed to the delayed angiogenic response and the prothrombotic phenotype observed in the PlGF^{-/-} animals.

2.2 INTRODUCTION

Neurons are highly sensitive to oxygen levels and a constant supply is necessary for their proper function and survival. However, high oxygen levels can also have a detrimental effect on neurons due to the formation of reactive oxygen species. Therefore, the oxygen balance in the brain is tightly controlled, via the cerebral blood flow (CBF), by the neurovascular unit, a functional group of cells that includes neurons, astrocytes, brain endothelial cells (BEC), vascular smooth muscle cells (VSMC) and pericytes (Hamel 2006). Under pathological conditions, such as stroke, interruption of cerebral blood flow leads to a reduction in oxygen delivery that results in neuronal death. In this situation, the brain has developed several adaptive mechanisms to protect and/or promote brain recovery including angiogenesis, the growth of new capillaries from preexisting vessels (LaManna *et al* 1992).

Angiogenesis is a complex process that occurs over several days after a hypoxic event (Krupinski *et al* 1994; LaManna *et al* 1992) and that involves all the cellular constituents of the neurovascular unit (del Zoppo 2010). Several angiogenic factors have been identified in the ischemic/hypoxic brain, however, the interplay between these factors and how they interact within the neurovascular unit, have not been completely characterized.

VEGF, a potent pro-angiogenic factor, is upregulated in the hypoxic/ischemic brain (Beck *et al* 2002; Kuo *et al* 1999). VEGF binds to the tyrosine kinase receptors VEGFR-1 and VEGFR-2 and to neuropilin-1 (NP-1) but VEGFR-2 is the primary transducer of VEGF signals, activating several intracellular signaling pathways including the Raf-Mek-Erk, involved in cell proliferation (Takahashi *et al* 1999), and the PI-3 kinase/Akt, involved in cell survival (Kilic *et al* 2006). In addition to having beneficial angiogenic and neuroprotective effects, VEGF is also a potent permeability factor and has been associated with brain edema during angiogenesis (Kilic *et al* 2006).

In contrast to the permeability effects of VEGF, PlGF, a homolog of VEGF (Maglione *et al* 1991), has been shown to play a role in vessel stabilization under pathological conditions (Autiero *et al* 2003a; Du *et al* 2010; Liu *et al* 2006; Luttun *et al* 2002c). Moreover, systemic delivery of transfected mesenchymal stem cells expressing PlGF in rats subjected to middle cerebral artery occlusion (MCAO) showed that PlGF significantly increased angiogenesis, without increasing cerebral edema (Liu *et al* 2006).

Under pathological conditions PlGF has been shown to synergistically enhance VEGF angiogenic activity in the systemic vascular system (Autiero *et al* 2003b; Carmeliet *et al* 2001) through various mechanisms: (i) displacing VEGF from VEGFR-1, thus increasing the availability of VEGF to bind and activate VEGFR-2 (Carmeliet *et al* 2001); (ii) heterodimerizing with VEGF (VEGF/PlGF) which leads to the activation and transmission of angiogenic signals through the VEGFR-2/VEGFR-1 heterodimer receptor complex (Autiero *et al* 2003b), and (iii) directly activating VEGFR-1 which, through transphosphorylation of VEGFR-2, enhances VEGFR-2 activity (Autiero *et al* 2003b). PlGF binding to VEGFR-1 has also been shown to induce its own signaling pathways, which result in the increased expression of c-Fos, FosB and Survivin (Adini *et al* 2002; Holmes and Zachary 2004).

While several lines of evidence indicate that hypoxia is a potent inducer of VEGF *in vitro* and *in vivo* (Ferrara *et al* 2003) studies investigating the effect of hypoxia on PlGF expression have rendered contradictory results, showing either no effect (Cao *et al* 1996b), PlGF upregulation (Cramer *et al* 2005), or PlGF downregulation (Ahmed *et al* 2000) depending on the cell type. However, the presence of endogenous PlGF mRNA and protein has been detected in the ischemic brain, suggesting an important role for PlGF during pathological conditions in the CNS (Beck *et al* 2002; Du *et al* 2010; Hayashi *et al* 2003). Recent studies have demonstrated the neuroprotective properties of PlGF in ischemic

conditions both *in vitro* and *in vivo* (Du *et al* 2010; Liu *et al* 2006). Collectively, these findings indicate that PlGF plays a role in hypoxic/ischemic brain, but the exact nature of its modulatory effect in hypoxia-induced brain angiogenesis remains unclear.

The purpose of this study was to investigate the role of PlGF in brain angiogenesis and blood brain barrier (BBB) permeability using PlGF^{-/-} animals. The current study shows that lack of PlGF results in a delayed angiogenic response to hypoxia, accumulation of fibrinogen in cerebral microvessels and vessel regression after 21 days of hypoxia.

2.3 MATERIALS AND METHODS

Whole body hypoxia and tissue collection

PlGF wild type and knockout mice (generated by Carmeliet *et al.* (2001) Vesalius Research Center, Belgium) were bred at the National Research Council of Canada (NRCC) Institute for Biological Sciences Animal Facility (Ottawa, ON, Canada). Experiments were approved by the NRCC animal care committee in accordance with Canadian Council on Animal Care (CCAC) guidelines. 19 day-old mice were housed in cages inside a normobaric hypoxic chamber (Forma Anaerobic System model 1024) at 10% O₂ and N₂ balance fed into the chamber. Oxygen levels were measured with MI-730 Micro-Oxygen Electrode (Microelectrodes, INC. Bedford, USA) and maintained at 10% O₂ inside the chamber. Normoxic control mice were housed in cages immediately adjacent to the hypoxic chamber, and were fed and changed on the same schedule as the hypoxic mice. At the beginning of the experiment and after each time point (7, 14, and 21 days) hypoxic and control mice were weighed and venous blood samples were collected for hematocrit. At the end of experimental period, mice were deeply anesthetized with 4% halothane B.P. (MTC Pharmaceuticals, Cambridge, ON, Canada) under an oxygen flow rate of 2 L/min, and

perfused transcardially with an 18-gauge needle connected to a syringe with cold heparinized saline. Brains from PIGF^{+/+} and PIGF^{-/-} hypoxic and control mice from each time point were dissected and immediately frozen in dry ice and stored at -80°C for cryosectioning.

Immunofluorescence

Brains were sectioned at 10 µm thickness using a cryostat (Jung CM3000; Leica, Richmond Hill, ON, Canada). Sections were placed on Superfrost Plus microscope slides (Fisher Scientific, Nepean, ON, Canada), fixed in ice-cold methanol for 10 min and washed twice in ice-cold phosphate-buffered saline (PBS). Sections were permeabilized by incubation in 0.25% Triton X-100 for 10 min, followed by three 5 min washes in PBS. Nonspecific staining was blocked by pre-incubation of sections in PBS containing 0.25% Triton X-100 and 10% normal goat serum or 1% bovine serum albumin for 1 hour at room temperature (RT). Incubation of primary antibodies were performed overnight at 4 °C, in PBS + 5 % goat serum. Primary antibodies and their dilutions used were as follows: monoclonal rat anti-mouse CD31/PECAM-1 (1:100; BD Bioscience, Mississauga, ON, Canada) for endothelial cells, polyclonal goat anti-rat VEGF (1:20; R&D Systems, Minneapolis, MN, USA) for mouse VEGF₁₂₀ and VEGF₁₆₄, polyclonal rabbit anti-nerve/glial antigen 2 (NG2) chondroitin sulfate proteoglycan (1:300; Millipore, Billerica, MA, USA), monoclonal mouse anti-human desmin (1:100; DakoCytomation, Burlington, ON, Canada) for pericytes, monoclonal mouse anti- α -smooth muscle actin (α -SMA) clone 1A4 (1:400; Sigma-Aldrich, Oakville, ON, Canada) for smooth muscle cells, polyclonal rabbit anti-human fibrinogen/FITC (1:60; DakoCytomation, Burlington, ON, Canada) for native fibrinogen as well as fibrinogen fragments D and E, and polyclonal rabbit anti-glial fibrillary

acidic protein (1:500; DakoCytomation, Burlington, ON, Canada) for astrocytes, rabbit polyclonal to human claudin-5 (1:100; abcam, abcam, MA, USA) for tight junction protein. Appropriate secondary Alexafluor[®] antibodies (invitrogen, Burlington, ON, Canada) were used at 1:500 dilution in PBS for 1 hour at RT. Samples were washed three times in PBS for 5 min and stained with Hoechst in PBS (Sigma-Aldrich, Oakville, ON, Canada) for 15 min. Sections were rinsed with PBS and coverslip mounted with a drop of Dako fluorescence mounting media. Omission of primary antibodies served as negative controls.

Quantitative analysis

Four mice per genotype (PlGF^{+/+} and PlGF^{-/-}), treatment condition (normoxia and hypoxia) and time point (7, 14, and 21 days) were analyzed (total of 24 mice per genotype). Three brain sections from the frontal cortex (Bregma 2.80mm, Bregma 1.70mm, and Bregma 1.18mm) were used per animal for immunofluorescent analysis. Double or triple immunostaining was performed in each section (see figure legends). Four images per section, two from the right and two from the left side of the cerebral cortex (12 images/animal) were taken using Olympus fluorescent microscope with a 10X objective, attached to Q-imaging Retiga EXi digital camera (Olympus Canada Inc, Markham, Canada). Total area, length, number and frequency distribution analysis of immunopositive cells for a particular marker were calculated from the three sections per animal using an image analysis program, Image-Pro[®] Plus (Olympus Canada Inc, Markham, Canada). The average of each parameter for the four mice per group was then calculated.

Gene expression analysis

PlGF and β -actin gene expression was analyzed in brain samples from PlGF^{+/+} and PlGF^{-/-} mice submitted to hypoxia for 7, 14 and 21 days by RT-PCR as described previously (Freitas-Andrade *et al* 2008). The sequences of the primers used were as follow: PlGF (accession no. NM_008827) 5' CAGCCAACATCACTATGCAG-3' (forward) and 5' GGGTGACGGTAATAAATACG-3' (reverse), yielding a 268-bp product; β -actin (accession no. NM_007393) 5'GGAGATTACTGCTCTGGCTC-3' (forward) and 5' GGACTCATCGTACTCCTGCT-3' (reverse), yielding a 131-bp product.

Protein expression analysis by Western-blot

Western blot analysis was used to determine levels of VEGF and PlGF in brain lysates. Briefly, brains were removed from PlGF^{+/+} and PlGF^{-/-} hypoxic mice, and immediately frozen in dry ice and stored in -80°C until further use. A section of the frontal cortex from each animal was isolated and placed into a tube with ice-cold RIPA lysis buffer (1%NP40 (IGEPAL), 0.5% Deoxycholate, 0.1% SDS and protease inhibitor 100 μ l/10ml RIPA in PBS), (Sigma-Aldrich Oakville, ON, Canada) and homogenized with an electric homogenizer at 4°C. The lysates were centrifuged at 14,000 rpm for 10 min at 4°C, and the supernatants were collected. Protein concentration was measured in each of the brain lysates by BCA Protein Assay Reagent Kit (Pierce Biotechnology, Rockford, IL, USA), and 50-100 μ g of protein were denatured in protein loading buffer for 5 min at 100°C, and then separated on a 15% sodium dodecyl sulfate-polyacrylamide gel. Separated proteins were then transferred to polyvinylidene difluoride (PVDF) membranes. Blocking was performed by incubating membranes in TBST (20 mM Tris-buffer [pH 8.0] and 150 mM NaCl with 0.1%

Tween-20) containing 5% BSA (Sigma-Aldrich Oakville, ON, Canada) for 1 h at room temperature. Membranes were then incubated overnight at 4°C with either primary rabbit polyclonal anti-human VEGF(A20) (Santa Cruz Biotechnology, Inc, CA, USA) diluted 1/100 in TBST containing 1% skim milk, or rabbit polyclonal human PlGF (abcam, MA, USA) diluted 1/100 in TBST containing 1% skim milk. The membranes were extensively washed with TBST and incubated for 1 h with Anti-Rabbit IgG (whole molecule)–Peroxidase antibody (Sigma-Aldrich Oakville, ON, Canada) diluted 1/5000 in TBST containing 5% skimmed milk. Immunoreactive proteins were visualized by Amersham ECL Plus Western Blotting Detection Reagents (GE Healthcare Buckinghamshire, UK). Membranes were also probed for β -actin protein expression using monoclonal anti- β -actin-peroxidase antibody (Sigma-Aldrich Oakville, ON, Canada) at a concentration of 1/50000, according to manufacturers' instruction.

Statistical analysis

Effects of hypoxia on PlGF^{+/+} and PlGF^{-/-} mice over the three time points were evaluated; two x three (genotype x time) ANOVAs were applied to detect differences between PlGF^{+/+} and PlGF^{-/-} hypoxic groups followed by Bonferroni post-test method. To detect differences among hypoxic and normoxic PlGF^{+/+} and PlGF^{-/-}, two x two (genotype x treatment) ANOVAs were applied followed by Bonferroni post test method with the statistical package GraphPad Prism® 5. P-values of < 0.05 were considered significant. All data are presented as mean $\pm$ s.e.m.

2.4 RESULTS

Effect of hypoxia on body weight and hematocrit in $PlGF^{+/+}$ and $PlGF^{-/-}$ mice.

To investigate the role of PlGF in hypoxia-induced brain angiogenesis, $PlGF^{+/+}$ and $PlGF^{-/-}$ mice were subjected to chronic hypoxia (10% oxygen), which mimics the effect of exposure to high altitude (~18000 ft).

Both $PlGF^{+/+}$ and $PlGF^{-/-}$ normoxic animals gained about 60% of the initial weight over the 21 days of the experiment, with no significant differences between the two genotypes (Fig. 1A). In contrast, $PlGF^{+/+}$ and $PlGF^{-/-}$ mice failed to gain weight when exposed to long-term hypoxia (7, 14 and 21 days), however, no differences were observed between the two genotypes (Fig. 1A).

$PlGF^{+/+}$ and $PlGF^{-/-}$ mice exposed to normoxia exhibited similar levels of hematocrit (~ 46%) over the 21 days of experiment (Fig. 1B). Under hypoxic conditions, both $PlGF^{+/+}$ and $PlGF^{-/-}$ animals developed polycythemia as indicated by the 2-fold increase in hematocrit levels in both genotypes (Fig. 1B).

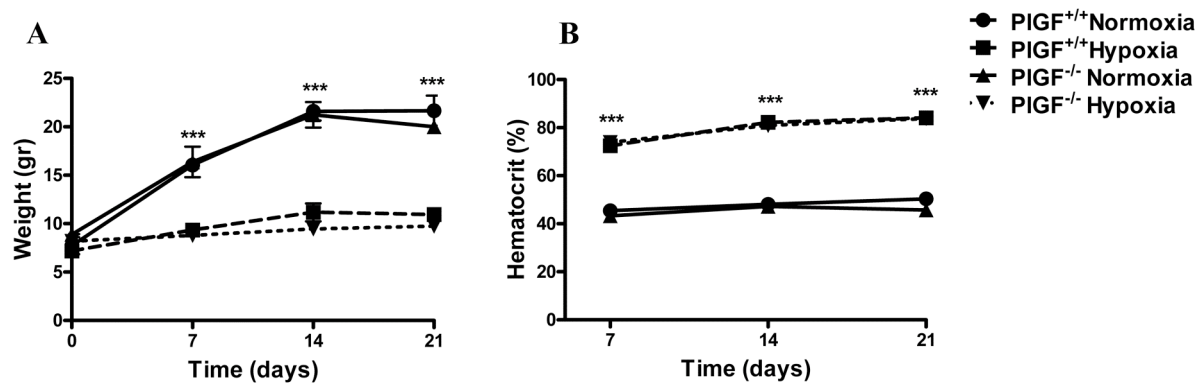


Figure 1 Effects of 7-, 14- and 21-day exposure to 10% hypoxia on body weight (A) and hematocrit (B) of PlGF^{+/+} and PlGF^{-/-} mice. Data presented are means $\pm$ s.e.m. of 4 mice per genotype and time point.

Effect of PlGF knockout on brain angiogenesis.

Brain angiogenesis was evaluated in $PlGF^{+/+}$ and $PlGF^{-/-}$ normoxic and hypoxic mice by quantifying the number, total length and total area of $CD31^{+}$ cells in frontal brain sections of the cerebral cortex. Data obtained from the analysis of both total area and total length of $CD31^{+}$ cells showed similar trends of changes in $PlGF^{+/+}$ and $PlGF^{-/-}$ animals in all conditions. The total area of $CD31^{+}$ cells is therefore presented as a representative measure of angiogenesis (Supplementary Figure 1, Appendix I). This parameter measures the changes in all three types of angiogenesis including sprouting, intercalated, and intussusceptive angiogenesis (Ward *et al* 2007).

Total area of $CD31^{+}$ cells was not significantly different in normoxic $PlGF^{+/+}$ and $PlGF^{-/-}$ mice at any time point (Fig. 2A-B). After 7 days exposure to hypoxia, $PlGF^{+/+}$ mice showed a significant $\sim 40\%$ increase in total area of $CD31^{+}$ cells compared to normoxic controls (Fig. 2A-B); however, no changes were observed in $PlGF^{-/-}$ animals under the same conditions (Fig. 2A-B). After 14 and 21 days both hypoxic $PlGF^{+/+}$ and $PlGF^{-/-}$ mice showed $\sim 40\%$ higher vessel area compared to their normoxic controls, with no significant differences between the two genotypes (Fig. 2A-B).

The total number of $CD31^{+}$ cells did not significantly change between the normoxic and hypoxic groups for either $PlGF^{+/+}$ or $PlGF^{-/-}$ mice at 7- or 14-day time points (data not shown). However, at 21 days of exposure to hypoxia, a significant $\sim 15\%$ increase in total number of $CD31^{+}$ cells was observed in both $PlGF^{+/+}$ and $PlGF^{-/-}$ mice (data not shown). In $PlGF^{+/+}$ mice subjected to 7 days of hypoxia, a greater number of vessels exhibited an increase in size and branching compared to either normoxic controls or the $PlGF^{-/-}$ counterparts (Fig. 2A).

To further evaluate the shift in vessels size, a frequency distribution analysis of CD31⁺ cells with area ranging between 0-100 μm^2 , 150-500 μm^2 , and >500 μm^2 was performed (Fig. 2C). In both PlGF^{+/+} and PlGF^{-/-} normoxic mice, the number of CD31⁺ vessels was similar and inversely proportional to the vessel size with ~3000 CD31⁺ vessels of 0-100 μm^2 , ~350 CD31⁺ vessels of 150-500 μm^2 and ~20 CD31⁺ vessels of >500 μm^2 (Fig. 2C).

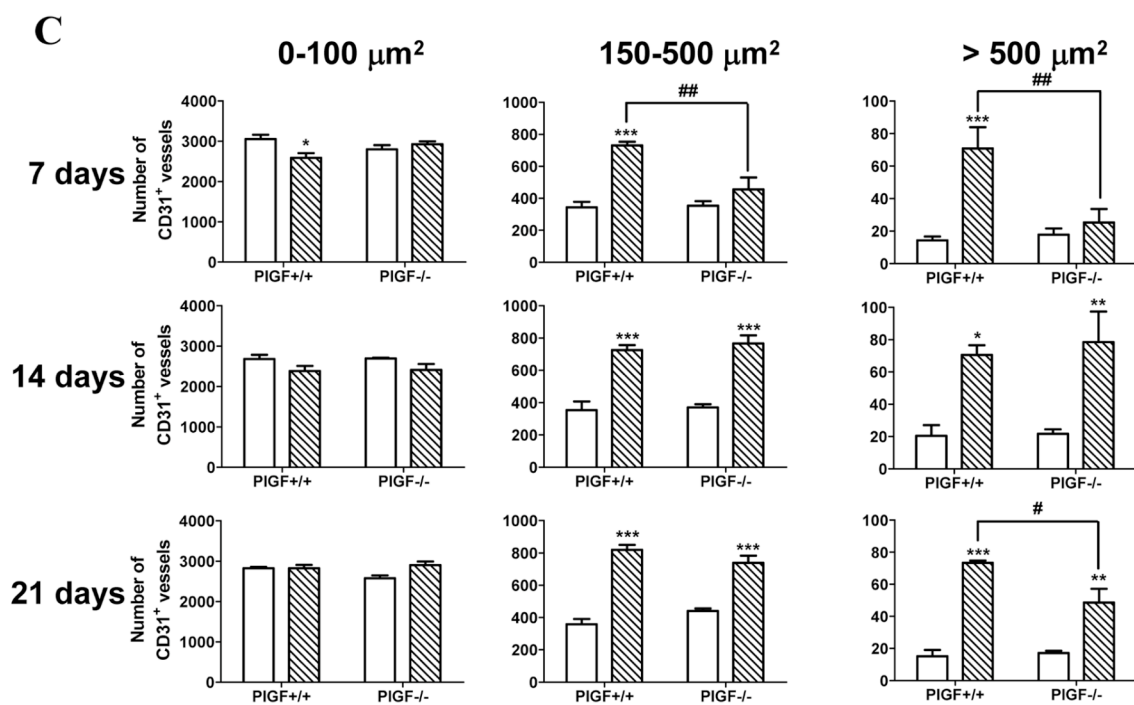
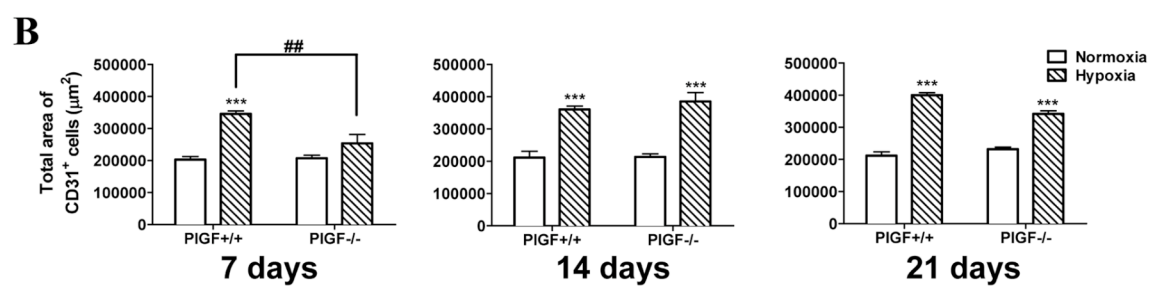
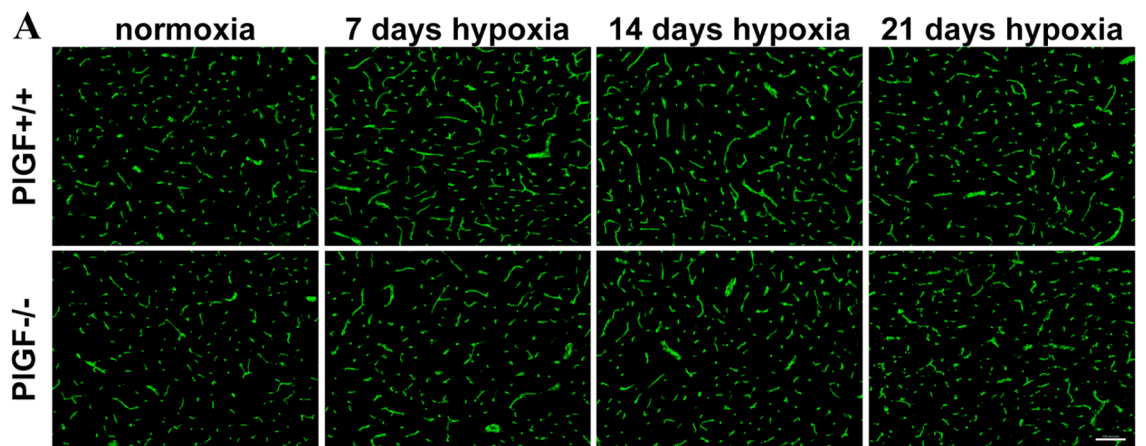
After 7 days of hypoxia, PlGF^{+/+} mice showed a significant decrease (~400 vessels) in the number of 0-100 μm^2 CD31⁺ vessels compared to normoxic controls (Fig. 2C). This was correlated with a significant increase (~400 vessels) in the number of CD31⁺ vessels measuring 150-500 μm^2 and >500 μm^2 in hypoxic PlGF^{+/+} mice compared to normoxic animals (Fig. 2C). Interestingly, no changes in the number of vessels were observed in any of the vessel-size distribution groups between hypoxic PlGF^{-/-} mice and its corresponding normoxic controls (Fig. 2C).

After a 14-day hypoxia, both PlGF^{+/+} and PlGF^{-/-} mice showed a small, non-significant, decrease in the number of 0-100 μm^2 CD31⁺ vessels but a significant increase in the number of 150-500 μm^2 and >500 μm^2 CD31⁺ vessels compared to their respective normoxic groups (Fig. 2C). No differences between hypoxic PlGF^{+/+} and PlGF^{-/-} was observed in either of the three vessel size distribution groups (0-100, 150-500, and >500 μm^2 ; Fig. 2C).

At day 21, a similar number of 0-100 μm^2 CD31⁺ vessels was measured in both PlGF^{+/+} and PlGF^{-/-} normoxic and hypoxic animals (Fig. 2C); a significant increase in the number of 150-500 μm^2 CD31⁺ vessels was observed in hypoxic PlGF^{+/+} and PlGF^{-/-} animals compared to their normoxic counterparts with no significant differences between the two

genotypes; a significant increase in the number of $>500 \mu\text{m}^2$ CD31⁺ vessels was also measured in hypoxic PlGF^{+/+} and PlGF^{-/-} animals compared to their normoxic counterparts; however PlGF^{-/-} mice demonstrated a significantly reduced number of $>500 \mu\text{m}^2$ CD31⁺ vessels compared to hypoxic PlGF^{+/+} animals (Fig. 2C).

Figure 2 (A) Representative images of sections of the cerebral cortex from $\text{PlGF}^{+/+}$ and $\text{PlGF}^{-/-}$ mice exposed to normoxia or 7-, 14-, or 21-day hypoxia. Sections were immunostained for the endothelial marker, CD31 (green). Scale bar = 100 μm . (B) Quantification of total area of CD31^+ cells after 7-, 14-, or 21-day normoxia or hypoxia as described in Material and Methods. (C) Number of CD31^+ vessels separated in size intervals of 0-100 μm^2 , 150-500 μm^2 and >500 μm^2 for each time point. White solid bars represent normoxic controls and hatched bars represent hypoxic groups. (See Appendix II, Table A2, page 160, for proportion of vessels in each size interval) Results expressed are means $\pm$ s.e.m. *** indicates significant difference ($p < 0.001$) between hypoxic groups and the corresponding normoxic control groups and ## indicates significant difference ($p < 0.01$) between hypoxic $\text{PlGF}^{+/+}$ and $\text{PlGF}^{-/-}$ groups (ANOVA followed by Bonferroni post-test).



Effect of PlGF knockout on pericyte and smooth muscle cell vessel coverage

PlGF has been shown to stimulate pericyte/VSMC recruitment under pathological ischemic conditions (Luttun *et al* 2002c; Takeda *et al* 2009). To investigate whether PlGF knockout affects pericyte/VSMC vessel coverage, total area of NG2⁺ and Desmin⁺ cells (pericyte markers) and α -SMA⁺ cells, (pericyte/VSMC marker) were evaluated in both PlGF^{+/+} and PlGF^{-/-} normoxic and hypoxic brain sections by immunofluorescence.

Immunofluorescent staining of PlGF^{+/+} and PlGF^{-/-} brain sections showed no morphological differences in NG2⁺, desmin⁺, and α -SMA⁺ cells in either normoxic or hypoxic conditions. NG2 and desmin markers stained the pericyte layer that was intimately apposed to CD31-labeled endothelial cells (Fig. 3A). NG2 stained vessels in a continuous pattern, while desmin staining was interrupted, showing frequent gaps and finger-like projections around the vessels (Fig 3A). α -SMA antibody selectively stained large microvessels (>500 μ m²) and did not stain the relatively smaller microvessels (Fig. 3A). α -SMA labeling showed a circumferentially arranged striated appearance that ran perpendicular to the longitudinal axis of the CD31-labeled vessels. The morphological features that were observed using the above pericyte and VSMC markers in this study are in agreement with those reported by other groups (Kurz *et al* 2008; Virgintino *et al* 2007).

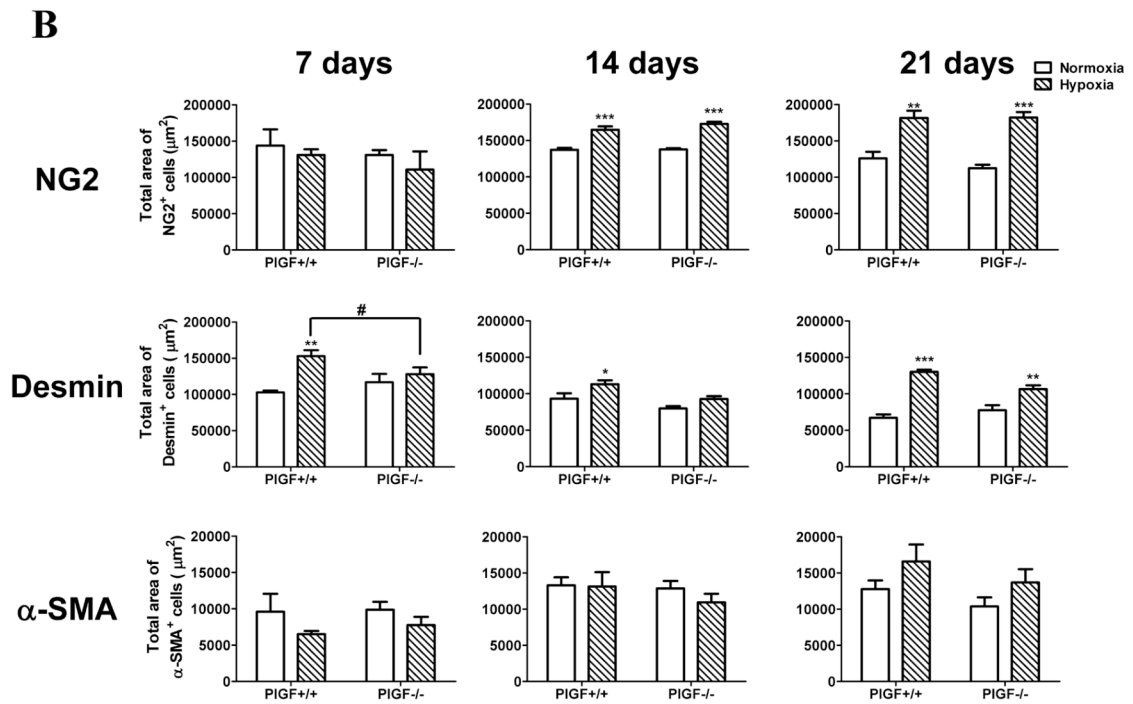
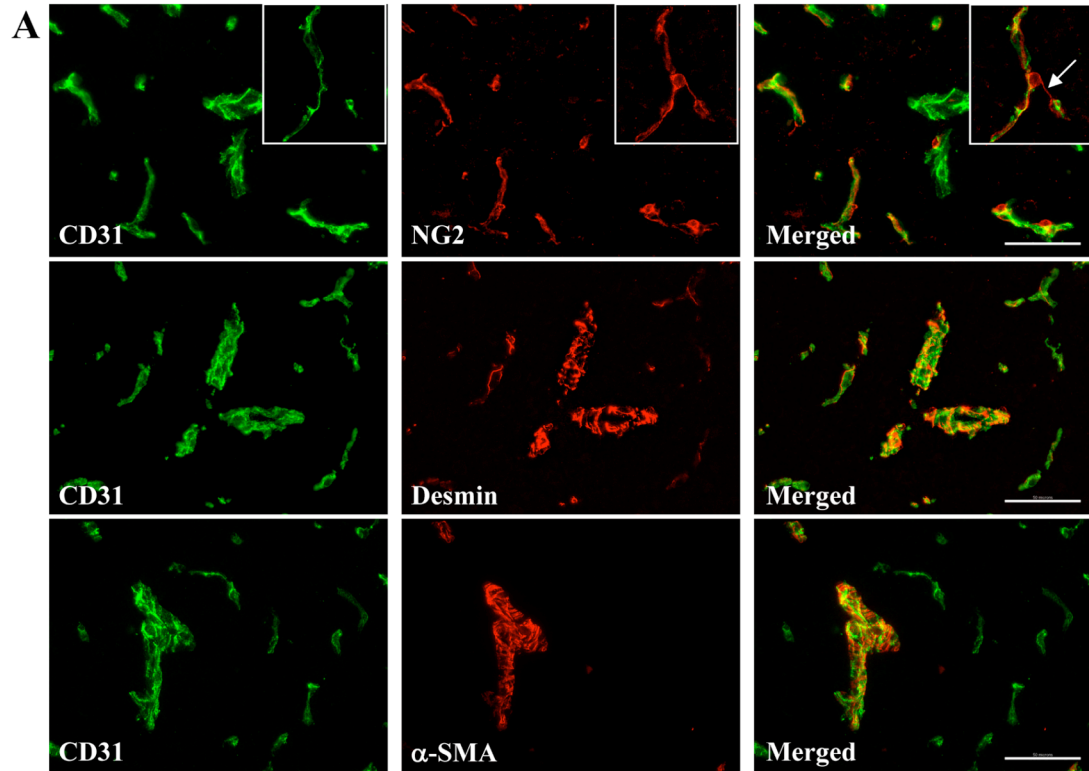
Quantitative analysis of total area covered by NG2⁺, desmin⁺, and α -SMA⁺ cells showed that NG2⁺ and desmin⁺ cells are 10-times more abundant than α -SMA⁺ cells in the brain vasculature (Fig 3B), which correlates with the observed selective staining of α -SMA⁺ cells in large (>500 μ m²) microvessels (Fig. 3A). No significant differences in total area covered by any of the three markers were observed between PlGF^{+/+} and PlGF^{-/-} mice under

normoxic conditions. Hypoxia did not affect the total area of α -SMA⁺ cells at any time point in either PlGF^{+/+} or PlGF^{-/-} mice.

Total area covered by NG2⁺ cells was not affected by 7 days hypoxia in either PlGF^{+/+} or PlGF^{-/-} animals, however, it was increased in both genotypes by ~20% and 30% after 14 and 21 days of hypoxia, respectively (Fig. 3B).

Total area covered by desmin⁺ cells was significantly higher in PlGF^{+/+} mice after 7, 14, and 21 days of hypoxia compared to the corresponding normoxic controls, while in PlGF^{-/-} mice the area was significantly higher than in the control group only after 21 days of hypoxia (Fig 3B).

Figure 3 (A) Double- immunofluorescence staining performed on sections of cerebral cortex from normoxic or hypoxic PIGF^{+/+} and PIGF^{-/-} mice, using the endothelial marker CD31 (green) with either of the following pericyte markers: NG2 (red), desmin (red) or VSMC marker α -SMA (red). Scale bar = 100 μ m. Insert in the right upper corner represents a magnified image of a NG2⁺ reactive pericyte. (B) Quantitative analysis of total area of NG2⁺, desmin⁺ and α -SMA⁺ cells as described in Material and Methods. White solid bars represent normoxic controls and hatched bars represent hypoxic groups. Results expressed are means $\pm$ s.e.m. * indicates significant difference (* p< 0.05, ** p<0.01, ***p<0.001) between hypoxic groups and the corresponding normoxic control groups and # indicates significant difference (p<0.05) between hypoxic PIGF^{+/+} and PIGF^{-/-} groups (ANOVA followed by Bonferroni post-test).



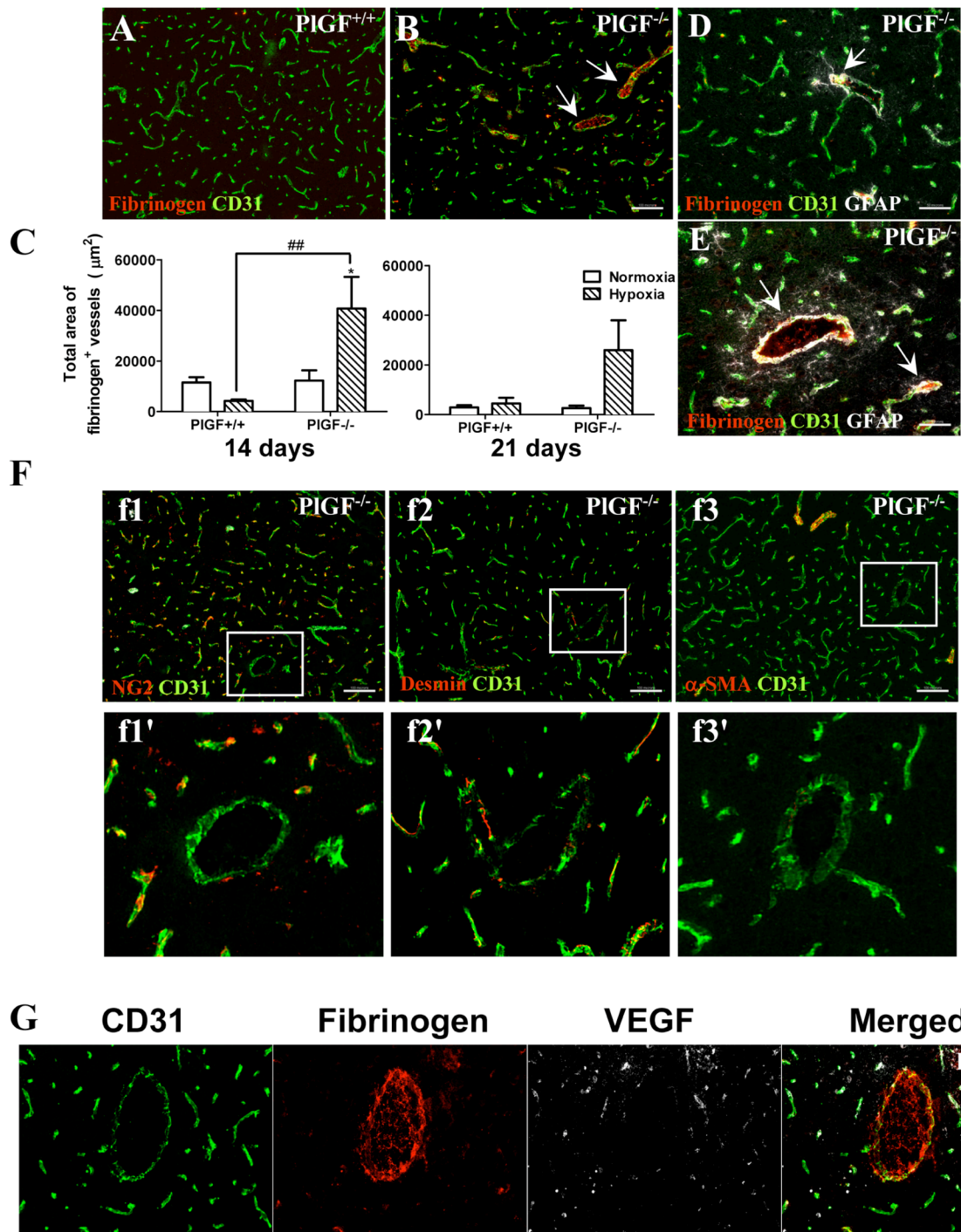
Effect of PlGF knockout on BBB permeability

Several lines of evidence indicated that PlGF plays a role in vessel stabilization under pathological conditions (Autiero *et al* 2003a; Du *et al* 2010; Liu *et al* 2006; Luttun *et al* 2002c). To evaluate whether PlGF knockout can affect vessel permeability, vessel extravasation of fibrinogen was investigated in frontal cortical brain sections of PlGF^{+/+} and PlGF^{-/-} normoxic and hypoxic mice by immunofluorescence. In all cases, fibrinogen colocalized with CD31⁺ vessels which consistently exhibited intraluminal fibrinogen accumulation (Fig. 4A, B, D, E and G) and a certain degree of fibrinogen extravasation (Fig. 4G).

After 7 days of either normoxia or hypoxia no significant differences in total area of fibrinogen⁺ vessels were observed in PlGF^{+/+} and PlGF^{-/-} animals (data not shown). After 14 days of hypoxia, the total area of fibrinogen⁺ vessels in PlGF^{-/-} mice was ~ 10-fold higher than in PlGF^{+/+} hypoxic mice (Fig. 4C). After 21 days, no significant differences in total area of fibrinogen⁺ cells was observed between the normoxic and hypoxic PlGF^{+/+} animals but a significant increase was still quantified in two out of four PlGF^{-/-} hypoxic mice compared to the normoxic group.

PlGF^{-/-} hypoxic mice exhibited a greater number of vessels (>500 μm^2 CD31⁺) with dilated lumen compared to those in PlGF^{+/+} hypoxic mice. These vessels frequently contained intraluminal fibrinogen accumulation (Fig. 4A, B, D, E and G), were surrounded by GFAP⁺ astrocytes (Fig. 4D and E) and completely lacked or had sparse NG2⁺, desmin⁺ and/or α -SMA⁺ cell coverage (Fig. 4F). In addition, these vessels presented very low VEGF expression compared to that of smaller vessels in the same sections (Fig. 4G).

Figure 4 (A-B) Double-immunofluorescence staining was performed on sections of cerebral cortex from normoxic and hypoxic $\text{PlGF}^{+/+}$ and $\text{PlGF}^{-/-}$ mice, using the endothelial marker CD31 (green) and fibrinogen (red). Arrows indicate the presence of fibrinogen in the lumen of $\text{PlGF}^{-/-}$ vessels after 14 days hypoxia (B). Scale bar = 100 μm . (C) Quantitative analysis of total area of fibrinogen⁺ microvessels from $\text{PlGF}^{+/+}$ and $\text{PlGF}^{-/-}$ mice exposed to 14- and 21- day normoxia or hypoxia. White solid bars represent normoxic controls and hatched bars represent hypoxic groups. Results expressed are means $\pm$ s.e.m. * indicates significant difference ($p < 0.05$) between hypoxic groups and the corresponding normoxic control groups, and ^{##} indicates significant difference ($p < 0.01$) between hypoxic $\text{PlGF}^{+/+}$ and $\text{PlGF}^{-/-}$ groups (ANOVA followed by Bonferroni post-test). (D-E) Triple-immunofluorescence for fibrinogen (red), endothelial cells (green) and astrocytes (white) on frozen sections of cerebral cortex from $\text{PlGF}^{-/-}$ mice exposed to 14 days of hypoxia. Scale bar = 50 μm . Arrows indicate reactive astrocytes surrounding fibrinogen⁺ vessels. (F) Double-immunofluorescence of sections of cerebral cortex from $\text{PlGF}^{-/-}$ mice exposed to 14-day hypoxia, using the endothelial marker CD31 (green) and pericyte/VSMC markers NG2, desmin and α -SMA (red). Boxes in f1, f2, f3 outline areas magnified in f1', f2', f3', showing the lack of pericyte/VSMC markers in enlarged microvessels ($>500 \mu\text{m}^2$ CD31⁺ vessel group). Scale bar = 100 μm . (G) Triple-immunofluorescence staining of fibrinogen (red), endothelial cells (green) and VEGF (white) in sections of cerebral cortex from $\text{PlGF}^{-/-}$ mice after 14 days of hypoxia. Scale bar = 50 μm .



The status of tight junction proteins in brain vessels was analyzed in $\text{PlGF}^{+/+}$ and $\text{PlGF}^{-/-}$ mice at 14 days of hypoxia, when fibrinogen accumulation was highly abundant in $\text{PlGF}^{-/-}$ animals. Claudin-5, the major interendothelial junctional protein limiting paracellular permeability at the BBB, exhibited a highly regular and organized immunoreactive arrangement in $\text{PlGF}^{+/+}$ vessels compared to the more disorganized and fragmented immunoreactivity pattern displayed in the $\text{PlGF}^{-/-}$ vessels (Fig 5). No significant changes were observed in ZO-1 expression between both genotypes (data not shown).

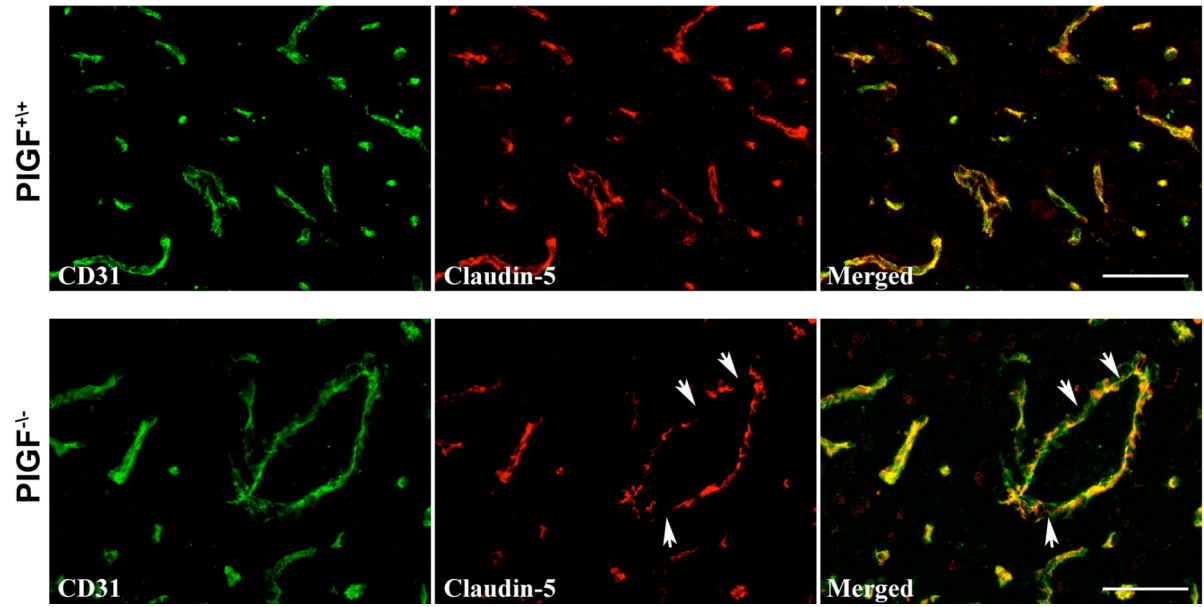


Figure 5 Double-immunofluorescence of sections of cerebral cortex from $PIGF^{+/+}$ and $PIGF^{-/-}$ mice exposed to 14-day hypoxia, using the endothelial marker CD31 (green) and tight junction marker claudin-5 (red). Arrows indicate areas of disruption of claudin-5. Scale bar = 50 μm .

Effect of hypoxia on PlGF and VEGF expression in PlGF^{+/+} and PlGF^{-/-} mice

Since the delay in angiogenic response in PlGF^{-/-} mice occurred during the first week of hypoxia, PlGF gene expression was analyzed in the brain homogenate of both genotypes during the course of the first 5 days of hypoxia (n=2 animals per time point). In PlGF^{+/+} mice, PlGF mRNA was expressed in the brain during normoxia as well as during the first five days of hypoxia. No PlGF expression was found in PlGF^{-/-} mice in either normoxia or hypoxia, as expected (Fig 6A). Although a slight increase in PlGF expression was observed in the wild type mice at specific time points during the course of hypoxia, the variability obtained between the two experiments do not allow us to firmly state that there is a significant change in PlGF expression during hypoxia in the brain of wild-type mice.

VEGF protein was upregulated during the first 5 days of hypoxia in PlGF^{+/+} animals, however, the peak of expression during those 5 days varied between 1 to 4 days in the two experiments performed. The expression of VEGF was consistently higher during hypoxia in PlGF^{+/+} animals compared to the PlGF^{-/-} mice (Fig 6B).

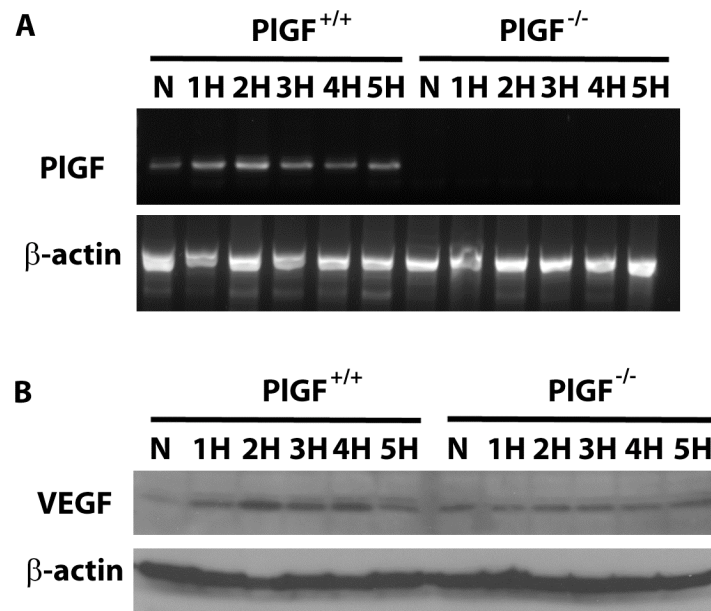


Figure 6 (A) Agarose gel showing PIGF and β -actin RT-PCR amplification products from frontal brain region of PIGF^{+/+} and PIGF^{-/-} mice exposed to 5 days of normoxia (N) or 1-5 days of hypoxia (H). (B) VEGF and β -actin protein expression analyzed by Western blot from PIGF^{+/+} and PIGF^{-/-} mice exposed to 5 days of normoxia (N) or 1-5 days of hypoxia (H).

2.5 DISCUSSION

In this study, the effect of PlGF in hypoxia-induced brain angiogenesis and BBB permeability was examined using PlGF^{+/+} and PlGF^{-/-} mice subjected to chronic hypoxia (10% oxygen) for 7, 14 and 21 days. PlGF knockout mice exhibited a delayed angiogenic response to mild hypoxia as well as fibrinogen accumulation and a certain degree of extravasation in a number of microvessels, some of which exhibited enlarged lumens. These fibrinogen⁺ microvessels presented claudin-5 disruption, were surrounded by reactive astrocytes, showed lack of both pericyte and VSMC coverage and VEGF expression, and regressed after 21 days of hypoxia.

Previous reports have shown that several adaptive mechanisms occur to protect the brain from the decrease in oxygen supply, including hyperventilation, an increase in packed red blood cell volume (hematocrit) and decrease in metabolism. Consistent with these reports, PlGF^{+/+} and PlGF^{-/-} mice exhibited an increase in hematocrit and failed to gain weight during the period of hypoxia (Harik *et al* 1996; LaManna *et al* 1992). However, no significant differences were found between the two genotypes.

Chronic hypoxia also promotes angiogenesis in the brain (Harik *et al* 1996; LaManna *et al* 1992). Among the factors contributing to the vascularization of the hypoxic brain, VEGF, angiopoietin-1 and integrins, play important roles (Milner *et al* 2008). PlGF expression has been shown to increase in the brain after middle cerebral artery occlusion (MCAO) (Beck *et al* 2002; Du *et al* 2010), but its contribution to chronic hypoxia-induced brain angiogenesis has not been fully elucidated.

In this study, 10% hypoxia promoted a rapid (from day 7) and sustained (until day 21) increase in vascularization of the cerebral cortex in wild-type mice, while in PlGF knockout animals angiogenesis was only measurable 14 days after hypoxia, reaching levels

similar to those obtained in the wild-type. This delay in angiogenic response to hypoxia in PlGF knockout mice is most likely due to both the lack of PlGF and the reduced levels of VEGF expression detected during the first 5 days of hypoxia compared to those in the PlGF^{+/+} animals. Although PlGF expression was only detected at the RNA but not at the protein level, this does not exclude the possibility that PlGF protein is produced in low amounts or expressed only in specific cells in the brain at quantities in the brain cortex homogenate beyond the detection limit by Western-blot. Experimental *in vitro* evidence obtained in our laboratory, indicated that mouse brain endothelial cells (MBEC) from wild-type mice express PlGF when subjected to 6 hours hypoxia followed by 14 hours reoxygenation (data not shown). As previously shown (Autiero *et al* 2003b; Park *et al* 1994), very low levels of PlGF can synergistically enhance the angiogenic response induced by sub-optimal concentrations of VEGF. As the angiogenic process is orchestrated by the activity of multiple growth factors, the lack of one of them may impact the timing of the response without affecting the final outcome.

Pericytes and VSMC play a role in vessel stabilization (Luttun *et al* 2002c), inhibit endothelial cell proliferation and migration (Orlidge and D'Amore 1987; Sato and Rifkin 1989) and promote BBB integrity (Armulik *et al* 2010). Under angiogenic conditions pericytes/VSMC interacts with endothelial cells and supports tube formation (Ozerdem and Stallcup 2003). The PlGF receptor, VEGFR-1, has been shown to be expressed in both pericytes and VSMC (Luttun *et al* 2002c). PlGF promotes vessel normalization, and reduces vascular leakage by inducing recruitment of VSMC and pericytes under ischemic conditions (Luttun *et al* 2002c). However, there are no cellular markers that are uniquely specific for pericytes, and none of them recognizes all pericytes; their expression is dynamic, varies between organs and developmental stages and have been shown to overlap as they are not

mutually exclusive in most pericytes (Armulik *et al* 2005). Desmin, NG2 and α -SMA are markers commonly used to identify pericytes and VSMC.

In both PlGF wild-type and knockout mice, the cerebrovascular coverage by NG2⁺ pericyte significantly increased after 14 days of hypoxia. This marker has been shown to specifically identify activated pericytes in angiogenic microvessels (Ozerdem and Stallcup 2003; Virgintino *et al* 2007). Since NG2 expression was still observed after 21 days of hypoxia, vascular remodeling at the pericyte level seems to be ongoing at least within this time frame.

Similarly, cerebrovascular coverage by desmin⁺ pericyte was significantly stimulated by hypoxia in wild-type mice, although occurring at an earlier time point (7 days) when compared to NG2⁺ cells. In PlGF^{-/-} animals, this process was delayed until day 21 of hypoxia, which correlates with the late angiogenic response observed in PlGF^{-/-} mice.

α -SMA cell staining was not affected by hypoxia in either PlGF^{+/+} or PlGF^{-/-} mice. This may indicate that the neo-vasculature has not reached full maturity within the 21 days time frame of this study. This is supported by the presence of activated pericytes (NG2⁺) 21 days after hypoxia and the selective association of α -SMA⁺ cells with larger microvessels ($\geq 500 \mu\text{m}^2$ CD31⁺ vessels).

Fibrinogen extravasation has been used as a marker of cerebrovascular permeability (del Zoppo 2008), and hypoxia has been shown to promote rapid microvascular thrombosis and fibrin deposition within the brain (Adhami *et al* 2006; del Zoppo 2008). Fibrinogen immunofluorescence was used in this study to investigate intra- and extra-vascular fibrinogen accumulation in PlGF^{+/+} and PlGF^{-/-} normoxic and hypoxic mice.

Despite no obvious and significant changes observed in pericyte/VSMC coverage between $\text{PlGF}^{+/+}$ and $\text{PlGF}^{-/-}$ hypoxic mice, $\text{PlGF}^{-/-}$ mice showed intravascular fibrinogen accumulation and extravasation after 14 days of exposure to hypoxia. This correlated with vascular disruption of the tight junction protein claudin-5 in $\text{PlGF}^{-/-}$ animals. Vessels with fibrinogen⁺ lumens, were surrounded by reactive astrocytes (GFAP⁺), and lacked pericyte/VSMC coverage. The size of these vessels were within the $>500\ \mu\text{m}^2$ CD31⁺ vessel group. Fibrinogen has been shown to inhibit neurite outgrowth, increase BBB permeability and endothelial cell disorganization, and promote neuroinflammation (del Zoppo 2008; Schachtrup *et al* 2007), and to induce astrocyte reactivity and scar formation (Schachtrup *et al* 2010). In $\text{PlGF}^{-/-}$ animals, a high proportion of the fibrinogen⁺ vessels exhibited relatively large lumen. This may be due to the lack of pericytes/VSMC coverage in these vessels as these cells play an important role in inhibiting endothelial cell proliferation (Orlidge and D'Amore 1987) and controlling the vascular tone (Diaz-Flores *et al* 2009). Moreover, supporting trophic factors such as VEGF were not expressed in the fibrinogen⁺ vessels, while it was present in the fibrinogen-free microvessels. We hypothesize that the delayed angiogenic process observed in $\text{PlGF}^{-/-}$ animals leads to an increase in hypoxic stress that promotes fibrinogen accumulation within pericyte-denuded cerebral microvessels (Adhami *et al* 2006) and gliosis (Schachtrup *et al* 2010), which ultimately may lead to vessel regression (Fig. 7). Interestingly, after 21 days of mild hypoxia, the overall extent of brain vascularization in these animals appears to be similar to that of $\text{PlGF}^{+/+}$ mice, indicating that, under these conditions, the lack of PlGF can be compensated against by other alternative factors/mechanisms in the CNS.

In conclusion, PlGF knockout results in a delay in angiogenic response, vascular instability and increase permeability in the brain under mild hypoxic conditions. This

phenotype may not be exclusively due to the lack of PlGF but to additional molecular changes [reduced VEGF expression, alterations in receptor expression (Freitas-Andrade *et al* 2008)] associated to the effect of the knockout. Since the chronic hypoxic conditions simulated in this study can be largely compensated by systemic changes such as hyperventilation, increased cerebral blood flow, reduced metabolism and increased hematocrit, a full evaluation of the impact that PlGF knockout may have in the CNS warrants further analysis in acute ischemic conditions such as stroke in which the severity of the insult may lead to more prominent effects.

2.6 DISCLOSURE/CONFLICT OF INTEREST

No duality of interest to declare

2.7 ACKNOWLEDGMENTS

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2.8 SUPPLEMENTARY INFORMATION

Supplementary information is available at the Journal of Cerebral Blood Flow and Metabolism website- www.nature.com/jcbfm. See Appendix I

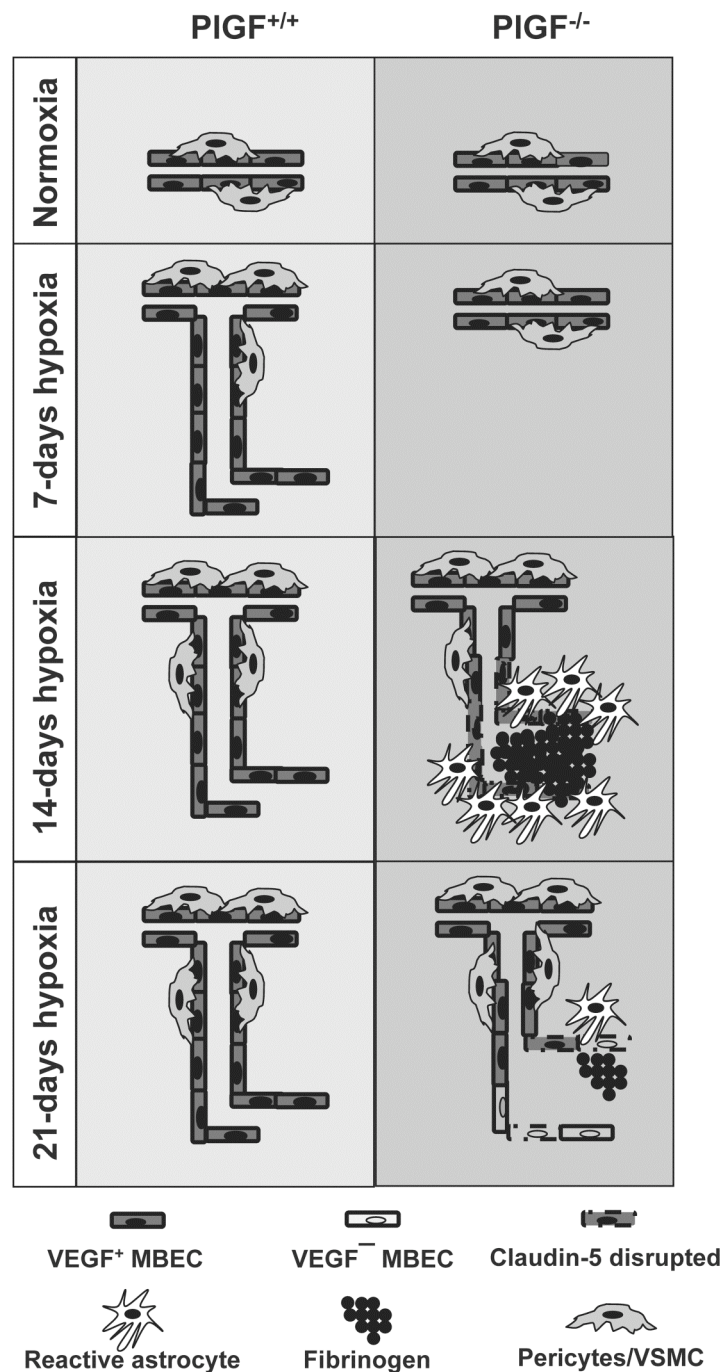


Figure 7 Schematic representation of the effect of hypoxia on brain angiogenesis in PIGF^{+/+} and PIGF^{-/-} mice. Under normoxic conditions, PIGF^{+/+} and PIGF^{-/-} mice exhibit similar brain vascularity. After 7 days hypoxia, an angiogenic response is induced in PIGF^{+/+} mice, while in PIGF^{-/-} mice this effect is delayed until 14 days of hypoxia. The delayed angiogenic response likely promotes hypoxic stress in PIGF^{-/-} mice which leads to fibrinogen accumulation and a small amount of extravasation. This affects vessel integrity as indicated by the lack of endothelial VEGF expression, pericyte coverage and disruption of the tight junction protein claudin-5 in the fibrinogen⁺ vessels which ultimately may result in vessel regression after 21-days of hypoxia.

PREFACE TO CHAPTER 3

The study presented in Chapter 2 shows the global effect of PlGF knockout on neurovascular remodeling and vascular stability during mild chronic hypoxia. Alterations in the phenotype of various components of the neurovascular unit were identified during the course of hypoxia in PlGF knockout mice. BEC exhibited an impaired capacity to initiate an early angiogenic response that over time resulted in vessel dysfunction characterized by lack of VEGF expression, reduced mural cell coverage and increased astrocyte reactivity. Studies described in Chapter 3 and 4 will dissect the individual contribution of various cellular constituents of the neurovascular unit to the overall effect of PlGF knockout on the impaired cerebrovascular remodeling process, observed *in vivo* during hypoxia. The study described in Chapter 3 will focus on the molecular mechanisms involved in the delayed angiogenic response to hypoxia of PlGF^{-/-} BEC. These studies will test the hypothesis that the impaired angiogenic response in PlGF^{-/-} BEC is due to alteration in VEGFR-1-dependent intracellular signaling associated with sprouting and branching morphogenesis. Preliminary data obtained in this study, shows that despite a significant increase in VEGF protein levels in PlGF^{-/-} BEC, these cells exhibited a delayed angiogenic response to hypoxia, underscoring the important role of PlGF in initiating the ‘angiogenic switch’ and the inability of VEGF to compensate for its absence. The delayed response was shown to be associated with an attenuation of ERK1/2 and GSK-3 β activation, two signaling transduction pathways involved in vascular sprouting and branching morphogenesis. Although data presented in Chapter 3 strongly corroborate the *in vivo* observation of a delayed angiogenic response in PlGF^{-/-} animals shown in Chapter 2, the study presented in this chapter is still incomplete and further analysis needs to be performed to confirm the relationship between PlGF-dependent activation of VEGFR-1 with ERK1/2 and GSK-3 β phosphorylation in BEC.

Pharmacological manipulation of BEC with selective VEGFR-1 antagonists will clarify this relationship, and its role in BEC sprouting and branching as final outcomes. To confirm the hypothesis sustained in this study that PlGF^{-/-} BEC reduced levels of phospho-GSK-3 β results in an attenuation of branching morphogenesis, impairment of α v β 3 integrin recruitment to focal adhesions in the cell membrane, shown to be implicated in this process, will be evaluated in PlGF^{+/+} and PlGF^{-/-} BEC by confocal microscopy. In addition, migration assay and cell death assay (i.e. TUNEL, Annexin V) will be performed to confirm the results observed in Matrigel experiments presented in this chapter.

CHAPTER 3

Impaired Sprouting and Branching in PlGF Deficient Brain Endothelial Cells Exposed to Hypoxia

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Contribution of the co-authors:

- Dr. Danica Stanimirovic and Dr. Maria Moreno provided scientific guidance and supervised project. Manuscript was written by Moises Freitas-Andrade and edited and revised by Dr. Maria Moreno and Dr. Danica Stanimirovic.
- Claudie Charlebois performed Western blots.
- Dr. Peter Carmeliet provided transgenic PlGF knockout and wild-type mice.

3.1 ABSTRACT

In ischemic conditions placental growth factor (PlGF) enhances vascular endothelial growth factor (VEGF) -driven angiogenesis in the peripheral vascular system. Here we report that PlGF knockout (PlGF^{-/-}) produces a phenotype in hypoxic brain endothelial cells (BEC) *in vitro*, characterized by impaired sprouting and branching. BEC from both genotypes proliferated at a similar rate under normoxic conditions. However, PlGF^{-/-} BEC seeded on Matrigel and subjected to hypoxia showed a delayed angiogenic response, compared to PlGF wild-type (PlGF^{+/+}) BEC. The ability of these cells to form tube-like structures, through sprouting and branching was impaired, despite the significantly higher VEGF protein levels expressed in these cells, compared to wild-type counterparts. Both PlGF and VEGF were upregulated in PlGF^{+/+} BEC under hypoxia, inducing a robust angiogenic response. Exogenous VEGF induced an angiogenic response in PlGF^{+/+} BEC, while PlGF^{-/-} BEC was unresponsive. The PlGF^{-/-} BEC phenotype was attributed to the overall reduction of ERK1/2 phosphorylation, a key intracellular modulator associated with sprouting, and an increase in GSK-3 β activity, an inhibitor of vessel branching, compared with PlGF^{+/+} BEC. Taken together, the delayed angiogenic response in hypoxic PlGF^{-/-} BEC was likely caused by the lack of PlGF activation of VEGFR-1-dependent intracellular signaling associated with sprouting and branching. This study provides new molecular insight into the role of PlGF in brain angiogenesis.

3.2 INTRODUCTION

Due to the high metabolic demand and low capacity for energy storage of the brain, neurons are highly sensitive to any disruption of blood flow. Brain angiogenesis, the formation of new capillaries from pre-existing vessels, is an important adaptive mechanism to restore impaired oxygen supply. Under hypoxic conditions (such as stroke) angiogenic factors and receptors are upregulated in hypoxic neurons, perivascular astrocytes, pericytes, and BEC in the affected tissue. The sprouting BEC degrade the surrounding basement membrane, migrate towards the hypoxic region, proliferate, and undergo capillary morphogenesis, and vessel maturation (Wang and Milner 2006). The end result is to restore blood flow, and increase oxygen delivery to the hypoxic tissue (Carmeliet 2003). Angiogenesis is a complex process involving tightly regulated interactions of several secreted pro- and anti-angiogenic proteins and receptors-many of these interactions are still unknown.

Several lines of evidence have demonstrated the critical function of vascular endothelial growth factor (VEGF), a multifunctional secreted dimeric glycoprotein (Roy *et al* 2006), in both angiogenesis and neuroprotection in the hypoxic brain. However, little is known about roles of other VEGF homologs such as placental growth factor (PlGF). Mice lacking even a single *Vegf* allele die at E11-E12 (Ferrara *et al* 1996), whereas lack of PlGF does not affect normal development in mice (Carmeliet *et al* 2001). Interestingly, PlGF deficient mice exhibit impaired angiogenesis, plasma extravasation and decreased collateral growth during ischemia, inflammation, wound healing and cancer in the peripheral vascular system (Carmeliet *et al* 2001). Recombinant PlGF homodimers potentiate the mitogenic and permeability effects of low, marginally efficacious concentrations of VEGF (Park *et al* 1994) indicating its potential role in enhancing VEGF-mediated angiogenesis. VEGF binds to two

tyrosine kinase receptors: VEGF receptor-1 (VEGFR-1) and VEGF receptor-2 (VEGFR-2). VEGF induces angiogenesis by activating primarily VEGFR-2, promoting vessel permeability (Quinn *et al* 1993), endothelial cell proliferation (Takahashi *et al* 1999), survival (Kilic *et al* 2006), and cell migration (Kanno *et al* 2000). In neurons, VEGFR-2 is associated with survival (Kilic *et al* 2006; Raab *et al* 2004), whereas in astrocytes it triggers both proliferation and survival (Freitas-Andrade *et al* 2008; Schmid-Brunclik *et al* 2008). PlGF only binds to VEGFR-1, and activation of this receptor is associated with induction of transcription factors FosB and c-Fos (Holmes and Zachary 2004), cell motility (Taylor *et al* 2010), vessel permeability (Vogel *et al* 2007), endothelial vascular morphogenesis (Funahashi *et al* 2010; Jones *et al* 2009; Mavria *et al* 2006) and cell survival (Cai *et al* 2003; Mavria *et al* 2006).

Under pathological angiogenesis, VEGFR-1 activation by PlGF enhances VEGFR-2 signaling in the systemic vascular system (Autiero *et al* 2003b; Carmeliet *et al* 2001; Luttun *et al* 2002a). PlGF has been shown to potentiate VEGF-driven angiogenesis by several mechanisms: (1) displacing VEGF from VEGFR-1, thus increasing the availability of VEGF to activate VEGFR-2; (2) activating VEGFR-1 which transphosphorylates VEGFR-2, thereby increasing VEGFR-2 phosphotyrosine levels; (3) inducing its own distinct VEGFR-1-dependent intracellular signaling; and (4) forming heterodimers with VEGF, which can activate intracellular signaling through VEGFR-1/VEGFR-2 heterodimer receptor complex (Autiero *et al* 2003b).

The role of PlGF in the brain under hypoxic conditions, in particular its effects on BEC is not fully elucidated. Simultaneous VEGF and PlGF upregulation in different cell types occurs after focal cerebral ischemia (Beck *et al* 2002). PlGF expression is detected in microvessels and interstitium of rat brain cortex after cerebral ischemic injury (Du *et al*

2010). *In vitro* experiments demonstrated that oxygen and glucose deprivation (OGD) induced PlGF protein expression in BEC, and that exogenous PlGF protected neurons subjected to OGD conditions (Du *et al* 2010). Collectively, these findings suggest that PlGF may be implicated in the repair and survival processes, specifically angiogenesis, in the brain under hypoxic conditions.

The goal of this study was to investigate, at cellular and molecular levels, the effects of PlGF knockout on the angiogenic response of BEC to *in vitro* hypoxia. Although in this study no morphological changes and proliferative differences were observed between PlGF wild-type (PlGF^{+/+}) and knockout (PlGF^{-/-}) BEC, the angiogenic response of PlGF^{-/-} BEC to hypoxia was delayed. This was associated with alterations in intracellular ERK1/2 and GSK-3 β signaling pathways involved in sprouting, and branching respectively in PlGF^{-/-} BEC.

3.3 MATERIALS AND METHODS

Cell cultures

Primary cultures of BEC from wild-type PlGF^{+/+} and PlGF^{-/-} knockout mice (generated by Carmeliet *et al.* 2001, Vesalius Research Center, Belgium) were isolated from 19-day-old mice, bred at the National Research Council of Canada (NRCC) Institute for Biological Sciences Animal Facility (Ottawa, ON, Canada). All procedures used in these studies were approved by the NRCC Animal Care Committee, in accordance with the Canadian Council on Animal Care (CCAC) guidelines. Mice were deeply anesthetized with 4% halothane B. P. (MTC Pharmaceuticals, Cambridge, ON, Canada) under the oxygen flow rate of 2 L/min and then killed by guillotine. Brains were removed and placed into ice-cold Dulbecco's modified Eagle's medium (DMEM) with antibiotic-antimycotic solution (Sigma-

Aldrich, Oakville, ON, Canada). The cerebellum, and the olfactory bulbs were removed and brain cortices (five brains per isolation) were homogenized with a Downs homogenizer and passed through 112 μm and 20 μm Nitex filter (Sefar Canada Inc., Scarborough, ON, Canada) each filtration step was followed by a rinse with 50 ml cold Hank's buffered salt solution (HBSS) (Sigma-Aldrich, Oakville, ON, Canada). The capillary fragments remaining on the 20 μm filter were removed by inserting the filter in 50 ml conical tube with cold HBSS and shaking the tubes gently. The vessel suspension was centrifuged at 4°C for 15 minutes at $1000 \times g$. The HBSS was carefully aspirated and the pellet was resuspended in 10 ml of 1 mg/ml Collagenase IV in DMEM at 37°C for 6 minutes, shaking periodically. The suspension was then centrifuged at 4°C for 10 min at $500 \times g$, the collagenase was aspirated and the fragments of microvessels were collected, and re-suspended in complete culture medium (DMEM, 10% heat-inactivated fetal bovine serum (Hyclone, Logan, UT, USA), 10% heat-inactivated human serum (Wisent Inc. St-Bruno, QC, Canada), 20% mouse melanoma conditioned media (MCM), 1X ITS™ premix insulin/transferrin/selenium and 25 $\mu\text{g}/\text{ml}$ endothelial cell growth supplement (BD Bioscience, Bedford, MA, USA), 12.5 U/ml heparin sodium (Sigma-Aldrich, Oakville, ON, Canada), 100 U/ml penicillin and 100 $\mu\text{g}/\text{ml}$ streptomycin (Sigma-Aldrich, Oakville, ON, Canada), pH 7.2-7.4). The suspension was then seeded in gelatin-coated 35mm Falcon® tissue culture dishes (Becton Dickinson labware, Franklin Lakes, NJ, USA). Cells were kept in culture in an atmosphere of 5% CO₂/95% air at 37°C, and at 80% confluence the cultures with ~ 95% BEC purity, determined by immunochemical staining for BEC-specific markers, were used at the third and fourth passage.

Immunofluorescence

PIGF^{+/+} and PIGF^{-/-} BEC were allowed to grow to confluence in 24-well plates. BEC were rinsed 2X with HBSS and fixed with 100% ice-cold methanol for 10 min at room temperature (RT). Cells were rinsed 4X with HBSS, and permeabilized with 0.1% Triton X-100 (EM Science Gibbstown, NJ, USA) in HBSS at RT for 10 min. Cells were rinsed and blocked with 4% serum in HBSS for 1 hr at RT and then incubated with polyclonal rabbit anti-human Von Willebrand Factor (Factor VIII related antigen) primary antibody (DAKO Glostrup, Denmark A/S) at 1:50 dilution for 1 hr at RT. Cells were then rinsed three times with HBSS and exposed to Alexafluor 568 goat anti-rabbit IgG secondary antibody (Invitrogen Burlington, ON, Canada) at 1:500 dilution for 30 min at RT. Cells were rinsed three times with HBSS followed by one rinse with H₂O spiked with DAPI (Sigma-Aldrich Oakville, ON, Canada), and visualized using Olympus IX-50 microscope. Procedure was repeated using polyclonal rabbit anti-GFAP primary antibody (DAKO Glostrup, Denmark A/S), 1:500 dilution, and fluorescein goat anti-rabbit IgG secondary antibody (Invitrogen Burlington, ON, Canada) at 1:500 dilution to detect astrocytes. Rabbit polyclonal ZO-1 primary antibody (Santa Cruz, Santa Cruz, CA, U.S.A) at a concentration of 1:100 and AlexaFluor 488 secondary antibody at a concentration of 1:500 to detect BBB tight junction protein. Mouse monoclonal anti- α -smooth muscle actin primary antibody (Sigma-Aldrich, Oakville, ON, Canada) at a concentration of 1:100 and Alexafluor 568 secondary antibody at a concentration of 1:500 were used to detect pericytes and smooth muscle cells. Omission of primary antibodies resulted in no staining.

Proliferation assay

Proliferation experiments were performed using CyQuant® cell proliferation assay kit (Molecular Probes, Eugene, OR, USA) following the protocol provided by the manufacturer, as previously described (Freitas-Andrade *et al* 2008). PIGF^{+/+} and PIGF^{-/-} BEC were plated in triplicate in 96-well plates at a density of 5000 cells/well. Cells were grown in DMEM + 1% FBS, cells were harvested at 24, 48, and 72 hr by gentle washing with HBSS, blotting microplates dry and storing at -80°C until analysis. Plates were thawed at 21°C and 200 µl of CyQuant GR dye/lysis buffer was added to each well for 5 min in the dark. Fluorescence values were measured using a cytofluorimeter plate reader (Bio-Tek FL600, Winooski, VT, USA) at 485 nm excitation/530 nm emission, and were directly proportional to cell numbers.

Matrigel in vitro angiogenesis assay

In vitro angiogenesis was assessed as previously described (Moreno *et al* 2006). Briefly, twenty four-well plates were coated with 250 µl of unpolymerized Matrigel and allowed to polymerize for 90 min at 37°C. PIGF^{+/+} and PIGF^{-/-} BEC (15000 cells) were suspended in DMEM and plated into Matrigel coated wells. Plates were then placed in a hypoxia chamber for 2, 3, and 4 hr, and at each time-point, plates were removed, and placed in a cell culture incubator for 14 hr. After each incubation phase contrast images of capillary-like networks (CLN) formation, by BEC were captured using an Olympus 1X50 microscope equipped with digital camera.

In vitro hypoxia experiment

Cells were grown to confluence in 35mm culture dishes and exposed to either normoxia (control), or 6 hr hypoxia (1% oxygen) or 6 hr hypoxia followed by 14 hr reoxygenation as previously described (Freitas-Andrade *et al* 2008). Briefly, for hypoxia experiments, glucose-containing DMEM was placed in the anaerobic chamber (Forma 1025 Anaerobic Chamber; ThermoForma, Marietta, OH, USA) and allowed to equilibrate for 2 hr prior to the start of the experiments. Oxygen levels were measured with MI-730 Micro-Oxygen Electrode (Microelectrodes, INC. Bedford, USA) and maintained at 1% O₂ inside the chamber. Cells were washed three times to eliminate FBS. The hypoxic PIGF^{+/+} and PIGF^{-/-} BEC groups were placed in the anaerobic chamber, and equilibrated DMEM was added to the cells in the chamber. PIGF^{+/+} and PIGF^{-/-} BEC were exposed to either 6 hr of hypoxia or 6 hr of hypoxia and returned to the cell culture incubator for an additional 14 hr reoxygenation. Normoxic controls were placed in the cell culture incubator in DMEM for the duration of the experiment 20 hr. After each treatment, conditioned media (CM) was collected, centrifuged at 1000 X g, supernatant was aliquoted, and stored in -20°C to be used for VEGF and PIGF protein expression by ELISA (R&D Systems Minneapolis, MN, USA) according to the manufacturers protocol.

Western blot

After each treatment, cells were washed with ice-cold HBSS and exposed to 250 µl RIPA lysis buffer (1%NP40 (IGEPAL), 0.5% Deoxycholate, 0.1% SDS and protease inhibitor 100µl/10ml RIPA in PBS), (Sigma-Aldrich Oakville, ON, Canada) for 20 min at 4°C. The cell lysates were centrifuged at 14,000 rpm for 10 min at 4°C, and the supernatants were collected. Protein concentration was measured in each of the cell lysates by BCA

Protein Assay Reagent Kit (Pierce Biotechnology, Rockford, IL, USA), and 20-50 µg of protein was denatured in protein loading buffer for 5 min at 100°C, and then separated on a 10% sodium dodecyl sulfate-polyacrylamide gel. Separated proteins were then transferred to polyvinylidene difluoride (PVDF) membranes. Blocking was performed by incubating membranes in TBST (20 mM Tris-buffer [pH 8.0] and 150 mM NaCl with 0.1% Tween-20) containing 5% BSA (Sigma-Aldrich Oakville, ON, Canada) for 1 hr at room temperature. Membranes were then incubated overnight at 4°C with primary antibodies against either p44/42 MAP Kinase (also known as ERK1/2) or phospho-p44/42 MAP Kinase (Cell Signaling Technology, MA, USA) diluted 1/1000 in TBST containing 1% BSA or glycogen synthase kinase-3β (GSK-3β) or phospho-GSK-3β (Cell Signaling Technology, MA, USA) diluted 1/1000 in TBST containing 5% BSA. Receptor expression was performed using the following antibodies: Neuropilin-1 (EMD Chemicals Inc., Darmstadt, Germany), VEGFR-1 and VEGFR-2 (Santa Cruz Biotechnology Inc., California, USA), 1/100 dilution in TBST containing 5% skimmed milk. The membranes were extensively washed with TBST and incubated for 1 hr with Anti-Rabbit IgG (whole molecule)–Peroxidase antibody (Sigma-Aldrich Oakville, ON, Canada) diluted 1/5000 in TBST containing 5% skimmed milk. Immunoreactive proteins were visualized by Amersham ECL Plus Western Blotting Detection Reagents (GE Healthcare Buckinghamshire, UK). Densitometry measurements of neuropilin-1, VEGFR-1, and VEGFR-2 western blots were performed using UN-SCAN-IT gel V6.1 (Silk Scientific, Inc. Orem, Utah, USA).

Statistical analysis

Unpaired two-tailed t test was used to compare normoxia and hypoxia $PIGF^{+/+}$ groups. Two-way ANOVA was used to compare between genotype and treatment, followed by Bonferroni post-test, using the statistical package GraphPad Prism® 5. P-values of < 0.05 were considered significant. All data are presented as mean $\pm$ s.e.m.

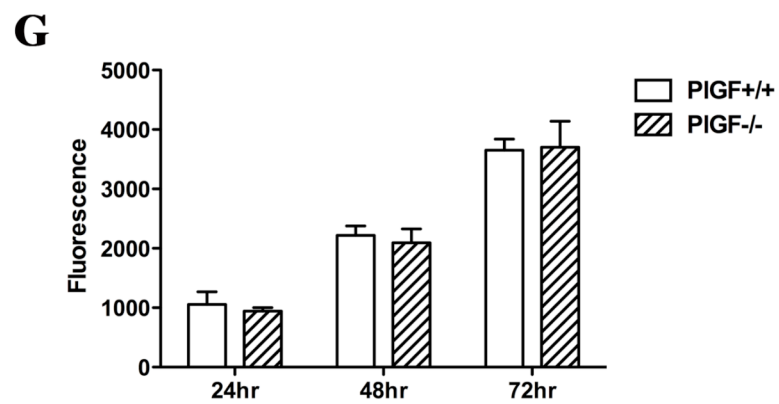
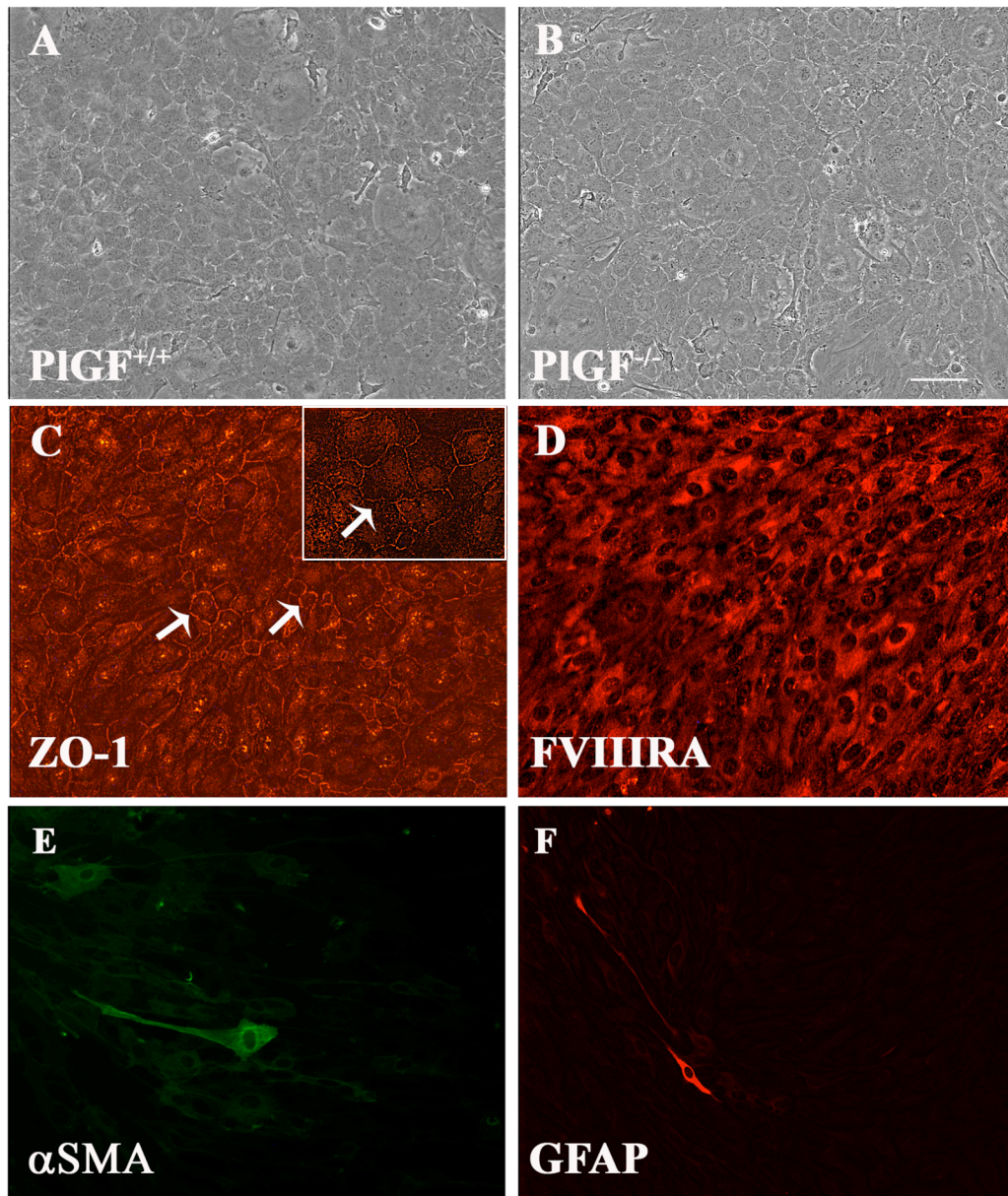
3.4 RESULTS

Characterization of isolated $PIGF^{+/+}$ and $PIGF^{-/-}$ BEC

Isolated $PIGF^{+/+}$ and $PIGF^{-/-}$ BEC cultures showed typical cobblestone morphology and expressed endothelial cell marker factor VIII related antigen, and phenotypical BEC tight junction marker ZO-1 (Figure 1A-D). $PIGF^{+/+}$ and $PIGF^{-/-}$ BEC cultures used in these experiments exhibited low levels (~ 5%) of contaminating cells such as astrocytes, pericytes and smooth muscle cells (Figure 1E and F).

No significant differences in proliferation were observed between $PIGF^{+/+}$ and $PIGF^{-/-}$ BEC grown under normoxic conditions (Figure 1G), in contrast to the previously reported increased proliferative capacity of $PIGF^{-/-}$ astrocytes under similar conditions (Freitas-Andrade *et al* 2008).

Figure 1 (A) Representative 20X micrograph showing PlGF^{+/+} mouse BEC and (B) PlGF^{-/-} mouse BEC in culture. Immunofluorescence staining of (C) endothelial cell marker factor VIII related antigen (Factor VIII RA), (D) BBB tight junction marker (ZO-1), cropped magnified insert (top right corner) shows ZO-1 staining detail, (E) astrocyte (GFAP) and (F) smooth muscle cell marker (α -SMA). (G) Quantification of PlGF^{+/+} (white bar) and PlGF^{-/-} (hatched bar) mouse BEC proliferation was determined with a CyQuant Proliferation Assay Kit. Each bar represents the average $\pm$ SEM of three experiments done in duplicates. Two-way ANOVA comparison was made between genotype and time. Scale bar = 50 μ m.



Angiogenic responses of $PlGF^{+/+}$ and $PlGF^{-/-}$ BEC to hypoxia

To determine whether PlGF knockout affects the ability of BEC to respond to hypoxia by angiogenic transformation, $PlGF^{+/+}$ and $PlGF^{-/-}$ BEC were seeded on Matrigel and exposed to 2, 3, or 4 hr hypoxia followed by 14 hr reoxygenation. The formation of CLN by BEC on Matrigel depends on the ability of these cells to migrate, sprout, and form complex tube-like structures through branching and cell-cell contact. After 2 hr hypoxia $PlGF^{-/-}$ BEC failed to form CLN, compared to $PlGF^{+/+}$ BEC (Figure 2). After 3 hr of hypoxia extensive CLN formation was observed in both $PlGF^{+/+}$ and $PlGF^{-/-}$ BEC (Figure 2). However, a large number of $PlGF^{-/-}$ BEC exhibited signs of apoptosis (high cytoplasmic vacuolization) and reduced migration capacity as suggested by the presence of larger areas of densely packed cells in the nodes of the $PlGF^{-/-}$ - derived CLN networks (Figure 2). After 4 hr exposure to hypoxia, the $PlGF^{-/-}$ BEC CLN have almost completely regressed compared to the wild-type BEC (Figure 2). Due to the presence of larger areas of densely packed cells in the nodes of the $PlGF^{-/-}$ - derived CLN networks, quantification of these experiments using image analysis software was difficult to perform. These experiments were performed 3 times with similar results.

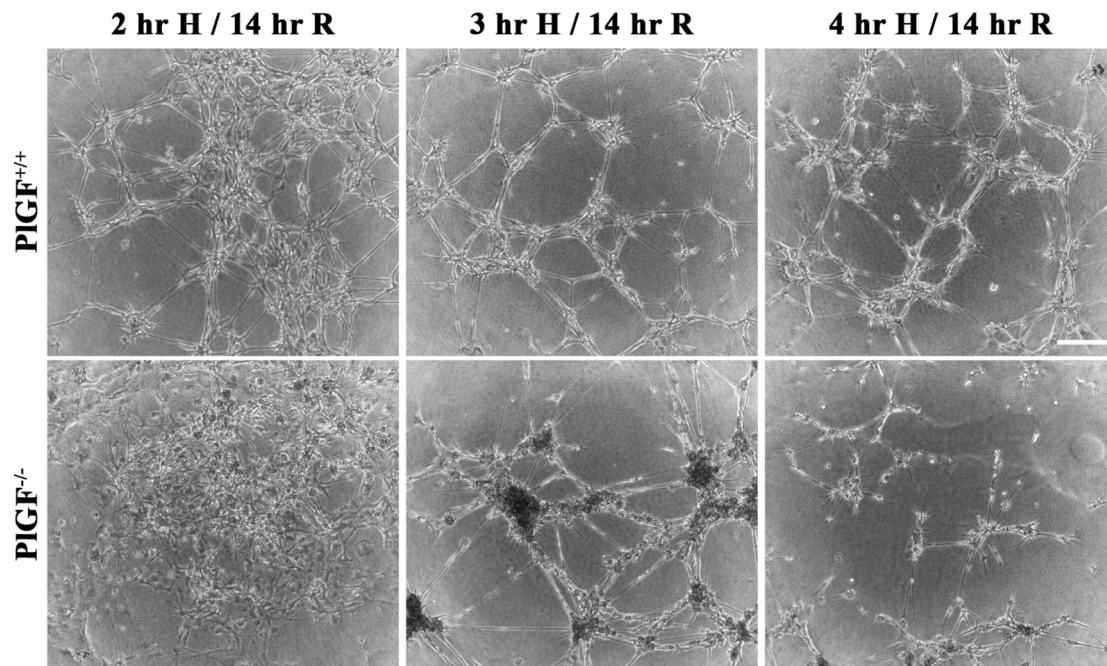


Figure 2. Capillary-like networks formation (CLN) induced by 2 hr, 3 hr, and 4 hr hypoxia (H) followed by 14 hr reoxygenation (R) on PIGF^{+/+} and PIGF^{-/-} mouse BEC seeded on Matrigel. Scale bar = 50 μ m. Representative experiment shown, of three independent experiments (see Appendix III, Figure A3, page 162, for experiments 2 and 3).

Hypoxia induced PlGF and VEGF secreted protein expression in PlGF^{+/+} and PlGF^{-/-} BEC

Exposure of PlGF^{+/+} BEC to 6 hr hypoxia followed by 14 hr reoxygenation induced an increase in secreted PlGF protein expression (2-fold) compared to normoxic controls (Figure 3A). As expected, no PlGF protein expression was detected in the conditioned media of either normoxic or hypoxic PlGF^{-/-} BEC. Hypoxia induced an increase in secreted VEGF in both PlGF^{+/+} and PlGF^{-/-} BEC; however, VEGF expression was 2-fold higher in hypoxic PlGF^{-/-} BEC compared to that in the wild-type counterparts (Figure 3B).

As previously described, PlGF^{-/-} BEC exhibited impaired angiogenesis in Matrigel (Figure 2), despite the observed increased expression of VEGF protein in hypoxic PlGF^{-/-} BEC. The unresponsive phenotype of PlGF^{-/-} BEC to VEGF was further confirmed by the inability of these cells to form CLN when exposed to 40 ng/ml of exogenous VEGF, compared to the robust capillary tube formation induced by VEGF in PlGF^{+/+} BEC (Figure 3C).

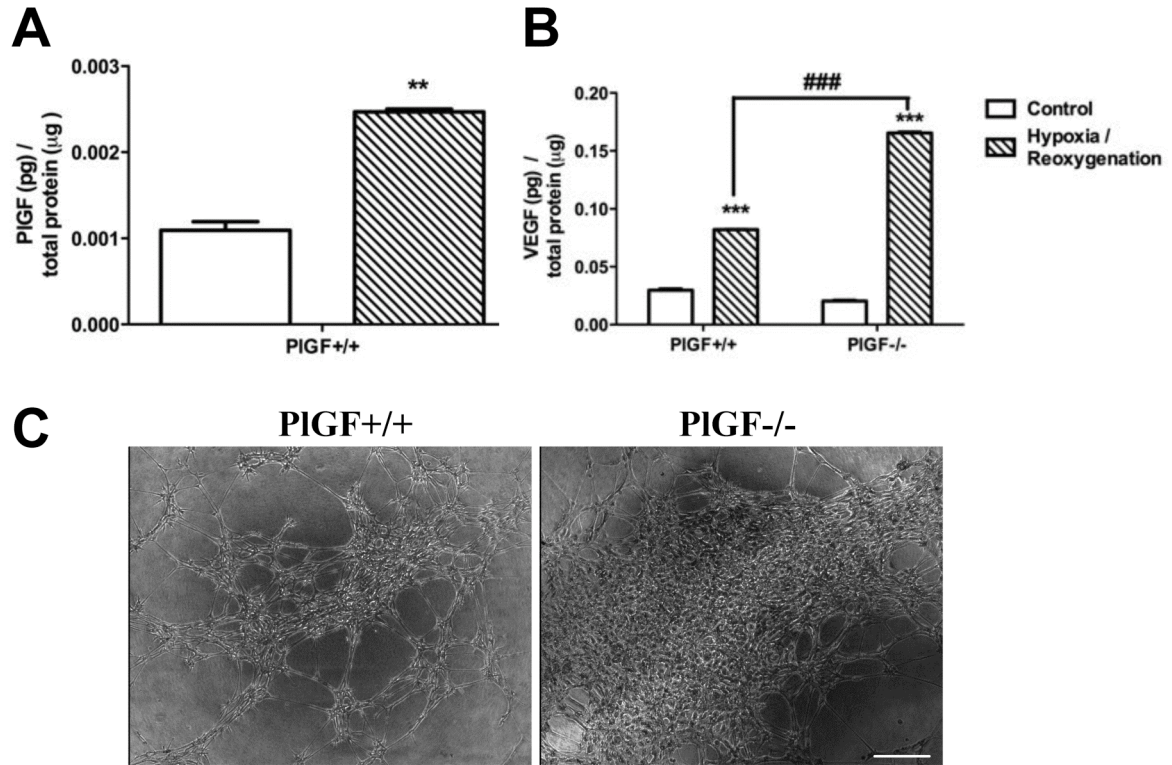


Figure 3. (A) PlGF protein expression, measured by ELISA, in conditioned media of PIGF^{+/+} and PIGF^{-/-} mouse BEC exposed to normoxia (white bar) or 6 hr hypoxia followed by 14 hr of reoxygenation (hatched bar). ** indicates significance (p<0.01,) between normoxia and hypoxia, after unpaired, two tailed t test (n=3). (B) VEGF protein expression, measured by ELISA, in conditioned media of PIGF^{+/+} and PIGF^{-/-} mouse BEC exposed to 20 hr normoxia or 6 hr hypoxia followed by 14 hr of reoxygenation. *** indicates significance (p<0.001, two-way ANOVA followed by Bonferroni post-test) between normoxia and hypoxia, and ### indicates significance (p<0.001, two-way ANOVA followed by Bonferroni post-test) between PIGF^{+/+} and PIGF^{-/-} during hypoxia (n=3). (C) Capillary-like networks formation (CLN) induced by 20 hr incubation of 40 ng/ml VEGF on PIGF^{+/+} and PIGF^{-/-} mouse BEC seeded on Matrigel. Representative experiment shown, of two independent experiments Scale bar = 50 μm.

Hypoxia induced NP-1, VEGFR-1, and VEGFR-2 receptor expression in $PIGF^{+/+}$ and $PIGF^{-/-}$ BEC

We previously reported that PIGF-knockout leads to changes in VEGFR-2 expression in astrocytes resulting in a specific astrocytic phenotype (Freitas-Andrade *et al* 2008). We therefore evaluated the expression levels of VEGFR-1, VEGFR-2, and the VEGFR-2 co-receptor neuropilin-1 in $PIGF^{+/+}$ and $PIGF^{-/-}$ BEC submitted to 6 h hypoxia alone or followed by 14 h reoxygenation (Figure 4A), to determine whether changes in receptor expression are implicated in the impaired angiogenic phenotype observed in PIGF knockout BEC. The results showed high inter-experimental variability in both genotypes, (Figure 4B). Suggesting that the unresponsiveness of $PIGF^{-/-}$ BEC might not be due to alterations in receptor expression.

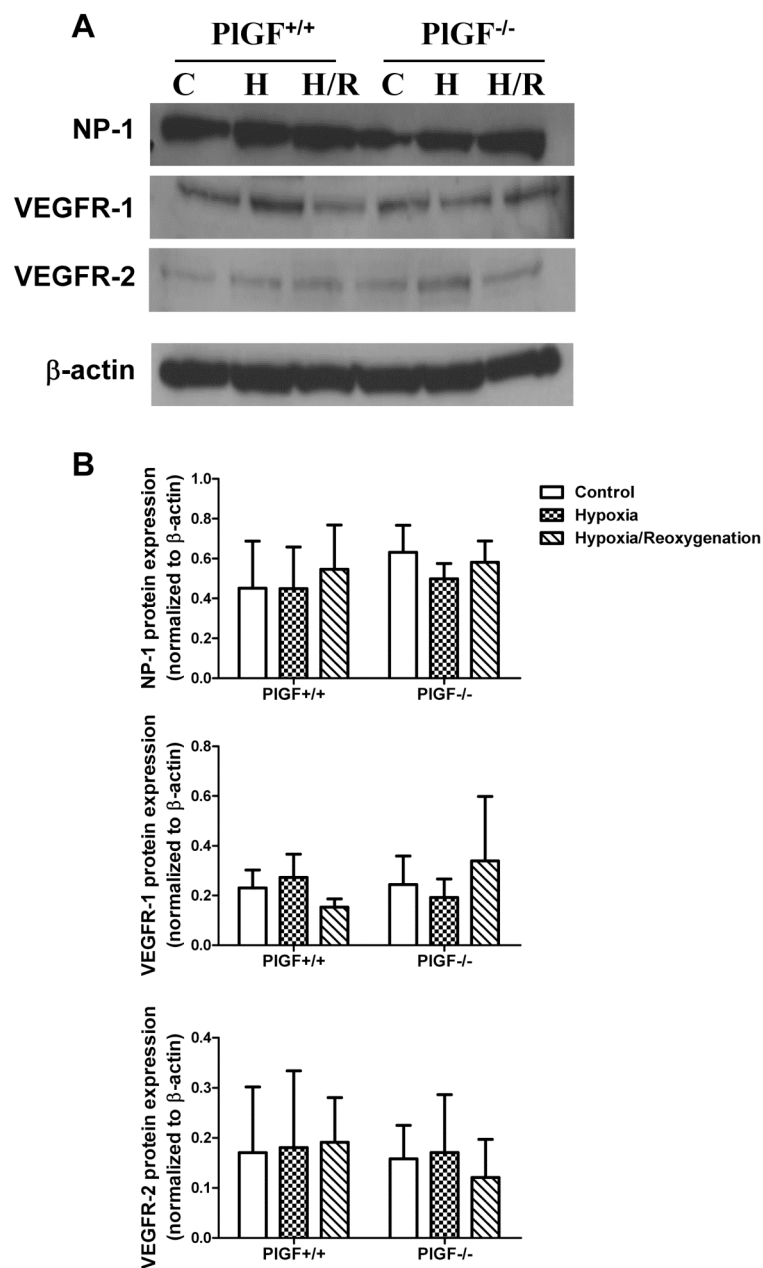


Figure 4. (A) Representative image of enhanced chemiluminescence signals from Western blots ($n = 3$) of BEC subjected to either 20 hr normoxia (C), 6 hr hypoxia (H), or 6 hr hypoxia followed by 14 hr reoxygenation (H/R). (B) Average densitometric scans of Western blots of NP-1, VEGFR-1, and VEGFR-2 normalized to β -actin.

Investigation of intracellular signaling pathways involved in sprouting and branching in $PIGF^{+/+}$ and $PIGF^{-/-}$ BEC exposed to either normoxic or hypoxic conditions

To investigate the potential cause of the delayed angiogenic response of $PIGF^{-/-}$ BEC to hypoxia, intracellular signaling pathways associated with endothelial cell sprouting, and branching morphogenesis were investigated in $PIGF^{+/+}$ and $PIGF^{-/-}$ BEC exposed to either 20 hr normoxia, 6 hr hypoxia or 6 hr hypoxia followed by 14 hr reoxygenation.

ERK1/2 signaling has been shown to be essential for capillary sprouting in human umbilical vein endothelial cells (HUVEC) (Mavria *et al* 2006; Zhou *et al* 2007). In this study, under normoxic conditions, phosphorylation levels of ERK1/2 were consistently lower in $PIGF^{-/-}$ BEC compared to $PIGF^{+/+}$ BEC controls in the two experiments performed (Figure 5A). ERK1/2 phosphorylation levels after 6 h of hypoxia showed high inter-experimental variability in both $PIGF^{+/+}$ and $PIGF^{-/-}$ BEC and, due to the small sample size (n=2), it was not possible to conclude whether hypoxia had effect on ERK1/2 phosphorylation within and between genotypes (Figure 5A). After 14 hr of reoxygenation, phospho-ERK1/2 levels in both $PIGF^{+/+}$ and $PIGF^{-/-}$ BEC were similar to those observed in the corresponding normoxic controls (Figure 5A). Total ERK1/2 protein expression was not affected by genotype or treatment (Figure 5A). Overall, these results indicate that the levels of phospho-ERK1/2 were in general lower in $PIGF^{-/-}$ than in $PIGF^{+/+}$ BEC, although more experiments are required to determine if these differences were statistically significant.

GSK-3 β activity has been recently shown to inhibit intracellular signaling involved in the formation of complex vascular networks (Jones *et al* 2009). $PIGF^{+/+}$ BEC exhibit higher levels of phospho-GSK-3 β compared to $PIGF^{-/-}$ BEC under normoxia, and either hypoxia alone or followed by reoxygenation. (Figure 5B). Total GSK-3 β protein expression was not affected by genotype or treatment (Figure 5B). However, more experiments are required

to determine if the differences in GSK-3 β phosphorylation levels observed between the two genotypes are statistically significant.

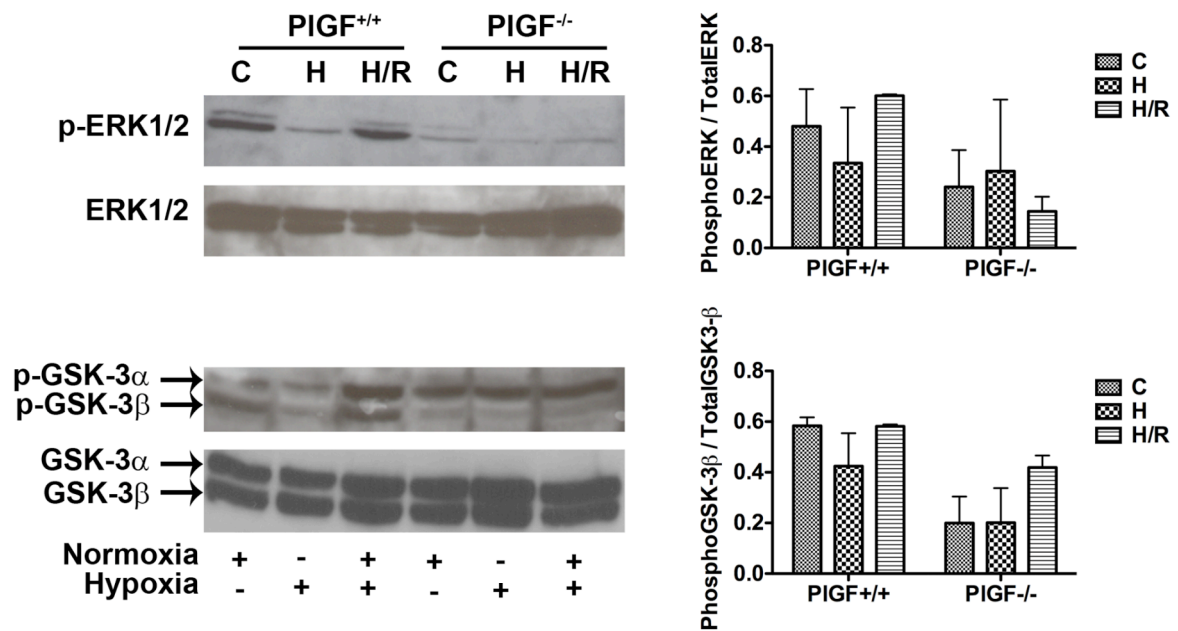


Figure 5. phospho-ERK1/2 (p-ERK1/2), total ERK1/2 (ERK1/2), phospho-GSK-3β (p-GSK-3β), and total GSK-3β (GSK-3β) protein levels analyzed by western blot in PIGF^{+/+} and PIGF^{-/-} mouse BEC subjected to either 20 hr normoxia (C), 6 hr hypoxia (H), or 6 hr hypoxia followed by 14 hr reoxygenation (H/R). Graphs show corresponding phospho-ERK1/2 and phospho-GSK-3β densitometric measurements of Western-blot gels from two independent experiments.

3.5 DISCUSSION

This study demonstrates that lack of PlGF affects the ability of BEC to sprout and form complex branch-like networks in hypoxic conditions, despite the fact that PlGF^{-/-} BEC secrete higher levels of VEGF protein compared to PlGF^{+/+} BEC. The data indicates that PlGF deficiency affects the interplay between PlGF and VEGF in hypoxic BEC. The delayed angiogenic response observed in hypoxic PlGF^{-/-} BEC was accompanied by the reduced phosphorylation of both ERK1/2 and GSK-3 β , two intracellular modulators that play an important role in sprouting and branching morphogenesis, respectively (Jones *et al* 2009; Mavria *et al* 2006; Zhou *et al* 2007).

The ability of endothelial cells to respond to hypoxia through angiogenesis is an important process in tissue repair and cell survival under pathological conditions, particularly in the CNS. This response involves the interaction of several factors and receptors that contribute to the angiogenic process (Carmeliet 2003; Ferrara *et al* 2003; Gerritsen *et al* 2003). The interaction between PlGF and VEGF contributing to angiogenic response under ischemic conditions has been previously reported (Autiero *et al* 2003b; Carmeliet *et al* 2001). PlGF acts synergistically with VEGF by promoting an inter- and intramolecular cross talk between VEGFR-1 and VEGFR-2 (Autiero *et al.*, 2003). VEGF/PlGF heterodimers or a combination of VEGF and PlGF homodimers have been shown to stimulate ischemic angiogenesis more potently than VEGF or PlGF alone (Autiero *et al* 2003b). The synergistic interaction between PlGF and VEGF is also supported by this study. PlGF^{-/-} BEC exhibited a reduced angiogenic response to hypoxia compared to the wild-type counterparts despite the fact that hypoxic PlGF^{-/-} BEC secreted higher levels of VEGF than PlGF^{+/+} BEC. This indicates that overexpression of VEGF was insufficient to compensate for the lack of PlGF in inducing an angiogenic response.

The reduced angiogenic response to hypoxia by PIGF^{-/-} BEC seeded on Matrigel was accompanied by alterations in cell morphology, including cell rounding, cytoplasmic vacuolization, and detachment from the extracellular matrix due to cell death. Consistent with this data, Cai and others showed that PIGF promotes and sustains *in vitro* CLN formation, and survival in bovine retinal endothelial cell (BREC) (Cai *et al* 2003). This effect is mediated by PIGF activation of VEGFR-1 (Cai *et al* 2003). PIGF has also been shown to induce ERK1/2 phosphorylation through VEGFR-1 in HUVEC (Arroyo *et al* 2004). ERK/MAPK signaling has been implicated in VEGF-mediated endothelial, cell survival (Gupta *et al* 1999) endothelial cell proliferation (Meadows *et al* 2001), and endothelial cell sprouting (Mavria *et al* 2006; Zhou *et al* 2007). In this study, despite the inter-experimental variability in ERK1/2 phosphorylation levels observed in normoxic and hypoxic experimental conditions, reduced ERK1/2 phosphorylation levels were reproducibly observed in PIGF^{-/-} BEC compared to PIGF^{+/+} BEC in both normoxia and hypoxia/reoxygenation in two independent experiments. This may suggest that both the morphological changes and the impaired ability of PIGF^{-/-} BEC to form CLN under hypoxia and reoxygenation may be in part effected through the reduced levels of phospho-ERK1/2 in these cells. However, due to the small sample size further experiments will be required to confirm this preliminary observation.

Interaction between ERK1/2 and GSK-3 β has been shown to mediate basic fibroblast growth factor-induced apoptosis in SK-N-MC neuroblastoma cells (Ma *et al* 2008). GSK-3 β was originally identified as a serine threonine kinase that phosphorylates glycogen synthase. However, several studies have demonstrated that GSK-3 β acts on a broad range of substrates and affects a diverse array of physiological pathways (Rayasam *et al* 2009). GSK-3 β has been shown to inhibit branching morphogenesis in HUVEC by inhibiting the Rab4a-regulated

route that carries $\alpha v\beta 3$ integrins from early endosomes to the plasma membrane (Jones *et al* 2009). VEGFR-1 activation, by either PlGF or VEGF, promotes branching morphogenesis by phosphorylating GSK-3 β , an event that leads to its inactivation (Jones *et al* 2009). The impaired capacity of PlGF^{-/-} BEC to form CLN under hypoxia or VEGF-stimulating conditions may correlate with the reduced levels of phosphorylated GSK-3 β in these cells compared to those in PlGF^{+/+} BEC. This functional impairment contrasts with the overexpression of VEGF in hypoxic BEC and indicates important synergistic effects of PlGF and VEGF in promoting branching morphogenesis.

PlGF^{-/-} BEC also exhibited substantially higher levels of phospho-GSK-3 α compared to PlGF^{+/+} BEC under normoxic and hypoxic conditions. GSK-3 α is a GSK-3 isoform that, despite exhibiting high homology in the catalytic domain with GSK-3 β , differs in the N- and C-terminal sequences and plays distinct roles in several biological processes (Rayasam *et al* 2009). While GSK-3 β has been associated with modulation of vascular branching (Jones *et al* 2009), the involvement of GSK-3 α in the process of angiogenesis is still unknown, and warrants further investigation.

Several studies show that VEGFR-1 plays an important role in endothelial cell sprouting and branching morphogenesis (Fong *et al* 1995; Kappas *et al* 2008; Kearney *et al* 2004; Odorisio *et al* 2002). It has been shown that VEGFR-1 homozygous knockout results in embryonic lethality. These mice fail to form an organized vascular network in the yolk sac, and exhibit vascular structures with abnormally large lumen (Fong *et al* 1995). Although VEGF can bind and activate VEGFR-1, the intracellular signaling induced by PlGF is distinct from that induced by VEGF (Autiero *et al* 2003b; Cai *et al* 2003). Our study

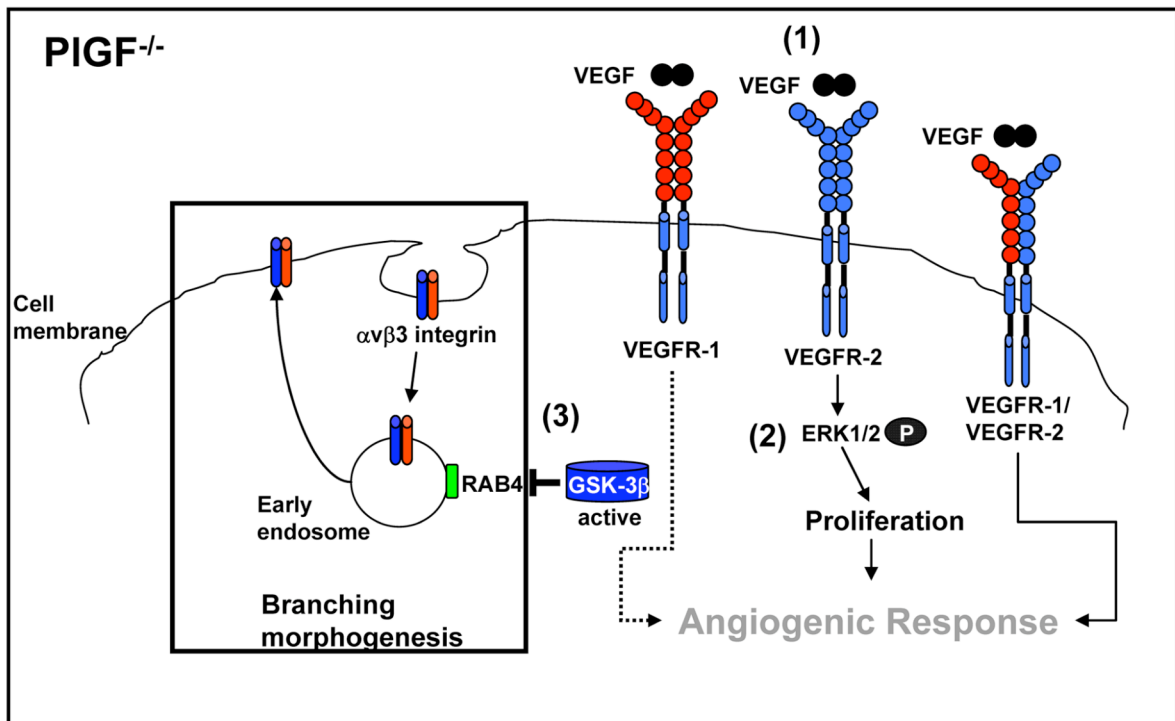
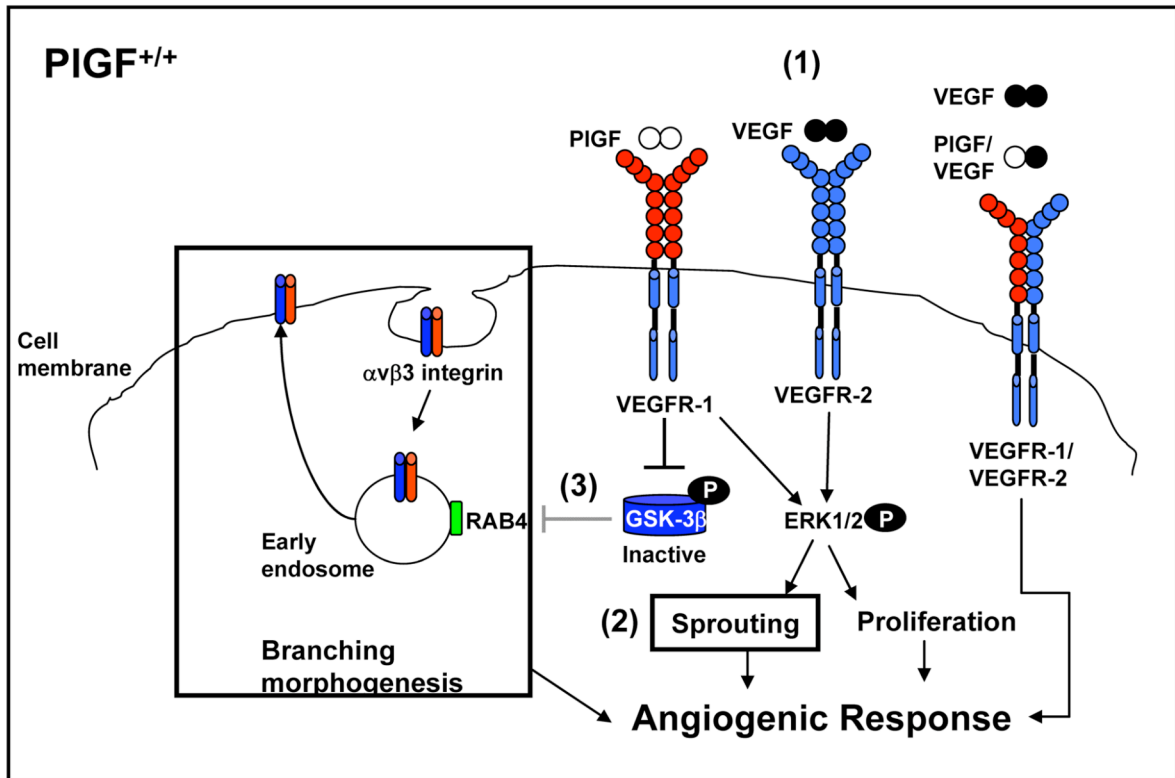
indicates that PlGF deficiency reduces BEC sprouting and branching, resulting in a delayed angiogenic response in PlGF knockout BEC.

We hypothesize that while hypoxic PlGF^{+/+} BEC express both VEGF and PlGF homodimers and VEGF/PlGF heterodimers, PlGF^{-/-} BEC only express VEGF homodimers. The balance of three dimeric forms has been shown to contribute to the robust angiogenic response of endothelial cells in hypoxic conditions (Autiero *et al* 2003b; Cai *et al* 2003; Carmeliet *et al* 2001; Park *et al* 1994). In PlGF^{-/-} BEC this balance is disrupted due to the lack of PlGF availability to induce VEGFR-1-dependent downstream signaling, despite an increase in VEGF expression (Figure 6). The data suggests that loss of PlGF decreases the responsiveness of BEC to hypoxia and exogenous VEGF, leading to a delayed angiogenic response and early CLN regression. Despite increased VEGF expression observed in the PlGF knockout BEC, lack of PlGF had a profound effect on the angiogenic response of these cells to hypoxia. This highlights the critical function of PlGF in pathological angiogenesis in the CNS. To our knowledge this is the first study to reveal alteration in intracellular signaling pathways related to vascular branching and sprouting mechanism induced by PlGF knockout in BEC.

3.6 ACKNOWLEDGMENTS

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Figure 6 Schematic model of the effects of PlGF knockout on BEC exposed to hypoxia. Hypoxic PlGF wild-type BEC secrete VEGF, PlGF and possibly VEGF/PlGF heterodimers (1); these dimeric forms bind to their receptors and induce specific intracellular signaling that contributes to the angiogenic response in BEC. Activation of VEGFR-1 by PlGF promotes sprouting and increased vascular networks, by inducing ERK1/2 signaling (2), and suppressing GSK-3 β -dependent inhibition of branching morphogenesis (3). VEGF promotes proliferation by activating VEGFR-2, and VEGF/PlGF heterodimers promote specific signaling through VEGFR-1/VEGFR-2 heterodimers. In contrast, PlGF^{-/-} BEC only produce VEGF in hypoxia (1). The functional consequence of VEGF is dependent on the production of PlGF in pathological conditions. Lack of PlGF reduces the diversity of the different ligands that could be produced, and reduces the diversity of intracellular signaling that occurs in these cells (2), (3). Proliferation is not affected in PlGF^{+/+} and PlGF^{-/-} BEC, since this is mediated by VEGF activation of VEGFR-2 in both genotypes.



PREFACE TO CHAPTER 4

Cerebral vascular remodeling under mild hypoxic conditions involves the complex interaction of the cellular constituents of the neurovascular unit (Dore-Duffy and LaManna 2007). Astrocytes are a major homeostatic component of the neurovascular unit, known to play an important regulatory role in different processes during physiological and pathological conditions. Under ischemic conditions neuronal survival has been shown to correlate with astrocyte survival, indicating a neuroprotective role of these cells. Under mild hypoxic conditions sprouting of new capillaries during angiogenesis has been shown to be closely matched with the formation of new astrocyte endfeet (Li *et al* 2010). VEGF, a key player in angiogenesis and neuroprotection, has also been shown to promote astrocyte reactivity, proliferation, and survival (Krum and Khaibullina 2003; Mani *et al* 2005; Schmid-Brunclik *et al* 2008), however, the role of its homolog PlGF in this processes is unknown. Our studies indicate that long-term hypoxia induces a pronounced increase in perivascular astrocyte reactivity in cortical areas of the brain, an effect that was significantly enhanced in PlGF-deficient mice (Appendix IV). Studies described in Chapter 4 focus on deciphering the effect of PlGF knockout on astrocyte proliferation and survival to OGD and the molecular mechanism involved. Chapter 4 shows that PlGF modulates VEGFR-2 expression in astrocytes both *in vitro* and *in vivo*, which results in a more proliferative and OGD-resistant phenotype in PlGF^{-/-} mice. Furthermore, we show that the classical antiapoptotic PI3K/Akt pathway is associated with astrocyte cell injury in OGD conditions.

CHAPTER 4

VEGFR-2-Mediated Increased Proliferation and Survival in Response to Oxygen and Glucose Deprivation (OGD) in PlGF Knockout Astrocytes

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Contribution of the co-authors:

- Dr. Danica Stanimirovic and Dr. Maria Moreno provided scientific guidance and supervised project. Manuscript was written by Moises Freitas-Andrade and edited and revised by Dr. Maria Moreno and Dr. Danica Stanimirovic.
- Dr. Peter Carmeliet provided transgenic PlGF knockout and wild-type mice, and provided critical review of the manuscript.

4.1 ABSTRACT

In hypoxic/ischemic conditions, astrocytes are involved in neuroprotection and angiogenesis. Vascular endothelial growth factor (VEGF) induces angiogenesis and exhibits neuroprotective and neurotrophic properties. However, the role of placental growth factor (PlGF), a VEGF homolog, in these processes is unclear. Therefore, proliferation and survival studies were performed on PlGF knockout (PlGF^{-/-}) and wild-type (PlGF^{+/+}) mouse astrocytes. A significant increase in cell proliferation and survival to oxygen and glucose deprivation (OGD) was observed in PlGF^{-/-} compared to PlGF^{+/+} astrocytes. Interestingly, no PlGF protein expression was detected in PlGF^{+/+} astrocytes and no changes in VEGF protein levels were observed between the two genotypes. Real-time PCR and immunocytochemistry showed overexpression of VEGF receptor-2 (VEGFR-2) in PlGF^{-/-} compared to PlGF^{+/+} astrocytes. Confocal microscopy revealed nuclear, membrane and cytoplasmic localization of VEGFR-2. *In vivo* overexpression of VEGFR-2 mRNA was also detected in PlGF^{-/-} compared to PlGF^{+/+} astrocytes. Stimulation with VEGF₁₆₅ resulted in increased proliferation in PlGF^{-/-} compared to PlGF^{+/+} astrocytes. This effect was blocked by the VEGFR-2 antagonist, VEGF₁₆₅b. The enhanced proliferation of PlGF^{-/-} astrocytes correlated with increased phospho-ERK-1/2 levels, while the resistance to OGD was independent of the PI3-K/Akt pathway. These results suggest that VEGFR-2 mediates the enhanced proliferative/OGD resistant phenotype observed in PlGF^{-/-} astrocytes.

4.2 INTRODUCTION

Astroglial cells play an important role in the homeostasis of the central nervous system (CNS) (Nedergaard *et al* 2003; Volterra and Meldolesi 2005) by providing a stable microenvironment in which different neuronal cell types can function properly (Volterra and Meldolesi 2005; Zonta *et al* 2003). Astrocytes have been shown to affect synaptic transmission, neuronal signaling, and neuronal plasticity (Grass *et al* 2004; Matthias *et al* 2003; Zhou and Kimelberg 2001). As a result of this intimate relationship with neurons, astrocytes have become highly heterogeneous and specialized and can play distinct regional roles within the brain (Bachoo *et al* 2004; Grass *et al* 2004; Matthias *et al* 2003; Seifert *et al* 2006; Wallraff *et al* 2004).

Astrocytes are one of the first responders to injury in the CNS; they proliferate, swell, accumulate glycogen and undergo fibrosis (Nedergaard *et al* 2003). Astrocytes remain viable longer under ischemic conditions than neurons, which are more sensitive to metabolic alterations (Xu *et al* 2001b; Zhao and Flavin 2000). Under hypoxic/ischemic conditions astrocytes regulate blood flow (Takano *et al* 2006), and secrete angiogenic, neurotrophic, and neuroprotective factors (Abbott *et al* 2006; Chow *et al* 2001). Therefore, astrocyte survival during ischemia appears to be crucial for preserving homeostasis and stimulating brain recovery. Similar to neurons, astrocytes display regional differences in responses to ischemic injury (Lukaszewicz *et al* 2002).

VEGF is the main inducer of angiogenesis (Carmeliet and Storkebaum 2002) and is secreted by astrocytes under hypoxic conditions (Chow *et al* 2001). VEGF is also neuroprotective and neurotrophic (Carmeliet and Storkebaum 2002); it can increase astrocyte proliferation and neurogenesis, as well as promote survival of both cell types (Silverman *et*

et al 1999; Zachary 2005). Inhibition of VEGF reduces angiogenesis and astrocyte proliferation in brain injury (Krum and Khaibullina 2003).

VEGF binds to the tyrosine kinase receptors VEGFR-1 and VEGFR-2 and to neuropilin-1 (NP-1). However, VEGFR-2 is the primary transducer of VEGF signals, through several intracellular signaling pathways including the Raf-Mek-Erk that promotes cell proliferation (Takahashi *et al* 1999) and the phosphatidylinositol 3'-kinase (PI3-K)/Akt that induces cell survival (Kilic *et al* 2006).

Placenta growth factor (PlGF), a member of the VEGF family that binds VEGFR-1 and NP-1, has been shown to enhance VEGF-induced pathological angiogenesis in the systemic vascular system (Autiero *et al* 2003a; Carmeliet *et al* 2001) through a complex intra- and intermolecular cross talk between VEGFR-1, VEGFR-2 and neuropilin-1 (Autiero *et al* 2003b; Carmeliet *et al* 2001; Shrager-Heled *et al* 2007). Simultaneous up-regulation of VEGF and PlGF has been observed after focal cerebral ischemia (Beck *et al* 2002), suggesting an important role for PlGF during pathological conditions in the CNS.

The purpose of this study was to determine whether PlGF knockout (PlGF^{-/-}) affects astrocyte proliferation and survival during hypoxic/ischemic conditions. PlGF^{-/-} astrocytes were found to be more proliferative and more resistant to oxygen and glucose deprivation (OGD) than the wild type (PlGF^{+/+}) astrocytes. This unique phenotype was attributed to VEGFR-2 upregulation in PlGF^{-/-} astrocytes.

4.3 MATERIALS AND METHODS

Cell cultures

Astrocytes from wild-type $PIGF^{+/+}$ and $PIGF^{-/-}$ knockout mice [previously generated by Dr. Peter. Carmeliet, Vesalius Research Center, Belgium, (Carmeliet *et al* 2001)] were isolated from 8-day-old mice, bred at the National Research Council of Canada (NRCC) Institute for Biological Sciences Animal Facility (Ottawa, ON, Canada). Mice were deeply anesthetized with 4% halothane B.P. (MTC Pharmaceuticals, Cambridge, ON, Canada) under the oxygen flow rate of 2 L/min and then sacrificed by guillotine. All procedures performed in these studies were approved by the NRCC Animal Care Committee. Brains were removed and placed into ice cold DMEM with antibiotic-antimycotic solution (Sigma-Aldrich, Oakville, ON, Canada). The cerebellum, and the olfactory bulbs were removed and brain cortices (4 brains per isolation) were then homogenized by passing through sequentially smaller gauge needles (18G 11/2, 23G 3/4, and 25G 5/8). Dispase 12 mg/ml, (Sigma-Aldrich Oakville, ON, Canada) in DMEM was added to the homogenate, and incubated at 37°C for 15 min. After incubation, the homogenate was filtered through a 53 μ m Nitex filter (Sefar Canada INC Scarborough, ON, Canada) into a 50 ml conical tube, and then centrifuged at 4°C for 10 min at 1500 rpm. The pellet was resuspended in astrocyte media containing 10% heat-inactivated fetal bovine serum (FBS, Hyclone Logan, UT, USA), 1X antibiotic-antimycotic, and DMEM, and the cells were plated on poly-L-lysine (Sigma-Aldrich Oakville, ON, Canada) coated 75 cm² culture flasks. After three days, cells were rinsed 3X with warm HBSS (Sigma-Aldrich Oakville, ON, Canada) and fed every 3-4 days thereafter. Cells were kept in culture in an atmosphere of 5% CO₂/ 95% air at 37°C. Experiments were performed using astrocytes from the first passage only. The purity of astrocyte cultures (>99%) was confirmed by positive immunostaining with a rabbit anti-

GFAP polyclonal antibody (DAKO, Hamburg, Germany). The absence of other contaminant cells such as endothelial cells and smooth muscle cells/pericytes was routinely confirmed by immunostaining with a polyclonal rabbit anti-human Factor VIII-related antigen antibody (DAKO, Hamburg, Germany), and a FITC-conjugated monoclonal anti- α -smooth muscle actin antibody (Sigma-Aldrich Oakville, ON, Canada), respectively. Astrocyte cultures were also free of neurons, since neurons do not survive passage and media/culture conditions used in this study.

Proliferation assay

Proliferation was measured using CyQuant® Cell Proliferation Assay Kit for cells in culture (Molecular Probes, Eugene, OR, USA) following the protocol provided by the manufacturer. Briefly, PIGF^{+/+} and PIGF^{-/-} astrocytes were plated in triplicate in 96 well plates at a density of 5000 cells/well. Cells were grown in DMEM + 1% FBS alone or in combination with either recombinant 20 ng/ml VEGF (R&D Systems, Minneapolis, MN, USA), or 20 ng/ml VEGF + 200 ng/ml VEGF-165b. VEGF165b is a natural splicing variant of VEGF165 that binds to VEGFR-2 with the same affinity, but without the ability to induce intracellular signaling (Bates et al., 2002). VEGF-165b was produced and generously provided by Dr. Yves Durocher (Biotechnology Research Institute, National Research Council Canada, Montreal, QC, Canada). Cells were harvested at 24, 48, and 72 h by washing with HBSS, blotting microplates dry and storing at -80°C until analysis. Plates were thawed at room temperature and 200 μ l of CyQUANT GR dye/lysis buffer was added to each well for 5 min in the dark. Fluorescence values were measured using a

cytofluorimeter plate reader (Bio-Tek FL600, Winooski, VT, USA) at 485 nm excitation/530 nm emission, and were directly proportional to cell numbers.

Cell viability assay

Nuclear staining with propidium iodide (PI) was used to assess the rate of cell death and to visualize changes in nuclear morphology characteristic of apoptosis in astrocytes submitted to OGD. PIGF^{+/+} and PIGF^{-/-} astrocytes were grown in 24-well plates until cells reached confluence. Before the start of each experiment, cells were rinsed 3 times with glucose free-Krebs' solution containing (in mM): NaCl, 118.3; KCl, 4.7; MgSO₄·7H₂O, 1.2; KH₂PO₄, 1.2; CaCl₂, 2.5; NaHCO₃. The OGD group was incubated in glucose-free Krebs' solution and placed in a hypoxic chamber (Forma 1025 Anaerobic Chamber, ThermoForma, Marietta, OH, USA) for 6, 10 and 14 h while the control normoxia group was incubated in Krebs solution supplemented with 0.5 mg/ml glucose and placed in a cell culture incubator for the same periods of time. In a set of experiments, astrocytes from both genotypes were treated with either 20 ng/ml VEGF, 100 ng/ml PIGF (R&D Systems, Minneapolis, MN, USA), or 100 nM Wortmannin (Calbiochem NJ, USA) during OGD conditions. At the end of the experiment, cells were incubated with 10 mg/ml PI for 15 min at 37°C in the dark, washed with HBSS, and then examined under fluorescent microscope (Olympus1x50, Carsen group INC Markham, ON, Canada). Images were captured by a digital video (Olympus U-CMT) and analyzed using the Northern Eclipse software v.5.0. Fluorescence levels (excitation 530 nm, emission 620 nm) were determined in the same plates using a cytofluorimeter (Cytofluor™ 2350, Millipore, Bedford, MA, USA). Fluorescent readings were then normalized with protein levels, measured by Lowry, from each of the plates.

Hypoxia experiments

PIGF^{+/+} and PIGF^{-/-} astrocytes were trypsinized using GIBCO trypsin (Invitrogen Burlington, ON, Canada) and plated on 35 mm culture dishes at a density of 6×10^4 cells/dish; cells were then allowed to grow and reach confluence. For hypoxia experiments, an appropriate volume of glucose-containing DMEM was placed in the anaerobic chamber and allowed to equilibrate for 2 h prior to the start of the experiments. Cells were washed three times with DMEM to eliminate FBS. The hypoxic PIGF^{+/+} and PIGF^{-/-} astrocyte groups were placed in the anaerobic chamber in equilibrated DMEM for 6 h (hypoxia) and returned to the cell culture incubator for additional 14 h (reoxygenation). Oxygen levels were measured with MI-730 Micro-Oxygen Electrode (Microelectrodes, INC. Bedford, NH, USA) and maintained between 0.5-1% inside the anaerobic chamber. The normoxic control PIGF^{+/+} and PIGF^{-/-} cell cultures were placed in the cell culture incubator in DMEM for 20 h. Cells were then processed appropriately for either RNA isolation or ELISA.

Gene expression analysis

Total RNA was isolated from samples using Trizol reagent (Invitrogen Burlington, ON, Canada) according to manufacturer's protocol. The RNA (0.5 µg) was primed with 10 pmol Anc-Oligo(dT)₁₂₋₁₈ primers (Cortec Kingston, ON, Canada) and reverse transcribed with 200 U SuperScript II Rnase H- Reverse Transcriptase (Invitrogen Burlington, ON, Canada), and 1 mM dNTPs (Invitrogen Burlington, ON, Canada) in a volume of 20 µl. Control reactions, lacking SuperScript enzyme, were run in parallel to check for genomic contamination. Primers for VEGF, PIGF, VEGFR-1, VEGFR-2, and β-actin (Table 1) were designed using Primer Express Software V2.0. (Applied Biosystems Foster City, USA) and purchased from Operon (Huntsville, AL, USA). Real-Time quantitative PCR (Q-PCR) was

carried out using SYBR[®] Green PCR Core Reagents Kit and the ABI Prism 7000 Sequence Detection System (Applied Biosystems, Foster City, USA). For each amplicon, the amount of target and endogenous reference (β -actin) was determined from a standard curve generated by serial dilution ranging from 1-1:1000. Complementary DNA representing 50 ng of total RNA was subjected to 40 cycles of PCR amplification. Samples were first incubated at 50°C for 2 min [AmpErase UNG incubation (SYBR[®] Green PCR Core Reagents Kit)], then 95°C for 15 min [AmpliTaq Gold activation (SYBR[®] Green PCR Core Reagents Kit)] followed by amplification. Each amplification cycle consisted of denaturation for 15 sec at 95°C and annealing/extension for 1 min at 60°C. To exclude non-specific products and primer-dimers, after the cycling protocol, a melting-curve analysis was performed by maintaining the temperature at 70°C for 30 sec, followed by a gradual increase in temperature to 98°C at a rate of 0.1°C/sec. The standard curve was amplified in triplicate during each experiment, and the amount of target gene was normalized to the endogenous reference (β -actin).

Table 4.1: Primers designed to amplify selected genes for Q-PCR analysis.

Gene	Forward primer (5'-3')	Reverse primer (5'-3')	Accession #	Amplicon length (bp)
NP-1	ACAGCAAGCGCAAA AGGTAA	GAGGTGAAAACGT CCGAAGCT	NM_008737	74
VEGFR-1	AGTGTGAACGGCTG CCCTAT	GCCAAATGCAGAG GCTTGAA	NM_010228	118
VEGFR-2	AGGTTTGCGTGCTC TTCACAGT	ACACGTAAGAGTC CGGAAGGAA	NM_010612	90
VEGF	CATCTTCAAGCCGT CCTGTGT	CTCCAGGGCTTCA TCGTTACA	NM_009505	66
β -actin	GGCGCTTTTGACTC AGGATT	GGGATGTTTGCTC CAACCAA	NM_007393	69
PIGF	CGTGGACTTTGACC GATACAAG	CGGCTCATCTCCC GTAGCT	NM_008827	65

Each set of forward and reverse primers was designed from NCBI published sequences corresponding to the provided accession number using the Primer Express Software v2.0.

RT-PCR for PlGF, GFAP and β -actin expression was performed using the following primers: PlGF (ACC# NM_008827), 5'-GGAGAGGCTATTGGGCTATGAC-3' (forward) and 5'-GGGTGACGGTAATAAATACG-3' (reverse), yielding a 268-bp product; GFAP (ACC # K01347), 5'-CGCGGCACGAACGAGTCC-3' (forward) and 5'-GTGTCCAGGCTGGTTTCTCG-3', yielding a 290-bp product and β -actin (ACC # NM_007393), 5'-GGAGATTACTGCTCTGGCTC-3' (forward) and 5'-GGACTCATCGTACTCCTGCT-3' (reverse), yielding a 131-bp product. The PCR amplification conditions were as follows: hot start at 94°C for 5 min, denaturation at 94°C for 40 sec, annealing at 56°C for 40 sec, and extension at 72 °C for 60 sec. After 40 cycles in these conditions, a final extension at 72 °C for 5 min was performed. Control reactions lacking the reverse transcriptase enzyme (NRT) were run in parallel for all samples to monitor for potential DNA contamination of the RNA samples. The PCR products were electrophoretically separated on a 1.2% agarose gel in Tris/borate/EDTA containing 0.5 mg/ml ethidium bromide and 100 bp DNA ladder to confirm the molecular size of the products.

ELISA

Conditioned media (CM) from normoxic or hypoxic PlGF^{+/+} and PlGF^{-/-} astrocytes were filtered through a Millex-GV (0.22 mm) sterilizing filter unit, consisting of a hydrophilic Durapore (PVDF) low protein binding membrane, and the levels of secreted PlGF and VEGF were respectively quantified using PlGF and VEGF colorimetric sandwich ELISA kits (R&D systems, Minneapolis, MN, USA) according to protocols provided by the

manufacturer. Each assay condition was run in duplicate; three independent experiments were performed.

Immunocytochemistry

PIGF^{+/+} and PIGF^{-/-} astrocytes were allowed to grow to confluence on coverslips in 24-well plates. Astrocytes were rinsed 2X with HBSS and fixed with 100% ice cold methanol for 10 min at room temperature (RT). Cells were rinsed 4X with HBSS, and permeabilized with 0.1% Triton X-100 (EM Science Gibbstown, NJ, USA) in HBSS at RT for 10 min. Cells were rinsed and blocked with 4% serum in HBSS for 1 h at RT and then incubated with polyclonal rabbit anti-Flk-1 primary antibody (Santa Cruz Biotechnology Santa Cruz, CA, U.S.A) at 1:50 dilution for 1 h at RT. Cells were then rinsed three times with HBSS and exposed to Alexafluor 568 goat anti-rabbit IgG secondary antibody (Invitrogen Burlington, ON, Canada) at 1:500 dilution for 30 min at RT. Finally, procedure was repeated using polyclonal rabbit anti-GFAP primary antibody (DAKO Glostrup, Denmark A/S), 1:500 dilution, and fluorescein goat anti-rabbit IgG secondary antibody (Invitrogen Burlington, ON, Canada) at 1:500 dilution. Cells were rinsed three times with HBSS followed by one rinse with H₂O. Coverslips were then removed from the wells, covered with DAKO mounting media, spiked with DAPI (Sigma-Aldrich Oakville, ON, Canada), placed on glass slides, and visualized using Olympus IX-50 microscope.

Confocal images using a Zeiss LSM 410 confocal microscope (Carl Zeiss Canada Ltd., Toronto, ON, Canada) and a plan-Apochromat 63x/1.4 objective were also obtained. Images of two fluoroprobes were captured simultaneously to exclude artifacts from sequential acquisition, using 488 and 568 nm excitation laser lines to detect FITC (BP505-550 emission) and Texas red/Alexa 568 fluorescence (LP590 emission), respectively. All

images were collected using the same laser power and pinhole size for the respective channels and were processed in an identical manner. Omission of primary antibodies resulted in no staining. No cross-reactivity was observed between the primary and non-corresponding secondary antibodies.

Laser-capture microdissection (LCM)

LCM was performed by combining two previously published protocols (Burbach *et al* 2004; Mojsilovic-Petrovic *et al* 2004). Briefly, mice were deeply anesthetized and then sacrificed by decapitation. Brains were removed, embedded in Tissue-Tec freezing medium (Miles Laboratories, Inc., Elkhart, IN, USA), immediately flash-frozen in 2-methyl-butane at -40 °C and stored at -80 °C until use. Brains were sectioned at 8µm thickness using a cryostat (Jung CM3000, Leica, Richmond Hill, ON, Canada). Sections were placed on Superfrost Plus microscope slides (Fisher Scientific, Nepean, ON, Canada), fixed in 75% acetone in PBS for 2 min, dried at 40 °C for 10 min followed by fast GFAP immunostaining (Burbach *et al* 2004). Sections were blocked for 3 min with 10% normal goat serum in PBS prepared with DEPC-treated water containing 0.3% TritonX-100. A primary rabbit anti-GFAP polyclonal antibody solution (DAKO, Hamburg, Germany, 1:50 in 10% BSA in PBS containing 0.1% Triton X-100) was applied onto the mounted section, incubated at 40 °C for 5 min in a humidified chamber, and rinsed three times for 15 sec each in PBS. Sections were then incubated with secondary antibody (Alexa568-labeled goat anti-rabbit 1:25, Invitrogen) for 2 min at room temperature, rinsed three times for 15 sec each in PBS and once in DEPC-treated water, then dehydrated by incubation in increasing concentrations of ethanol (75%, 96%, 100%) for 30 sec each followed by 5 min incubation in xylene. Sections were allowed to dry at room temperature, placed in a desiccating chamber at 40°C for 5 min and

immediately subjected to LCM. Isolation of GFAP expressing astrocytes was performed using a Pixcell II Laser-capture Microscope (Arcturus, Mountain View, CA, USA) and high sensitivity (HS) caps (CapSure LCM Caps, Arcturus, CA, USA). Three thousand laser shots (laser spot size of 7.5 μm and laser pulse of 1 ms duration) corresponding to roughly 3000 astrocytes were picked from each section, for a total of 6000 shots/slide. The 6000 astrocytes (i.e., each slide) were considered one sample. RNA was then extracted from each sample as previously described (Mojsilovic-Petrovic *et al* 2004). An Absolutely RNA Microprep Kit (Stratagene, La Jolla, CA, USA) was used according to protocols supplied by the manufacturer. Briefly, the astrocytes were lysed in a lysis buffer and loaded onto an RNA-binding spin cup. After washing with 70% ethanol and low-salt buffer, DNase I was added to the cup and incubated at 37 °C for 15 min. The cup was then sequentially washed with high- and low-salt buffers, and RNA was eluted from the cup with two 30 μl volumes of elution buffer. RNA was then lyophilized, and finally dissolved in 10 μl of DEPC treated water. RNA concentration was assessed by measuring optical density of the extracts at 260-280 nm. Reverse transcriptase reaction was performed, and VEGFR-2, GFAP and β -actin expression levels were determined by Q-PCR from each sample.

Western Blot

Cells were grown to confluence, and after three days of incubation in astrocyte media, cells were treated for 10 min with either 20 ng/ml VEGF165 or 200 ng/ml VEGF165b alone or were pre-incubated with VEGF165b for 15 min before adding VEGF165. Control groups were submitted to the same experimental conditions but without receiving any drug. Another set of astrocytes were subjected to OGD time-course (0-8 h) in

glucose-free Kreb's solution. After treatments, cultures were washed with ice-cold HBSS and exposed to 250 µl RIPA lysis buffer (1%NP40 (IGEPAL), 0.5% Deoxycholate, 0.1% SDS and protease inhibitor 100µl/10ml RIPA in PBS), (Sigma-Aldrich Oakville, ON, Canada) for 20 min at 4°C. The cell lysates were centrifuged at 14,000 rpm for 10 min at 4°C, and the supernatants were collected. Protein concentration was measured in each of the cell lysates by BCA Protein Assay Reagent Kit (Pierce Biotechnology, Rockford, IL, USA), and 20 µg of protein was denatured in protein loading buffer for 5 min at 100°C, and then separated on a 10% sodium dodecyl sulfate-polyacrylamide gel. Separated proteins were then transferred to polyvinylidene difluoride (PVDF) membranes. Blocking was performed by incubating membranes in TBST (20 mM Tris-buffer [pH 8.0] and 150 mM NaCl with 0.1% Tween-20) containing 5% BSA (Sigma-Aldrich Oakville, ON, Canada) for 1 h at room temperature. Membranes were then incubated overnight at 4°C with primary antibodies against either Phospho-Akt, Akt, Phospho-p44/42 MAP Kinase, or p44/42 MAP Kinase (all from Cell Signaling Technology, MA, USA) diluted 1/1000 in TBST containing 1% BSA. The membranes were washed with TBST and incubated for 1 h with Anti-Rabbit IgG (whole molecule)–Peroxidase antibody (Sigma-Aldrich Oakville, ON, Canada) diluted 1/5000 in TBST containing 5% skimmed milk. Immunoreactive proteins were visualized by Amersham ECL Plus Western Blotting Detection Reagents (GE Healthcare Buckinghamshire, UK).

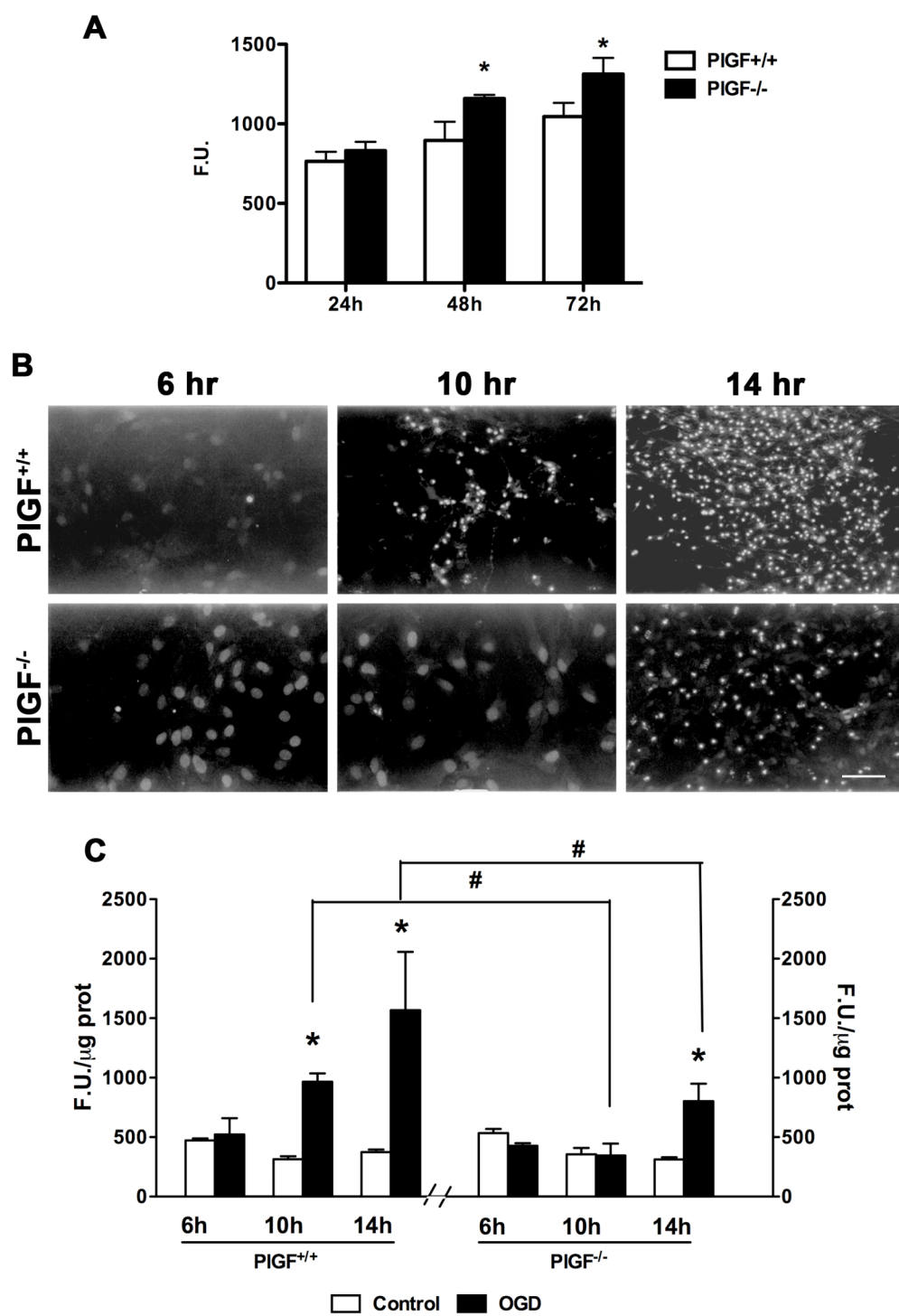
4.4 RESULTS

Effect of PlGF knockout on mouse astrocyte proliferation and survival

Under basal (control) conditions, PlGF^{-/-} astrocytes showed significantly higher proliferation rates at 48 and 72 h time points than their wild-type counterparts (Fig. 1A). To

investigate the effect of PlGF knockout on cell survival, PlGF^{+/+} and PlGF^{-/-} mouse astrocytes were exposed to OGD for 6, 10 and 14 h and cell death was quantified at each time point by cell staining with propidium iodide. As shown in Fig. 1 (B and C), the number of PI stained nuclei increased with longer exposure of astrocytes to OGD. However, PlGF^{-/-} astrocytes were more resistant than PlGF^{+/+} astrocytes to 10 h and 14 h OGD as shown by lower number of PI positive, picnotic nuclei (Fig. 1B) and lower PI fluorescence readings (Fig. 1C). No significant cell death was observed in the control (normoxic) group at any time point (Fig. 1C).

Figure 1. (A) $\text{PIGF}^{+/+}$ and $\text{PIGF}^{-/-}$ astrocyte proliferation. Astrocytes were grown in DMEM + 1% FBS for 24 h, 48 h and 72 h. Each bar represents the average $\pm$ SEM of three experiments done in triplicates. * indicates significance ($p < 0.05$, ANOVA followed by Newman-Keuls) between $\text{PIGF}^{+/+}$ and $\text{PIGF}^{-/-}$ at each time point. (B-C) $\text{PIGF}^{+/+}$ and $\text{PIGF}^{-/-}$ astrocyte cell viability to OGD. Astrocytes were exposed to OGD for the indicated periods of time. Control group was incubated in Kreb's solution + glucose in normoxic condition for the same periods of time. At the end of each time point, propidium iodide was added to cultures, washed, and then examined for incorporated fluorescence by either fluorescence microscopy (B) or cytofluorimeter (C). Scale bar: 50 μm . Each bar represents the average $\pm$ SEM of four experiments done in triplicates. * indicates significance ($p < 0.05$, ANOVA followed by Newman-Keuls) between normoxic control and OGD groups within each genotype for the corresponding time point. # indicates significance ($p < 0.05$, ANOVA followed by Newman-Keuls) between $\text{PIGF}^{+/+}$ and $\text{PIGF}^{-/-}$ OGD groups.



VEGF and PlGF expression in PlGF^{+/+} and PlGF^{-/-} astrocytes

PlGF expression was confirmed in wild-type astrocytes by RT-PCR; no expression was detected in the knockout animals (Fig. 2A). PlGF mRNA expression levels increased 2.5-fold in PlGF^{+/+} astrocytes exposed to 6 h hypoxia compared to those in astrocytes submitted to normoxic conditions, and returned to basal levels after 14 h reoxygenation (Fig. 2B). However, PlGF protein was not detected in cells by Western-blot (data not shown) or in cell conditioned media by ELISA (sensitivity: 1.4-1.84 pg/mL) in PlGF^{+/+} astrocytes exposed to either a 6 h hypoxia and 14 h reoxygenation or to a 20 h hypoxia and 3 days reoxygenation (data not shown).

The effect of PlGF knockout on VEGF expression was also investigated. VEGF mRNA (Fig. 2C) and protein (Fig. 2D) expression levels were highly up-regulated by hypoxia in both PlGF^{+/+} and PlGF^{-/-} astrocytes; no significant differences in VEGF mRNA and protein levels were found between the two genotype groups in either normoxic or hypoxic conditions. VEGF mRNA expression returned to control levels after 14 h reoxygenation in both PlGF^{+/+} and PlGF^{-/-} astrocytes (Fig. 2C).

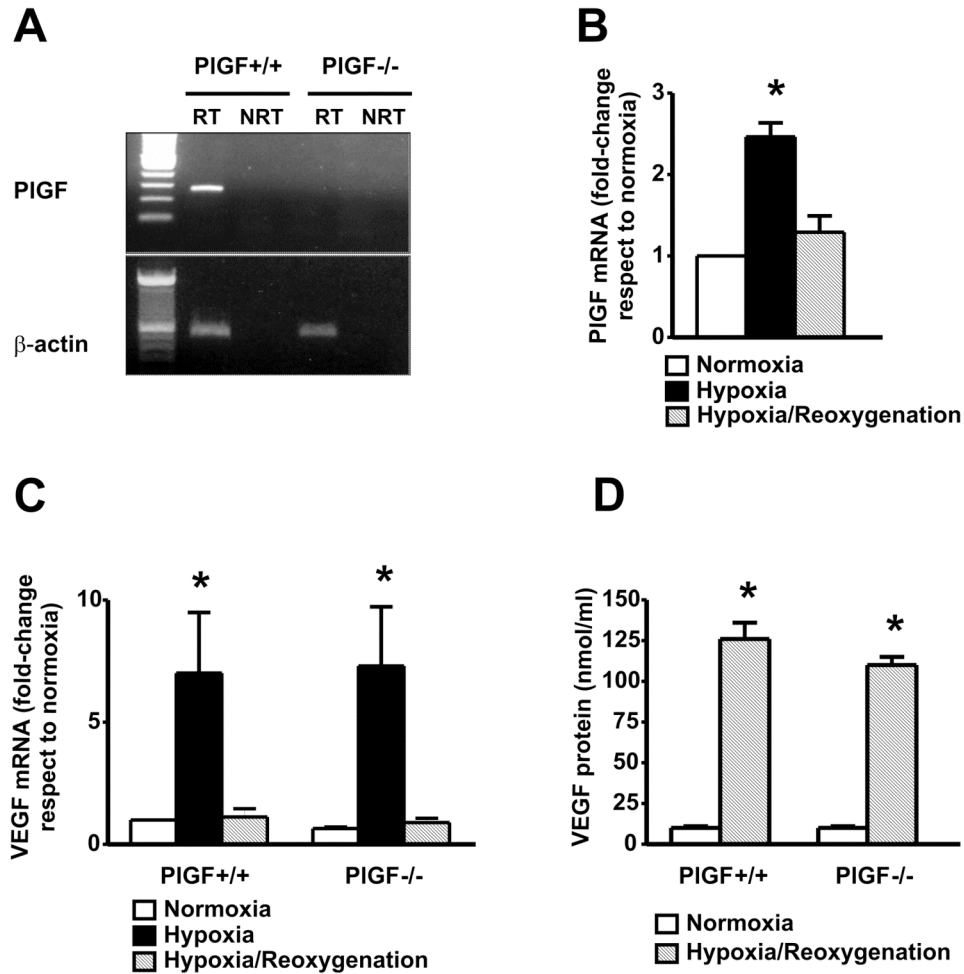


Figure 2. (A) Representative agarose gel showing PIGF and β -actin RT-PCR amplification products (RT) and their non-RT (NRT) negative controls, from PIGF^{+/+} and PIGF^{-/-} astrocytes. (B) Q-PCR analysis of PIGF mRNA expression in PIGF^{+/+} mouse astrocytes submitted to 20 h normoxia, 6 h hypoxia and 6 h hypoxia/14 h reoxygenation. Each bar represents the average $\pm$ SEM of four experiments done in duplicates. * indicates significance ($p < 0.05$, ANOVA followed by Newman-Keuls) with respect to normoxia. (C) VEGF expression levels quantified by Q-PCR and by (D) ELISA in PIGF^{+/+} and PIGF^{-/-} astrocytes after 20 h normoxia, 6 hypoxia and 6 h normoxia/14 h reoxygenation. Each bar represents the average $\pm$ SEM of four experiments done in duplicates. * indicates significance ($p < 0.05$, ANOVA followed by Newman-Keuls) with respect to normoxia.

VEGF/PlGF receptor expression in $PlGF^{+/+}$ and $PlGF^{-/-}$ astrocytes

To investigate whether changes in VEGF/PlGF receptor expression could be responsible for the more proliferative and OGD resistant phenotype of $PlGF^{-/-}$ astrocytes, the expression of VEGFR-1/flt-1, VEGFR-2/flk-1 and neuropilin-1 (NP-1) was analyzed in both genotypes by Q-PCR. The results revealed significantly higher (~2-fold) levels of VEGFR-2 mRNA in $PlGF^{-/-}$ compared to those in $PlGF^{+/+}$ astrocytes during normoxic conditions (Fig. 3A). After 6 h hypoxia and 14 h reoxygenation, a significant increase (~1.5-fold) in VEGFR-2 mRNA expression was detected in $PlGF^{+/+}$ astrocytes compared to the normoxic counterparts. No increases in VEGFR-2 mRNA levels were observed in $PlGF^{-/-}$ astrocytes exposed to hypoxia/reoxygenation compared with their respective normoxic controls (Fig. 3A). Contrary to VEGFR-2, VEGFR-1 and NP-1 expression levels were not significantly different between genotypes in either normoxic or hypoxic conditions (data not shown).

Immunocytochemistry studies also confirmed higher levels of VEGFR-2 protein expression in $PlGF^{-/-}$ compared to $PlGF^{+/+}$ astrocytes in culture (Fig. 3B). A more detailed analysis of VEGFR-2 cellular localization was performed using confocal microscopy. Serial Z-sections revealed heterogeneous localization of VEGFR-2 immunostaining in cell cultures; receptor immunoreactivity was detected in the cytoplasm of both cell bodies and processes as well as in nuclei. In a small number of cells, receptor immunoreactivity appeared to be located in clusters in the plasma membrane (Fig. 3C). The cellular localization of VEGFR-2 was similar in $PlGF^{+/+}$ and $PlGF^{-/-}$ astrocyte, although the levels of expression were higher in $PlGF^{-/-}$ astrocytes.

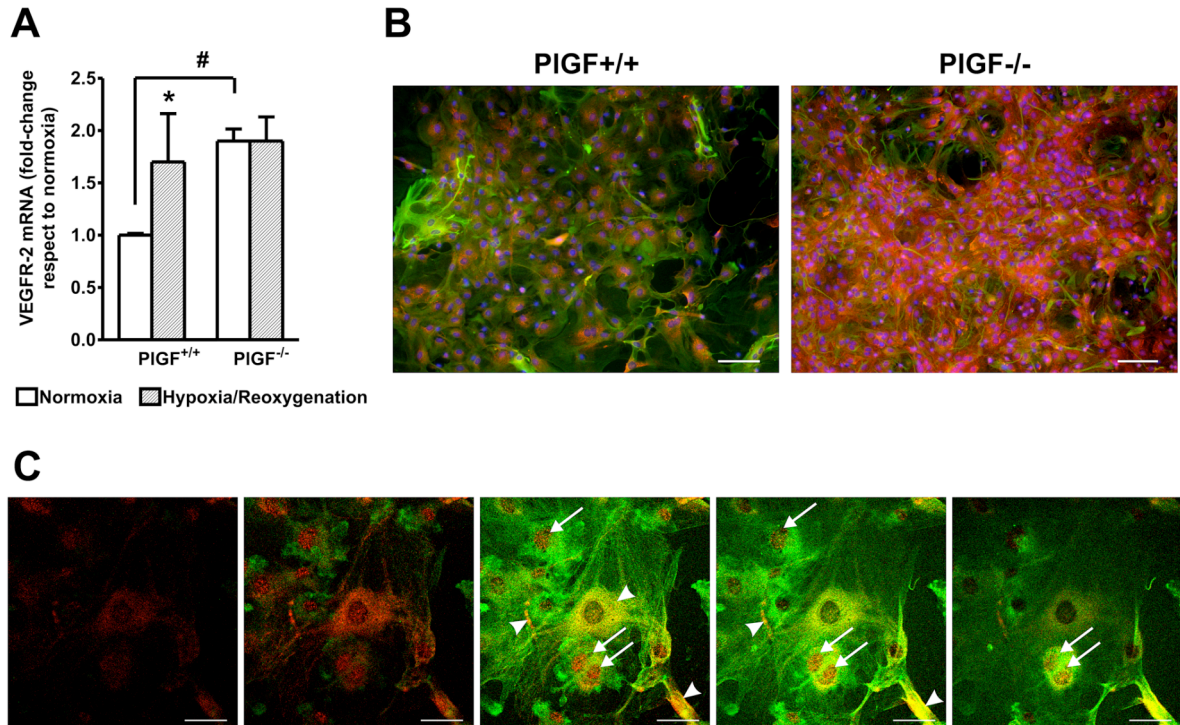


Figure 3. (A) VEGFR-2 mRNA expression levels quantified by Q-PCR in PIGF^{+/+} and PIGF^{-/-} astrocytes submitted to either 20 h normoxia or 6 h hypoxia/14 h reoxygenation. Each bar represents the average $\pm$ SEM of four experiments done in duplicates. * indicates significance ($p < 0.05$, ANOVA followed by Newman-Keuls) between normoxia and hypoxia and, # indicates significance ($p < 0.05$, ANOVA followed by Newman-Keuls) between PIGF^{+/+} and PIGF^{-/-} astrocytes under normoxic conditions. (B) Micrographs of PIGF^{+/+} and PIGF^{-/-} astrocytes in culture immunostained for VEGFR-2 (red), the astrocyte specific marker GFAP (green) and nuclear marker DAPI (blue). Scale bar: 100 μ m (C) Confocal microscopy serial z-sections of PIGF^{-/-} astrocytes in culture immunostained for VEGFR-2 (red) and GFAP (green). Arrows indicate nuclear VEGFR-2 expression; arrow heads indicate VEGFR-2 expression in plasma membrane, cell body and processes. Scale bar: 10 μ m.

Effect of PlGF knockout on VEGFR-2 expression in vivo

The overexpression of VEGFR-2 observed in PlGF^{-/-} astrocytes grown in culture was also confirmed *in vivo*. Astrocytes from PlGF^{+/+} and PlGF^{-/-} mice under normoxic conditions were isolated by LCM from brain sections stained for the astrocyte marker GFAP (Fig. 4A). Total mRNA was obtained from pools of astrocytes isolated from 8 sections of either frontal (Bregma +1 and +2mm) or parietal-occipital (Bregma -2 and -3 mm) cortex. Similar to astrocytes in culture, VEGFR-2 expression was approximately 4.5-fold higher in PlGF^{-/-} compared to PlGF^{+/+} astrocytes isolated from the frontal cortex (Fig. 4C). However, VEGFR-2 expression was not significantly different between PlGF^{+/+} and PlGF^{-/-} astrocytes isolated from the parietal-occipital region (Fig. 4D).

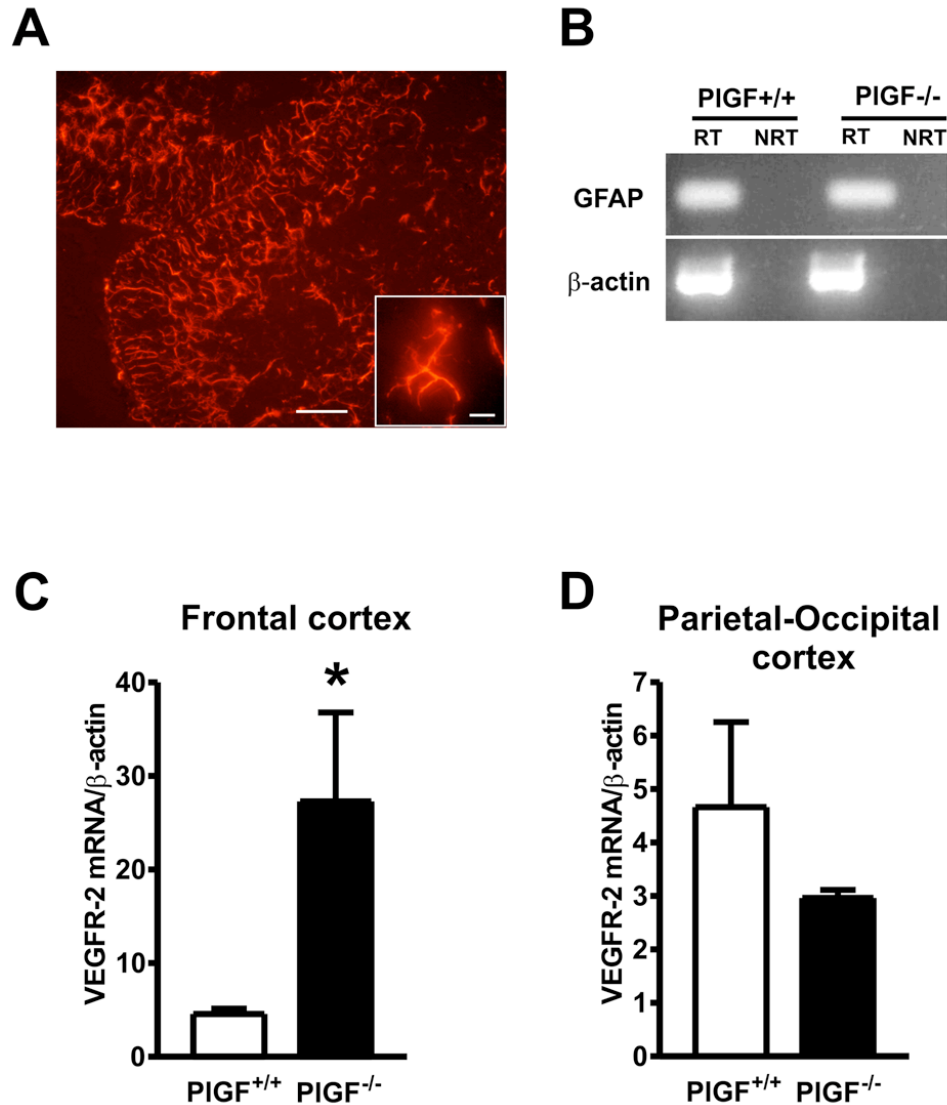


Figure 4. (A) Fast GFAP immunostaining of astrocytes in coronal brain tissue sections prepared for LCM. Scale bar: 100 μ m. Overlay image shows the typical star-shape morphology of astrocytes captured by LCM. Scale bar: 10 μ m. (B) Representative agarose gel showing GFAP and β -actin RT-PCR amplification products (RT) and their non-RT (NRT) negative controls, from PIGF^{+/+} and PIGF^{-/-} astrocytes captured by LCM. (C) VEGFR-2 mRNA expression levels quantified by Q-PCR in PIGF^{+/+} and PIGF^{-/-} LCM captured astrocytes from frontal cortex (Bregma +1 and +2mm) (C) and parietal-occipital cortex (Bregma -2 and -3 mm) (D). Each bar represents the average $\pm$ SEM of two mice of each genotype, with 8 sections per mouse analyzed in each region. * indicates significance ($p < 0.05$, ANOVA followed by Newman-Keuls) between PIGF^{+/+} and PIGF^{-/-} astrocytes.

Effects of VEGFR-2 agonist/antagonist in $PlGF^{+/+}$ and $PlGF^{-/-}$ astrocyte proliferation and ERK-1/2 phosphorylation

Since $PlGF^{-/-}$ astrocytes expressed significantly higher levels of VEGFR-2 compared to $PlGF^{+/+}$ mice, studies were performed to elucidate whether VEGFR-2- induced signaling pathways might be implicated in the increased proliferation rates observed in $PlGF^{-/-}$ astrocytes. Stimulation with 20 ng/ml VEGF165 for 24 and 48 h induced a more pronounced increase in proliferation in $PlGF^{-/-}$ compared to $PlGF^{+/+}$ astrocytes (Fig. 5A); This effect was blocked by co-incubation of VEGF165 with the VEGFR-2 antagonist VEGF165b (200 ng/ml) (Fig. 5A).

VEGFR-2 activation of Raf-Mek-Erk intracellular signaling, previously described to promote cell proliferation (Takahashi *et al* 1999), was determined by Western blot analysis. Higher basal levels of phosphorylated extracellular-signal-regulated kinase (Erk-1/2), also known as p42/44 MAPK, were observed in $PlGF^{-/-}$ compared to $PlGF^{+/+}$ astrocytes (Fig. 5B). These results are in agreement with the higher basal mRNA levels of VEGFR-2 observed in $PlGF^{-/-}$ compared to $PlGF^{+/+}$ astrocytes (Fig. 3A). Stimulation of astrocytes with 20 ng/ml VEGF increased phospho-Erk-1/2 levels in $PlGF^{+/+}$ but not in $PlGF^{-/-}$ mice, which suggests that, in $PlGF^{-/-}$ astrocytes, Erk-1/2 phosphorylation levels might be maximally activated in basal conditions. VEGF 165b (200 ng/ml) blocked the Erk-1/2 phosphorylation induced by 20 ng/ml of VEGF in both $PlGF^{+/+}$ and $PlGF^{-/-}$ astrocytes. Exposure of astrocytes to 200 ng/ml VEGF165b alone resulted in induction of Erk-1/2 phosphorylation in $PlGF^{+/+}$ but not in $PlGF^{-/-}$ astrocytes (Fig. 5B). This partial agonist effect of VEGF165b has been recently reported in human umbilical vein endothelial cells (Cebe Suarez *et al* 2006) and has been attributed to the inability of VEGF165b to bind neuropilin (Kawamura *et al* 2008), a receptor known to enhance VEGFR-2 signaling (Shraga-Heled *et al* 2007) and/or to its potential

interaction with VEGFR-1 (Glass *et al* 2006). No changes in total Erk-1/2 protein levels were detected among groups (Fig. 5B).

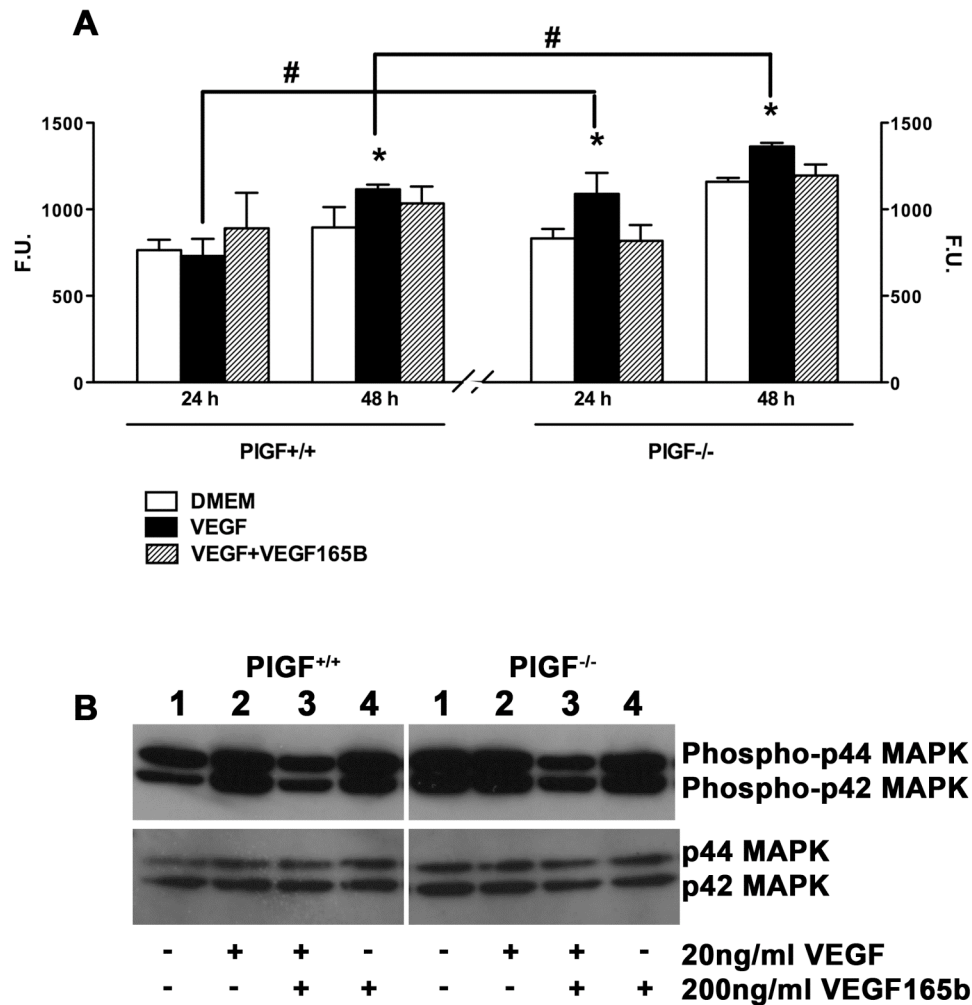


Figure 5. (A) PIGF^{+/+} and PIGF^{-/-} astrocyte proliferation. Astrocytes were treated with either DMEM + 1% FBS alone (control) or supplemented with 20 ng/ml VEGF165, 20 ng/ml VEGF165+ 200 ng/ml VEGF165b or 200 ng/ml VEGF165b for 24 and 48 h. Each bar represents the average $\pm$ SEM of three experiments done in triplicates. * indicates significance ($p < 0.05$, ANOVA followed by Newman-Keuls) relative to control (CTR) # indicates significance ($p < 0.05$, ANOVA followed by Newman-Keuls) relative to the corresponding time-point/treatment in the PIGF^{+/+} astrocyte group. (B) Phospho-p42/p44-MAPK and total p42/p44-MAPK protein levels analyzed by Western blot in PIGF^{+/+} and PIGF^{-/-} astrocytes in control conditions or stimulated with 20 ng/ml VEGF, 20 ng/ml VEGF +200 ng/ml VEGF165b, or VEGF165b alone.

Survival signaling pathways in $PlGF^{+/+}$ and $PlGF^{-/-}$ astrocytes exposed to OGD

As $PlGF^{-/-}$ astrocytes were more resistant to OGD and expressed significantly higher levels of VEGFR-2 compared to $PlGF^{+/+}$ astrocytes, survival signaling pathways associated with VEGFR-2 activation were investigated. Stimulation of $PlGF^{+/+}$ and $PlGF^{-/-}$ astrocytes with 20 ng/ml VEGF165 and 100 ng/ml $PlGF$ significantly reduced cell death after 10 h OGD in both genotypes (Fig. 6A). VEGF neuroprotection against hypoxia has been attributed to the VEGFR-2-activated PI3-K/Akt antiapoptotic signaling pathway (Kilic *et al* 2006). In contrast, incubation of $PlGF^{+/+}$ and $PlGF^{-/-}$ astrocytes with, the PI3-K inhibitor, Wortmannin (100 nM), potently inhibited OGD-induced cell death in both genotypes (Fig. 6A). In addition, Akt phosphorylation was significantly higher in $PlGF^{+/+}$ compared to $PlGF^{-/-}$ astrocytes at 3-8 h of OGD (Fig. 6B). In $PlGF^{+/+}$ astrocytes, Akt phosphorylation increased during OGD compared to control, reaching maximum levels at 7-8 h OGD. In contrast, Akt phosphorylation levels in $PlGF^{-/-}$ astrocytes did not change under OGD conditions compared to control (Fig. 6B). No changes in total Akt protein were detected among groups (Fig. 6B).

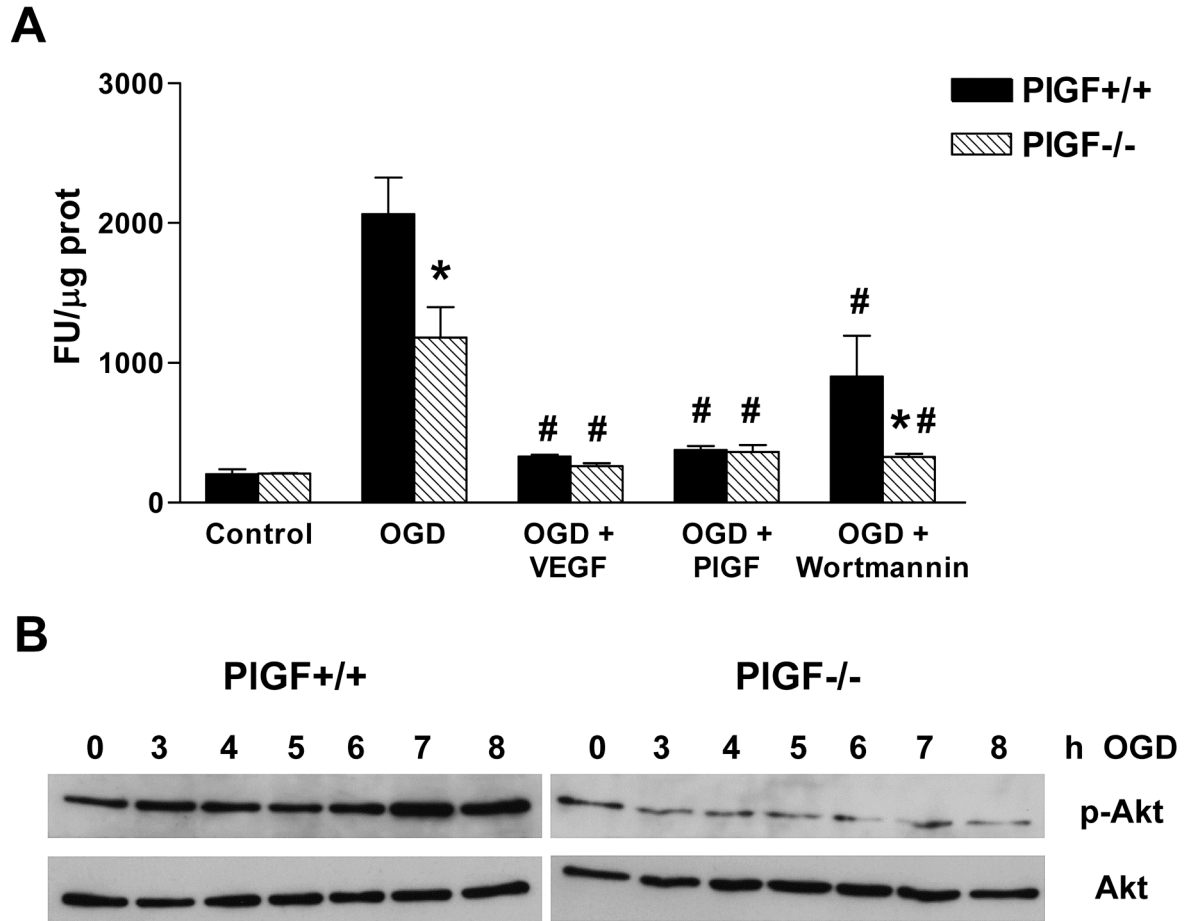


Figure 6. (A) PIGF^{+/+} and PIGF^{-/-} astrocyte cell viability to OGD. Astrocytes were incubated in glucose-free Kreb's solution and exposed to OGD for 10 h in the absence or presence of either 20 ng/ml VEGF165, 100 ng/ml PIGF or 100 nM Wortmannin. Control group was incubated in Kreb's solution + glucose for 10 h in normoxic conditions. * indicates significance ($p < 0.05$, ANOVA followed by Newman-Keuls) relative to the corresponding PIGF^{+/+} control group (CTR) # indicates significance ($p < 0.05$, ANOVA followed by Newman-Keuls) relative to the corresponding OGD group. (B) Phospho-Akt and total Akt protein levels analyzed by Western blot in PIGF^{+/+} and PIGF^{-/-} astrocytes subjected to 3-8 h of OGD (Densitometric analysis of Western blots is shown in Appendix V, Figure A5, Page 166).

4.5 DISCUSSION

The results of the present study indicate that PlGF^{-/-} astrocytes are more proliferative and more resistant to OGD than their PlGF^{+/+} counterparts. This distinctive phenotype was linked to an increase in VEGFR-2 expression in PlGF^{-/-} astrocytes *in vitro* and *in vivo*. Signaling studies confirmed the involvement of VEGFR-2 in the enhanced proliferative phenotype of PlGF knockout astrocytes through a mechanism involving Erk signaling pathways. The enhanced PlGF^{-/-} astrocyte resistance to OGD was PI3-K/Akt independent; furthermore, and contrary to neurons, this pathway was shown to promote astrocyte cell injury during ischemic conditions.

The more proliferative and OGD resistant phenotype of PlGF^{-/-} astrocytes was most likely independent of an autocrine loop involving PlGF interaction with astrocyte receptors since no detectable protein levels of PlGF were found in wild-type astrocytes in either normoxic or hypoxic conditions. Gollmer et al. (2000) reported no detection of PlGF mRNA levels in either human normal brain tissues or different grades of astrocytomas by Northern blot analysis, although PlGF expression was detected by PCR using the same tissues (Gollmer *et al* 2000). In mice, no PlGF protein was also detected by immunohistochemistry in normal brain (Beck et al., 2002). However, after middle cerebral artery occlusion (MCAO), expression of PlGF was found in astrocytes surrounding the infarct area, with a peak of expression at day 3 after MCAO (Beck *et al* 2002). Under the milder and shorter hypoxic/OGD conditions applied in our studies, increased PlGF mRNA levels were observed in wild-type astrocytes, however this did not correlate with changes in protein expression. While a body of evidence indicates that hypoxia is a potent inducer of VEGF *in vitro* and *in vivo* (Shweiki *et al* 1992), studies investigating the effect of hypoxia on PlGF expression have rendered contradictory results, showing either no effect (Cao *et al* 1996a) PlGF

upregulation (Cramer *et al* 2005) or PlGF downregulation (Ahmed *et al* 2000) depending on the cell type.

The possibility of VEGF being differentially upregulated in PlGF^{-/-} astrocytes, thus influencing their survival and proliferation through an autocrine loop, was also ruled out since no significant differences in VEGF mRNA and protein expression levels were detected between the two genotypes in either normoxia or hypoxia.

VEGF-induced proliferation and survival is mediated by activation of either VEGFR-1 (Mani *et al* 2005) or VEGFR-2 (Kilic *et al* 2006; Takahashi *et al* 1999) alone or in collaboration with NP-1 (Shintani *et al* 2006). In this study, no significant differences in VEGFR-1 and NP-1 mRNA expression were detected between PlGF^{+/+} and PlGF^{-/-} astrocytes in normoxic or hypoxic conditions; in contrast, the basal VEGFR-2 mRNA levels were ~2-fold higher in the PlGF knockout astrocytes compared to the wild-type. The increased VEGFR-2 mRNA expression observed in cultured PlGF^{-/-} astrocytes was also confirmed *in vivo* in GFAP positive astrocytes isolated from frontal brain sections using LCM. Interestingly, no significant changes were detected in the parietal-occipital regions between the two genotypes. This may reflect the heterogeneity of the astroglial cell populations present in different brain regions, as previously described (Bachoo *et al* 2004; Wallraff *et al* 2004).

The upregulation of VEGFR-2 in PlGF^{-/-} astrocytes was also confirmed by immunocytochemistry and by demonstrated elevation of phosphorylated ERK-1/2, a VEGFR2 downstream signaling molecule. Phosphorylation of ERK/MAPK pathway upon activation of VEGFR-2 has been shown to play a critical role in cell proliferation (Takahashi, 1999). Studies reported here show a direct correlation between increased overall expression of VEGFR-2, elevated Erk-1/2 phosphorylation levels and higher proliferative rates in PlGF^{-/-}

^{-/-} compared to PlGF^{+/+} astrocytes. Furthermore, stimulation with VEGF induced a more pronounced proliferation in PlGF^{-/-} than in PlGF^{+/+} astrocytes, an effect blocked by VEGF165b, a natural splicing variant of VEGF165 (Bates *et al* 2002). This isoform was found to act as a natural VEGF165 antagonist since it binds VEGFR-2 (Woolard *et al.*, 2004) with the same affinity as VEGF165 but without the ability to stimulate downstream signaling pathways (Woolard *et al* 2004). Recently, VEGF165b was also shown to exert a weak and transient partial agonist activity on VEGFR-2 (Cebe Suarez *et al* 2006). In two separate studies, this effect has been attributed to the lack of VEGF165b binding to NP (Kawamura *et al* 2008) or to its agonist effect on VEGFR-1 (Glass *et al* 2006). Although in this study VEGF165b effectively blocked VEGF-induced astrocyte responses, it also demonstrated partial agonist properties in the absence of VEGF165. In these cells, the combined application of VEGF165 and VEGF165b appeared to actively suppress VEGFR-2 downstream signaling.

Confocal studies to determine VEGFR-2 cellular localization in astrocytes indicate that the receptor appears to form clusters in the cell membrane and processes; abundant VEGFR-2 immunoreactivity was also found in the cytoplasm and nuclei of many cells. Recent reports have shown that VEGFR-2 can internalize and extend its proliferative signaling activity for longer periods within intracellular compartments (Lampugnani *et al* 2006); it has also been shown that phosphorylated VEGFR-2 can be translocated to the nucleus by a process mediated by caveolae or VEGFR-1 (Blazquez *et al* 2006; Feng *et al* 1999; Santos *et al* 2007). Although no significant differences in VEGFR-2 cellular localization were observed between PlGF^{+/+} and PlGF^{-/-} astrocytes, the overall upregulation of VEGFR-2 in PlGF^{-/-} astrocytes also translated into higher intracellular levels of the

receptor; this may contribute to the enhanced proliferative phenotype of PlGF^{-/-} astrocytes as the intracellular receptor has longer signal transduction capacity.

VEGF protective effects against cell death through VEGFR-2 have been primarily associated with the activation of anti-apoptotic pathways involving PI3-K/Akt (Kilic, 2006). In this study, VEGF treatment significantly reduced cell death in both PlGF^{+/+} and PlGF^{-/-} astrocytes exposed to OGD. However, this effect was PI3-K/Akt-independent since incubation of astrocytes with the PI3-K inhibitor, Wortmannin, attenuated cell death during OGD. In addition, Akt phosphorylation was significantly higher in PlGF^{+/+} compared to PlGF^{-/-} astrocytes during OGD, despite the fact that PlGF^{+/+} astrocytes were less resistant to ischemia. One previous report has also indicated that the protective effect of VEGF against hypoxia can involve PI3-K/Akt independent pathways (Svensson *et al* 2002), although the specific antiapoptotic signaling molecules have yet to be characterized. In this study PI3-K/Akt pathway was not responsible for astrocyte protection against OGD but rather promoted cell injury during ischemia. This finding is in disagreement with the classically described PI3-K/Akt antiapoptotic pathway in neurons and other cell types (Brunet *et al* 2001). Interestingly, Jiang *et al.*, (2003) found that LY294002, a PI3-K inhibitor, delayed ischemia-induced astrocyte cell death, which correlated with elevation of bcl-2 expression levels (Jiang *et al* 2003). These results support our observations and suggest a potential involvement of PI3-K/Akt signaling pathway in astrocyte cell injury.

Similarly to VEGF, PlGF treatment significantly reduced OGD-induced astrocyte cell death. This effect may have been exerted through direct activation of VEGFR-1, which has been previously shown to promote cell survival (Cai *et al* 2003) or through VEGFR-1 crosstalk with VEGFR-2, as reported by Autiero *et al.*, 2003. Although no PlGF expression was found in postnatal (P8) astrocytes in the wild-type mice, this protein may have been

produced during embryonic development and contribute to the controlled expression of VEGFR-2. As PlGF promotes astrocyte cell survival, the lack of PlGF in the knockout mice may have resulted in the development of an alternative protective mechanism through overexpression of VEGFR-2.

This study indicates that PlGF, directly or indirectly, modulates VEGFR-2 expression in astrocytes both *in vitro* and *in vivo*, which results in a more proliferative and OGD-resistant phenotype that may enhance neuronal protection in the ischemic brain. The enhanced proliferation of the PlGF knockout astrocytes was mediated through Erk activation, while the survival properties were PI3-K/Akt independent. Furthermore, our studies indicate that, contrary to what was previously described for neurons, the classical antiapoptotic PI3-K/Akt pathway promotes astrocyte cell injury in ischemic conditions. The opposite outcomes of the PI3-K/Akt signaling pathway activation in neurons and astrocytes highlight the need for a more comprehensive analysis of the differential impact on astrocytes and neurons that specific therapeutic treatments targeting this pathway may have in the ischemic brain.

4.6 ACKNOWLEDGMENTS

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CHAPTER 5: GENERAL DISCUSSION

The brain is vitally dependent on a continuous supply of oxygen to maintain its function. In mild hypoxic conditions where oxygen availability is reduced, for example in high altitude, systemic adaptations are initiated. In prolonged exposure to hypoxia, compensatory mechanisms triggered at the level of the tissue, such as increased glucose metabolism, decreased oxidative capacity, and microvascular remodeling (angiogenesis) are induced (LaManna 2007). Angiogenesis is initiated in the brain within the first week of exposure to chronic mild hypoxia; the end result of this adaptive mechanism is an attempt to restore partial oxygen pressure in the tissue back to baseline levels in order to balance oxygen delivery and tissue oxygen consumption (LaManna 2007). Several studies have demonstrated that an age-dependent decline in cerebral capillary density and CBF occurs in the normal brain (Amano *et al* 1982; Bell and Ball 1990; Jucker *et al* 1990; Kety 1956; Melamed *et al* 1980). Using thick sections of postmortem human visual cortex, Bell *et al.* (1990) showed an age dependent decrease in capillary density that was similar in both normal aged and demented aged subjects (Bell and Ball 1990). Similarly, regional CBF measured by the ¹³³Xenon inhalation method demonstrated that decline in regional CBF is a progressive phenomenon which begins at an early age (Melamed *et al* 1980). These vascular changes have been shown to be correlated with a decline in growth hormone and insulin-like growth factor 1, in rodents (Sonntag *et al* 1997). Taken together, this may affect the ability of the old-age subjects to mount an adequate pro-angiogenic response after an insult and is associated with detrimental effects after stroke and in neurodegenerative diseases such as Alzheimer's disease (Bell *et al* 2009; Farkas *et al* 2000; Hachinski 2011; Krupinski *et al* 1994; Popa-Wagner *et al* 2010; Ratan *et al* 2007; Zlokovic 2008). However, despite

evidence that angiogenesis is critical for brain recovery and long-term health (Chopp *et al* 2007; Du *et al* 2011; Krupinski *et al* 1994; Luo *et al* 2011; Morimoto *et al* 2010; Teng *et al* 2008; Zhang *et al* 2010; Zlokovic 2008), therapeutic strategies to promote angiogenesis, and thereby reduce brain damage are lacking (Moskowitz *et al* 2010).

VEGF has been classically considered the principal driver of angiogenesis both in the peripheral and the CNS vasculature. However, to date, little is known about the biological functions of PlGF in the brain, particularly under hypoxic conditions. Most of what is known about the actions of this protein comes from studies performed on the peripheral vasculature (Adini *et al* 2002; Carmeliet *et al* 2001; Chaballe *et al* 2011; Duits *et al* 2006; Foyouzi *et al* 2006; Gigante *et al* 2006; Khurana *et al* 2005). However, exogenous application of PlGF has been shown to promote angiogenesis and neuroprotection in brain ischemia (Du *et al* 2010; Liu *et al* 2006) but the role of endogenously produced PlGF in the CNS has not yet been explored under physiological or pathological conditions.

Several studies have shown that PlGF does not play a critical role during developmental angiogenesis, however it has an important synergistic effect to enhance VEGF-induced angiogenesis under pathological conditions (Autiero *et al* 2003b; Carmeliet *et al* 2001; Luttun *et al* 2002c). Preliminary *in vitro* studies showed that PlGF can act synergistically with VEGF to induce an angiogenic response in human BEC (Autiero *et al* 2003b), suggesting its potential capacity to promote cerebral angiogenesis. However, the functional contribution of endogenous PlGF to hypoxia-induced brain angiogenesis is unknown. Furthermore, while the effects of VEGF on non-endothelial cell types such as astrocytes have been intensively studied, very little is known about the effects of PlGF in glial cells.

Hypoxia is a major regulator of VEGF expression. However, studies investigating the effect of oxygen levels on PlGF expression in different cell types have rendered contradictory results. Whereas Yonekura *et al.*, (1999) showed no expression of PlGF in dermal microvascular endothelial cells and bovine retinal pericytes cultured under various oxygen tensions (Yonekura *et al* 1999), direct correlation between PlGF expression with both hyperoxia (Ahmed *et al* 2000; Shyu *et al* 2008) and hypoxia (Cramer *et al* 2005; Green *et al* 2001; Nomura *et al* 1998; Torry *et al* 2009) has been demonstrated in other cell types in peripheral tissues. PlGF mRNA and protein expression has also been detected in the CNS by different groups under ischemic conditions (Beck *et al* 2002; Du *et al* 2010; Hayashi *et al* 2003) but with significant discrepancies in its spatial and temporal localization. These discrepancies are likely due to the use of different animal species (mouse and rat), strains of animals (BalbC and CD1 mice), and/or experimental conditions (permanent versus reversible MCAO). Moreover, the effect of mild chronic hypoxia (10% oxygen) on PlGF expression in the brain is unknown.

Our study indicates an overall low expression of PlGF in the brain of PlGF wild-type mice. PlGF expression was only detectable at the mRNA level in both brain homogenates and astrocytes in culture. In BEC, low concentrations of PlGF secreted protein were detected in the conditioned media, however, PlGF expression levels were significantly upregulated by hypoxia. These results indicate that i) BEC are the major source of PlGF in the mouse brain under hypoxic conditions and ii) PlGF can act in an autocrine fashion on BEC receptors, thus maximizing its synergistic effect with VEGF. This is in agreement with the presence of a heparin-binding domain in the PlGF sequence (Cao *et al* 1997), which allows the protein to bind to HSPGs in the ECM and cell surface, increasing its local concentration in the proximity of the receptor.

Contrary to PlGF, high levels of VEGF expression were detected in brain homogenates *in vivo*, and in BEC and astrocytes *in vitro*. VEGF protein expression was consistently lower in the brain of PlGF^{-/-} mice compared to that in PlGF^{+/+} animals during both normoxic and hypoxic conditions. At the cellular level, however, while no changes in VEGF protein expression were observed in hypoxic astrocytes between the two genotypes, VEGF was significantly upregulated in PlGF^{-/-} hypoxic BEC compared to the PlGF^{+/+} counterparts. All this suggests that the overall reduction in VEGF expression observed in the brain of PlGF^{-/-} animals must be caused by down-regulation of VEGF in other cellular components of the brain (e.g. neurons or pericytes), a question that remains to be investigated.

The differential effect of PlGF knockout on VEGF expression in the various cell types that constitute the neurovascular unit renders it difficult to predict the response of the cerebrovasculature to hypoxia. Overall, the low expression levels of PlGF found in the brain indicate that PlGF is most likely not the principal driver of angiogenesis in the CNS but rather a potential collaborator of VEGF, as it has been previously shown by other groups in the peripheral vascular system (Carmeliet *et al* 2001).

In vivo studies demonstrated a significant delay in the angiogenic response to hypoxia in PlGF^{-/-} mice compared to PlGF^{+/+} animals. This impaired angiogenic phenotype was further corroborated *in vitro* using mouse brain endothelial cells. While the *in vivo* studies would suggest that the delayed angiogenic response is mainly due to the combined effect of the lack of PlGF and the overall reduction of VEGF expression in the brain, the *in vitro* studies using BEC in the absence of any other component of the neurovascular unit, indicate that the main factor contributing to this delay is the lack of PlGF. As previously discussed, PlGF^{-/-} BEC exhibited a delayed angiogenic response to hypoxia despite expressing higher

levels of VEGF compared to PlGF^{+/+} BEC. This indicates that overexpression of VEGF is not sufficient to compensate for the lack of PlGF during hypoxic remodelling. PlGF^{-/-} BEC not only exhibited impaired response to endogenous VEGF but they also failed to respond to exogenous VEGF. This suggests that although PlGF levels are significantly lower than those of VEGF in the brain, PlGF is an important factor in the angiogenic switch that, in combination with VEGF, tips the balance towards an immediate angiogenic response to hypoxia. This ‘accelerating’ angiogenic effect of PlGF can be attributed to its synergistic interaction with VEGF at low concentrations. Studies performed in the peripheral vascular system also support this notion. For instance, Ziche et al., (1997), showed that although VEGF, PlGF and bFGF have similar angiogenic potency, both VEGF and PlGF induce a faster angiogenic response than bFGF (Ziche *et al* 1997). As the angiogenic process is orchestrated by the activity of multiple growth factors, the lack of one of them may impact the timing of the response without affecting the final outcome. Studies by Zhao et al. (2004) also suggest that PlGF can act as a molecular switch that increases the sensitivity of the cell to hypoxia by enhancing their responsiveness to lower concentrations of VEGF (Zhao *et al* 2004), the result of this being an early angiogenic response to hypoxia. Furthermore, Sand et al. (2011), indicate that the synergistic effect of VEGF and PlGF is highly dependent on their relative concentrations, and while low concentrations (~40 ng/ml) of PlGF enhance the lung microvascular endothelial cell migration induced by VEGF, higher concentrations of PlGF (160 ng/ml) fail to stimulate this response (Sands *et al* 2011), confirming previous studies by Carmeliet et al. (2001). All together, this shows that low concentrations of PlGF in combination with VEGF are sufficient to induce a profound biological response during angiogenesis

The molecular mechanisms responsible for the impaired angiogenic response to hypoxia observed in $\text{PlGF}^{-/-}$ mice were further investigated. No significant changes in VEGF/PlGF receptor expression were detected between the two genotypes either in normoxic or hypoxic conditions, however, preliminary data shows that the phosphorylation levels of ERK1/2 and GSK-3 β signalling transduction pathways involved in sprouting (Mavria *et al* 2006) and branching morphogenesis (Jones *et al* 2009), were attenuated, although not completely abolished.

In addition to the delayed angiogenic response observed in $\text{PlGF}^{-/-}$ animals, other vascular abnormalities were also detected in a number of vessels during the second week of hypoxia, such as enlargement of the vascular lumen accompanied by intravascular fibrinogen accumulation and extravasation. Furthermore, accumulation and extravasation of fibrinogen was associated with a disorganized and fragmented pattern of expression of claudin-5 in $\text{PlGF}^{-/-}$ vascular endothelium, indicative of BBB disruption. Fibrinogen positive vessels were in close proximity to reactive astrocytes and lack both the endothelial trophic factor VEGF and a stabilizing perivascular SMC/pericyte layer, jointly contributing to “unstable” vascular phenotype that, after the course of 21-days of hypoxia, resulted in vascular regression.

As previously described, fibrinogen accumulation in $\text{PlGF}^{-/-}$ hypoxic mice may have resulted from the increased hypoxic stress experienced by these mice within the first week, since hypoxia promotes fibrinogen clot formation (Adhami *et al* 2006; Lawson *et al* 1997). Alternatively, endothelial cells have been shown to be highly sensitive to hemodynamic shear stress, which is known to induce changes in endothelial cell morphology and gene expression through mechanotransduction mechanisms (Davies and Tripathi 1993). These changes are more acute in regions where flow disturbances occur, near arterial branches,

bifurcations and curvatures, resulting in complex spatiotemporal shear stresses, which under certain conditions can promote thrombotic susceptibility (Davies 2009). The delayed angiogenic response of $\text{PlGF}^{-/-}$ hypoxic mice may induce a compensatory increase in CBF, promoting endothelial cell activation specifically in regions of the microvessel where flow disturbances occur, leading to fibrinogen accumulation and vessel dysfunction.

In the peripheral vascular system, treatment of $\text{PlGF}^{-/-}$ mice with recombinant PlGF has been shown to promote smooth muscle cell growth and vascular stabilization through activation of VEGFR-1 (Luttun *et al* 2002c). Moreover, the PlGF-stimulated vessels did not exhibit the classical vascular edema and fibrin deposition observed in vessels treated with VEGF (Luttun *et al* 2002c). All this indicates that the aberrant vascular phenotype observed in the $\text{PlGF}^{-/-}$ mice can be rescued by administration of exogenous PlGF.

Astrocytes, compared to other CNS cell types, can survive and function in chronic hypoxic conditions for extended periods of time. Astrocytes exposed to hypoxia become reactive and increase the expression of the intermediate filament GFAP (Li *et al* 2007). However, under ischemic conditions, hypoxic astrocytes become highly vulnerable to the acidosis resulting from the accumulation of lactic acid during anaerobic glycolysis (Chen and Swanson 2003). Previous studies have shown the importance of astrocytes during ischemia for neuronal survival. They modulate ion balance, induce antioxidants and free radical scavengers, protect neurons from glutamate neurotoxicity and secrete angiogenic and neurotrophic factors. Collectively these astrocytic functions have been shown to positively influence immediate, as well as long-term, recovery post-stroke (Zhao and Rempe 2010).

Based on the importance of astrocytes for neuronal survival under ischemic conditions, we investigated the effect of PlGF knockout in hypoxia-induced astrocyte reactivity and OGD-induced astrocyte cell death.

Exposure to chronic mild hypoxic conditions induced astrocyte reactivity in the brain of both $\text{PIGF}^{+/+}$ and $\text{PIGF}^{-/-}$ mice (Appendix II), an effect that is consistent with previous studies (Li *et al* 2010) using a similar hypoxic animal model. However, prolonged hypoxia (21 days) had a more pronounced effect on astrocyte reactivity in $\text{PIGF}^{-/-}$ mice compared to $\text{PIGF}^{+/+}$ animals (Appendix II). When exposed to OGD conditions, PIGF -deficient astrocytes were more resistant to cell death than the wild-type astrocytes. This phenotype was associated with increased expression of VEGFR-2 detected in both $\text{PIGF}^{-/-}$ astrocytes in culture and in laser-captured microdissected $\text{PIGF}^{-/-}$ astrocytes from frontal brain sections (Freitas-Andrade *et al* 2008). Previous studies have demonstrated that VEGF-induced cell survival effects are associated with VEGFR-2 activation of the classic PI3K/Akt anti-apoptotic pathway (Jin *et al* 2000). However, in our study, pharmacological manipulation of this signalling pathway revealed that the OGD-resistant phenotype of $\text{PIGF}^{-/-}$ astrocytes is PI3K/Akt-independent. Moreover, the activity of this intracellular pathway had a detrimental effect on astrocyte survival (Freitas-Andrade *et al* 2008). The association of PI3K/Akt activation with neuronal survival (Jin *et al* 2000) has been the focus of several clinical studies targeting neuroprotection (Xiong *et al* 2010). For instance, pharmaceutical agents, such as the cholesterol-lowering statin, simvastatin, that induce phosphorylation of Akt and GSK-3 β (Wu *et al* 2008) have been associated, based on the National Institutes of Health Stroke Scale (NIHSS), with a significant improvement in overall recovery after traumatic brain injury. This effect was observed three days after stroke in the groups that received the drug. However, a non-significant increase in patient mortality and infection was also reported (Montaner *et al* 2008). Our study demonstrating the detrimental effect of activation of PI3K/Akt signaling pathway on astrocyte survival suggests that the short-term beneficial effect exerted by simvastatin directly on neurons may be counteracted in long term

by the loss of homeostatic properties of the injured astrocytes. The dual effect of PI3K/Akt signal transduction pathway on neurons and astrocytes should be therefore considered for the development of future neuroprotective therapeutic treatments.

Collectively, these studies reflect the need to more precisely understand the molecular mechanisms and factors involved in brain angiogenesis in order to develop more effective treatments for rehabilitation after hypoxic/ischemic conditions such as brain trauma or stroke.

Angiogenesis is initiated soon after a stroke event, and is a natural protective mechanism in the brain to restore oxygen and nutrients to the ischemic tissue (Beck and Plate 2009). Several studies have shown that stimulating angiogenesis post-stroke can improve neurological function (Beck and Plate 2009).

This thesis demonstrates that under mild hypoxic conditions PlGF is an important factor for the ‘angiogenic switch’ in the hypoxic brain. This is consistent with the reported pro-angiogenic role of PlGF in the peripheral vascular system under ischemic conditions (Luttun *et al* 2002c). The *in vivo* model of chronic mild hypoxia used in this thesis presents advantages compared to other more invasive models such as MCAO to mimic stroke in rodents. For instance, this model allows for several animals to be simultaneously exposed to exactly the same degree of hypoxia. In addition, compared to the destructive effect of focal or global cerebral ischemia, chronic mild hypoxia does not induce tissue damage and neurological deficits and is rather adaptive in nature (Li *et al* 2010). It has been shown that this model closely resembles the environmental conditions of high altitude where angiogenesis is induced as an adaptive response to chronic mild hypoxia. Furthermore, previous studies have suggested that normal physiological angiogenesis in the brain, such as the one that occurs under chronic mild hypoxia, is regulated by similar mechanisms as those

during pathological angiogenesis in brain tumors or hypoxia/ischemia (Carmeliet and Storkebaum 2002; Milner *et al* 2008). Therefore, the data extracted from these experiments can serve as a proof of principle that PlGF is critical in initiating angiogenesis under pathological situations characterized by hypoxia, including stroke. However, acute injury from ischemia is highly complex involving the expression of a plethora of factors associated with cell death, inflammation, edema, and scarring (Ratan *et al* 2007). These factors are not represented in the *in vivo* paradigm used in this thesis; although similar angiogenic mechanisms may be involved, this is a limitation of the model when extrapolating to more severe pathological hypoxic conditions.

To address this issue, in this thesis we have also performed *in vitro* studies mimicking more severe hypoxic conditions, such as OGD and 1% oxygen to evaluate the effect of lack of PlGF on astrocyte survival and BEC angiogenic response, respectively. These studies corroborate the findings obtained *in vivo* under milder hypoxic conditions and further support the hypothesis that the molecular mechanisms involved in ‘physiological adaptive’ angiogenesis highly correlate with those governing pathological angiogenic processes.

VEGF has been investigated as a potential treatment for stroke due to its potent proangiogenic (Yang *et al* 2010) and neuroprotective (Jin *et al* 2000; Matsuzaki *et al* 2001) properties. However, VEGF is also known to stimulate vessel permeability (Kilic *et al* 2006), which augments brain edema after stroke (van Bruggen *et al* 1999). These conflicting effects have raised several concerns about VEGF’s use as a therapeutic candidate (Manoonkitiwongsa 2011), and highlight the need to investigate other potential VEGF family members, such as PlGF, that exhibit similar proangiogenic properties without the vascular permeability effects (Carmeliet 2003).

This study provides evidence of PlGF as an important contributor in cerebral vascular remodelling under hypoxic conditions. We have also shown that exogenous PlGF is as effective as VEGF in protecting astrocytes from injury due to exposure to OGD. To what extent these pleiotropic activities of PlGF may be exploited for therapeutic application, for example in patients suffering global cerebral ischemia in cardiac arrest or focal stroke, is a challenging and outstanding question. Although the results presented in this thesis contribute new insight into several biological mechanisms of PlGF in the hypoxic brain, more preclinical studies are needed to evaluate the possible therapeutic effects of PlGF in pathological conditions involving hypoxia in the CNS. A summary of the key findings presented in this thesis is shown in Figure 5.1.

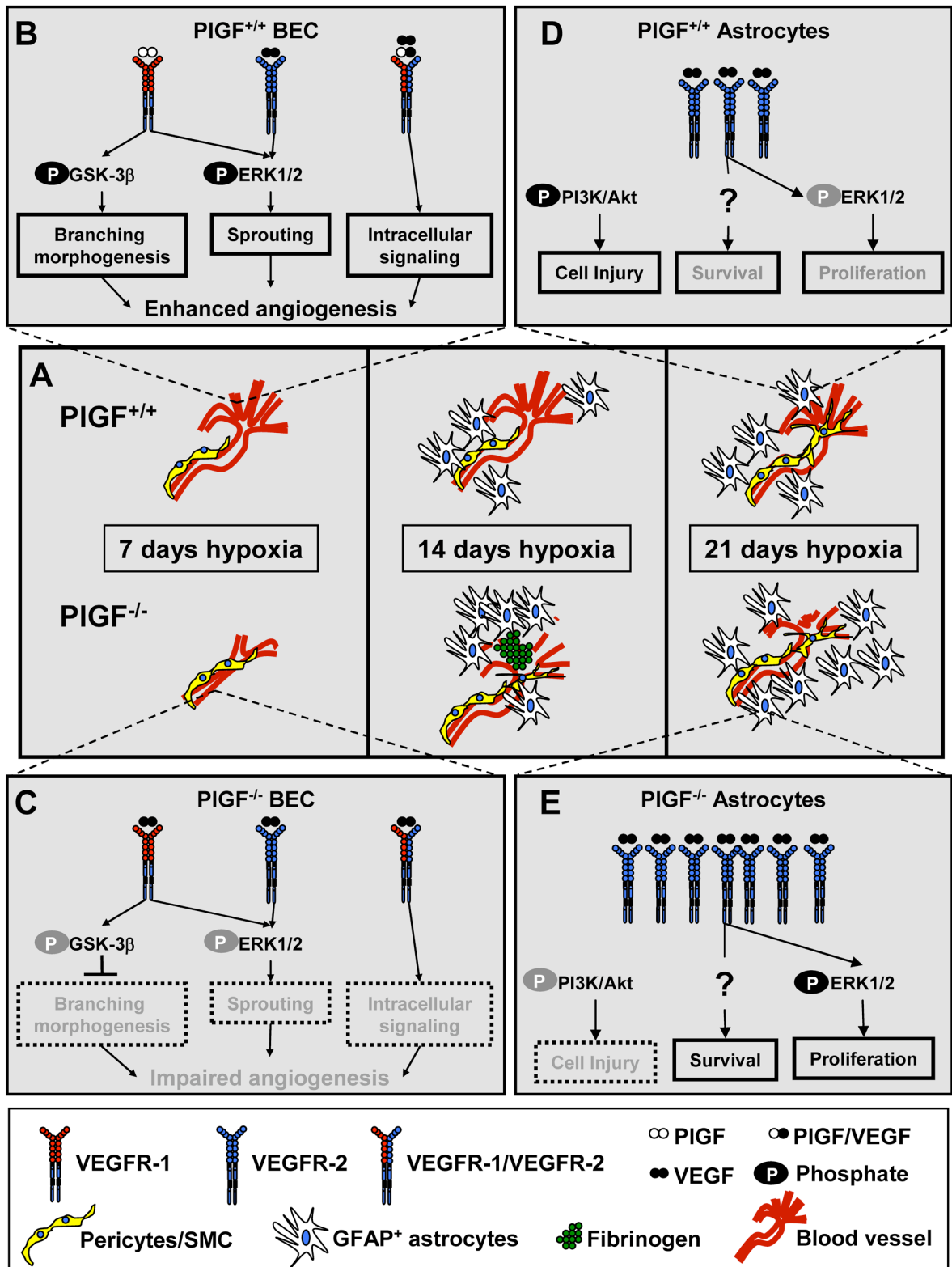
5.1 Future studies

The findings presented in this thesis demonstrate that PlGF is a critical factor in initiating the adaptive cerebrovascular remodeling process in the brain exposed to mild chronic hypoxia. However, future studies investigating whether the delayed angiogenic response of PlGF knockout would lead to increased neuronal death in mice subjected to more severe hypoxic conditions or to ischemia would highlight importance of PlGF for tissue damage outcomes in pathological conditions characterized by hypoxia.

PlGF knockout was shown to increase astrocyte proliferation and survival to OGD conditions *in vitro*. Whether this unique phenotype may prevent or reduce brain damage due to the delayed angiogenic response of PlGF^{-/-} mice to mild chronic hypoxia, needs to be investigated. Since astrocytes have been shown to play a dual role in the process of brain recovery after trauma by promoting neuronal survival through secretion of neurotrophic factors and by inhibiting neurite outgrowth through the formation of the glial scar (Chen and

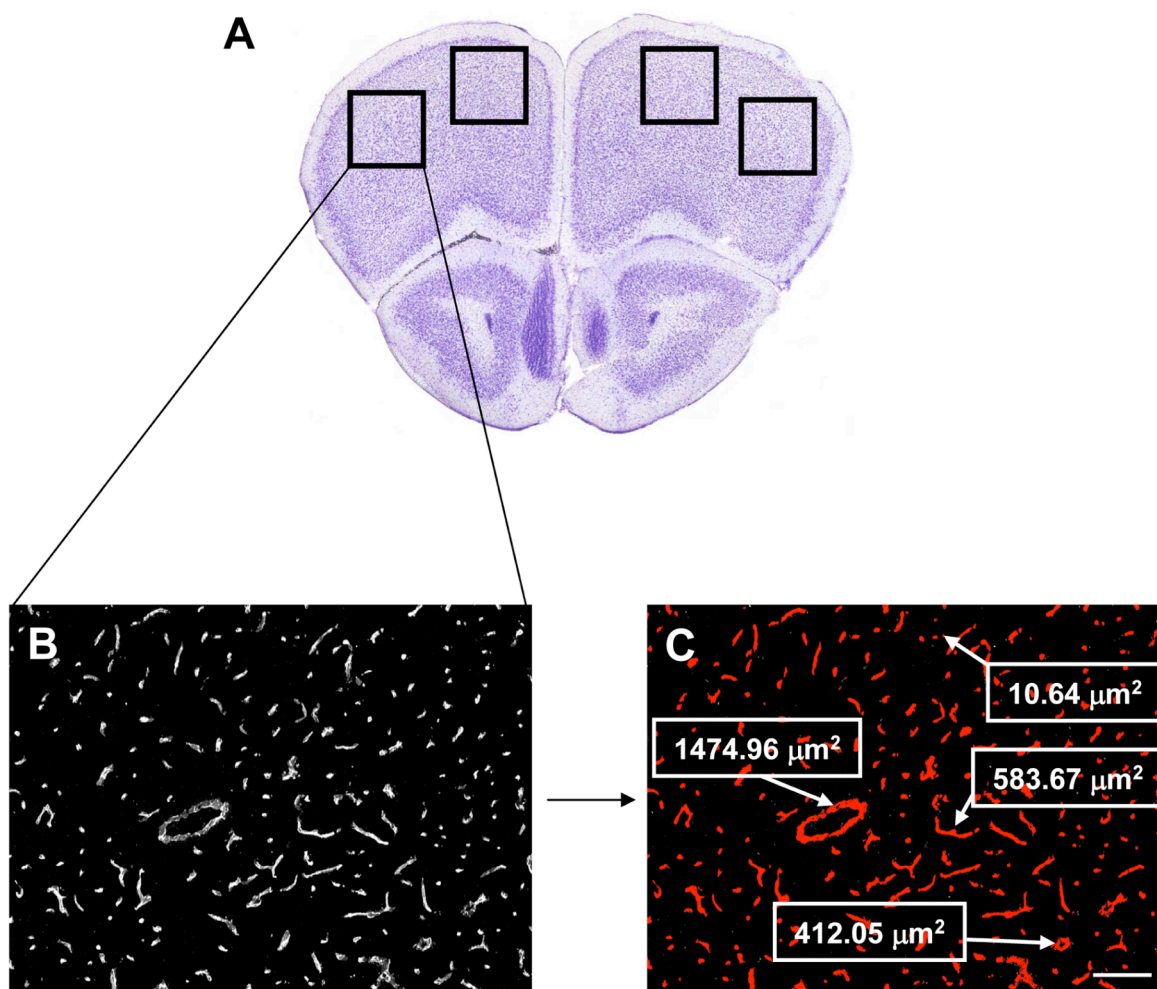
Swanson 2003), the ultimate effect of the increased proliferation and resistance of PlGF knockout astrocytes on brain recovery after stroke requires further evaluation.

Figure 5.1 Schematic representation of the effect of hypoxia on brain angiogenesis in $\text{PlGF}^{+/+}$ and $\text{PlGF}^{-/-}$ mice. After 7 days of hypoxia, an angiogenic response is induced in $\text{PlGF}^{+/+}$ mice (A), this is due to BEC secretion of VEGF, PlGF and possibly VEGF/PlGF heterodimers. These dimeric forms bind to their receptors and induce specific intracellular signaling that contributes to the angiogenic response in BEC (B). Activation of VEGFR-1 by PlGF promotes sprouting and increased vascular networks, by inducing ERK1/2 and GSK-3 β phosphorylation (B). In $\text{PlGF}^{-/-}$ mice this effect is delayed until 14 days of hypoxia (A). The mechanism is likely due to lack of PlGF, which reduces the diversity of the different ligands that could be produced, and reduces the diversity of intracellular signaling that occurs in $\text{PlGF}^{-/-}$ BEC despite increased VEGF expression (C). The delayed angiogenic response likely promotes hypoxic stress in $\text{PlGF}^{-/-}$ mice, which leads to vascular accumulation of fibrinogen and extravasation (A). This affects vessel integrity, which ultimately may result in vessel regression after 21-days of hypoxia (A). Astrocyte reactivity was shown to be significantly higher in $\text{PlGF}^{-/-}$ mice *in vivo* after 21 days of hypoxia (A). *In vitro* studies show that $\text{PlGF}^{-/-}$ astrocytes express higher levels of VEGFR-2 leading to a more proliferative and resistant phenotype, to OGD exposure, compared to wild-type astrocytes (D and E).



APPENDICES

Appendix I



Supplementary Figure 1 Example of the analysis performed by the image analysis program, Image-Pro® Plus (A-C). Representative image of one of the three sections used from the frontal cortex (Bregma 2.80mm); the outlined boxes represent the four 10X images taken from the cerebral cortex (A). A grey scale representation of one of the 10X images immunostained with the CD31 vessel marker and analyzed by the image analysis software (B). An example of the “intensity range selection” analysis performed by Image-Pro® Plus; the areas in red delineate the surface of the CD31⁺ vessels measured by the software. Some examples of microvessel size (area) are denoted in the figure, largest object is 1474.96 μm^2 and the smallest object is 10.64 μm^2 . Scale bar = 100 μm (C).

Appendix II

7 days	PIGF+/+		PIGF-/-		
	N	H	N	H	
	0-100 μm^2	89.5	76.3	88.2	85.9
	150-500 μm^2	10.0	21.5	11.1	13.3
> 500 μm^2	0.4	2.0	0.5	0.7	

14 days	PIGF+/+		PIGF-/-		
	N	H	N	H	
	0-100 μm^2	87.7	74.9	87.2	74.0
	150-500 μm^2	11.5	22.8	12.0	23.5
> 500 μm^2	0.6	2.2	0.6	2.3	

21 days	PIGF+/+		PIGF-/-		
	N	H	N	H	
	0-100 μm^2	88.3	76.0	84.9	78.7
	150-500 μm^2	11.1	22.0	14.4	19.9
> 500 μm^2	0.4	1.9	0.5	1.3	

Table A2 (A) Proportion of vessels (percentages with respect to total number of vessels) within each size interval (0-100 μm^2 , 150-500 μm^2 and >500 μm^2) for each time point. N = normoxia and H = hypoxia

Appendix III

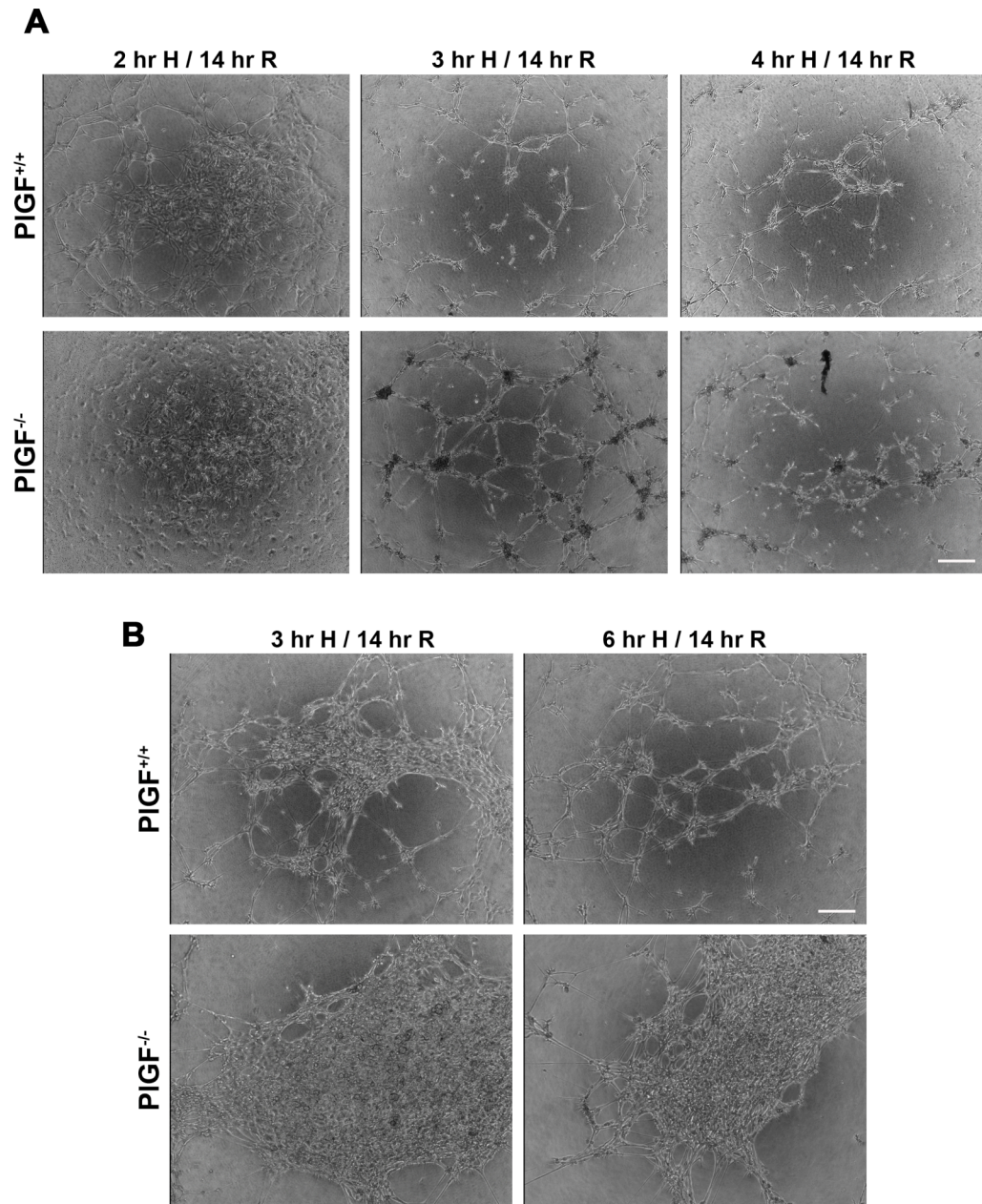


Figure A3 (A) Capillary-like networks (CLN) induced by 2 hr, 3 hr, and 4 hr hypoxia (H) followed by 14 hr reoxygenation (R), and (B) 3 hr and 6 hr hypoxia (H) followed by 14 hr reoxygenation (R) on PIGF^{+/+} and PIGF^{-/-} mouse BEC seeded on Matrigel. In both experiments PIGF^{-/-} BEC show a delayed angiogenic response to hypoxia. Scale bar = 50 μ m.

Appendix IV

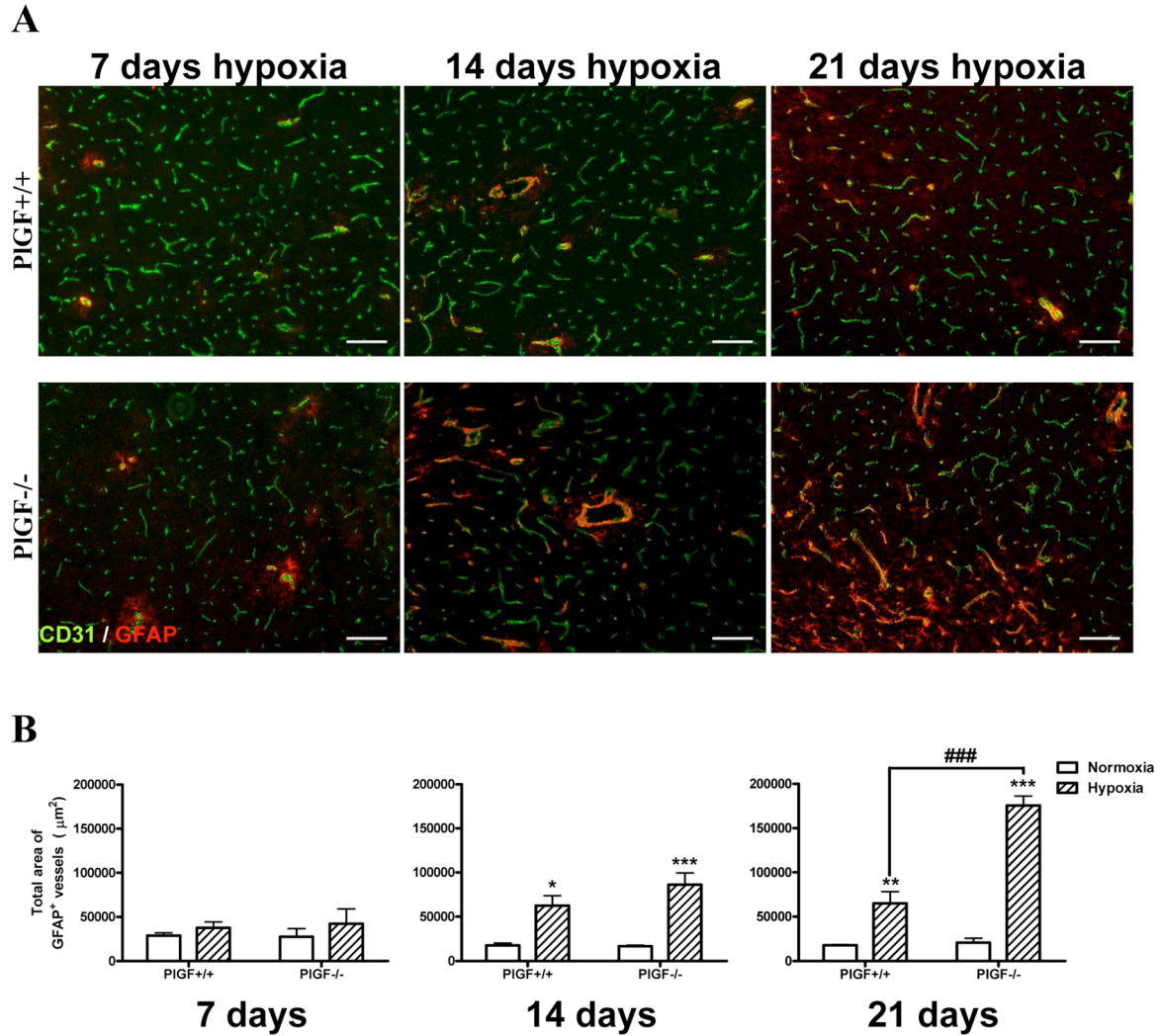


Figure A4 (A) Representative images of sections of the cerebral cortex from PIGF^{+/+} and PIGF^{-/-} mice exposed to normoxia or 7-, 14-, or 21-day hypoxia. Sections were immunostained for the endothelial marker, CD31 (green) and astrocyte marker GFAP (red). Scale bar = 100 μm. (B) Quantification of total area of GFAP⁺ cells after 7-, 14-, or 21-day normoxia or hypoxia as described in Material and Methods. Results expressed are means ± s.e.m. *** indicates significant difference (p < 0.001) between hypoxic groups and the corresponding normoxic control groups and ## indicates significant difference (p < 0.01) between hypoxic PIGF^{+/+} and PIGF^{-/-} groups (ANOVA followed by Bonferroni post-test).

Appendix V

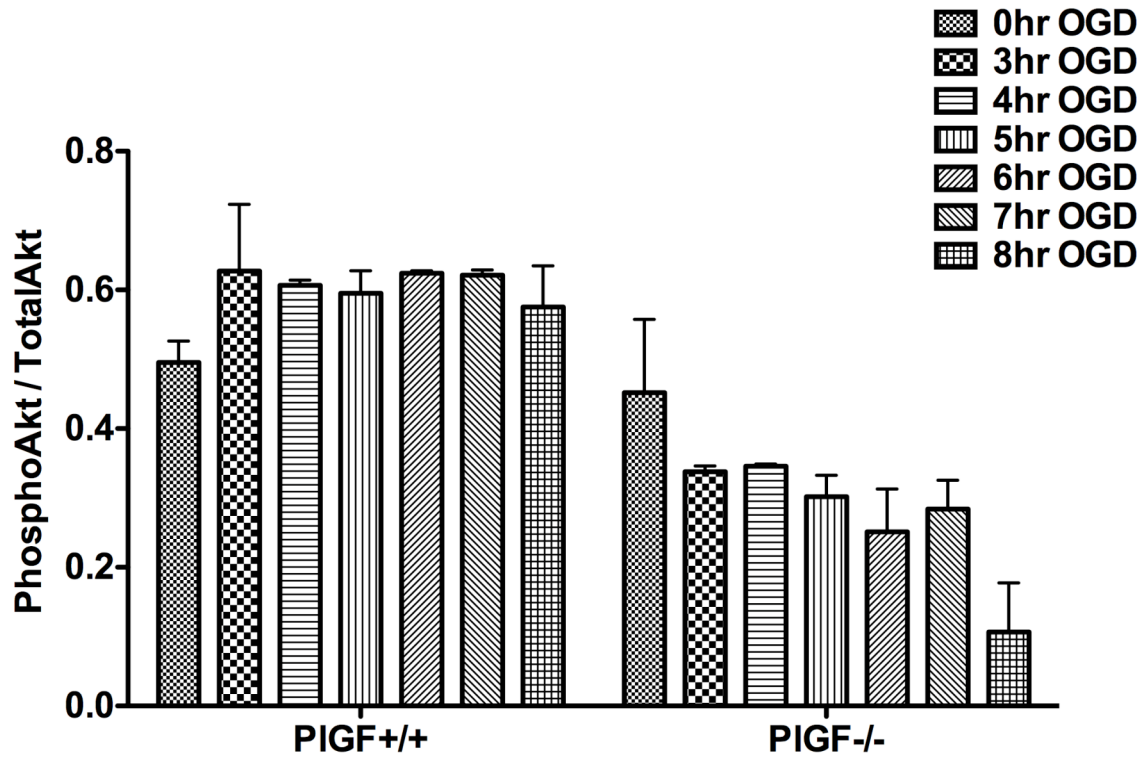


Figure A5 (A) Densitometric analysis of Akt phosphorylation in PIGF^{+/+} and PIGF^{-/-} astrocytes exposed to OGD time course. Results expressed are means $\pm$ s.e.m of two independent experiments.

Appendix VI

Isolation and Culture of Mouse Astrocytes

Day1:

1. Autoclave: 1 Filter/extraction, (plus one extra filter), two times two sets of tweezers and 1 scissors, 53um membrane. All should be autoclaved for 20min steam, 15min dry.
2. Mouse Astrocyte Media (MAS): 500ml DME+50ml Australian FBS (10%)+5ml 100X antibiotic/antimycotic
3. In 500ml HBSS add 5ml 100X antibiotic/antimycotic store at 4C
4. Prepare Dispase 12mg/ml, trypsin, and Poly-L-lysine

Day2:

1. Coat 250ml culture dish with 10ml Poly-L-lysine let it sit for 1hr. Rinse Poly-L-lysine 2X with 10ml HBSS.
2. Fill three petri dishes with HBSS +antibiotic/antimycotic (Step 3 day1) and place them on ice.
3. Put Ice packs in laminar flow hood place three 35mm dish on ice pack. Fill 2 with 10ml DME
4. Warm MAS and Dispase in 37C water bath
5. Spray 8 day old mouse with ETOH and cut off head. Rinse head in each Petri dish (step2 day2). Do this for 4-5 mice. Bring last dish with heads to hood.
6. Remove brain and transfer brain to dish with DME
7. Remove Olfactory Bulb, Superior and Inferior Colliculus, and Cerebellum. Keep Cerebral cortex (Fig. 1).
8. Transfer cortex to second DME dish.
9. With 10ml syringe suck up brain and put 18G 1 1/2 needle on syringe flush out brain.
10. Repeat step 9 with 23G 3/4 needle, and again with a 25G 5/8 needle but this time flush homogenized brain into 3rd (empty) 35mm dish.

11. Add 1ml Dispase to homogenized brain and incubate at 37C for 15-20 min NO longer.
12. Remove dish from incubator and pass through a 53um filter. Rinse dish with 10ml DME and pass through filter do this 2X.
13. Pour filtrate in 50ml conical tube, and spin for 10min at 1500rpm, 4C
14. Resuspend each pellet in 10ml MAS for plating taking care to break up clumps with gentle pipetting. Cells are plated in one T-80.

Day3:

Wash with pre-warmed HBSS 3X 10ml each rinse. Then add 10 ml pre-warmed MAS.

After initial wash change media every 3-4 days

Once cells are well established, 7-14 days after plating, trypsinize 1:3

Cells can be frozen in liquid nitrogen in 90% FBS + 10% DMSO.

Note!! These cells lose angiogenic markers after passage 2

ISOLATION AND CULTIVATION OF MOUSE BRAIN MICROVASCULAR ENDOTHELIAL CELLS

Prepare in Advance

Instruments: Autoclave Day Before or allow to cool several hours before use.

Gross dissection: 1. big scissors for head
 2. small scissors for brain
 3. two pairs of forceps

Laminar Flow Hood dissection 1. small scissors for dissection
 2. forceps for dissection
 3. forceps for filter papers

Filter Units: - 2 of them per cell type

- Nitex filter papers. Have 2 or 3 of each just in case but don't repeat autoclaving too many times – will deteriorate with repeat autoclaving
- cut to size with template. One is 112 μ m size, other is 20 μ m

Homogenizer – one of each per cell type

Media

DME	28.3ml
Human Serum, heat inactivated	5ml
FBS, heat inactivated	5ml
MCM media	10ml need to filter
ECGS, 1.5mg/ml	0.75ml need to filter. Endothelial Cell Growth Serum
ITS, 1000x	0.25ml (Insulin/Transferin/Selenium)
Heparin, 2500 units/ml	0.2ml need to filter Used to inhibit pericyte growth
Penicillin-Streptomycin solu. 100x	0.5ml
Total Volume	50ml

Day Of:

DME + Antibiotics/Antimycotics (AB/AM): 20-40 mls DME, depending on # of pups. Add 200ul AB/AM to 20mls or 400ul to 40mls

Collagenase Mixture: 10-12 mls DME + 10-12mg Collagensase IV.

Needs to be sterile filtered before use.

Gelatin coating: 0.5% in HBSS. Use 1ml for each well in 6well plate. Coat before beginning isolation and remove just before plating. Don't let dry and no need for rinsing. Minimum coating time is 5min. Also need to coat T-25 cell culture flask at time of passage.

Dissection and Isolation

1. Anesthetize mice. Cut head off and remove brain from 19 day old mice and place in 50mL Epp tube with cold DME+ antibiotic/antimycotic (on ice). Can use mice between 16-19 days old. Generally younger mice yield more cells. Five brains per dish of DME.

2. Put dish in laminar flow hood and pour brains into a 100mm dish with cold straight DME (on ice pack). Using sterile forceps, transfer brain to 60mm dish filled with approx. 10mls straight DME.
3. Dissect specimen: Remove Olfactory Bulb, Superior and Inferior Colliculus and Cerebellum. Keep Cerebral cortex. Transfer dissected brains to 2nd 60mm dish with approx. 10mls straight DME.
4. Using 10ml syringe suck up brains and inject into 10ml Wheaton Dows homogenizer. Using Teflon pestel homogenize brain manually until mixture becomes pink and no large brain chunks are left. Up and down approx 20x
5. Place Nitex filters into units. Pour homogenate directly into unit with the 112 μ m filter. Pour 10mls of cold HBSS into the homogenizer to rinse and add to filter unit. Pour an additional 50mls of cold HBSS into unit. If collecting vessels 112-360 μ m or smooth muscle cells for culture using sterile forceps place Nitex filter into sterile tube of 40mls cold HBSS.
6. Pass filtrate through second filter unit with 20 μ m Nitex filter. Add another 50mls of cold HBSS on top. NOTE: this filtrate will take time to pass filter. Gentle vacuum may be applied if necessary. Using sterile forceps remove Nitex filter from unit and place into tube of 40mls cold HBSS. This fraction is used for HBEC microvascular endothelial cultures.
7. Gently invert tube containing filter approx 20x to remove all vessels from the mesh. Remove mesh from tube.

8. Centrifuge the microvessl suspension at 2200 rpm (or 1000xg) in the Beckman J6B at 4 degrees Celsius for 15 minutes.
9. Carefully aspirate the HBSS supernatant without disrupting the pellet and add 10-12 ml of cold Collagenase IV (1mg/ml in base media plain DME or M199). Aspirate with 10ml pipet a few times to resuspend pellet and incubate at 37~ for 6 minutes shaking periodically (NOT LONGER!!)
10. Centrifuge the suspension at 1500 rpm (or 500xg) in Beckman J6B at 4 degrees for 10 min.
11. Aspirate the collagenase and resuspend the pellet in desired volume (1.5ml in 1 well of 6 well plate) of initial primary media for plating taking care to break up clumps with gentle pipetting. Cells are plated on 0.5% gelatin coated plastic. Fill other 5 wells in 6 well plate with HBSS or sterile water. Can consider plating into smaller well plates and observing growth. Then collect 2 best wells (least contaminated) for placement into T25. For 5 pups or less put into 12well plate.

Purmycin: Add 100ul of 80ug/ml in DME to 6 well plate and 50ul if using 12 well. Should be removed approx 30hrs later by replacing media with fresh.

12. Cells are fed four days after plating. Note!! NO WASHING. Continue feeding every 3-4 days , full fluid changes
13. Once cells are well established, usually 10-15 days after plating, trypsinize with Endothelial Trypsin and passage into T25 flask or larger well plate.

14. Passage at 1:3 split ratio from then on, depending on culture growth. Cells can be frozen in liquid nitrogen in 90% FBS + 10% DMSO.
15. Observe cells daily to note growth and morphology.

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REFERENCE

- Abbott NJ, Ronnback L, Hansson E (2006) Astrocyte-endothelial interactions at the blood-brain barrier. *Nat Rev Neurosci* 7:41-53
- Adhami F, Liao G, Morozov YM, Schloemer A, Schmithorst VJ, Lorenz JN, Dunn RS, Vorhees CV, Wills-Karp M, Degen JL, Davis RJ, Mizushima N, Rakic P, Dardzinski BJ, Holland SK, Sharp FR, Kuan CY (2006) Cerebral ischemia-hypoxia induces intravascular coagulation and autophagy. *Am J Pathol* 169:566-83
- Adini A, Kornaga T, Firoozbakht F, Benjamin LE (2002) Placental growth factor is a survival factor for tumor endothelial cells and macrophages. *Cancer Res* 62:2749-52
- Ahmed A, Dunk C, Ahmad S, Khaliq A (2000) Regulation of placental vascular endothelial growth factor (VEGF) and placenta growth factor (PlGF) and soluble Flt-1 by oxygen--a review. *Placenta* 21 Suppl A:S16-24
- Al Ahmad A, Gassmann M, Ogunshola OO (2009) Maintaining blood-brain barrier integrity: pericytes perform better than astrocytes during prolonged oxygen deprivation. *J Cell Physiol* 218:612-22
- Al Ahmad A, Taboada CB, Gassmann M, Ogunshola OO (2010) Astrocytes and pericytes differentially modulate blood-brain barrier characteristics during development and hypoxic insult. *J Cereb Blood Flow Metab*
- Amano T, Meyer JS, Okabe T, Shaw T, Mortel KF (1982) Stable xenon CT cerebral blood flow measurements computed by a single compartment--double integration model in normal aging and dementia. *J Comput Assist Tomogr* 6:923-32
- Anderson CM, Nedergaard M (2003) Astrocyte-mediated control of cerebral microcirculation. *Trends Neurosci* 26:340-4; author reply 4-5
- Anderson MF, Blomstrand F, Blomstrand C, Eriksson PS, Nilsson M (2003) Astrocytes and stroke: networking for survival? *Neurochem Res* 28:293-305
- Armulik A, Abramsson A, Betsholtz C (2005) Endothelial/pericyte interactions. *Circ Res* 97:512-23
- Armulik A, Genove G, Mae M, Nisancioglu MH, Wallgard E, Niaudet C, He L, Norlin J, Lindblom P, Strittmatter K, Johansson BR, Betsholtz C (2010) Pericytes regulate the blood-brain barrier. *Nature* 468:557-61
- Arroyo J, Torry RJ, Torry DS (2004) Differential regulation of placenta growth factor (PlGF)-mediated signal transduction in human primary term trophoblast and endothelial cells. *Placenta* 25:379-86

Augustin HG, Koh GY, Thurston G, Alitalo K (2009) Control of vascular morphogenesis and homeostasis through the angiopoietin-Tie system. *Nat Rev Mol Cell Biol* 10:165-77

Autiero M, Luttun A, Tjwa M, Carmeliet P (2003a) Placental growth factor and its receptor, vascular endothelial growth factor receptor-1: novel targets for stimulation of ischemic tissue revascularization and inhibition of angiogenic and inflammatory disorders. *J Thromb Haemost* 1:1356-70

Autiero M, Waltenberger J, Communi D, Kranz A, Moons L, Lambrechts D, Kroll J, Plaisance S, De Mol M, Bono F, Kliche S, Fellbrich G, Ballmer-Hofer K, Maglione D, Mayr-Beyrle U, Dewerchin M, Dombrowski S, Stanimirovic D, Van Hummelen P, Dehio C, Hicklin DJ, Persico G, Herbert JM, Communi D, Shibuya M, Collen D, Conway EM, Carmeliet P (2003b) Role of PlGF in the intra- and intermolecular cross talk between the VEGF receptors Flt1 and Flk1. *Nat Med* 9:936-43

Bachoo RM, Kim RS, Ligon KL, Maher EA, Brennan C, Billings N, Chan S, Li C, Rowitch DH, Wong WH, DePinho RA (2004) Molecular diversity of astrocytes with implications for neurological disorders. *Proc Natl Acad Sci U S A* 101:8384-9

Bamforth SD, Kniesel U, Wolburg H, Engelhardt B, Risau W (1999) A dominant mutant of occludin disrupts tight junction structure and function. *J Cell Sci* 112 (Pt 12):1879-88

Basilico C, Moscatelli D (1992) The FGF family of growth factors and oncogenes. *Adv Cancer Res* 59:115-65

Bates DO, Cui TG, Doughty JM, Winkler M, Sugiono M, Shields JD, Peat D, Gillatt D, Harper SJ (2002) VEGF165b, an inhibitory splice variant of vascular endothelial growth factor, is down-regulated in renal cell carcinoma. *Cancer Res* 62:4123-31

Bazzoni G, Dejana E (2004) Endothelial cell-to-cell junctions: molecular organization and role in vascular homeostasis. *Physiol Rev* 84:869-901

Beall CM, Brittenham GM, Strohl KP, Blangero J, Williams-Blangero S, Goldstein MC, Decker MJ, Vargas E, Villena M, Soria R, Alarcon AM, Gonzales C (1998) Hemoglobin concentration of high-altitude Tibetans and Bolivian Aymara. *Am J Phys Anthropol* 106:385-400

Beck H, Acker T, Puschel AW, Fujisawa H, Carmeliet P, Plate KH (2002) Cell type-specific expression of neuropilins in an MCA-occlusion model in mice suggests a potential role in post-ischemic brain remodeling. *J Neuropathol Exp Neurol* 61:339-50

Beck H, Semisch M, Culmsee C, Plesnila N, Hatzopoulos AK (2008) Egr-1 regulates expression of the glial scar component phosphacan in astrocytes after experimental stroke. *Am J Pathol* 173:77-92

- Beck H, Plate KH (2009) Angiogenesis after cerebral ischemia. *Acta Neuropathol* 117:481-96
- Bell MA, Ball MJ (1990) Neuritic plaques and vessels of visual cortex in aging and Alzheimer's dementia. *Neurobiol Aging* 11:359-70
- Bell RD, Deane R, Chow N, Long X, Sagare A, Singh I, Streb JW, Guo H, Rubio A, Van Nostrand W, Miano JM, Zlokovic BV (2009) SRF and myocardin regulate LRP-mediated amyloid-beta clearance in brain vascular cells. *Nat Cell Biol* 11:143-53
- Bell RD, Winkler EA, Sagare AP, Singh I, LaRue B, Deane R, Zlokovic BV (2010) Pericytes control key neurovascular functions and neuronal phenotype in the adult brain and during brain aging. *Neuron* 68:409-27
- Bellik L, Vinci MC, Filippi S, Ledda F, Parenti A (2005) Intracellular pathways triggered by the selective FLT-1-agonist placental growth factor in vascular smooth muscle cells exposed to hypoxia. *Br J Pharmacol* 146:568-75
- Blazquez C, Cook N, Micklem K, Harris AL, Gatter KC, Pezzella F (2006) Phosphorylated KDR can be located in the nucleus of neoplastic cells. *Cell Res* 16:93-8
- Bondarenko A, Chesler M (2001) Rapid astrocyte death induced by transient hypoxia, acidosis, and extracellular ion shifts. *Glia* 34:134-42
- Brunet A, Datta SR, Greenberg ME (2001) Transcription-dependent and -independent control of neuronal survival by the PI3K-Akt signaling pathway. *Curr Opin Neurobiol* 11:297-305
- Burbach GJ, Dehn D, Nagel B, Del Turco D, Deller T (2004) Laser microdissection of immunolabeled astrocytes allows quantification of astrocytic gene expression. *J Neurosci Methods* 138:141-8
- Cai J, Ahmad S, Jiang WG, Huang J, Kontos CD, Boulton M, Ahmed A (2003) Activation of vascular endothelial growth factor receptor-1 sustains angiogenesis and Bcl-2 expression via the phosphatidylinositol 3-kinase pathway in endothelial cells. *Diabetes* 52:2959-68
- Cao R, Xue Y, Hedlund EM, Zhong Z, Tritsarlis K, Tondelli B, Lucchini F, Zhu Z, Dissing S, Cao Y (2010) VEGFR1-mediated pericyte ablation links VEGF and PlGF to cancer-associated retinopathy. *Proc Natl Acad Sci U S A* 107:856-61
- Cao Y, Chen H, Zhou L, Chiang MK, Anand-Apte B, Weatherbee JA, Wang Y, Fang F, Flanagan JG, Tsang ML (1996a) Heterodimers of placenta growth factor/vascular endothelial growth factor. Endothelial activity, tumor cell expression, and high affinity binding to Flk-1/KDR. *J Biol Chem* 271:3154-62

- Cao Y, Linden P, Shima D, Browne F, Folkman J (1996b) In vivo angiogenic activity and hypoxia induction of heterodimers of placenta growth factor/vascular endothelial growth factor. *J Clin Invest* 98:2507-11
- Cao Y, Ji WR, Qi P, Rosin A, Cao Y (1997) Placenta growth factor: identification and characterization of a novel isoform generated by RNA alternative splicing. *Biochem Biophys Res Commun* 235:493-8
- Cao Y (2009) Positive and negative modulation of angiogenesis by VEGFR1 ligands. *Sci Signal* 2:re1
- Cardoso FL, Brites D, Brito MA (2010) Looking at the blood-brain barrier: molecular anatomy and possible investigation approaches. *Brain Res Rev* 64:328-63
- Carmeliet P (1999) Basic Concepts of (Myocardial) Angiogenesis: Role of Vascular Endothelial Growth Factor and Angiopoietin. *Curr Interv Cardiol Rep* 1:322-35
- Carmeliet P (2000) Mechanisms of angiogenesis and arteriogenesis. *Nat Med* 6:389-95
- Carmeliet P, Moons L, Luttun A, Vincenti V, Compernelle V, De Mol M, Wu Y, Bono F, Devy L, Beck H, Scholz D, Acker T, DiPalma T, Dewerchin M, Noel A, Stalmans I, Barra A, Blacher S, Vandendriessche T, Ponten A, Eriksson U, Plate KH, Foidart JM, Schaper W, Charnock-Jones DS, Hicklin DJ, Herbert JM, Collen D, Persico MG (2001) Synergism between vascular endothelial growth factor and placental growth factor contributes to angiogenesis and plasma extravasation in pathological conditions. *Nat Med* 7:575-83
- Carmeliet P, Storkebaum E (2002) Vascular and neuronal effects of VEGF in the nervous system: implications for neurological disorders. *Semin Cell Dev Biol* 13:39-53
- Carmeliet P (2003) Angiogenesis in health and disease. *Nat Med* 9:653-60
- Carmeliet P (2005a) Angiogenesis in life, disease and medicine. *Nature* 438:932-6
- Carmeliet P (2005b) VEGF as a key mediator of angiogenesis in cancer. *Oncology* 69 Suppl 3:4-10
- Cebe Suarez S, Pieren M, Cariolato L, Arn S, Hoffmann U, Bogucki A, Manlius C, Wood J, Ballmer-Hofer K (2006) A VEGF-A splice variant defective for heparan sulfate and neuropilin-1 binding shows attenuated signaling through VEGFR-2. *Cell Mol Life Sci* 63:2067-77
- Chaballe L, Close P, Sempels M, Delstanche S, Fanielle J, Moons L, Carmeliet P, Schoenen J, Chariot A, Franzen R (2011) Involvement of placental growth factor in Wallerian degeneration. *Glia* 59:379-96
- Chen Y, Swanson RA (2003) Astrocytes and brain injury. *J Cereb Blood Flow Metab* 23:137-49

Choi JS, Kim HY, Cha JH, Choi JY, Chun MH, Lee MY (2007) Upregulation of vascular endothelial growth factor receptors Flt-1 and Flk-1 in rat hippocampus after transient forebrain ischemia. *J Neurotrauma* 24:521-31

Chopp M, Zhang ZG, Jiang Q (2007) Neurogenesis, angiogenesis, and MRI indices of functional recovery from stroke. *Stroke* 38:827-31

Chow J, Ogunshola O, Fan SY, Li Y, Ment LR, Madri JA (2001) Astrocyte-derived VEGF mediates survival and tube stabilization of hypoxic brain microvascular endothelial cells in vitro. *Brain Res Dev Brain Res* 130:123-32

Clauss M, Weich H, Breier G, Knies U, Rockl W, Waltenberger J, Risau W (1996) The vascular endothelial growth factor receptor Flt-1 mediates biological activities. Implications for a functional role of placenta growth factor in monocyte activation and chemotaxis. *J Biol Chem* 271:17629-34

Cramer M, Nagy I, Murphy BJ, Gassmann M, Hottiger MO, Georgiev O, Schaffner W (2005) NF-kappaB contributes to transcription of placenta growth factor and interacts with metal responsive transcription factor-1 in hypoxic human cells. *Biol Chem* 386:865-72

Cross MJ, Dixelius J, Matsumoto T, Claesson-Welsh L (2003) VEGF-receptor signal transduction. *Trends Biochem Sci* 28:488-94

Cuevas P, Gutierrez-Diaz JA, Reimers D, Dujovny M, Diaz FG, Ausman JI (1984) Pericyte endothelial gap junctions in human cerebral capillaries. *Anat Embryol (Berl)* 170:155-9

Dai J, Rabie AB (2007) VEGF: an essential mediator of both angiogenesis and endochondral ossification. *J Dent Res* 86:937-50

Daneman R, Zhou L, Kebede AA, Barres BA (2010) Pericytes are required for blood-brain barrier integrity during embryogenesis. *Nature* 468:562-6

Davies PF, Tripathi SC (1993) Mechanical stress mechanisms and the cell. An endothelial paradigm. *Circ Res* 72:239-45

Davies PF (2009) Hemodynamic shear stress and the endothelium in cardiovascular pathophysiology. *Nat Clin Pract Cardiovasc Med* 6:16-26

De Smet F, Segura I, De Bock K, Hohensinner PJ, Carmeliet P (2009) Mechanisms of vessel branching: filopodia on endothelial tip cells lead the way. *Arterioscler Thromb Vasc Biol* 29:639-49

de Vries C, Escobedo JA, Ueno H, Houck K, Ferrara N, Williams LT (1992) The fms-like tyrosine kinase, a receptor for vascular endothelial growth factor. *Science* 255:989-91

Dehouck MP, Meresse S, Delorme P, Fruchart JC, Cecchelli R (1990) An easier, reproducible, and mass-production method to study the blood-brain barrier in vitro. *J Neurochem* 54:1798-801

Deitmer JW, Rose CR (1996) pH regulation and proton signalling by glial cells. *Prog Neurobiol* 48:73-103

del Zoppo GJ (2008) Virchow's triad: the vascular basis of cerebral injury. *Rev Neurol Dis* 5 Suppl 1:S12-21

del Zoppo GJ (2009) Relationship of neurovascular elements to neuron injury during ischemia. *Cerebrovasc Dis* 27 Suppl 1:65-76

del Zoppo GJ (2010) The neurovascular unit in the setting of stroke. *J Intern Med* 267:156-71

Diaz-Flores L, Gutierrez R, Madrid JF, Varela H, Valladares F, Acosta E, Martin-Vasallo P, Diaz-Flores L, Jr. (2009) Pericytes. Morphofunction, interactions and pathology in a quiescent and activated mesenchymal cell niche. *Histol Histopathol* 24:909-69

Dimmeler S, Fleming I, Fisslthaler B, Hermann C, Busse R, Zeiher AM (1999) Activation of nitric oxide synthase in endothelial cells by Akt-dependent phosphorylation. *Nature* 399:601-5

DiPalma T, Tucci M, Russo G, Maglione D, Lago CT, Romano A, Saccone S, Della Valle G, De Gregorio L, Dragani TA, Viglietto G, Persico MG (1996) The placenta growth factor gene of the mouse. *Mamm Genome* 7:6-12

DiSalvo J, Bayne ML, Conn G, Kwok PW, Trivedi PG, Soderman DD, Palisi TM, Sullivan KA, Thomas KA (1995) Purification and characterization of a naturally occurring vascular endothelial growth factor.placenta growth factor heterodimer. *J Biol Chem* 270:7717-23

Donnini S, Machein MR, Plate KH, Weich HA (1999) Expression and localization of placenta growth factor and PlGF receptors in human meningiomas. *J Pathol* 189:66-71

Dore-Duffy P, LaManna JC (2007) Physiologic angiodynamics in the brain. *Antioxid Redox Signal* 9:1363-71

Dore-Duffy P, Wang X, Mehedi A, Kreipke CW, Rafols JA (2007) Differential expression of capillary VEGF isoforms following traumatic brain injury. *Neurol Res* 29:395-403

Drogat B, Auguste P, Nguyen DT, Bouche-careilh M, Pineau R, Nalbantoglu J, Kaufman RJ, Chevet E, Bikfalvi A, Moenner M (2007) IRE1 signaling is essential for ischemia-induced vascular endothelial growth factor-A expression and contributes to angiogenesis and tumor growth in vivo. *Cancer Res* 67:6700-7

Du H, Li P, Pan Y, Li W, Hou J, Chen H, Wang J, Tang H (2010) Vascular endothelial growth factor signaling implicated in neuroprotective effects of placental growth factor in an in vitro ischemic model. *Brain Res* 1357:1-8

Du Y, Shi L, Li J, Xiong J, Li B, Fan X (2011) Angiogenesis and improved cerebral blood flow in the ischemic boundary area were detected after electroacupuncture treatment to rats with ischemic stroke. *Neurol Res* 33:101-7

Duits AJ, Rodriguez T, Schnog JJ (2006) Serum levels of angiogenic factors indicate a pro-angiogenic state in adults with sickle cell disease. *Br J Haematol* 134:116-9

el-Bacha RS, Minn A (1999) Drug metabolizing enzymes in cerebrovascular endothelial cells afford a metabolic protection to the brain. *Cell Mol Biol (Noisy-le-grand)* 45:15-23

Eliceiri BP, Klemke R, Stromblad S, Cheresh DA (1998) Integrin alphavbeta3 requirement for sustained mitogen-activated protein kinase activity during angiogenesis. *J Cell Biol* 140:1255-63

Eriksson A, Cao R, Pawliuk R, Berg SM, Tsang M, Zhou D, Fleet C, Tritsarlis K, Dissing S, Leboulch P, Cao Y (2002) Placenta growth factor-1 antagonizes VEGF-induced angiogenesis and tumor growth by the formation of functionally inactive PlGF-1/VEGF heterodimers. *Cancer Cell* 1:99-108

Escudero-Esparza A, Martin TA, Douglas-Jones A, Mansel RE, Jiang WG (2010) PGF isoforms, PLGF-1 and PGF-2 and the PGF receptor, neuropilin, in human breast cancer: prognostic significance. *Oncol Rep* 23:537-44

Esser S, Lampugnani MG, Corada M, Dejana E, Risau W (1998) Vascular endothelial growth factor induces VE-cadherin tyrosine phosphorylation in endothelial cells. *J Cell Sci* 111 (Pt 13):1853-65

Farkas E, De Jong GI, Apro E, De Vos RA, Steur EN, Luiten PG (2000) Similar ultrastructural breakdown of cerebrocortical capillaries in Alzheimer's disease, Parkinson's disease, and experimental hypertension. What is the functional link? *Ann N Y Acad Sci* 903:72-82

Feng Y, Venema VJ, Venema RC, Tsai N, Caldwell RB (1999) VEGF induces nuclear translocation of Flk-1/KDR, endothelial nitric oxide synthase, and caveolin-1 in vascular endothelial cells. *Biochem Biophys Res Commun* 256:192-7

Ferrara N, Carver-Moore K, Chen H, Dowd M, Lu L, O'Shea KS, Powell-Braxton L, Hillan KJ, Moore MW (1996) Heterozygous embryonic lethality induced by targeted inactivation of the VEGF gene. *Nature* 380:439-42

Ferrara N, Gerber HP, LeCouter J (2003) The biology of VEGF and its receptors. *Nat Med* 9:669-76

- Fischer S, Wobben M, Marti HH, Renz D, Schaper W (2002) Hypoxia-induced hyperpermeability in brain microvessel endothelial cells involves VEGF-mediated changes in the expression of zonula occludens-1. *Microvasc Res* 63:70-80
- Fong GH, Rossant J, Gertsenstein M, Breitman ML (1995) Role of the Flt-1 receptor tyrosine kinase in regulating the assembly of vascular endothelium. *Nature* 376:66-70
- Forsyth RJ (1996) Astrocytes and the delivery of glucose from plasma to neurons. *Neurochem Int* 28:231-41
- Forsythe JA, Jiang BH, Iyer NV, Agani F, Leung SW, Koos RD, Semenza GL (1996) Activation of vascular endothelial growth factor gene transcription by hypoxia-inducible factor 1. *Mol Cell Biol* 16:4604-13
- Foyouzi N, Norwitz ER, Tsen LC, Buhimschi CS, Buhimschi IA (2006) Placental growth factor in the cerebrospinal fluid of women with preeclampsia. *Int J Gynaecol Obstet* 92:32-7
- Freitas-Andrade M, Carmeliet P, Stanimirovic DB, Moreno M (2008) VEGFR-2-mediated increased proliferation and survival in response to oxygen and glucose deprivation in PlGF knockout astrocytes. *J Neurochem* 107:756-67
- Fulton D, Gratton JP, McCabe TJ, Fontana J, Fujio Y, Walsh K, Franke TF, Papapetropoulos A, Sessa WC (1999) Regulation of endothelium-derived nitric oxide production by the protein kinase Akt. *Nature* 399:597-601
- Funahashi Y, Shawber CJ, Vorontchikhina M, Sharma A, Outtz HH, Kitajewski J (2010) Notch regulates the angiogenic response via induction of VEGFR-1. *J Angiogenesis Res* 2:3
- Furuse M, Hirase T, Itoh M, Nagafuchi A, Yonemura S, Tsukita S (1993) Occludin: a novel integral membrane protein localizing at tight junctions. *J Cell Biol* 123:1777-88
- Gerber HP, Dixit V, Ferrara N (1998a) Vascular endothelial growth factor induces expression of the antiapoptotic proteins Bcl-2 and A1 in vascular endothelial cells. *J Biol Chem* 273:13313-6
- Gerber HP, McMurtrey A, Kowalski J, Yan M, Keyt BA, Dixit V, Ferrara N (1998b) Vascular endothelial growth factor regulates endothelial cell survival through the phosphatidylinositol 3'-kinase/Akt signal transduction pathway. Requirement for Flk-1/KDR activation. *J Biol Chem* 273:30336-43
- Gerber HP, Ferrara N (2003) The role of VEGF in normal and neoplastic hematopoiesis. *J Mol Med* 81:20-31
- Gerhardt H, Golding M, Fruttiger M, Ruhrberg C, Lundkvist A, Abramsson A, Jeltsch M, Mitchell C, Alitalo K, Shima D, Betsholtz C (2003) VEGF guides angiogenic sprouting utilizing endothelial tip cell filopodia. *J Cell Biol* 161:1163-77

- Gerritsen ME, Tomlinson JE, Zlot C, Ziman M, Hwang S (2003) Using gene expression profiling to identify the molecular basis of the synergistic actions of hepatocyte growth factor and vascular endothelial growth factor in human endothelial cells. *Br J Pharmacol* 140:595-610
- Giannelli G, Falk-Marzillier J, Schiraldi O, Stetler-Stevenson WG, Quaranta V (1997) Induction of cell migration by matrix metalloprotease-2 cleavage of laminin-5. *Science* 277:225-8
- Gigante B, Morlino G, Gentile MT, Persico MG, De Falco S (2006) Plgf^{-/-}eNos^{-/-} mice show defective angiogenesis associated with increased oxidative stress in response to tissue ischemia. *Faseb J* 20:970-2
- Glass CA, Harper SJ, Bates DO (2006) The anti-angiogenic VEGF isoform VEGF165b transiently increases hydraulic conductivity, probably through VEGF receptor 1 in vivo. *J Physiol* 572:243-57
- Gollmer JC, Ladoux A, Gioanni J, Paquis P, Dubreuil A, Chatel M, Frelin C (2000) Expression of vascular endothelial growth factor-b in human astrocytoma. *Neuro Oncol* 2:80-6
- Gonzalez-Mariscal L, Tapia R, Huerta M, Lopez-Bayghen E (2009) The tight junction protein ZO-2 blocks cell cycle progression and inhibits cyclin D1 expression. *Ann N Y Acad Sci* 1165:121-5
- Gordon GR, Choi HB, Rungta RL, Ellis-Davies GC, MacVicar BA (2008) Brain metabolism dictates the polarity of astrocyte control over arterioles. *Nature* 456:745-9
- Grass D, Pawlowski PG, Hirrlinger J, Papadopoulos N, Richter DW, Kirchhoff F, Hulsmann S (2004) Diversity of functional astroglial properties in the respiratory network. *J Neurosci* 24:1358-65
- Green CJ, Lichtlen P, Huynh NT, Yanovsky M, Laderoute KR, Schaffner W, Murphy BJ (2001) Placenta growth factor gene expression is induced by hypoxia in fibroblasts: a central role for metal transcription factor-1. *Cancer Res* 61:2696-703
- Greenberg JI, Shields DJ, Barillas SG, Acevedo LM, Murphy E, Huang J, Scheppke L, Stockmann C, Johnson RS, Angle N, Cheresch DA (2008) A role for VEGF as a negative regulator of pericyte function and vessel maturation. *Nature* 456:809-13
- Gupta K, Kshirsagar S, Li W, Gui L, Ramakrishnan S, Gupta P, Law PY, Hebbel RP (1999) VEGF prevents apoptosis of human microvascular endothelial cells via opposing effects on MAPK/ERK and SAPK/JNK signaling. *Exp Cell Res* 247:495-504
- Hachinski V (2011) Stroke and Alzheimer disease: fellow travelers or partners in crime? *Arch Neurol* 68:797-8

- Hamel E (2006) Perivascular nerves and the regulation of cerebrovascular tone. *J Appl Physiol* 100:1059-64
- Hangai M, Kitaya N, Xu J, Chan CK, Kim JJ, Werb Z, Ryan SJ, Brooks PC (2002) Matrix metalloproteinase-9-dependent exposure of a cryptic migratory control site in collagen is required before retinal angiogenesis. *Am J Pathol* 161:1429-37
- Harik N, Harik SI, Kuo NT, Sakai K, Przybylski RJ, LaManna JC (1996) Time-course and reversibility of the hypoxia-induced alterations in cerebral vascularity and cerebral capillary glucose transporter density. *Brain Res* 737:335-8
- Hattori K, Heissig B, Wu Y, Dias S, Tejada R, Ferris B, Hicklin DJ, Zhu Z, Bohlen P, Witte L, Hendriks J, Hackett NR, Crystal RG, Moore MA, Werb Z, Lyden D, Rafii S (2002) Placental growth factor reconstitutes hematopoiesis by recruiting VEGFR1(+) stem cells from bone-marrow microenvironment. *Nat Med* 8:841-9
- Hauser S, Weich HA (1993) A heparin-binding form of placenta growth factor (PlGF-2) is expressed in human umbilical vein endothelial cells and in placenta. *Growth Factors* 9:259-68
- Hawkins BT, Davis TP (2005) The blood-brain barrier/neurovascular unit in health and disease. *Pharmacol Rev* 57:173-85
- Hayashi T, Noshita N, Sugawara T, Chan PH (2003) Temporal profile of angiogenesis and expression of related genes in the brain after ischemia. *J Cereb Blood Flow Metab* 23:166-80
- Hayashi Y, Nomura M, Yamagishi S, Harada S, Yamashita J, Yamamoto H (1997) Induction of various blood-brain barrier properties in non-neural endothelial cells by close apposition to co-cultured astrocytes. *Glia* 19:13-26
- Hellstrom M, Phng LK, Hofmann JJ, Wallgard E, Coultas L, Lindblom P, Alva J, Nilsson AK, Karlsson L, Gaiano N, Yoon K, Rossant J, Iruela-Arispe ML, Kalen M, Gerhardt H, Betsholtz C (2007) Dll4 signalling through Notch1 regulates formation of tip cells during angiogenesis. *Nature* 445:776-80
- Henno A, Blacher S, Lambert CA, Deroanne C, Noel A, Lapiere C, de la Brassinne M, Nusgens BV, Colige A (2010) Histological and transcriptional study of angiogenesis and lymphangiogenesis in uninvolved skin, acute pinpoint lesions and established psoriasis plaques: an approach of vascular development chronology in psoriasis. *J Dermatol Sci* 57:162-9
- Hiratsuka S, Minowa O, Kuno J, Noda T, Shibuya M (1998) Flt-1 lacking the tyrosine kinase domain is sufficient for normal development and angiogenesis in mice. *Proc Natl Acad Sci U S A* 95:9349-54
- Holmes DI, Zachary I (2004) Placental growth factor induces FosB and c-Fos gene expression via Flt-1 receptors. *FEBS Lett* 557:93-8

Iadecola C (2004) Neurovascular regulation in the normal brain and in Alzheimer's disease. *Nat Rev Neurosci* 5:347-60

Ishida A, Murray J, Saito Y, Kanthou C, Benzakour O, Shibuya M, Wijelath ES (2001) Expression of vascular endothelial growth factor receptors in smooth muscle cells. *J Cell Physiol* 188:359-68

Iyer S, Acharya KR (2002) Role of placenta growth factor in cardiovascular health. *Trends Cardiovasc Med* 12:128-34

Janzer RC, Raff MC (1987) Astrocytes induce blood-brain barrier properties in endothelial cells. *Nature* 325:253-7

Jiang Z, Zhang Y, Chen XQ, Lam PY, Yang H, Xu Q, Yu AC (2003) Apoptosis and activation of Erk1/2 and Akt in astrocytes postischemia. *Neurochem Res* 28:831-7

Jin KL, Mao XO, Greenberg DA (2000) Vascular endothelial growth factor: direct neuroprotective effect in in vitro ischemia. *Proc Natl Acad Sci U S A* 97:10242-7

Jones MC, Caswell PT, Moran-Jones K, Roberts M, Barry ST, Gampel A, Mellor H, Norman JC (2009) VEGFR1 (Flt1) regulates Rab4 recycling to control fibronectin polymerization and endothelial vessel branching. *Traffic* 10:754-66

Jucker M, Battig K, Meier-Ruge W (1990) Effects of aging and vincamine derivatives on pericapillary microenvironment: stereological characterization of the cerebral capillary network. *Neurobiol Aging* 11:39-46

Kanno S, Oda N, Abe M, Terai Y, Ito M, Shitara K, Tabayashi K, Shibuya M, Sato Y (2000) Roles of two VEGF receptors, Flt-1 and KDR, in the signal transduction of VEGF effects in human vascular endothelial cells. *Oncogene* 19:2138-46

Kappas NC, Zeng G, Chappell JC, Kearney JB, Hazarika S, Kallianos KG, Patterson C, Annex BH, Bautch VL (2008) The VEGF receptor Flt-1 spatially modulates Flk-1 signaling and blood vessel branching. *J Cell Biol* 181:847-58

Kawamura H, Li X, Goishi K, van Meeteren LA, Jakobsson L, Cebe-Suarez S, Shimizu A, Edholm D, Ballmer-Hofer K, Kjellen L, Klagsbrun M, Claesson-Welsh L (2008) Neuropilin-1 in regulation of VEGF-induced activation of p38MAPK and endothelial cell organization. *Blood* 112:3638-49

Kearney JB, Kappas NC, Ellerstrom C, DiPaola FW, Bautch VL (2004) The VEGF receptor flt-1 (VEGFR-1) is a positive modulator of vascular sprout formation and branching morphogenesis. *Blood* 103:4527-35

Kendall RL, Wang G, Thomas KA (1996) Identification of a natural soluble form of the vascular endothelial growth factor receptor, FLT-1, and its heterodimerization with KDR. *Biochem Biophys Res Commun* 226:324-8

Kety SS (1956) Human cerebral blood flow and oxygen consumption as related to aging. *Res Publ Assoc Res Nerv Ment Dis* 35:31-45

Keyt BA, Nguyen HV, Berleau LT, Duarte CM, Park J, Chen H, Ferrara N (1996) Identification of vascular endothelial growth factor determinants for binding KDR and FLT-1 receptors. Generation of receptor-selective VEGF variants by site-directed mutagenesis. *J Biol Chem* 271:5638-46

Khurana R, Moons L, Shafi S, Luttun A, Collen D, Martin JF, Carmeliet P, Zachary IC (2005) Placental growth factor promotes atherosclerotic intimal thickening and macrophage accumulation. *Circulation* 111:2828-36

Kilic E, Kilic U, Wang Y, Bassetti CL, Marti HH, Hermann DM (2006) The phosphatidylinositol-3 kinase/Akt pathway mediates VEGF's neuroprotective activity and induces blood brain barrier permeability after focal cerebral ischemia. *FASEB J* 20:1185-7

Klein B, Kuschinsky W, Schrock H, Vetterlein F (1986) Interdependency of local capillary density, blood flow, and metabolism in rat brains. *Am J Physiol* 251:H1333-40

Kocharyan A, Fernandes P, Tong XK, Vaucher E, Hamel E (2008) Specific subtypes of cortical GABA interneurons contribute to the neurovascular coupling response to basal forebrain stimulation. *J Cereb Blood Flow Metab* 28:221-31

Krueger M, Bechmann I (2010) CNS pericytes: concepts, misconceptions, and a way out. *Glia* 58:1-10

Krum JM, Khaibullina A (2003) Inhibition of endogenous VEGF impedes revascularization and astroglial proliferation: roles for VEGF in brain repair. *Exp Neurol* 181:241-57

Krum JM, Mani N, Rosenstein JM (2008) Roles of the endogenous VEGF receptors flt-1 and flk-1 in astroglial and vascular remodeling after brain injury. *Exp Neurol* 212:108-17

Krupinski J, Kaluza J, Kumar P, Kumar S, Wang JM (1994) Role of angiogenesis in patients with cerebral ischemic stroke. *Stroke* 25:1794-8

Kuo NT, Benhayon D, Przybylski RJ, Martin RJ, LaManna JC (1999) Prolonged hypoxia increases vascular endothelial growth factor mRNA and protein in adult mouse brain. *J Appl Physiol* 86:260-4

Kurz H, Fehr J, Nitschke R, Burkhardt H (2008) Pericytes in the mature chorioallantoic membrane capillary plexus contain desmin and alpha-smooth muscle actin: relevance for non-sprouting angiogenesis. *Histochem Cell Biol* 130:1027-40

Lacal PM, Failla CM, Pagani E, Odorisio T, Schietroma C, Falcinelli S, Zambruno G, D'Atri S (2000) Human melanoma cells secrete and respond to placenta growth factor and vascular endothelial growth factor. *J Invest Dermatol* 115:1000-7

LaManna JC, Vendel LM, Farrell RM (1992) Brain adaptation to chronic hypobaric hypoxia in rats. *J Appl Physiol* 72:2238-43

LaManna JC, Cordisco BR, Knuese DE, Hudetz AG (1994) Increased capillary segment length in cerebral cortical microvessels of rats exposed to 3 weeks of hypobaric hypoxia. *Adv Exp Med Biol* 345:627-32

LaManna JC, Chavez JC, Pichiule P (2004) Structural and functional adaptation to hypoxia in the rat brain. *J Exp Biol* 207:3163-9

LaManna JC (2007) Hypoxia in the central nervous system. *Essays Biochem* 43:139-51

Lambrechts D, Carmeliet P (2006) VEGF at the neurovascular interface: therapeutic implications for motor neuron disease. *Biochim Biophys Acta* 1762:1109-21

Lampugnani MG, Orsenigo F, Gagliani MC, Tacchetti C, Dejana E (2006) Vascular endothelial cadherin controls VEGFR-2 internalization and signaling from intracellular compartments. *J Cell Biol* 174:593-604

Lawson CA, Yan SD, Yan SF, Liao H, Zhou YS, Sobel J, Kisiel W, Stern DM, Pinsky DJ (1997) Monocytes and tissue factor promote thrombosis in a murine model of oxygen deprivation. *J Clin Invest* 99:1729-38

LeCouter J, Lin R, Ferrara N (2002) Endocrine gland-derived VEGF and the emerging hypothesis of organ-specific regulation of angiogenesis. *Nat Med* 8:913-7

Lee SW, Kim WJ, Choi YK, Song HS, Son MJ, Gelman IH, Kim YJ, Kim KW (2003) SSeCKS regulates angiogenesis and tight junction formation in blood-brain barrier. *Nat Med* 9:900-6

Li L, Lundkvist A, Andersson D, Wilhelmsson U, Nagai N, Pardo AC, Nodin C, Stahlberg A, Aprico K, Larsson K, Yabe T, Moons L, Fotheringham A, Davies I, Carmeliet P, Schwartz JP, Pekna M, Kubista M, Blomstrand F, Maragakis N, Nilsson M, Pekny M (2007) Protective role of reactive astrocytes in brain ischemia. *J Cereb Blood Flow Metab*

Li L, Welser JV, Dore-Duffy P, del Zoppo GJ, LaManna JC, Milner R (2010) In the hypoxic central nervous system, endothelial cell proliferation is followed by astrocyte activation, proliferation, and increased expression of the alpha 6 beta 4 integrin and dystroglycan. *Glia* 58:1157-67

Li W, Shen W, Gill R, Corbly A, Jones B, Belagaje R, Zhang Y, Tang S, Chen Y, Zhai Y, Wang G, Wagle A, Hui K, Westmore M, Hanson J, Chen YF, Simons M, Singh J (2006) High-resolution quantitative computed tomography demonstrating selective enhancement of

medium-size collaterals by placental growth factor-1 in the mouse ischemic hindlimb. *Circulation* 113:2445-53

Lindahl P, Johansson BR, Leveen P, Betsholtz C (1997) Pericyte loss and microaneurysm formation in PDGF-B-deficient mice. *Science* 277:242-5

Liu H, Honmou O, Harada K, Nakamura K, Houkin K, Hamada H, Kocsis JD (2006) Neuroprotection by PlGF gene-modified human mesenchymal stem cells after cerebral ischaemia. *Brain* 129:2734-45

Lukaszewicz AC, Sampaio N, Guegan C, Benchoua A, Couriaud C, Chevalier E, Sola B, Lacombe P, Onteniente B (2002) High sensitivity of protoplasmic cortical astroglia to focal ischemia. *J Cereb Blood Flow Metab* 22:289-98

Luo C, Yi B, Fan W, Chen K, Gui L, Chen Z, Li L, Feng H, Chi L (2011) Enhanced angiogenesis and astrocyte activation by ecdysterone treatment in a focal cerebral ischemia rat model. *Acta Neurochir Suppl* 110:151-5

Luttun A, Brusselmans K, Fukao H, Tjwa M, Ueshima S, Herbert JM, Matsuo O, Collen D, Carmeliet P, Moons L (2002a) Loss of placental growth factor protects mice against vascular permeability in pathological conditions. *Biochem Biophys Res Commun* 295:428-34

Luttun A, Tjwa M, Carmeliet P (2002b) Placental growth factor (PlGF) and its receptor Flt-1 (VEGFR-1): novel therapeutic targets for angiogenic disorders. *Ann N Y Acad Sci* 979:80-93

Luttun A, Tjwa M, Moons L, Wu Y, Angelillo-Scherrer A, Liao F, Nagy JA, Hooper A, Priller J, De Klerck B, Compennolle V, Daci E, Bohlen P, Dewerchin M, Herbert JM, Fava R, Matthys P, Carmeliet G, Collen D, Dvorak HF, Hicklin DJ, Carmeliet P (2002c) Revascularization of ischemic tissues by PlGF treatment, and inhibition of tumor angiogenesis, arthritis and atherosclerosis by anti-Flt1. *Nat Med* 8:831-40

Luttun A, Autiero M, Tjwa M, Carmeliet P (2004) Genetic dissection of tumor angiogenesis: are PlGF and VEGFR-1 novel anti-cancer targets? *Biochim Biophys Acta* 1654:79-94

Ma C, Bower KA, Chen G, Shi X, Ke ZJ, Luo J (2008) Interaction between ERK and GSK3 β mediates basic fibroblast growth factor-induced apoptosis in SK-N-MC neuroblastoma cells. *J Biol Chem* 283:9248-56

Mac Gabhann F, Popel AS (2004) Model of competitive binding of vascular endothelial growth factor and placental growth factor to VEGF receptors on endothelial cells. *Am J Physiol Heart Circ Physiol* 286:H153-64

Maglione D, Guerriero V, Viglietto G, Delli-Bovi P, Persico MG (1991) Isolation of a human placenta cDNA coding for a protein related to the vascular permeability factor. *Proc Natl Acad Sci U S A* 88:9267-71

Maglione D, Guerriero V, Viglietto G, Ferraro MG, Aprelikova O, Alitalo K, Del Vecchio S, Lei KJ, Chou JY, Persico MG (1993) Two alternative mRNAs coding for the angiogenic factor, placenta growth factor (PlGF), are transcribed from a single gene of chromosome 14. *Oncogene* 8:925-31

Maltepe E, Schmidt JV, Baunoch D, Bradfield CA, Simon MC (1997) Abnormal angiogenesis and responses to glucose and oxygen deprivation in mice lacking the protein ARNT. *Nature* 386:403-7

Mamluk R, Gechtman Z, Kutcher ME, Gasiunas N, Gallagher J, Klagsbrun M (2002) Neuropilin-1 binds vascular endothelial growth factor 165, placenta growth factor-2, and heparin via its b1b2 domain. *J Biol Chem* 277:24818-25

Mani N, Khaibullina A, Krum JM, Rosenstein JM (2005) Astrocyte growth effects of vascular endothelial growth factor (VEGF) application to perinatal neocortical explants: receptor mediation and signal transduction pathways. *Exp Neurol* 192:394-406

Manoonkitiwongsa PS (2011) Critical questions for preclinical trials on safety and efficacy of vascular endothelial growth factor-based therapeutic angiogenesis for ischemic stroke. *CNS Neurol Disord Drug Targets* 10:215-34

Marti HH, Risau W (1998) Systemic hypoxia changes the organ-specific distribution of vascular endothelial growth factor and its receptors. *Proc Natl Acad Sci U S A* 95:15809-14

Marti HJ, Bernaudin M, Bellail A, Schoch H, Euler M, Petit E, Risau W (2000) Hypoxia-induced vascular endothelial growth factor expression precedes neovascularization after cerebral ischemia. *Am J Pathol* 156:965-76

Matsuzaki H, Tamatani M, Yamaguchi A, Namikawa K, Kiyama H, Vitek MP, Mitsuda N, Tohyama M (2001) Vascular endothelial growth factor rescues hippocampal neurons from glutamate-induced toxicity: signal transduction cascades. *Faseb J* 15:1218-20

Matthias K, Kirchhoff F, Seifert G, Hutmacher K, Matyash M, Kettenmann H, Steinhauser C (2003) Segregated expression of AMPA-type glutamate receptors and glutamate transporters defines distinct astrocyte populations in the mouse hippocampus. *J Neurosci* 23:1750-8

Mavria G, Vercoulen Y, Yeo M, Paterson H, Karasarides M, Marais R, Bird D, Marshall CJ (2006) ERK-MAPK signaling opposes Rho-kinase to promote endothelial cell survival and sprouting during angiogenesis. *Cancer Cell* 9:33-44

Mazzone M, Dettori D, Leite de Oliveira R, Loges S, Schmidt T, Jonckx B, Tian YM, Lanahan AA, Pollard P, Ruiz de Almodovar C, De Smet F, Vinckier S, Aragonés J, Debackere K, Luttun A, Wyns S, Jordan B, Pisacane A, Gallez B, Lampugnani MG, Dejane E, Simons M, Ratcliffe P, Maxwell P, Carmeliet P (2009) Heterozygous deficiency of PHD2 restores tumor oxygenation and inhibits metastasis via endothelial normalization. *Cell* 136:839-51

- McAllister MS, Krizanac-Bengez L, Macchia F, Naftalin RJ, Pedley KC, Mayberg MR, Marroni M, Leaman S, Stanness KA, Janigro D (2001) Mechanisms of glucose transport at the blood-brain barrier: an in vitro study. *Brain Res* 904:20-30
- Meadows KN, Bryant P, Pumiglia K (2001) Vascular endothelial growth factor induction of the angiogenic phenotype requires Ras activation. *J Biol Chem* 276:49289-98
- Melamed E, Lavy S, Bentin S, Cooper G, Rinot Y (1980) Reduction in regional cerebral blood flow during normal aging in man. *Stroke* 11:31-5
- Migdal M, Huppertz B, Tessler S, Comforti A, Shibuya M, Reich R, Baumann H, Neufeld G (1998) Neuropilin-1 is a placenta growth factor-2 receptor. *J Biol Chem* 273:22272-8
- Millauer B, Wизigmann-Voos S, Schnurch H, Martinez R, Moller NP, Risau W, Ullrich A (1993) High affinity VEGF binding and developmental expression suggest Flk-1 as a major regulator of vasculogenesis and angiogenesis. *Cell* 72:835-46
- Milner R, Campbell IL (2002) Developmental regulation of beta1 integrins during angiogenesis in the central nervous system. *Mol Cell Neurosci* 20:616-26
- Milner R, Hung S, Erokwu B, Dore-Duffy P, LaManna JC, del Zoppo GJ (2008) Increased expression of fibronectin and the alpha5beta1 integrin in angiogenic cerebral blood vessels of mice subject to hypobaric hypoxia. *Mol Cell Neurosci* 38:43-52
- Mironov V, Hritz MA, LaManna JC, Hudetz AG, Harik SI (1994) Architectural alterations in rat cerebral microvessels after hypobaric hypoxia. *Brain Res* 660:73-80
- Mojsilovic-Petrovic J, Nesic M, Pen A, Zhang W, Stanimirovic D (2004) Development of rapid staining protocols for laser-capture microdissection of brain vessels from human and rat coupled to gene expression analyses. *J Neurosci Methods* 133:39-48
- Montaner J, Chacon P, Krupinski J, Rubio F, Millan M, Molina CA, Hereu P, Quintana M, Alvarez-Sabin J (2008) Simvastatin in the acute phase of ischemic stroke: a safety and efficacy pilot trial. *Eur J Neurol* 15:82-90
- Moreno MJ, Ball M, Andrade MF, McDermid A, Stanimirovic DB (2006) Insulin-like growth factor binding protein-4 (IGFBP-4) is a novel anti-angiogenic and anti-tumorigenic mediator secreted by dibutyryl cyclic AMP (dB-cAMP)-differentiated glioblastoma cells. *Glia* 53:845-57
- Morimoto T, Yasuhara T, Kameda M, Baba T, Kuramoto S, Kondo A, Takahashi K, Tajiri N, Wang F, Meng J, Ji YW, Kadota T, Maruo T, Kinugasa K, Miyoshi Y, Shingo T, Borlongan CV, Date I (2010) Striatal Stimulation Nurtures Endogenous Neurogenesis and Angiogenesis in Chronic-Phase Ischemic Stroke Rats. *Cell Transplant*
- Moskowitz MA, Lo EH, Iadecola C (2010) The science of stroke: mechanisms in search of treatments. *Neuron* 67:181-98

- Mulligan SJ, MacVicar BA (2004) Calcium transients in astrocyte endfeet cause cerebrovascular constrictions. *Nature* 431:195-9
- Ndubizu OI, Tsipis CP, Li A, LaManna JC (2010) Hypoxia-inducible factor-1 (HIF-1)-independent microvascular angiogenesis in the aged rat brain. *Brain Res* 1366:101-9
- Nedergaard M, Ransom B, Goldman SA (2003) New roles for astrocytes: redefining the functional architecture of the brain. *Trends Neurosci* 26:523-30
- Neufeld G, Kessler O, Herzog Y (2002) The interaction of Neuropilin-1 and Neuropilin-2 with tyrosine-kinase receptors for VEGF. *Adv Exp Med Biol* 515:81-90
- Neuwelt EA, Bauer B, Fahlke C, Fricker G, Iadecola C, Janigro D, Leybaert L, Molnar Z, O'Donnell ME, Povlishock JT, Saunders NR, Sharp F, Stanimirovic D, Watts RJ, Drewes LR (2011) Engaging neuroscience to advance translational research in brain barrier biology. *Nat Rev Neurosci* 12:169-82
- Nishioku T, Dohgu S, Takata F, Eto T, Ishikawa N, Kodama KB, Nakagawa S, Yamauchi A, Kataoka Y (2009) Detachment of brain pericytes from the basal lamina is involved in disruption of the blood-brain barrier caused by lipopolysaccharide-induced sepsis in mice. *Cell Mol Neurobiol* 29:309-16
- Nishiyama H, Knopfel T, Endo S, Itohara S (2002) Glial protein S100B modulates long-term neuronal synaptic plasticity. *Proc Natl Acad Sci U S A* 99:4037-42
- Nitta T, Hata M, Gotoh S, Seo Y, Sasaki H, Hashimoto N, Furuse M, Tsukita S (2003) Size-selective loosening of the blood-brain barrier in claudin-5-deficient mice. *J Cell Biol* 161:653-60
- Nomura M, Yamagishi S, Harada S, Yamashita T, Yamashita J, Yamamoto H (1998) Placenta growth factor (PlGF) mRNA expression in brain tumors. *J Neurooncol* 40:123-30
- O'Reilly MS, Holmgren L, Shing Y, Chen C, Rosenthal RA, Cao Y, Moses M, Lane WS, Sage EH, Folkman J (1994) Angiostatin: a circulating endothelial cell inhibitor that suppresses angiogenesis and tumor growth. *Cold Spring Harb Symp Quant Biol* 59:471-82
- O'Reilly MS, Boehm T, Shing Y, Fukai N, Vasios G, Lane WS, Flynn E, Birkhead JR, Olsen BR, Folkman J (1997) Endostatin: an endogenous inhibitor of angiogenesis and tumor growth. *Cell* 88:277-85
- Odorisio T, Schietroma C, Zaccaria ML, Cianfarani F, Tiveron C, Tatangelo L, Failla CM, Zambruno G (2002) Mice overexpressing placenta growth factor exhibit increased vascularization and vessel permeability. *J Cell Sci* 115:2559-67
- Orlidge A, D'Amore PA (1987) Inhibition of capillary endothelial cell growth by pericytes and smooth muscle cells. *J Cell Biol* 105:1455-62

Ozerdem U, Stallcup WB (2003) Early contribution of pericytes to angiogenic sprouting and tube formation. *Angiogenesis* 6:241-9

Padden M, Leech S, Craig B, Kirk J, Brankin B, McQuaid S (2007) Differences in expression of junctional adhesion molecule-A and beta-catenin in multiple sclerosis brain tissue: increasing evidence for the role of tight junction pathology. *Acta Neuropathol* 113:177-86

Papapetropoulos A, Fulton D, Mahboubi K, Kalb RG, O'Connor DS, Li F, Altieri DC, Sessa WC (2000) Angiopoietin-1 inhibits endothelial cell apoptosis via the Akt/survivin pathway. *J Biol Chem* 275:9102-5

Park JE, Keller GA, Ferrara N (1993) The vascular endothelial growth factor (VEGF) isoforms: differential deposition into the subepithelial extracellular matrix and bioactivity of extracellular matrix-bound VEGF. *Mol Biol Cell* 4:1317-26

Park JE, Chen HH, Winer J, Houck KA, Ferrara N (1994) Placenta growth factor. Potentiation of vascular endothelial growth factor bioactivity, in vitro and in vivo, and high affinity binding to Flt-1 but not to Flk-1/KDR. *J Biol Chem* 269:25646-54

Peppiatt CM, Howarth C, Mobbs P, Attwell D (2006) Bidirectional control of CNS capillary diameter by pericytes. *Nature* 443:700-4

Pichiule P, LaManna JC (2002) Angiopoietin-2 and rat brain capillary remodeling during adaptation and deadaptation to prolonged mild hypoxia. *J Appl Physiol* 93:1131-9

Pizurki L, Zhou Z, Glynos K, Roussos C, Papapetropoulos A (2003) Angiopoietin-1 inhibits endothelial permeability, neutrophil adherence and IL-8 production. *Br J Pharmacol* 139:329-36

Popa-Wagner A, Pirici D, Petcu EB, Mogoanta L, Buga AM, Rosen CL, Leon R, Huber J (2010) Pathophysiology of the vascular wall and its relevance for cerebrovascular disorders in aged rodents. *Curr Neurovasc Res* 7:251-67

Praidou A, Androudi S, Brazitikos P, Karakiulakis G, Papakonstantinou E, Dimitrakos S (2010) Angiogenic growth factors and their inhibitors in diabetic retinopathy. *Curr Diabetes Rev* 6:304-12

Quaeghebeur A, Lange C, Carmeliet P (2011) The neurovascular link in health and disease: molecular mechanisms and therapeutic implications. *Neuron* 71:406-24

Quinn TP, Peters KG, De Vries C, Ferrara N, Williams LT (1993) Fetal liver kinase 1 is a receptor for vascular endothelial growth factor and is selectively expressed in vascular endothelium. *Proc Natl Acad Sci U S A* 90:7533-7

- Raab S, Beck H, Gaumann A, Yuce A, Gerber HP, Plate K, Hammes HP, Ferrara N, Breier G (2004) Impaired brain angiogenesis and neuronal apoptosis induced by conditional homozygous inactivation of vascular endothelial growth factor. *Thromb Haemost* 91:595-605
- Ratan RR, Siddiq A, Smirnova N, Karpisheva K, Haskew-Layton R, McConoughey S, Langley B, Estevez A, Huerta PT, Volpe B, Roy S, Sen CK, Gazaryan I, Cho S, Fink M, LaManna J (2007) Harnessing hypoxic adaptation to prevent, treat, and repair stroke. *J Mol Med* 85:1331-8
- Rayasam GV, Tulasi VK, Sodhi R, Davis JA, Ray A (2009) Glycogen synthase kinase 3: more than a namesake. *Br J Pharmacol* 156:885-98
- Reiss Y, Droste J, Heil M, Tribulova S, Schmidt MH, Schaper W, Dumont DJ, Plate KH (2007) Angiopoietin-2 impairs revascularization after limb ischemia. *Circ Res* 101:88-96
- Ribatti D (2008) The discovery of the placental growth factor and its role in angiogenesis: a historical review. *Angiogenesis* 11:215-21
- Risau W (1997) Mechanisms of angiogenesis. *Nature* 386:671-4
- Roncal C, Buysschaert I, Chorianopoulos E, Georgiadou M, Meilhac O, Demol M, Michel JB, Vinckier S, Moons L, Carmeliet P (2008) Beneficial effects of prolonged systemic administration of PlGF on late outcome of post-ischaemic myocardial performance. *J Pathol* 216:236-44
- Rousseau S, Houle F, Landry J, Huot J (1997) p38 MAP kinase activation by vascular endothelial growth factor mediates actin reorganization and cell migration in human endothelial cells. *Oncogene* 15:2169-77
- Roy H, Bhardwaj S, Yla-Herttuala S (2006) Biology of vascular endothelial growth factors. *FEBS Lett* 580:2879-87
- Sanchez A, Wadhwani S, Grammas P (2010) Multiple neurotrophic effects of VEGF on cultured neurons. *Neuropeptides* 44:323-31
- Sands M, Howell K, Costello CM, McLoughlin P (2011) Placenta growth factor and vascular endothelial growth factor B expression in the hypoxic lung. *Respir Res* 12:17
- Santos SC, Miguel C, Domingues I, Calado A, Zhu Z, Wu Y, Dias S (2007) VEGF and VEGFR-2 (KDR) internalization is required for endothelial recovery during wound healing. *Exp Cell Res* 313:1561-74
- Sato Y, Rifkin DB (1989) Inhibition of endothelial cell movement by pericytes and smooth muscle cells: activation of a latent transforming growth factor-beta 1-like molecule by plasmin during co-culture. *J Cell Biol* 109:309-15

- Schachtrup C, Lu P, Jones LL, Lee JK, Lu J, Sachs BD, Zheng B, Akassoglou K (2007) Fibrinogen inhibits neurite outgrowth via beta 3 integrin-mediated phosphorylation of the EGF receptor. *Proc Natl Acad Sci U S A* 104:11814-9
- Schachtrup C, Ryu JK, Helmrick MJ, Vagena E, Galanakis DK, Degen JL, Margolis RU, Akassoglou K (2010) Fibrinogen triggers astrocyte scar formation by promoting the availability of active TGF-beta after vascular damage. *J Neurosci* 30:5843-54
- Schinkel AH (1999) P-Glycoprotein, a gatekeeper in the blood-brain barrier. *Adv Drug Deliv Rev* 36:179-94
- Schmid-Brunclik N, Burgi-Taboada C, Antoniou X, Gassmann M, Ogunshola OO (2008) Astrocyte responses to injury: VEGF simultaneously modulates cell death and proliferation. *Am J Physiol Regul Integr Comp Physiol* 295:R864-73
- Schmidt M, Dogan C, Birdir C, Kuhn U, Gellhaus A, Kimmig R, Kasimir-Bauer S (2009) Placental growth factor: a predictive marker for preeclampsia? *Gynakol Geburtshilfliche Rundsch* 49:94-9
- Schomber T, Kopfstein L, Djonov V, Albrecht I, Baeriswyl V, Strittmatter K, Christofori G (2007) Placental growth factor-1 attenuates vascular endothelial growth factor-A-dependent tumor angiogenesis during beta cell carcinogenesis. *Cancer Res* 67:10840-8
- Seifert G, Schilling K, Steinhauser C (2006) Astrocyte dysfunction in neurological disorders: a molecular perspective. *Nat Rev Neurosci* 7:194-206
- Shalaby F, Rossant J, Yamaguchi TP, Gertsenstein M, Wu XF, Breitman ML, Schuh AC (1995) Failure of blood-island formation and vasculogenesis in Flk-1-deficient mice. *Nature* 376:62-6
- Shehab SA, Cronly-Dillon JR, Nona SN, Stafford CA (1990) Preferential histochemical staining of protoplasmic and fibrous astrocytes in rat CNS with GFAP antibodies using different fixatives. *Brain Res* 518:347-52
- Shintani Y, Takashima S, Asano Y, Kato H, Liao Y, Yamazaki S, Tsukamoto O, Seguchi O, Yamamoto H, Fukushima T, Sugahara K, Kitakaze M, Hori M (2006) Glycosaminoglycan modification of neuropilin-1 modulates VEGFR2 signaling. *Embo J* 25:3045-55
- Shiu YT, Li S, Marganski WA, Usami S, Schwartz MA, Wang YL, Dembo M, Chien S (2004) Rho mediates the shear-enhancement of endothelial cell migration and traction force generation. *Biophys J* 86:2558-65
- Shraga-Heled N, Kessler O, Prahst C, Kroll J, Augustin H, Neufeld G (2007) Neuropilin-1 and neuropilin-2 enhance VEGF121 stimulated signal transduction by the VEGFR-2 receptor. *Faseb J* 21:915-26

Shweiki D, Itin A, Soffer D, Keshet E (1992) Vascular endothelial growth factor induced by hypoxia may mediate hypoxia-initiated angiogenesis. *Nature* 359:843-5

Shyu KG, Hung HF, Wang BW, Chang H (2008) Hyperbaric oxygen induces placental growth factor expression in bone marrow-derived mesenchymal stem cells. *Life Sci* 83:65-73

Silverman WF, Krum JM, Mani N, Rosenstein JM (1999) Vascular, glial and neuronal effects of vascular endothelial growth factor in mesencephalic explant cultures. *Neuroscience* 90:1529-41

Sobue K, Yamamoto N, Yoneda K, Hodgson ME, Yamashiro K, Tsuruoka N, Tsuda T, Katsuya H, Miura Y, Asai K, Kato T (1999) Induction of blood-brain barrier properties in immortalized bovine brain endothelial cells by astrocytic factors. *Neurosci Res* 35:155-64

Soker S, Takashima S, Miao HQ, Neufeld G, Klagsbrun M (1998) Neuropilin-1 is expressed by endothelial and tumor cells as an isoform-specific receptor for vascular endothelial growth factor. *Cell* 92:735-45

Soker S, Miao HQ, Nomi M, Takashima S, Klagsbrun M (2002) VEGF165 mediates formation of complexes containing VEGFR-2 and neuropilin-1 that enhance VEGF165-receptor binding. *J Cell Biochem* 85:357-68

Sonntag WE, Lynch CD, Cooney PT, Hutchins PM (1997) Decreases in cerebral microvasculature with age are associated with the decline in growth hormone and insulin-like growth factor 1. *Endocrinology* 138:3515-20

Staton CA, Kumar I, Reed MW, Brown NJ (2007) Neuropilins in physiological and pathological angiogenesis. *J Pathol* 212:237-48

Svensson B, Peters M, König HG, Poppe M, Levkau B, Rothermundt M, Arolt V, Kogel D, Prehn JH (2002) Vascular endothelial growth factor protects cultured rat hippocampal neurons against hypoxic injury via an antiexcitotoxic, caspase-independent mechanism. *J Cereb Blood Flow Metab* 22:1170-5

Swanson RA, Farrell K, Stein BA (1997) Astrocyte energetics, function, and death under conditions of incomplete ischemia: a mechanism of glial death in the penumbra. *Glia* 21:142-53

Takahashi T, Ueno H, Shibuya M (1999) VEGF activates protein kinase C-dependent, but Ras-independent Raf-MEK-MAP kinase pathway for DNA synthesis in primary endothelial cells. *Oncogene* 18:2221-30

Takano T, Tian GF, Peng W, Lou N, Libionka W, Han X, Nedergaard M (2006) Astrocyte-mediated control of cerebral blood flow. *Nat Neurosci* 9:260-7

Takeda Y, Uemura S, Iwama H, Imagawa K, Nishida T, Onoue K, Takemoto Y, Soeda T, Okayama S, Somekawa S, Ishigami K, Takaoka M, Kawata H, Kubo A, Horii M, Nakajima

- T, Saito Y (2009) Treatment with recombinant placental growth factor (PlGF) enhances both angiogenesis and arteriogenesis and improves survival after myocardial infarction. *Circ J* 73:1674-82
- Tammela T, Enholm B, Alitalo K, Paavonen K (2005) The biology of vascular endothelial growth factors. *Cardiovasc Res* 65:550-63
- Taylor AP, Leon E, Goldenberg DM (2010) Placental growth factor (PlGF) enhances breast cancer cell motility by mobilising ERK1/2 phosphorylation and cytoskeletal rearrangement. *Br J Cancer* 103:82-9
- Teng H, Zhang ZG, Wang L, Zhang RL, Zhang L, Morris D, Gregg SR, Wu Z, Jiang A, Lu M, Zlokovic BV, Chopp M (2008) Coupling of angiogenesis and neurogenesis in cultured endothelial cells and neural progenitor cells after stroke. *J Cereb Blood Flow Metab* 28:764-71
- Thomas M, Augustin HG (2009) The role of the Angiopoietins in vascular morphogenesis. *Angiogenesis* 12:125-37
- Tong XK, Hamel E (1999) Regional cholinergic denervation of cortical microvessels and nitric oxide synthase-containing neurons in Alzheimer's disease. *Neuroscience* 92:163-75
- Torry RJ, Tomanek RJ, Zheng W, Miller SJ, Labarrere CA, Torry DS (2009) Hypoxia increases placenta growth factor expression in human myocardium and cultured neonatal rat cardiomyocytes. *J Heart Lung Transplant* 28:183-90
- van Bruggen N, Thibodeaux H, Palmer JT, Lee WP, Fu L, Cairns B, Tumas D, Gerlai R, Williams SP, van Lookeren Campagne M, Ferrara N (1999) VEGF antagonism reduces edema formation and tissue damage after ischemia/reperfusion injury in the mouse brain. *J Clin Invest* 104:1613-20
- Verkhratsky A, Steinhauser C (2000) Ion channels in glial cells. *Brain Res Brain Res Rev* 32:380-412
- Virgintino D, Girolamo F, Errede M, Capobianco C, Robertson D, Stallcup WB, Perris R, Roncali L (2007) An intimate interplay between precocious, migrating pericytes and endothelial cells governs human fetal brain angiogenesis. *Angiogenesis* 10:35-45
- Vogel C, Bauer A, Wiesnet M, Preissner KT, Schaper W, Marti HH, Fischer S (2007) Flt-1, but not Flk-1 mediates hyperpermeability through activation of the PI3-K/Akt pathway. *J Cell Physiol* 212:236-43
- Volterra A, Meldolesi J (2005) Astrocytes, from brain glue to communication elements: the revolution continues. *Nat Rev Neurosci* 6:626-40
- von Tell D, Armulik A, Betsholtz C (2006) Pericytes and vascular stability. *Exp Cell Res* 312:623-9

Wallraff A, Odermatt B, Willecke K, Steinhauser C (2004) Distinct types of astroglial cells in the hippocampus differ in gap junction coupling. *Glia* 48:36-43

Wang J, Milner R (2006) Fibronectin promotes brain capillary endothelial cell survival and proliferation through $\alpha 5 \beta 1$ and $\alpha v \beta 3$ integrins via MAP kinase signalling. *J Neurochem* 96:148-59

Ward NL, Moore E, Noon K, Spassil N, Keenan E, Ivanco TL, LaManna JC (2007) Cerebral angiogenic factors, angiogenesis, and physiological response to chronic hypoxia differ among four commonly used mouse strains. *J Appl Physiol* 102:1927-35

Woolard J, Wang WY, Bevan HS, Qiu Y, Morbidelli L, Pritchard-Jones RO, Cui TG, Sugiono M, Waine E, Perrin R, Foster R, Digby-Bell J, Shields JD, Whittles CE, Mushens RE, Gillatt DA, Ziche M, Harper SJ, Bates DO (2004) VEGF165b, an inhibitory vascular endothelial growth factor splice variant: mechanism of action, in vivo effect on angiogenesis and endogenous protein expression. *Cancer Res* 64:7822-35

Wu H, Lu D, Jiang H, Xiong Y, Qu C, Li B, Mahmood A, Zhou D, Chopp M (2008) Simvastatin-mediated upregulation of VEGF and BDNF, activation of the PI3K/Akt pathway, and increase of neurogenesis are associated with therapeutic improvement after traumatic brain injury. *J Neurotrauma* 25:130-9

Xiao Z, Kong Y, Yang S, Li M, Wen J, Li L (2007) Upregulation of Flk-1 by bFGF via the ERK pathway is essential for VEGF-mediated promotion of neural stem cell proliferation. *Cell Res* 17:73-9

Xiong Y, Mahmood A, Chopp M (2010) Angiogenesis, neurogenesis and brain recovery of function following injury. *Curr Opin Investig Drugs* 11:298-308

Xu J, Rodriguez D, Peticlec E, Kim JJ, Hangai M, Moon YS, Davis GE, Brooks PC (2001a) Proteolytic exposure of a cryptic site within collagen type IV is required for angiogenesis and tumor growth in vivo. *J Cell Biol* 154:1069-79

Xu K, Lamanna JC (2006) Chronic hypoxia and the cerebral circulation. *J Appl Physiol* 100:725-30

Xu L, Sapolsky RM, Giffard RG (2001b) Differential sensitivity of murine astrocytes and neurons from different brain regions to injury. *Exp Neurol* 169:416-24

Yamagata K, Tagami M, Nara Y, Fujino H, Kubota A, Numano F, Kato T, Yamori Y (1997) Faulty induction of blood-brain barrier functions by astrocytes isolated from stroke-prone spontaneously hypertensive rats. *Clin Exp Pharmacol Physiol* 24:686-91

Yancopoulos GD, Davis S, Gale NW, Rudge JS, Wiegand SJ, Holash J (2000) Vascular-specific growth factors and blood vessel formation. *Nature* 407:242-8

Yang JP, Liu HJ, Liu XF (2010) VEGF promotes angiogenesis and functional recovery in stroke rats. *J Invest Surg* 23:149-55

Yang W, Ahn H, Hinrichs M, Torry RJ, Torry DS (2003a) Evidence of a novel isoform of placenta growth factor (PlGF-4) expressed in human trophoblast and endothelial cells. *J Reprod Immunol* 60:53-60

Yang Y, Ge W, Chen Y, Zhang Z, Shen W, Wu C, Poo M, Duan S (2003b) Contribution of astrocytes to hippocampal long-term potentiation through release of D-serine. *Proc Natl Acad Sci U S A* 100:15194-9

Yonekura H, Sakurai S, Liu X, Migita H, Wang H, Yamagishi S, Nomura M, Abedin MJ, Unoki H, Yamamoto Y, Yamamoto H (1999) Placenta growth factor and vascular endothelial growth factor B and C expression in microvascular endothelial cells and pericytes. Implication in autocrine and paracrine regulation of angiogenesis. *J Biol Chem* 274:35172-8

Zachary I (2005) Neuroprotective role of vascular endothelial growth factor: signalling mechanisms, biological function, and therapeutic potential. *Neurosignals* 14:207-21

Zhang P, Li J, Liu Y, Chen X, Lu H, Kang Q, Li W, Gao M (2010) Human embryonic neural stem cell transplantation increases subventricular zone cell proliferation and promotes peri-infarct angiogenesis after focal cerebral ischemia. *Neuropathology*

Zhang W, Mojsilovic-Petrovic J, Andrade MF, Zhang H, Ball M, Stanimirovic DB (2003) The expression and functional characterization of ABCG2 in brain endothelial cells and vessels. *Faseb J* 17:2085-7

Zhang ZG, Zhang L, Jiang Q, Zhang R, Davies K, Powers C, Bruggen N, Chopp M (2000) VEGF enhances angiogenesis and promotes blood-brain barrier leakage in the ischemic brain. *J Clin Invest* 106:829-38

Zhao B, Cai J, Boulton M (2004) Expression of placenta growth factor is regulated by both VEGF and hyperglycaemia via VEGFR-2. *Microvasc Res* 68:239-46

Zhao G, Flavin MP (2000) Differential sensitivity of rat hippocampal and cortical astrocytes to oxygen-glucose deprivation injury. *Neurosci Lett* 285:177-80

Zhao Y, Rempe DA (2010) Targeting astrocytes for stroke therapy. *Neurotherapeutics* 7:439-51

Zhou HJ, Zhang HN, Tang T, Zhong JH, Qi Y, Luo JK, Lin Y, Yang QD, Li XQ (2010) Alteration of thrombospondin-1 and -2 in rat brains following experimental intracerebral hemorrhage. Laboratory investigation. *J Neurosurg* 113:820-5

Zhou M, Kimelberg HK (2001) Freshly isolated hippocampal CA1 astrocytes comprise two populations differing in glutamate transporter and AMPA receptor expression. *J Neurosci* 21:7901-8

Zhou Q, Heinke J, Vargas A, Winnik S, Krauss T, Bode C, Patterson C, Moser M (2007) ERK signaling is a central regulator for BMP-4 dependent capillary sprouting. *Cardiovasc Res* 76:390-9

Ziche M, Maglione D, Ribatti D, Morbidelli L, Lago CT, Battisti M, Paoletti I, Barra A, Tucci M, Parise G, Vincenti V, Granger HJ, Viglietto G, Persico MG (1997) Placenta growth factor-1 is chemotactic, mitogenic, and angiogenic. *Lab Invest* 76:517-31

Zlokovic BV (2008) The blood-brain barrier in health and chronic neurodegenerative disorders. *Neuron* 57:178-201

Zonta M, Angulo MC, Gobbo S, Rosengarten B, Hossmann KA, Pozzan T, Carmignoto G (2003) Neuron-to-astrocyte signaling is central to the dynamic control of brain microcirculation. *Nat Neurosci* 6:43-50