UNIVERSITY OF LIEGE

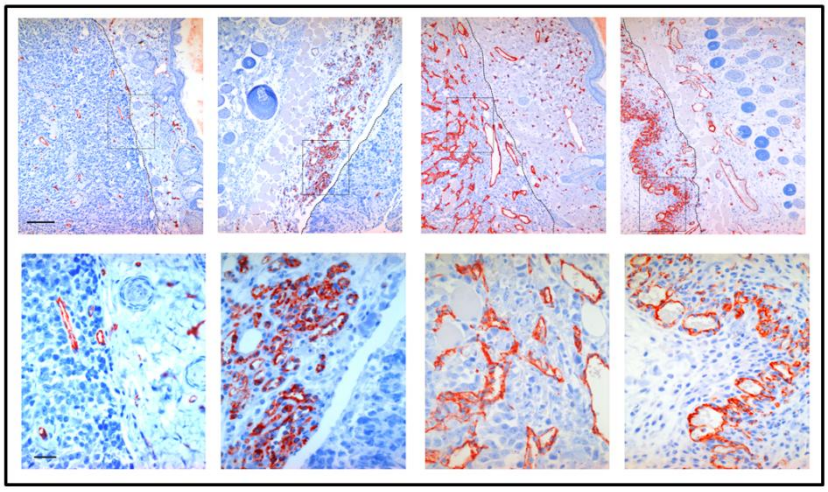
FACULTY OF MEDICINE

LABORATORY OF CONNECTIVE TISSUES BIOLOGY

Professor A. Colige

Doctor C. Lambert

Cooperation between carboxy-terminal domains of VEGF-A dictates its biological properties *in vitro* and *in vivo*



DELCOMBEL Romain

Thesis presented to obtain the degree of

PhD in Pharmaceutical and Biomedical Sciences

Academic year 2012-2013

UNIVERSITY OF LIEGE

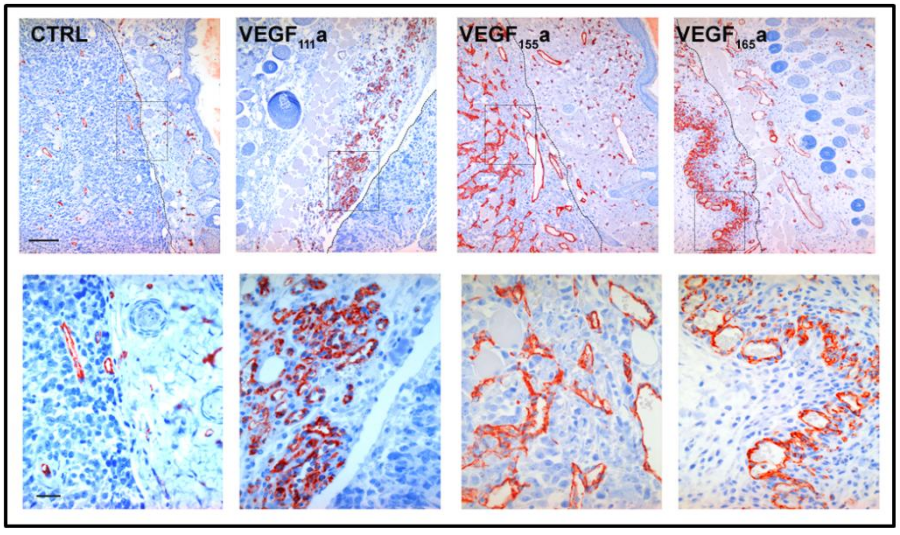
FACULTY OF MEDICINE

LABORATORY OF CONNECTIVE TISSUES BIOLOGY

Professor A. Colige

Doctor C. Lambert

Cooperation between carboxy-terminal domains of VEGF-A dictates its biological properties *in vitro* and *in vivo*



Blood vessels staining (anti-CD31 antibody) in tumours formed by HEK293 cells (either control or expressing $VEGF_{111}a$, $VEGF_{155}a$ and $VEGF_{165}a$) and in adjacent healthy tissues (upper panel: 10x magnification, lower panel: 40x magnification).

DELCOMBEL Romain

Thesis presented to obtain the degree of

PhD in Pharmaceutical and Biomedical Sciences

Academic year 2012-2013

REMERCIEMENTS

De mes premiers pas au laboratoire à la finalisation de l'écriture de ma thèse, je n'ai pu m'empêcher de comparer mon projet doctoral à la réalisation d'un film. Durant ce long métrage de 4 années, j'ai eu l'opportunité d'incarner le rôle d'un réalisateur à l'image de « Ferrand », interprété par François Truffaut lui-même dans son chef d'œuvre « La nuit Américaine ». Tout aussi passionné par la recherche contre le cancer que l'est Ferrand par le cinéma, j'ai atteint le but final de mon doctorat à travers un parcours semé d'embûches sans jamais perdre mon sens de l'humour.

A l'instar du film, la réalisation de ma thèse s'est effectuée sur le plateau principal qui est le Laboratoire de Biologie des Tissus Conjonctifs au CHU de Liège. Mais il y a également eu des scènes tournées en extérieur à l'image de la « Nouvelle Vague ». Par extérieur, j'entends ici le passage dans d'autres laboratoires en Belgique mais aussi en France et en Angleterre. Bien que certaines scènes ont été coupées au montage, je n'oublierai pas d'exprimer ma gratitude aux personnes avec qui des collaborations n'ont pas donné les résultats escomptés. Car, tout comme dans le 7ème art, la confrontation à l'échec au cours d'une thèse fait partie du quotidien. Truffaut lui-même dit dans une célèbre scène du long-métrage : *Un tournage de film [une thèse] ça ressemble exactement au trajet d'une diligence au Far West. D'abord, on espère faire un bon voyage et puis, très vite, on en vient à se demander si on arrivera à destination*. Néanmoins un sujet passionnant est toujours une source de motivation pour arriver au bout.

Enfin, tout comme dans « La Nuit Américaine », où le film se construit en dehors des plateaux et des lieux de tournage, le doctorat se joue aussi en dehors du labo. Dans ces circonstances, l'entourage y tient une place primordiale.

Dans cette logique, je vais donc remercier d'abord mes superviseurs qui ont été pour moi des producteurs passionnés par leur art : tantôt enthousiastes selon les résultats tantôt sceptiques à quelques « idées farfelues ». Mes pensées vont à mes deux producteurs principaux, Alain Colige et Charles Lambert, sans qui ce travail n'aurait jamais été aussi abouti. En poursuivant dans la métaphore, je pense également aux producteurs associés qui m'ont entouré tout au long de ce travail : Betty Nusgens et Christophe Deroanne pour les nombreuses idées échangées et leurs encouragements. Je n'oublie pas non plus l'auteur des précédentes recherches qui ont inspiré l'écriture du projet à l'origine de ma thèse: Pierre Mineur. Ses travaux ont été à l'image d'un fait marquant, source d'inspiration de mon scénario.

Un travail de réalisation est avant tout un travail d'équipe. Un bon doctorat est comme « la nuit Américaine » : on y joue le premier rôle, mais le film ne serait être bon sans les autres acteurs et membres de l'équipe de tournage. Prenons en exemple, le film « Docteur Foldingue » où Eddy Murphy joue tous les rôles. Comment le qualifier autrement que de « Mauvais Nanard » ? Tout comme Ferrand, je me suis très vite aperçu que pour entreprendre et terminer un doctorat le plus brillamment possible, j'avais besoin de m'entourer de gens compétents en qui j'exprimais une pleine confiance. Je pense ici en premier lieu à Lauriane Janssen avec qui j'ai démarré mon projet et qui m'a soutenu techniquement, « ingénieurement » et intellectuellement pendant tout son déroulement. Il me faut aussi citer Céline Hendricks qui a fabriqué de belles cellules, primordiales dans ce travail et bien sûre l'ensemble de mes collègues qui m'ont entouré. Ils ont été mes aînés et modèles (mes « Alfred Hitchcock », « Jean Renoir » ou autre « Roberto Rosselini ») qui m'ont tant inspiré : Nancy Garbacki, Audrey Courtois, Alexia Hulin, Audrey Stultiens, Thibault Neutelings, Audrey Henno, Marie-Jeanne Niks, Victor Tateu, Zakia Belaid Choucair et Mourad Bekhouche. Je ne veux pas oublier mes pairs contemporains ainsi que les futurs espoirs de la recherche scientifiques (nos DHP : Doctorant à Haut Potentiel) avec qui j'ai échangé de nombreuses idées et partagé de fructueux points de vue : Yves Delforges, Maude Gabriel, Jérôme Willems et Laura Dupont. Je remercie également les experts de l'histologie avec qui j'ai eu le plaisir de travailler et partager mes repas : Marie-Paule Defresne, Chantal Humblet, Aurore Beaulieu, Géraldine Poncin et Delphine Delneuville.

Pour assistants techniques, j'ai eu la chance de côtoyer de réels super-héros de la biologie pro-active : le Super-Toto (Antoine Heyeres, expert de la Q-RT-PCR, parfois plus de la Q que de la RT-PCR) et la Super-Cocotte (Audrey Hoffman, reine de l'histo). Je remercie également Christine, qui outre son importante aide administrative, m'a aussi transmis sagesse et leçons de vie.

Je ne saurai m'empêcher de citer les gens qui ont apporté leur savoir-faire et expériences pour élargir les domaines de compétence de mon projet. J'exprime ici ma gratitude à Roger Vassy, Anna Starzec et Oualid Haddad qui m'ont fait découvrir les merveilles du Biacore. J'en profite pour remercier les autres personnes qui m'ont accueilli pour utiliser cette technique, offrant un « effet spécial » à ma publication : Nathalie Charnaux et Didier Letourneur. Avant de quitter Paris, j'aimerais remercier Nadine Suffee qui m'a apporté son expérience tant dans la publication de son travail dans le journal « Angiogenesis » que dans la rédaction de sa thèse. Je remercie également Melissa Gammons, Nick-Beazley Long et David Bates qui m'ont accueilli à Bristol pour réaliser les expériences sur les yeux. Je remercie les membres du Laboratoire de Biologie des Tumeurs et du Développement, pour le prêt de matériel (merci Céline Gérard et Charlotte Erpicum), mais surtout Nor Eddine

Sounni et Agnès Noël avec qui j'ai fait de belles souris bleues. Je remercie Maryse Delehedde, Beatrice Eymin et Martine Applanat qui m'ont fait profiter de leurs expériences lors d'une récente collaboration pour la première et lors d'échanges scientifiques pour les secondes. Enfin je remercie toutes les personnes du CHU, du GIGA, de l'animalerie centrale (Fabrice Olivier, Luc Duwez et Pierre Drion) et de la faculté de médecine vétérinaire qui m'ont permis de mener à bien ce travail.

Je m'attèlerai maintenant à remercier mon épouse, ma famille et mes proches pour leur soutien durant ces 4 longues années. Jennifer partage ma vie depuis près de 5 ans ; son amour et le bonheur qu'elle m'apporte ont été une aide quotidienne. Je remercie mes parents pour l'éducation qu'ils m'ont donnée, l'environnement qu'ils m'ont offert depuis ma naissance et pour m'avoir donné la possibilité de partir à Liège faire mes études. Je remercie mes frères, Cyril et Hugo, compagnons de ma jeunesse et excellents camarades comme lors nos dernières vacances. J'exprime ma gratitude à mes grands-parents, Janine et Bernard, avec qui je partage une relation privilégiée et qui ont été une oreille attentive tout au long de ma thèse. Je me permets ici de saluer le remarquable travail de recherche sur les guérisons miraculeuses de Lourdes que mon grand-père a effectué ces 5 dernières années. Ce dernier a été publié dans un journal scientifique international et c'est un grand honneur pour moi de lui rendre hommage en le citant dans ma thèse : (Francois et al., 2012). Mes pensées vont à mes témoins de mariage (Antoine, Emmanuel, Marion et Thomas) que je ne regrette pas d'avoir choisi tant ils sont des amis fidèles. Je n'oublie pas le reste de ma famille (Jimou y compris) et de mes amis qui comptent beaucoup pour moi.

Je remercie tous les spectateurs qui seront présents dans la salle pour assister à la présentation de ce travail. Sans attendre une palme, un lion ou un ours d'or, j'espère que les membres du jury apprécieront la qualité scientifique de mon manuscrit et qu'ils pourront ressentir à travers sa lecture toute la passion, l'énergie et le plaisir que j'ai eu à le réaliser. Je souhaite aussi que les scientifiques qui assisteront à ma défense la décriront à leurs collègues (lors de leur prochaine pause-café) telle une critique dithyrambique dans « Les cahiers du cinéma ». J'exprime ma gratitude à tous mes amis présents ce jour en les invitant lors de l'apéritif qui suivra la projection. Me féliciteront-ils en me mettant une cote « cinq étoiles» comme dans le magazine « Première » ?

Pour clore le parallélisme avec le cinéma, un support financier au projet est toujours nécessaire, c'est pourquoi je remercie les organismes de soutien à la recherche sans qui ce travail n'aurait pu ni débuter ni s'achever : la Fondation contre le cancer et la Région Wallonne.

TABLE OF CONTENTS

ABBREVIATIONS LIST

Ala:	Alanine
ALT:	Alanine Transaminase
AMD:	Age-related Macular Degeneration
Ang:	Angiopoietin
Arg:	Arginine
Asn:	Asparagine
Asp:	Aspartic acid
AST:	Aspartate Transaminase
BSA :	Bovine Serum Albumin
CNV:	Choroidal Neovascularization
Cys:	Cysteine
Dll:	Delta-like ligand
dpf:	day post fertilization
DSS:	Distal Splice Site
DV:	Draining Veins
E:	Exon
EC:	Endothelial Cells
ECM:	Extra-Cellular Matix
EGF:	Epidermal Growth Factor
EPC:	Endothelial Progenitors Cells
ERK:	Extracellular signal-regulated kinases phosphorylation
ESM:	Endothelial-cell Specific Molecule
FA:	Feeder Arteries
FAK:	Focal Adhesion Kinase
FBS:	Fœtal Bovine Serum
FGF:	Fibroblast Growth Factor
GAG:	GlycosAminoGlycan
Gln:	Glutamic acid
Glu:	Glutamine
GMP:	Glomeroïd Microvascular Proliferation
H:	Heparin
h:	hour (s)
HIF:	Hypoxia-Inductible Factor
His:	Histidine
HRP:	HorseRadish Peroxydase
HSPG:	Heparan Sulfate ProteoGlycans
HUVEC:	Human Umbilical Vein Endothelial Cells
Ig:	Immunoglobulin
IGF:	Insulin-like Growth Factor
IP:	Intraperitoneal
IU:	International Unit

IV:	Intraveinous
Ka:	Constant of association
Kd:	Constant of dissociation
kD:	kiloDalton
LDH:	Lactate DeHydrogenase
Leu:	Leucine
Lys:	Lysine
M:	Molar
MAPK:	Mitogen-Activated Protein Kinase
min:	minute (s)
MMP:	Matrix MetalloProteinase
MV:	Mother Vessels
NO:	Nitric Oxide
NRP:	Neuropilin
OD:	Optical Density
OIR:	Oxygen Induced Retinopathy
PAEC:	Porcine Aortic Endothelial Cells
PBS:	Phosphate Buffered Saline
PBT:	PBS-BSA 0,5%-tween-80
PC:	Pericytes
PDGF:	Platelet-Derived Growth Factor
Phe:	Phylalanine
PlGF:	Placental Growth Factor
Pro:	Proline
PSS:	Proximal Splice Site
ROP:	Retinopathy of Prematurity
RPE:	Retinal Pigment Epithelial cells
RU:	Response Unit
s:	second
SC:	Subcutaneous
SDS-PAGE:	Sodium Dodecyl Sulfate-PolyAcrylamide Gel Electrophoresis
Ser:	Serine
SF:	Splicing Factor
sNRP:	soluble Neuropilin
SPR:	Surface Plasmon Resonance
SR-proteins:	Serine and Arginine rich proteins
TGF:	Transforming Growth Factor
Thr:	Threonine
Tie:	Tyrosine kinase with immunoglobulin-like and EGF-like domains
TKI:	Tyrosine Kinase Inhibitor
TNF:	Tumor Necrosis Factor
Trp:	Tryptophan
Tyr:	Tyrosine
uPA:	urokinase-type Plasminogen Activator
VEGF:	Vascular Endothelial Growth Factor
VEGF-R:	Vascular Endothelial Growth Factor Receptor

VM:	Vascular Malformation
VPF:	Vascular Permeability Factor
VSMC:	Vascular Smooth Muscle Cell
WT1:	Wilms Tumour protein 1
α-SMA:	Alpha Smooth Muscle Actin

GENERAL INTRODUCTION

I- Processes of novel vessels formation

I-1 Generalities

I-1-1 Blood vessels types

Nearly 100.000 kilometers of blood vessels and capillaries cover the entire volume of the human body. In a highly organized circuit, blood oxygenated in the lungs is pulsed from the heart to the organs by aorta, arteries, arterioles and finally capillaries that allow exchanges with the extra-vascular compartment. Blood flows then into veinules and veins up to the lung to enable its reoxygenation.

The inner lining of all types of blood vessels is the endothelium formed by vascular endothelial cells (Mehta and Malik, 2006). Capillaries are the simplest vessels. They can be considered as immature or mature depending on the presence of a continuous basement membrane and of mural cells, referred to as pericytes (Gerhardt and Betsholtz, 2003; Sims, 1991) (Fig 1A) that play critical functions in the stabilization of the vessels (Benjamin et al., 1998; Dor et al., 2002). The intermediate (arterioles and veinules) and large (arteries and veins) vessels are surrounded by one or many layers of vascular smooth muscle cells (VSMC) (Gerhardt and Betsholtz, 2003) located at the interface between the endothelium and the surrounding tissue. This localization close to endothelial cells gives VSMC a crucial role in angiogenesis, notably by secreting growth factors (Allt and Lawrenson, 2001; Sims, 1986).

I-1-2 Blood vessels origin

The development of the blood vascular system is one of the earliest events in embryogenesis, all other organs depending on vascular supply for delivery of nutrients and oxygen and for clearance of wastes. During embryonic development, the nascent vascular network is formed through two processes (Fig 1B). In the first one, identified as vasculogenesis, new vessels form *de novo* via the assembly of mesoderm-derived precursors (hemangioblasts differentiating in angioblasts) that evolve into a primitive vascular labyrinth (Eichmann et al., 1997; Isner and Asahara, 1999). In the second process, referred to as angiogenesis, the pre-existing vasculature progressively spreads through a mechanism of vessel sprouting (also called capillary branching) (Karamysheva, 2008). These two processes, together with progressive remodelling mechanisms will finally end up into a highly organized and stereotyped vascular network of large vessels ramifying into smaller ones (Carmeliet, 2000; Potente et al., 2011) (Fig 1B).

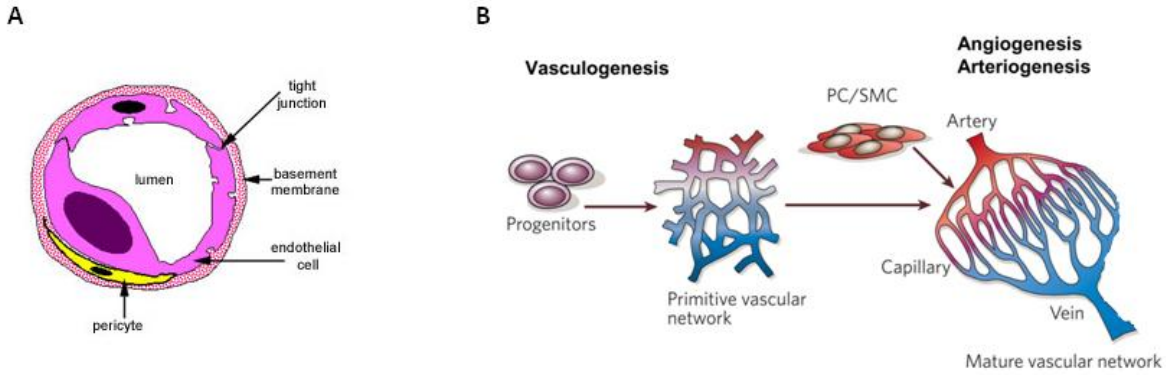


Figure 1 : Development of the vascular system
(A) Mature capillaries are formed by endothelial cells surrounded by a basement membrane and partly covered by pericytes. **(B)** Vessels development during embryogenesis. By vasculogenesis, endothelial progenitors (hemangioblasts) give rise to a primitive vascular labyrinth of arteries and veins. During the subsequent step of angiogenesis, the network extends and pericytes (PCs) and smooth muscle cells (SMCs) cover nascent endothelial channels. ***From (Carmeliet, 2005)***

I-1-3 Blood vessel formation in adulthood

In adulthood in physiological conditions, the formation of new blood vessels is highly regulated and restricted to some defined situations such as embryonic implantation, wound healing and menstrual cycles for example. Excessive or abnormal angiogenesis is a key process in cancer, ocular or inflammatory diseases but is also implicated in pathological conditions such as asthma, diabetes, cirrhosis, multiple sclerosis, endometriosis, obesity and autoimmune diseases (Carmeliet, 2005). By contrast, insufficient angiogenesis is observed in ischemic disorders, pre-eclampsia and chronic ulcers.

Angiogenic sprouting (Fig 2A) is the main, but not the unique, mechanism of blood vessel formation in the adult (Adams and Alitalo, 2007; Carmeliet and Jain, 2011). Indeed, endothelial precursors cells (EPC) can be mobilized from bone marrow or from tissue niches and incorporated into the walls of growing blood vessels where they differentiate into endothelial cells. This process corresponds to the previously mentioned vasculogenesis (Fig 2B) (Kirton and Xu, 2010). Intussusception is another process allowing the spreading of the vascular network. It involves transluminal tissue pillars development and fusion within a mother blood vessel, thus delineating two new daughter vessels (Burri et al., 2004; Kurz et al., 2003) (Fig 2 C).

I-1-4 Blood vessel development in cancer

Interactions between cancer cells and their microenvironment are crucial for promoting tumour growth and invasiveness. Together with degradation and remodelling of the peritumoural extracellular matrix, development of blood vessels is a key factor regulating

cancer progression (Ferrara and Kerbel, 2005) by allowing the supply of sufficient amounts of oxygen and nutrients (Folkman, 1995). It is usually considered that the growth of tumours beyond 2-3 mm^3 and the formation of metastases are largely dependent on angiogenesis (Folkman, 1972). Recently, additional mechanisms of vascularization have been identified in tumour environment (Carmeliet and Jain, 2011) (Fig 2D-F). For vessel co-option, appearing usually in well-vascularized tissues, cancer cells initially associate with and grow preferentially along pre-existing normal vessels. This favours tumour growth without any actual angiogenic response. By contrast, in vascular mimicry and cancer stem cell differentiation, cancer cells directly participate in blood vessel formation by differentiating and functioning as endothelial cells. These different processes may exist concomitantly in the same tumour or may be selectively involved in a specific tumour type or host environment.

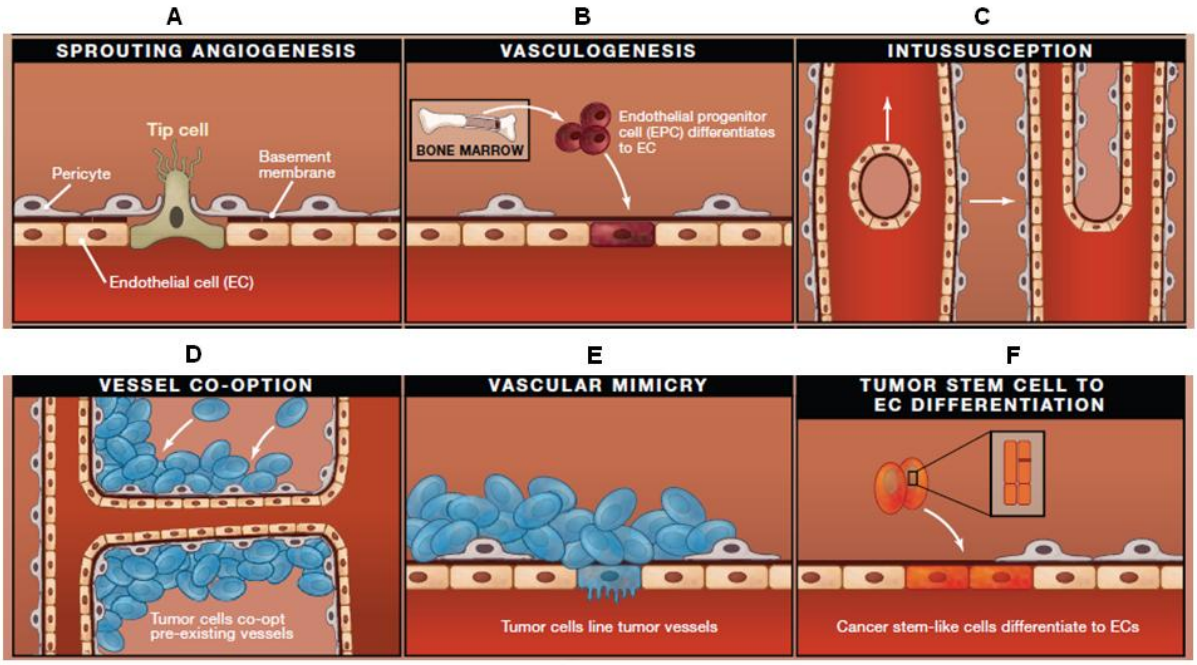


Figure 2 : Blood vessels formation in normal tissues and tumours.
Blood vessels can be formed by sprouting angiogenesis (**A**) and by vasculogenesis **(B)**, a process involving the recruitment of bone-marrow-derived endothelial progenitor cells (EPCs) that differentiate into endothelial cells (ECs). Intussusception refers to a mechanism where a vessel is split in two vessels of smaller diameter (**C**). Tumours cells can grow along the pre-existing vessels by a mechanism referred to as vessel co-option (**D**) or can participate to tumour vessel formation together with endothelial cells (vascular mimicry, **E**). Endothelial cells with cytogenetic abnormalities and probably deriving from putative cancer stem cells have also been described in the literature (**F**). Unlike normal tissues, which use only angiogenesis, vasculogenesis and intussusception (**A-C**), blood supply in tumours can rely on any of the six modes of vessel formation (**A-F**). ***From (Jain and Carmeliet, 2012)***

I-2 Cellular and molecular mechanisms of angiogenesis

Angiogenesis is initiated by various types of stimuli, including hypoxia, inflammation and mechanical solicitation such as shear stress (Milkiewicz et al., 2006). In hypoxic conditions, the cellular level of HIF1α (Hypoxia-Inducible Factor 1α) is increased, resulting in vascular endothelial growth factor-A (VEGF-A or VEGF) synthesis and secretion, which, in turn, participates to the recruitment of blood vessels towards hypoxic tissues (Kelly et al., 2003).

The process of sprouting angiogenesis involves several sequential steps, each requiring the coordinated action of mechanisms involving the activation of a large set of ligands and receptors (Karamysheva, 2008). The overall process is depicted in Figure 3.

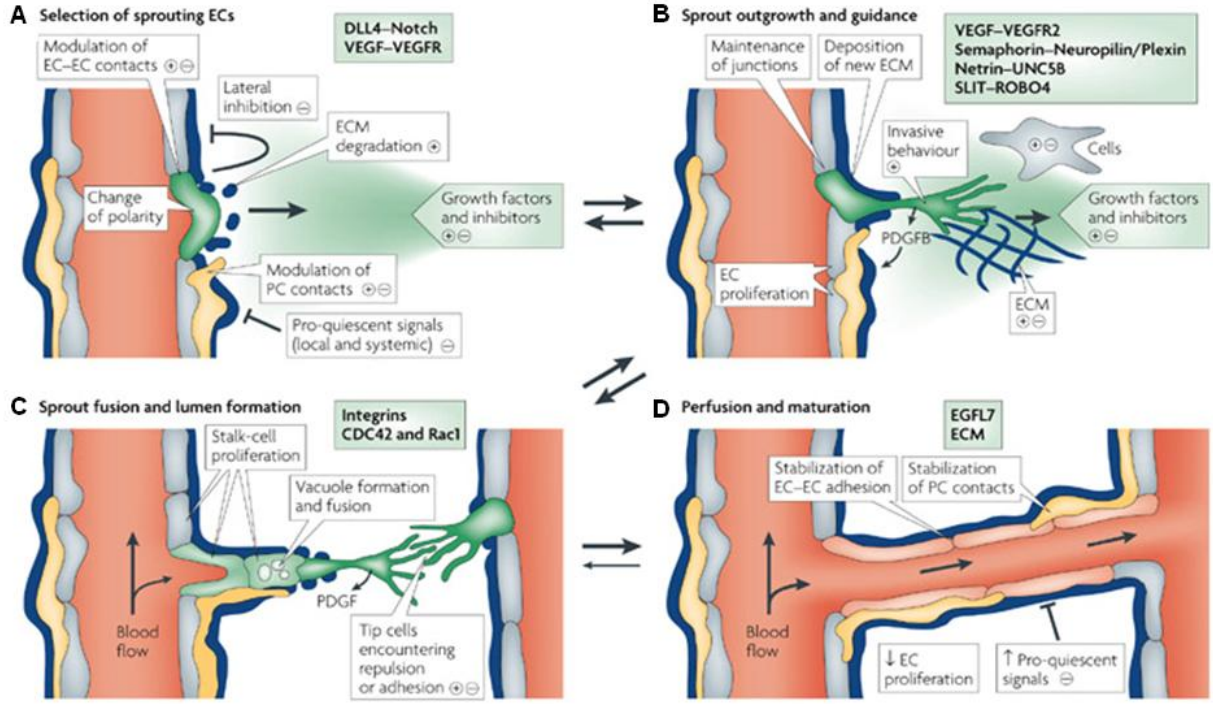


Figure 3 : Angiogenic sprouting
(A) Sprouting angiogenesis is promoted when pro-angiogenic processes (+) overwhelm anti-angiogenic (-) ones. In these conditions, some endothelial cells (EC) (green) are selected among others (grey) to become "tip cells" able to sprout. Sprouting is composed of several crucial steps: the adoption of an apico-basal polarity, the induction of motility and invasiveness, the modulation of the contacts with adjacent cells (other EC and pericytes, PC) and the degradation of the surrounding matrix (ECM). **(B)** The growing EC sprout is guided by a gradient of growth factors, such as VEGF. Other cues may include attractive (+) or repulsive (-) signals from matrix and cells found in the tissue environment. Then, the "tip cells" secrete PDGF to induce the recruitment of PC to the newly formed vessel. These cells allow to maintain EC-EC junctions and to prevent excessive vessel permeability. **(C)** Adhesive and repulsive interactions occur when "tip cells" meet each other. This controls the fusion of adjacent sprouts and the formation of the new vessel. **(D)** Fusion processes at the EC-EC interfaces establish a continuous lumen. As blood flow improves oxygen delivery the hypoxia-induced pro-angiogenic signals are reduced. Blood circulation also promotes the final maturation processes consisting in stabilization of cell junctions, matrix deposition and tight PC attachment. ***From (Adams and Alitalo, 2007)***

I-2-1 Increase of vascular permeability

VEGF secretion controls the initial step of angiogenesis by inducing dilatation and increased permeability of the vessels. These two processes involve nitric oxide pathway (NO, see paragraph VI-2*)* and the remodelling and destabilization of the junctions between adjacent endothelial cells (Dejana et al., 2009). This results in the release of plasma proteins in the ECM to form a provisional scaffold permissive for the migration of endothelial cells (Potente et al., 2011). Prior to endothelial cells migration, mature vessels need to be destabilized. This process is induced by Angiopoietin 2 (Ang 2) and includes VSMC detachment and degradation of the surrounding matrix by several proteolytic enzymes, such as MMP (Matrix Metalloproteinases). These proteases further release and/or activate growth factors sequestered within the extracellular matrix (Thomas and Augustin, 2009).

I-2-2 Endothelial cell proliferation and migration

Some endothelial cells within the vessel wall are elected to initiate the newly formed vessel formation. These cells are named “tip cells” because they migrate and lead the growing sprout. The neighbouring endothelial cells form the “stalk” of the vascular sprout, proliferating and staying behind to maintain the integrity and perfusion of the growing vascular bed (Gerhardt et al., 2003).

The “tip” or “stalk” phenotype is mainly regulated by the VEGF-VEGFR2 and Notch-Dll4 pathways. First, VEGF-A signalling via VEGF-R2 (VEGF-Receptor 2) induces the chemotactism and the invasiveness of the “tip cell”. It also stimulates Dll4 (Delta-like ligand 4) expression in “tip cells” which activates Notch receptors in adjacent cells, which is responsible for the induction of the “stalk cell” phenotype (Liu et al., 2003; Sainson et al., 2005; Uyttendaele et al., 2001). As Notch activation also induces Dll4, this mechanism propagates the Dll4-Notch-mediated lateral inhibition along the developing vessels, limiting therefore the number of “tip cells” and precluding anarchic vascular development (Hellstrom et al., 2007; Suchting et al., 2007).

In addition to motility features, “tip cells” are also characterized by a proteolytic phenotype responsible for invasiveness. Indeed, they secrete MMPs that are able to breakdown the basement membrane and the surrounding matrix. “Tip cells” also possess numerous filipodial protrusions sensing in their environment the gradients of different attractive (eg. VEGF) and repulsive cues allowing tightly regulated guidance of the sprout (Gerhardt et al., 2003). When a “tip cell” moves forward up to a “tip cell” of another sprout or to existing capillaries, it loses its motile phenotype and establishes strong EC-EC adhesive interactions (Karamysheva, 2008). During all these processes, “stalk cells”, controlled by the VEGF-

VEGFR2 axis, maintain vessel integrity by proliferating and maintaining contacts together and with the leading tip cell (Adams and Alitalo, 2007; Adams and Eichmann, 2010; Chung et al., 2010; Eilken and Adams, 2010; Herbert and Stainier, 2011; Potente et al., 2011).

I-2-3 Lumen formation

The establishment of blood flow requires the formation of a lumen, which may occur within the sprout before or after they have joined with other vessels. Initially, forming blood vessels consist of multicellular rods of endothelial cells that are interconnected by uniform junctions. Thus, the formation of the lumen needs a lateral redistribution of adhesion molecules and the acquisition of an apico-basal polarity. This process is further expanded by several mechanisms that may be cell or situation dependent. For instance, we can mention modifications of cell shape or the formation and the coalescence of intracellular vacuoles that fuse with the apical surface of the cells delimitating the lumen (Herbert and Stainier, 2011).

I-2-4 Vessels stabilization and maturation

The generation of a lumen and the onset of blood circulation improve oxygen delivery which consequently decreases the local VEGF production (Karamysheva, 2008). This resolution phase is associated with the interruption of endothelial cells proliferation. Endothelial cells secrete platelet-derived growth factor-B (PDGF-B), which promotes recruitment of pericytes to nascent vessels (Hellstrom et al., 1999; Lindahl et al., 1997). Pericytes establish then direct contacts with endothelial cells in the immature vessels (Gerhardt and Betsholtz, 2003). Vessel maturation relies also partly on transforming growth factor β (TGFβ) signalling which stimulates mural cell formation (Gerhardt and Betsholtz, 2003) and also promotes the production of extracellular matrix molecules.

Several other signalling pathways are further implicated in these maturation processes, such as Ang1 ligand/Tie2 receptor (Tyrosine kinase with Immunoglobulin-like and EGF-like domains 2) signalling (Holash et al., 1999; Ramsauer and D'Amore, 2002; Suri et al., 1996). Finally, secretion of specific extracellular matrix molecules by endothelial cells and surrounding cells results in the formation of a subendothelial basement membrane and the transition to a quiescent state (Adams and Alitalo, 2007; Chung et al., 2010; Potente et al., 2011).

I-3 Specific aspects of cancer blood vessels

Pathological angiogenesis relies on many similar inducers, regulators and mechanisms as those involved in physiological neo-vessels formation. A striking difference is however

observed at the end of the process. In physiological conditions, perfusion of the new vessel initiates cascades that will contribute to its final maturation and terminate angiogenesis. By contrast, during pathological conditions, such as tumour angiogenesis, the angiogenic cascade is persistent and generates an anarchic formation of blood vessels (Sitohy et al., 2012). This specific aspect is driven by VEGF over-expression and gives rise to 6 different types of vessels by angiogenesis and arterio-venogenesis (Fig 4) (Nagy et al., 2010; Nagy et al., 2007). In the first process, small capillaries and veinules are transformed in "*mother vessels*", enlarged vessels devoid of pericyte and basement membrane. This structure can then evolve into three types of "*daughter vessels*":

- "*Glomeroïd Microvascular Proliferation*" units, which are poorly ordered structures composed of proliferative endothelial cells, pericytes and macrophages;
- "*capillaries*", obtained by a process of intussusception
- "*vascular malformation*", a mother vessel which acquired a dense coat of VSMC.

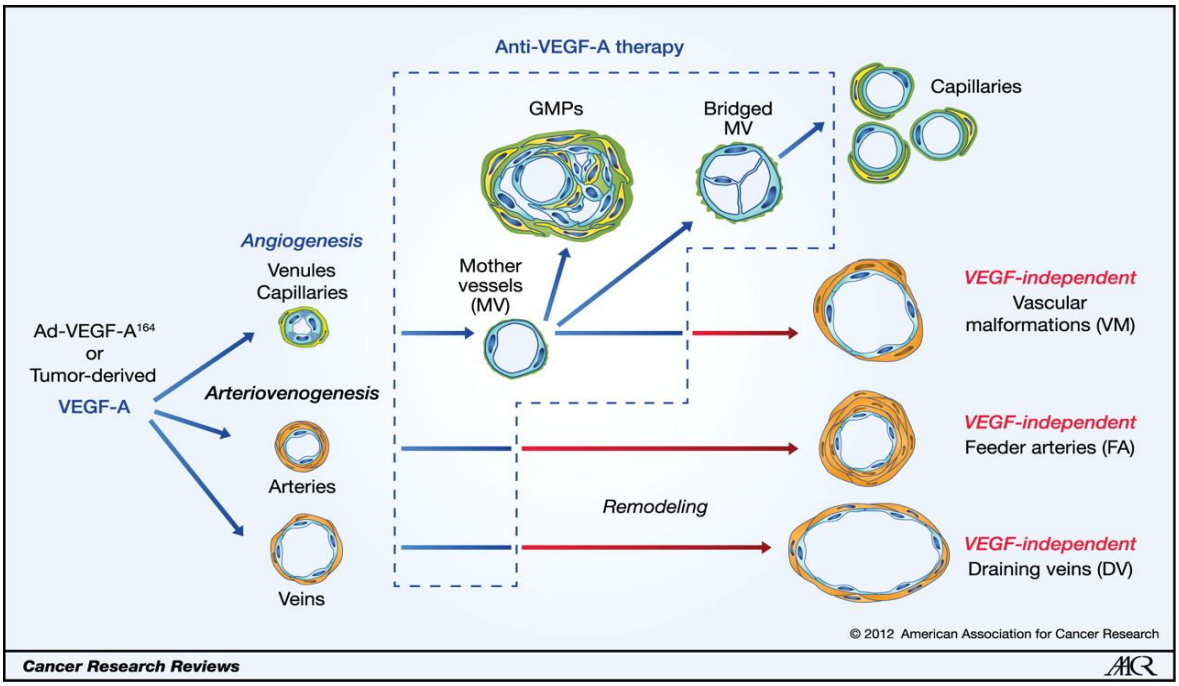


Figure 4 : Blood vessel types observed in tumours or after implantation of adenovirus expressing VEGF$_{164}$a

VEGF expressed by tumour cells is responsible for the formation of typical cancer blood vessels. "Mother vessels" (MV), derived from capillaries and veinules, give rise to "Glomeroïd Microvascular Proliferation" (GMPs) units, "Vessel Malformations" (VM) and, by a process of splitting, new "Capillaries". Remodelling of arteries and veins generates respectively "Feeder arteries" (FA) and '"Draining Veins" (DV). These latter vessel types, as well as "Vessel Malformation" and Capillaries, are surrounded by VSMCs (orange) and/or pericytes (yellow), a sign of maturation conferring resistance to anti-VEGF therapy. By contrast, "Mother vessels" and "Glomeroïd Microvascular Proliferation" units have been described to be sensitive to anti-VEGF therapy (Dashed line box). ***From (Sitohy et al., 2012)***.

Arteries and veins are also affected during tumour angiogenesis. They undergo an enlargement resulting from proliferation of endothelial cells and VSMC to give birth to *feeder arteries* and *draining veins*. These vascular patterns determine resistance or sensitivity to anti-VEGF therapy (see chapter VII).

II- Alternative splicing

In Eukaryotes, most of the genes are transcribed as pre-mRNA composed by an alternation of coding (exons) and non-coding (introns) sequences (Berget et al., 1977; Chow et al., 1977). During a maturation step, introns are then spliced out while exons are joined together to form mature mRNA. This process is referred to as constitutive splicing when it remains unchanged in any condition. For more than 90 % of the genes, however, such as VEGF-A for example (Munaut et al., 2010), the pre-mRNA can give rise to several mRNA and proteins through a process of alternative splicing of defined sequences (Hilmi et al., 2012; Wang et al., 2008). This highly regulated mechanism can lead to a variety of combinations such as exclusion of one, several or part of exons or retention of sequences that are intronic in other transcripts (Fig 5). The different mRNA variants can then encode different proteins that may possess distinct or, sometimes, opposite functions.

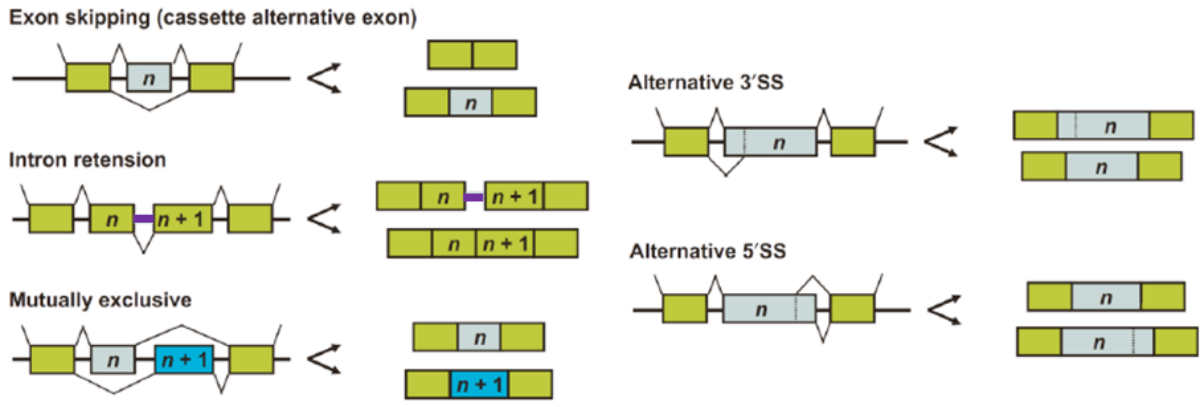


Figure: 5 Mechanisms of alternative splicing

Constitutive splicing refers to a mechanism leading always to an identical process of intron excision and exon joining. Introns are represented by black or violet lines and exons by green, grey or blue boxes, respectively. Alternative splicing of pre-mRNA can generate several isoforms (variants) of mRNA and proteins. Several mechanisms of alternative splicing can affect pre-mRNA such as “cassette exon” splicing (skipping of one or several complete exons), “intron retention” (a situation where the newly included sequence (violet line) can be formally considered as an intron for other variants, “mutually exclusive exons” (one exon (grey) or the other (blue) is present, but never both together) and “alternative 3’ or 5’ splice site (SS)” selection. ***From (Miura et al., 2012)***

Several regulatory cascades and factors control the alternative splicing process. For instance, Akt (Blaustein et al., 2005) or SR-proteins kinases, SRPK1 and SRPK 2 (Nowak et al., 2010), can act as potent regulators by phosphorylating the SR-proteins (Serine and Arginine rich proteins) which induces their translocation to the nucleus where they can affect splicing decisions (Stamm, 2008).

During cancer formation and progression, pre-mRNA of many genes undergo alternative splicing to give rise to novel variants conferring specific advantages to tumour cells (Miura et al., 2012; Skotheim and Nees, 2007). Furthermore, many chemotherapeutic agents can alter alternative splicing (Sumanasekera et al., 2008). An interesting example is the generation of a novel VEGF variant, $VEGF_{111}$, by treating various tumour cells with cytotoxic drugs (Mineur et al., 2007). This example has a direct link with our work and will be therefore detailed below (see paragraph IV-8).

III- Vascular Endothelial Growth Factor-A

III-1 Discovery and generalities

VEGF-A, usually referred to as VEGF, is a glycoprotein described by N. Ferrara (Ferrara and Henzel, 1989) in the conditioned culture medium of pituitary gland cells as a factor able to trigger endothelial cells proliferation. It was also previously identified as VPF (Vascular Permeability Factor), a factor secreted by numerous tumour cells lines (Senger et al., 1983) or as FSdGF (Follicular Stellate-derived Growth Factor) (Gospodarowicz et al., 1989) and Vasotropin (Plouet and Moukadiri, 1990b; Plouet et al., 1989).

VEGF-A is now considered as the main growth factor regulating both physiological and pathological angiogenesis (Ferrara, 2004; Ferrara and Alitalo, 1999). Its expression is crucial for development as inactivation of a single VEGF allele in mice causes early embryonic mortality due to deficient endothelial cell proliferation, defective blood vessels formation and consequently numerous vascular abnormalities (Carmeliet et al., 1996; Ferrara et al., 1996; Gerber et al., 1999).

VEGF-A is a member of the VEGF family which also includes VEGF-B, -C, -D and PlGF (Placental Growth Factor). VEGF-E and -F are also considered as being members of this family although they are not of mammalian origin but produced from the pox virus genome or present in some snake venom, respectively (Ogawa et al., 1998; Pieren et al., 2006; Yamazaki et al., 2005). The specific functions of each of these VEGF are largely, but not

only, dictated by their affinity for three receptors and two co-receptors (Fig 6). For example, most of the activities of VEGF-A rely on its capacity to interact with VEGF-R1, VEGF-R2 and, according to the isoform, to neuropilins 1 and 2. VEGF-B and PlGF bind to VEGF-R1 and NRP1 but not VEGF-R2 (de Vries et al., 1992; Neufeld et al., 2002; Soker et al., 1998). VEGF-C and VEGF-D acts through VEGF-R2 and VEGF-R3 to drive lymphangiogenesis (Koch et al., 2011).

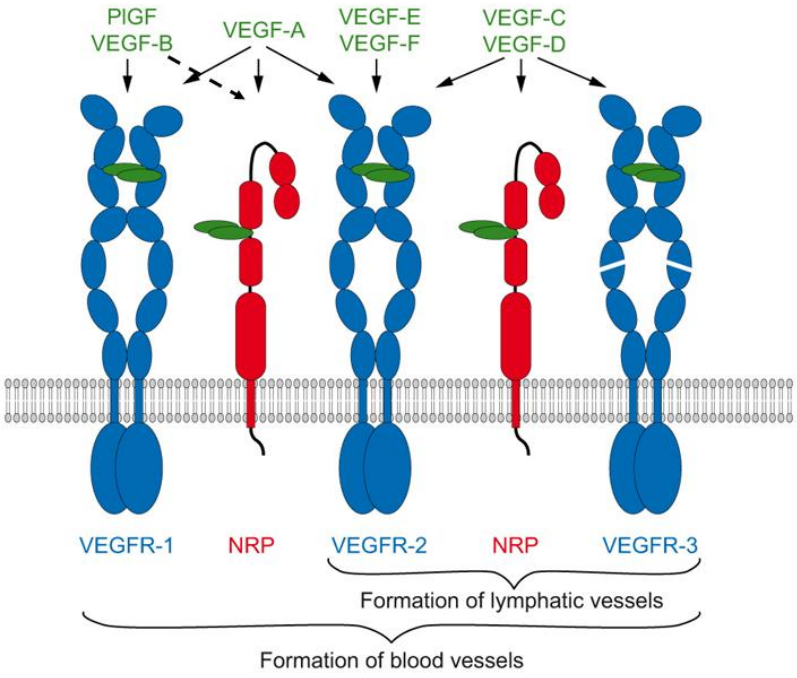


Figure 6 : VEGF family members and their receptors

VEGF-A, -B, -C, -D, -E and -F and PlGF are soluble growth factors regulating cell phenotype through binding to different receptors (VEGF-R1, VEGF-R2, VEGF-R3) and co-receptors (NRP and proteoglycans (not shown here)). A short summary of what is known about the (co-)receptors is provided elsewhere (paragraph VI-3, VI-4 and VI-5). Although a single VEGF member can bind to several (co-)receptors, redundancy between their roles is limited. VEGF: Vascular Endothelial Growth Factor, VEGF-R: VEGF-Receptors, NRP: Neuropilins 1 or 2, PlGF: Placental Growth Factor. ***From (Berger and Ballmer-Hofer, 2011)***

III-2 General structure of VEGF

VEGF-A gene contains 8 exons (Ferrara et al., 2003). After transcription, the pre-mRNA gives rise to several mRNA coding for VEGF-A variants possessing some specific properties. The first 4 exons (E1-E4) are present in all mRNA variants while the terminal exons (E5-E8) can be alternatively spliced by mechanisms of cassette exon skipping, partial exon retention and alternative 3' splice sites (Fig 5, Fig 8). The nature of the regulations influencing the alternative splicing of the VEGF-A pre-mRNA remains poorly understood (Gout et al., 2012). The resulting proteins are identified by a code corresponding to the

number of amino acid present in the molecule after cleavage of the signal peptide and a letter, “a” or “b”, indicating the nature of the terminal exon, either E8a or E8b, encoding the last six amino acids (Fig 8).

As the most critical domains required for the correct folding of the VEGF molecules are located in the sequence encoded by exons 1 to 4, it is generally accepted that all these variants possess a similar general organization. VEGF is active only as a dimer formed by two monomers positioned in a head-to-tail manner (Muller et al., 1997; Pieren et al., 2006) (Fig 7). Disulfide bridges between Cys residues encoded by exons 3 and 4 are crucial for the proper folding of individual monomers (intra-chain bridges involving Cys^{57} and Cys^{102}, Cys^{61} and Cys^{104}, Cys^{26} and Cys^{68}) but are also required for stabilizing the dimer (inter-chain bridges, Cys^{51} and Cys^{60}). Formation of dimers by two monomers of different size has been demonstrated (Keyt et al., 1996a) but their properties have not been thoroughly studied (Kurz et al., 1998; Morbidelli et al., 1997). In the same context, VEGF-A/PlGF (DiSalvo et al., 1995) and VEGF-A/VEGF-B (Olofsson et al., 1996) heterodimers have been also identified, adding an additional level of complexity in the study of the functions of the individual family members.

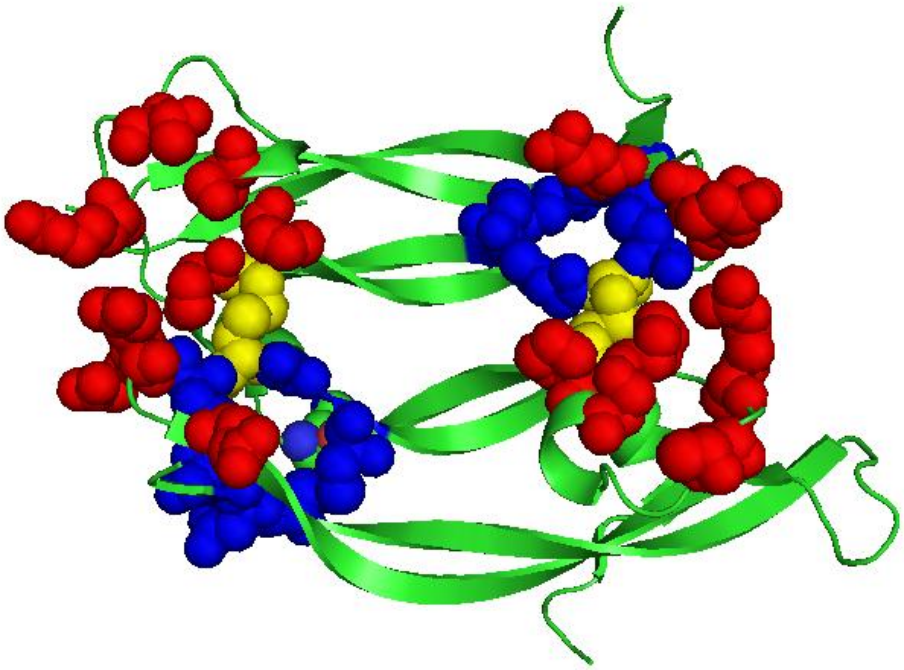


Figure 7 : 3D-Structure of VEGF dimer

VEGF, in this case VEGF-E used as a model, are composed of 2 monomers linked by 2 disulfides bonds (yellow) in head-to-tail manner. In VEGF-A, Cys^{51} and Cys^{60} are the two critical residues involved in the formation of these disulfide bonds. Each monomer contains a “Cystin-Knot” structure (blue) where Cys^{57} and Cys^{102}, Cys^{61} and Cys^{104}, Cys^{26} and Cys^{68} form intra-chain bonds. The binding sites to VEGF-R2 are drawn here in red.

III-3 Regulation of VEGF-A expression

VEGF-A is expressed in almost all healthy tissues where it plays a crucial role for blood vessels homeostasis (Berse et al., 1992; Ferrara et al., 1992; Ferrara et al., 1991b). Besides alternative splicing, its expression is also controlled at the levels of transcription and mRNA stability (Neufeld et al., 1999).

VEGF is up regulated in tumours and many other pathological situations such as diabetes, rheumatoid arthritis and cardiovascular diseases (Ferrara and Davis-Smyth, 1997). It is also overexpressed in several eye diseases characterized by abnormal blood vessel formation, as, for example, age-related macular degeneration (AMD) (Kvanta et al., 1996), retinopathy of prematurity (ROP) and diabetic proliferative retinopathy (Aiello et al., 1994). High levels of VEGF are detected in vitreous and aqueous humors in patients with retinal and anterior segment pathologies (Aiello et al., 1994). The concentration is even more severely increased in patients presenting neovascular symptoms.

The VEGF expression is driven by hypoxia during development (Levy et al., 1995; Shweiki et al., 1992; Stone et al., 1995) but also during post-natal neovascularization and in ischemic conditions (Damert et al., 1997; Dor et al., 2001; Forsythe et al., 1996; Ikeda et al., 1995; Iyer et al., 1998; Shweiki et al., 1992). HIF-1 is the main factor implicated in this process (Kelly et al., 2003). The molecule is formed as a heterodimer composed of two subunits HIF-1α and HIF-1β (Chung and Ferrara, 2011). In normoxic conditions, HIF-1α is maintained at low level by a constant degradation driven by von Hippel-Lindau tumor suppressor protein. On the contrary, a decrease of the oxygen concentration or the activation of the oncogene v-src promotes its expression and association with HIF-1β (Strieter, 2005). The complete heterodimer translocates then in the nucleus and binds to a hypoxia response element hosted in the promoter of the VEGF-A gene which induces its mRNA transcription and production of VEGF protein (Tanimoto et al., 2003). Alternatively, VEGF upregulation can also be induced by several growth factors, including EGF, TGF, IGF-1, FGF and PDGF as well as by oncogenes, including activated ras (Neufeld et al., 1999; Okada et al., 1998; Rak et al., 1995).

IV- VEGF variants

Alternative splicing of the pre-mRNA gives rise to several isoforms of VEGF proteins (Fig 8) that possess specific characteristics regarding diffusibility (Ferrara, 2010), receptor activation (Soker et al., 2002), vessels maturation (Mineur et al., 2007) and pro- or anti-angionenic functions (Bates et al., 2002).

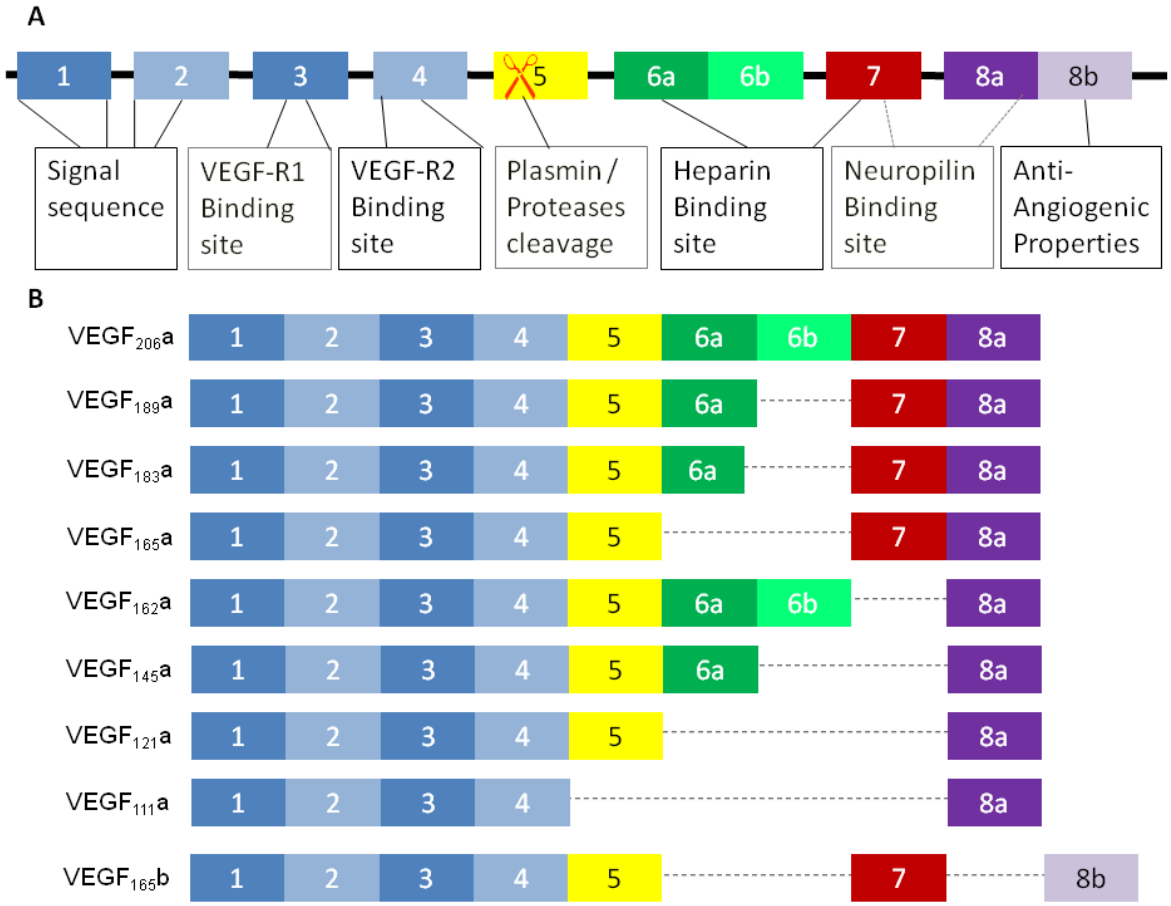


Figure 8 : Schematic representation of the different VEGF isoforms

Alternative splicing of the VEGF-A pre-mRNA gives rise to several isoforms of mRNA and proteins. **(A)** Exons (numbered from 1 to 8b) and introns are represented by boxes and lines, respectively. The biological functions associated with the protein domains encoded by different exons are provided. **(B)** Drawing of the main isoforms of VEGF. The amino-terminal portion (encoded by exons 1 to 4) is found in all known variants while the carboxy-terminal composition is highly variable. $VEGF_{165}b$ is a particular variant where the six amino acids encoded by exon 8a are replaced by six amino acids encoded by the alternative exon 8b. $VEGF_{111}a$ is the only variant which does not possess the exon 5 encoded domain where main sites of proteolytic cleavages are located.

VEGF variants are differentially expressed depending on tissue and life stages (Bacic et al., 1995; Ng et al., 2001). $VEGF_{121}a$ and $VEGF_{165}a$ are usually the most abundant variants (Ferrara et al., 1991a). In some circumstances, however, the proportion of other variants can be significantly increased. As an example, $VEGF_{189}a$ is the dominant isoform in the uterus when progesterone is highly expressed (Ancelin et al., 2002).

It has been suggested that the combine expression of $VEGF_{121}a$, $VEGF_{165}a$ and $VEGF_{189}a$ is crucial to induce a VEGF gradient favouring a correct sprouting angiogenesis and vessel maturation (Grunstein et al., 2000). This hypothesis has been later nuanced. Even though it has been confirmed that the expression of only $VEGF_{120}a$ or $VEGF_{188}a$ (the murine analogous of $VEGF_{121}a$ and $VEGF_{189}a$, respectively) is not sufficient to allow a good

vascular development (Carmeliet et al., 1999), the combination of both variants (Ruhrberg et al., 2002) or the solely expression of $VEGF_{165}$a seems to be sufficient during embryogenesis (Stalmans et al., 2002).

IV-1 $VEGF_{165}$a (E1-E5 ; E7; E8a)

$VEGF_{165}$a was the first identified VEGF molecule and is still the most investigated VEGF variant (Ferrara and Henzel, 1989; Harper and Bates, 2008). As a result the characterizations of the VEGF properties described in the literature mostly concern $VEGF_{165}$a. Its mRNA is formed by exons 1 to 5 (E1-E5), E7 and E8a. It binds to VEGF-R1, VEGF-R2, NRP1 (see chapter VI) and NRP2 but also to HSPG. It displays intermediate affinities for components of the extracellular matrix conferring it with an optimal "diffusibility" and availability for endothelial cell (Ferrara, 2010). This is illustrated by the fact that mice expressing exclusively the $VEGF_{164}$ variant (analogous to human $VEGF_{165}$a) display a normal development (Carmeliet et al., 1999; Maes et al., 2004; Stalmans et al., 2002).

IV-2 $VEGF_{121}$a (E1-E5 ; E8a)

As $VEGF_{165}$a, $VEGF_{121}$a is an abundant isoform. It binds with high affinity to VEGF-R1 and VEGF-R2 (Gitay-Goren et al., 1992; Kaplan et al., 1997; Leung et al., 1989). It differs from $VEGF_{165}$a by the absence of E7-encoded domain, which strongly reduces its affinity for heparin, proteoglycans and several components of the ECM (Cohen et al., 1995; Houck et al., 1992). As a consequence $VEGF_{121}$a is able to stimulate endothelial cells and to be active at distance from its site of secretion (Ferrara, 2010; Grunstein et al., 2000). Interestingly, its binding properties to NRP1 remain controversial being described as binder and non binder (Cebe Suarez et al., 2006; Herve et al., 2008; Pan et al., 2007b; Parker et al., 2012; Soker et al., 1996). This latter point has been investigated in this work.

The exclusive expression of $VEGF_{120}$a (the murine analogous of $VEGF_{121}$a) during the embryonic life is not sufficient to allow a correct foetus development (Carmeliet et al., 1999), with about 50 % of pups dying perinatally because of impaired myocardial angiogenesis and ischemic cardiomyopathy. In the retina, it has been also noticed that $VEGF_{120/120}$ mice have a reduced number and shorter filopodia at the surface of "tips cells" (Gerhardt et al., 2003), larger capillaries, reduced vessels branching and delays in recruitment of mural cells (Ruhrberg et al., 2002; Stalmans et al., 2002). Similarly, overexpression of $VEGF_{120}$a during development decreases branching and increases the number of endothelial cells per capillary (Ruhrberg et al., 2002).

IV-3 $VEGF_{189}a$ (E1-E5 ; E6a ; E7; E8a)

In addition to the sequences present in $VEGF_{165}a$, $VEGF_{189}a$ contains also the 24 amino acids encoded by exon 6a (Keck et al., 1989). As this domain is highly enriched in Arg and Lys basic residues, it largely explains why $VEGF_{189}a$ binds strongly to heparin and heparan sulphate proteoglycans and is almost only found either trapped at the cell surface or immobilized in the ECM (Houck et al., 1992; Tischer et al., 1991). This variant is therefore considered as a reservoir of VEGF becoming active and available for endothelial cells only after cleavage (Ferrara, 2010; Houck et al., 1992), treatment by heparin (Plouet et al., 1997) and/or degradation of the ECM (Park et al., 1993). As expected from these properties, expression of $VEGF_{189}a$ by tumour cells seems to induce angiogenesis only locally (Grunstein et al., 2000). Although $VEGF_{189}a$ was initially reported to be active only after cleavage within the exon6-encoded sequence, by plasmin or urokinase for example (Plouet et al., 1997), more recent studies describe however its effect as an uncleaved molecule on the induction of endothelial cell proliferation and migration, on the formation of dilated blood vessels *in vivo* (Herve et al., 2005; Herve et al., 2008) and as an inducer of vascular permeability (Ancelin et al., 2002). Interestingly, $VEGF_{189}a$ binds to NRP1 with a higher affinity and a slower dissociation rate than $VEGF_{165}a$, probably leading to the formation of more stable tri-molecular complexes (VEGF-R2, $VEGF_{189}a$, NRP1) and to an increased VEGF-R2 signalling that could be related to the formation of large vessels (Herve et al., 2008; Miao et al., 2000). Finally, $VEGF_{189}a$ presents the remarkable property of binding to fibronectin and vitronectin (via $\alpha_v\beta_5$ and $\alpha_5\beta_1$) and stimulating the recruitment of pericytes (Tozer et al., 2008), which plays a crucial role during tumour implantation and growth (Oshika et al., 2000).

In a mouse model, overexpression of $VEGF_{188}a$ (the murine analogous of $VEGF_{189}a$) induces an excess of endothelial cells filopodia favouring hyperbranching of blood vessels during development and a reduction of the number of cells per capillary (Ruhrberg et al., 2002). These latter data are in contrast with those observed in tumour model expressing $VEGF_{189}a$ where the blood vessels are dilated. Finally, in a model of mice expressing only $VEGF_{188}a$, cartilage and bone developments are impaired (Maes et al., 2004).

IV-4 $VEGF_{206}a$ (E1-E5 ; E6a ; E6b ; E7; E8a)

$VEGF_{206}a$ is considered as the full length variant as its mRNA contains all the exons (E1 to E8a). Similarly to $VEGF_{189}a$, from which it differs only by the presence of the 17 amino acids encoded by E6b (Lange et al., 2003), it remains largely cell and matrix associated (Park et al., 1993) and can induce vascular permeability. Furthermore, when attached to the ECM, it

is also able to stimulate endothelial cells proliferation (Houck et al., 1991; Park et al., 1993). Its expression is mainly restricted to the pre-natal period (Houck et al., 1991; Jakeman et al., 1993).

IV-5 $VEGF_{145}$a (E1-E5; E6a ; E8a)

$VEGF_{145}$a has been identified in tumourigenic cell lines established from the female reproductive system but also in ovine placenta and foetal membranes, penis, kidney and skin (Burchardt et al., 1999; Charnock-Jones et al., 1993; Cheung et al., 1995; Poltorak et al., 1997; Whittle et al., 1999). It is secreted by some cancer cells lines in the medium and can be purified using a heparin-Sepharose affinity chromatography. $VEGF_{145}$a displays a higher affinity for matrix components than $VEGF_{165}$a (Kawamura et al., 2008b; Lange et al., 2003; Poltorak et al., 1997). Interestingly this does not seem to be related to interactions with heparin, because heparitinase treatment does not release $VEGF_{145}$a from ECM (Poltorak et al., 1997).

$VEGF_{145}$a binds to VEGF-R2 and to NRP2 but probably not to NRP1 (Gluzman-Poltorak et al., 2000; Poltorak et al., 1997) although this is still debated (Kawamura et al., 2008b). It induces endothelial cells proliferation and migration, although less efficiently than $VEGF_{165}$a. When transfected in tumour cells, it induces a significant blood vessels invasion *in vivo* (Kawamura et al., 2008b; Poltorak et al., 2000; Poltorak et al., 1997).

IV-6 $VEGF_{162}$a (E1-E5 ; E6a ; E6b ; E8a)

$VEGF_{162}$a was identified in human ovarian carcinoma cell line (Lange et al., 2003). It induces HUVECs mitogenesis with an intermediate activity as compared to $VEGF_{165}$a and $VEGF_{145}$a. It also triggers the formation of blood vessels *in vivo*. Interestingly, its affinity for HSPG is lower than the affinity of $VEGF_{145}$a, suggesting that the amino acid sequence encoded by E6b has some inhibitory effect (Lange et al., 2003).

IV-7 $VEGF_{183}$a (E1-E5 ; E6a truncated ; E7; E8a)

Although ubiquitously expressed (Jingjing et al., 2000), $VEGF_{183}$ has been discovered in the eye where it is synthesized by Müller cells (glial cell of the retina). Its expression is up-regulated under hypoxic conditions (Jingjing et al., 1999). It differs from $VEGF_{189}$a by the absence of the 6 amino acids encoded by the 3'-end of E6a, due to the presence of a conserved alternative splicing donor site (Lei et al., 1998). Except for a slightly reduced affinity for some extracellular components, it is considered to possess the same properties than $VEGF_{189}$a (Jingjing et al., 2001).

IV-8 $VEGF_{111}a$ (E1-E4 ; E8a)

$VEGF_{111}a$ is the only VEGF that does not possess the E5-encoded domain (Lambert et al., 2008; Mineur et al., 2007) as the entire sequence between E4 and E8a is spliced out (Fig 8).

It is the shortest and the more recently described variant. It was identified in many cells types upon "stress" in culture by either UV-B irradiation, exposure to chemotherapeutic drugs (Mineur et al., 2007) or cold shock and rewarming (Neutelings et al., 2013, in revision).

When produced in mammalian cells, $VEGF_{111}a$ is secreted under its glycosylated form while, interestingly, a significant part of the other variants remain always non glycosylated (Mineur et al., 2007). This might influence its biological properties although it was never demonstrated.

$VEGF_{111}a$ has pro-angiogenic activities (Fig 9). It initiates the phosphorylation of VEGF-R2 and the activation of the downstream ERK1/2 (Extracellular signal-regulated kinases phosphorylation 1/2) signalling pathway. It stimulates the proliferation of endothelial cells in culture and vasculogenesis in a model of embryoid body formation *in vitro*. It signals also through VEGF-R1 as illustrated by its capacity to induce monocyte migration. Its activity was also studied in a model of tumoural angiogenesis using HEK293 cells expressing a single VEGF variant and implanted in nude mice. In these conditions, $VEGF_{121}a$ and $VEGF_{165}a$ were shown to promote the formation of enlarged blood vessels inside the tumour mass. By sharp contrast, expression of $VEGF_{111}a$ did not modify the intra-tumoural angiogenesis but induced the formation of a dense network of functional capillaries in the peritumoural tissue. Beside demonstrating a strong activity *in vivo*, these observations also suggested that $VEGF_{111}a$ is probably able to diffuse almost freely without any interaction with the ECM.

As another interesting and very specific property resulting from the absence of the E5-encoded sequence, $VEGF_{111}a$ is resistant to degradation by plasmin, an enzyme with broad spectrum substrates, and by the cocktail of proteases present in skin ulcer exudates (Mineur et al., 2007). All these characteristics make $VEGF_{111}a$ a variant with many potential clinical applications and implications in various pathologies. In acute or chronic ischemia (heart infarction, stroke, skin ulcer, tissues grafting, ...) its use could potentially be beneficial for the patients by preventing endothelial cell apoptosis and stimulating the formation of new blood vessels. By contrast its endogenous expression could be detrimental in diseases where abnormal or excessive angiogenesis is observed (cancer, arthritis, ...).

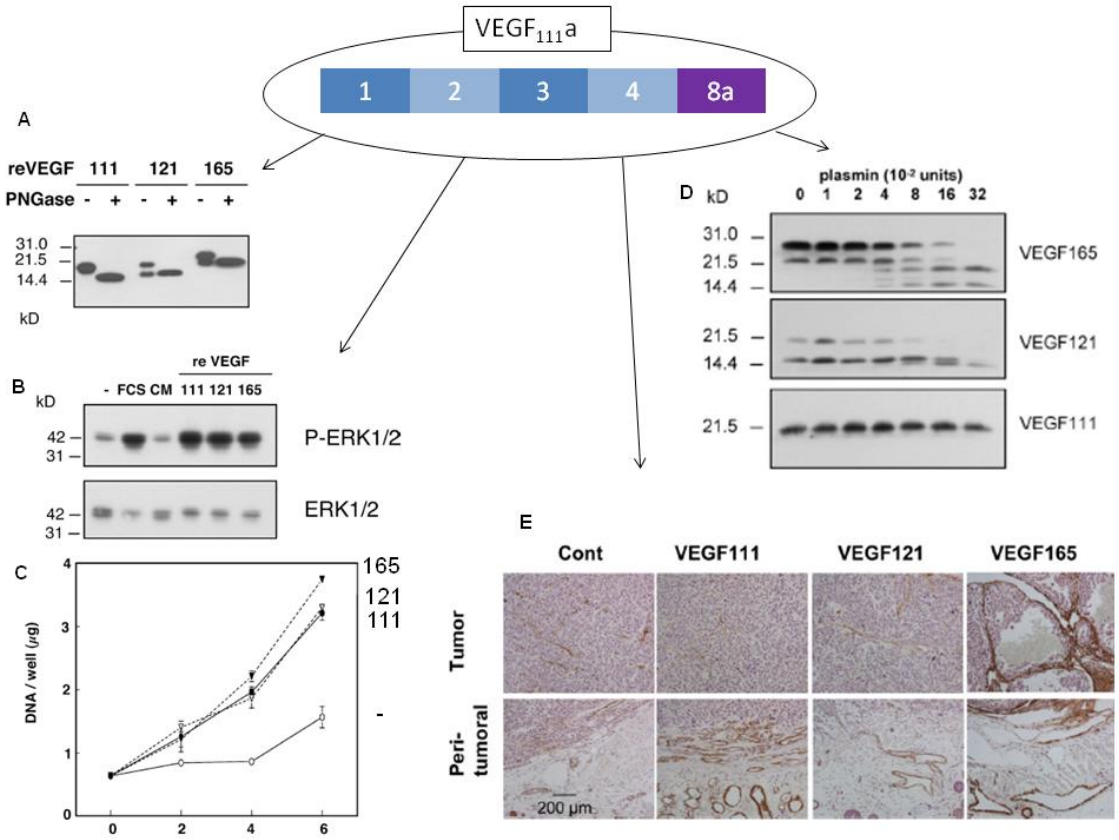


Figure 9 : Characterizations of $VEGF_{111}a$

(A) As demonstrated by western-blot analyses of VEGF variants treated with Peptide-N-Glycosidase F (PNGase), $VEGF_{111}a$ is secreted only as a glycosylated protein while the "classical" variants ($VEGF_{121}a$ and $VEGF_{165}a$), are identified as glycosylated and non glycosylated polypeptides. $VEGF_{111}a$ is as efficient as $VEGF_{121}a$ and $VEGF_{165}a$ **(B)** for stimulating the phosphorylation of ERK1/2 and **(C)** the proliferation of endothelial cell ($VEGF_{111}a$, black circle, $VEGF_{121}a$, open triangle, $VEGF_{165}a$, black triangle and control, open circle). **(D)** $VEGF_{111}a$ displays an increased resistance to proteolytic degradation by plasmin. **(E)** As compared to $VEGF_{121}a$ and $VEGF_{165}a$ that induce intratumoural vascularization, $VEGF_{111}a$ stimulates the formation of a dense network of small capillaries in the surrounding normal skin. ***From (Mineur et al., 2007)***

IV-9 $VEGF_{xxx}b$ variants

IV-9-1 Discovery, generalities and expression

A study of VEGF mRNA isoforms in kidneys led to the identification of a new class of variants reported as anti-angiogenic and characterized by the presence of an alternative exon 8b (Bates et al., 2002; Bates and Jones, 2003). Although a recent controversy questioning their existence/biological significance (Catena et al., 2010; Harris et al., 2012), we felt that their description and study were nonetheless fully relevant to our work for three independent reasons:

- There are tens of publications from several laboratories describing their expression;

- Due to their properties, their use as recombinant proteins is promising for treating human pathologies, even if they are not significantly produced as physiological native proteins;
- Their characterization and comparison with other variants provide fundamental new knowledge concerning the function of the different domains alternatively present in the various VEGF variants.

In their original publication, Bates and co-workers found that this new variant was expressed in podocytes in normal kidneys but was down-regulated in tumour samples and cell lines (Bates et al., 2002; Cui et al., 2004). By sequencing, they identified a new terminal coding sequence that is present in the other variants but only in the 3'-UTR (Xu et al., 2011b). This new sequence, referred to as exon 8b (E8b) (Woolard et al., 2004), codes for six amino acids and a stop codon followed by a 3'-UTR. The alternative splicing mechanism leading to its formation has been studied. It has been shown that VEGF with E8b or E8a are generated as a result of the alternative use of 2 different splice sites (Fig 10). Beside $VEGF_{165}b$ (E1-E5; E7; E8b) described in the original publication (Bates et al., 2002), others have been identified such as $VEGF_{121}b$ (E1-E5 ; E8b), $VEGF_{145}b$ (E1-E5 ; E6a ; E8b) and $VEGF_{189}b$ (E1-E5 ; E6a ; E7; E8b) (Cui et al., 2004; Harper and Bates, 2008; Miller-Kasprzak and Jagodzinski, 2008; Rennel et al., 2009b; Woolard et al., 2004). As already mentioned, VEGF variants possessing E8a or the alternative E8b are referred to as $VEGF_{xxx}a$ or $VEGF_{xxx}b$, respectively. The expression of this new class of variants was reported to be significant and it was even postulated that they could be more abundant in healthy tissues than the $VEGF_{xxx}a$ isoforms (Bevan et al., 2008; Pritchard-Jones et al., 2007; Rennel et al., 2008; Varey et al., 2008; Woolard et al., 2004), which is however another controversy in the field.

$VEGF_{xxx}a$ and $VEGF_{xxx}b$ isoforms are generated by the selection of a proximal (PSS) or a distal (DSS) splice site, the latter being localized 66 bp further downstream in the terminal exon 8 (see Fig 10) (Woolard et al., 2004). Cascades of molecules, such as growth factors and splicing factors, are implicated in the choice of the splicing site. Studies in primary epithelial cell, podocytes and endothelial cells demonstrate that factors such as IGF1 and TNFα (Tumor Necrosis Factor α) favour the PSS, which leads to the synthesis of $VEGF_{xxx}a$, while TGF-β1 favours DSS and secretion of $VEGF_{xxx}b$ (Nowak et al., 2008).

IGF induces the PKC (Protein Kinase C) pathway (Fig 10A) and SRPK1 (Serine/Arginine Protein Kinase 1) activation, which induces the phosphorylation and the translocation of ASF/SF2 (also known SRSF1: Serine/Arginine-rich Splicing Factor 1) in the nucleus where it participates to the preferential selection of the PSS (Nowak et al., 2010). By contrast (Fig

10B), TGF-β1 activates successively p38 and Clk1 (CDC-like kinase 1) pathways, which induces the phosphorylation of SRp55 (also known SRSF6) allowing its binding to the VEGF pre-mRNA, which favours the selection of the DSS and the synthesis of $VEGF_{xxx}b$ (Manetti et al., 2011; Nowak et al., 2008). Endostatin and the E2F1 transcription factor could also participate to the selection of the DSS by triggering the activation of the splicing factor SC35 (Li et al., 2011; Merdzhanova et al., 2008; Merdzhanova et al., 2010). However, a positive correlation between the expression of $VEGF_{xxx}a$ and E2F1 has been identified in colorectal cancer samples, suggesting that the relationship between these two factors is probably complex (Diaz et al., 2008). Finally, WT1 (Wilms Tumour protein 1) can bind to SRPK1 and down-regulate its expression, which represses the expression of $VEGF_{xxx}a$. Interestingly, in Deny Drash Syndome, a urogenital disorder, WT1 is expressed as a mutated form unable to bind and to inhibit SRPK1 (Amin et al., 2011; Schumacher et al., 2007), which stimulates an overexpression of $VEGF_{xxx}a$ responsible for renal failure.

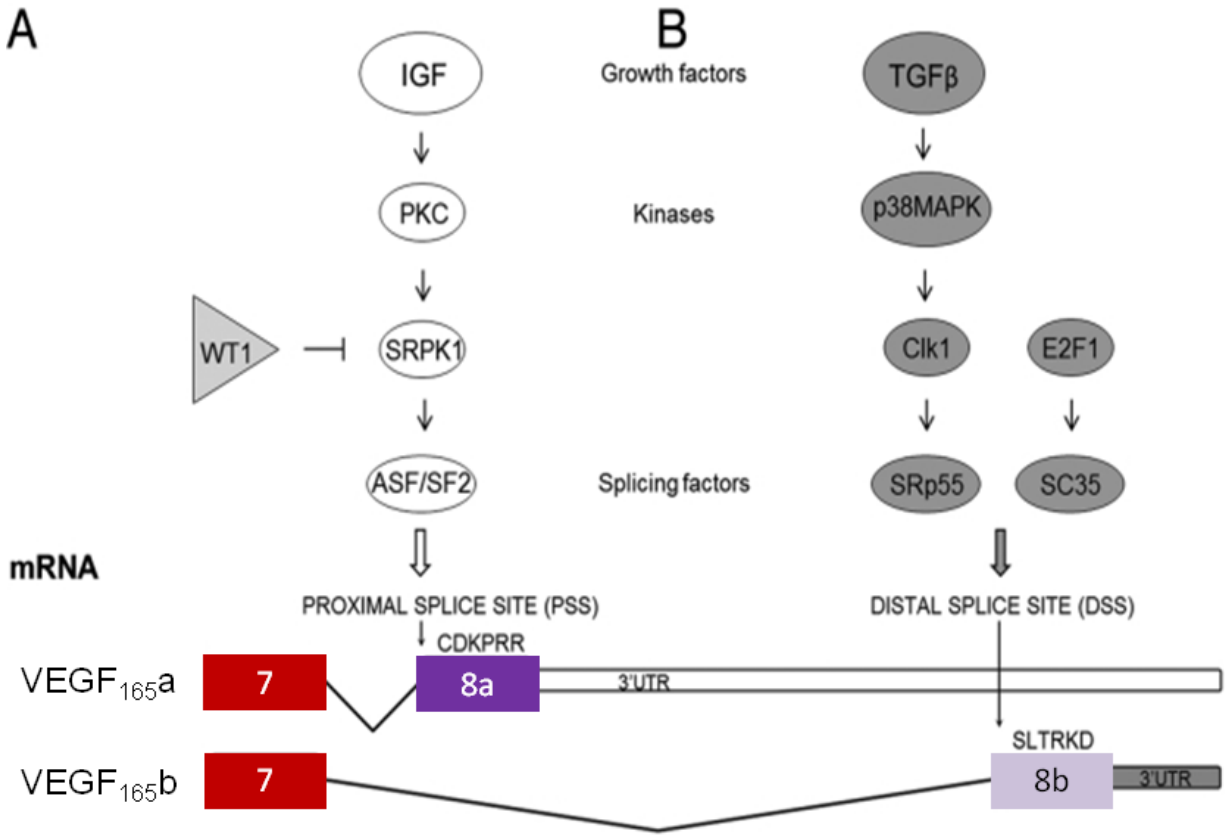


Figure 10 : Regulation of the alternative splicing in the terminal exon of the VEGF-A pre-mRNA

Several growth factors, kinases and splicing factors are implicated in pathways regulating the selection of a proximal **(A)** or a more distal **(B)** splice site, which leads to the synthesis of either $VEGF_{xxx}a$ or $VEGF_{xxx}b$ isoforms, respectively. ***From (Peiris-Pages, 2012)***

$VEGF_{165}b$ (E1-E5; E7; E8b) is the most investigated $VEGF_{xxx}b$ isoform. It has been produced as a recombinant protein (Fig 11A) (Bates et al., 2002; Catena et al., 2010). It is efficiently secreted (Leung et al., 1989), forms dimers (Bates et al., 2002; Woolard et al., 2004), and has high affinities for VEGF receptors (Hua et al., 2010). An antibody targeting the 6 amino acids encoded by E8b has been produced and used to detect, by immunohistochemistry or ELISA, the presence of $VEGF_{165}b$ in kidney and plasma samples (Bills et al., 2009; Woolard et al., 2004).

The 6 amino acids encoded by E8b (Ser-Leu-Thr-Arg-Lys-Asp or "SLTRKD") largely differ from those derived from E8a (Cys-Asp-Lys-Pro-Arg-Arg or "CDKPRR") (Bates and Jones, 2003), which has potential functional implications. In $VEGF_{165}a$, the Cys residue encoded by E8a is expected to form a disulfide bond with Cys^{146} (derived from E7) (Claffey et al., 1995). This brings the two terminal Arg encoded by E8a close to the basic residues derived from E6, which forms a highly basic 3D-domain. By sharp contrast, the domain encoded by E8b does not contain a Cys and ends with an Asp acidic residue (Cebe-Suarez et al., 2008), two features that could strongly modify the 3D-conformation and the properties of $VEGF_{165}b$ as compared to $VEGF_{165}a$.

$VEGF_{165}b$ binds to VEGF-R1 and VEGF-R2 (Fig 11B and 11C) (Hua et al., 2010; Woolard et al., 2004) but not to NRP1, heparin or HSPG (Cebe Suarez et al., 2006; Kawamura et al., 2008b). Surprisingly, however, it has been shown in independent studies that endothelial cells treatment by $VEGF_{165}b$ induces only a faint and transient phosphorylation of VEGF-R2 and a very limited activation of ERK and Akt downstream signalling pathways (Catena et al., 2010; Cebe Suarez et al., 2006; Magnussen et al., 2010; Manetti et al., 2011; Woolard et al., 2004). Other studies have further shown that impaired phosphorylation of VEGF-R2 affects several Tyr residues (Cebe Suarez et al., 2006; Kawamura et al., 2008b; Magnussen et al., 2010). The involved mechanism is not yet fully understood. According to a first hypothesis, it could be related to the lack of binding of $VEGF_{xxx}b$ isoforms to NRP1. This hypothesis is however hampered by the fact that $VEGF_{xxx}a$ variants that have no affinity for NRP1 are however strong inducers of VEGF-R2 phosphorylation. Another more appealing hypothesis suggests that $VEGF_{165}b$ would not be able to dictate the perfect orientation of the two VEGF-R2 molecules forming a functional dimer, which would affect tyrosine "trans-phosphorylation" and explain the quasi absence of activation of downstream pathways (Fig 11D). In this context it is not surprising that $VEGF_{165}b$ is not able to trigger endothelial cells proliferation (Fig 11E), migration (Fig 11F) and tube formation *in vitro* or angiogenesis (Fig 12A) and vascular dilatation *in vivo* (Bates et al., 2002; Manetti et al., 2011; Rennel et al., 2008). The

low level of activation of these cascades seems however sufficient to have a biological role since $VEGF_{165}b$ treatments promote survival of endothelial cell *in vitro* (Bevan et al., 2008; Magnussen et al., 2010; Woolard et al., 2004). Besides this protective function it was reported that $VEGF_{165}b$ is able to repress the $VEGF_{xxx}a$-induced angiogenesis by a mechanism that would involve a competition for VEGF-R2.

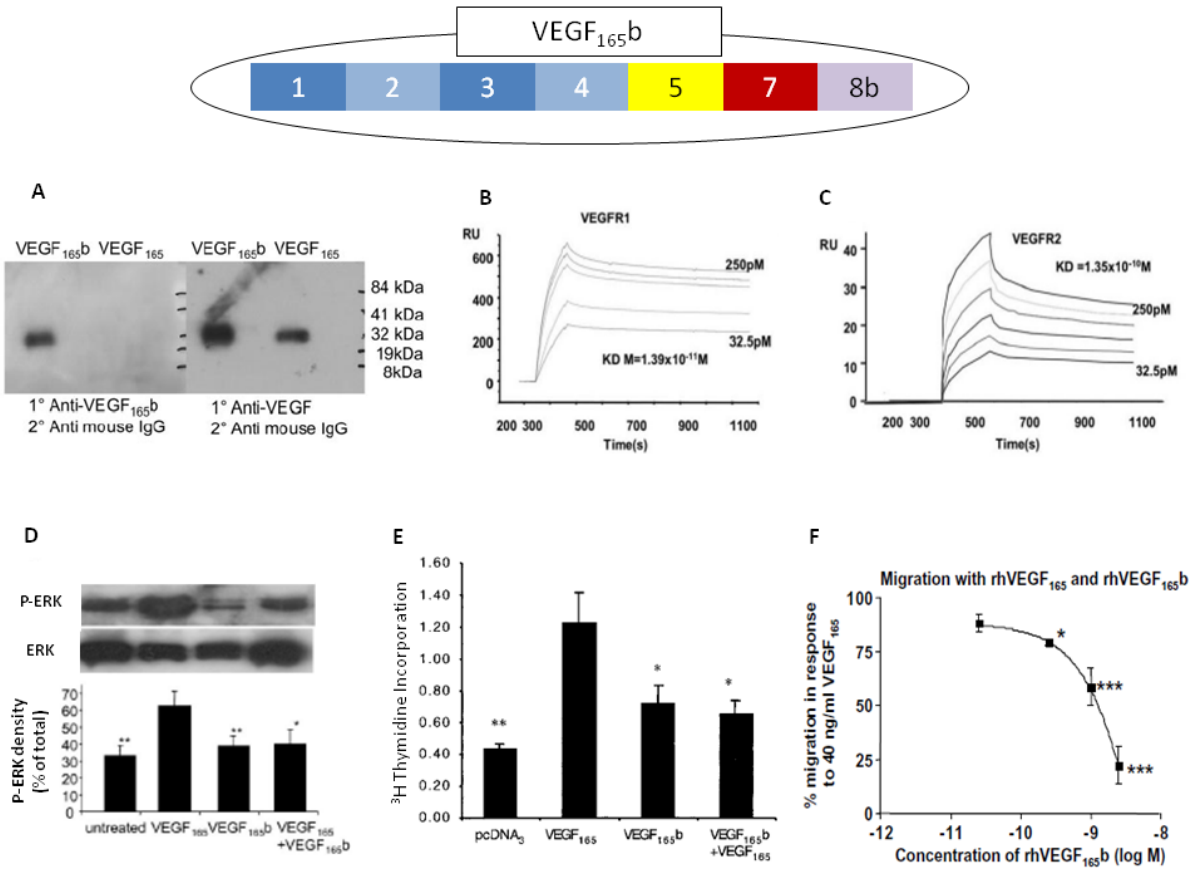


Figure 11 : *In vitro* effect of $VEGF_{165}b$ on angiogenesis

$VEGF_{165}a$ and $VEGF_{165}b$ can both be evidenced by Westen Blotting using an antibody targeting the N-terminus of the molecules, while only $VEGF_{165}b$ can be visualized by an antibody raised against the E8b-derived sequence **(A)**. $VEGF_{165}b$ is able to bind to VEGF-R1 **(B)** and VEGF-R2 **(C)** as demonstrated by Surface Plasmon Resonance. **(D)** However, experiments performed *in vitro* on endothelial cells demonstrated that $VEGF_{165}b$ cannot activate ERK pathway and inhibits the $VEGF_{165}a$-induced phosphorylation as shown by western-blot and the corresponding quantification. **(E)** $VEGF_{165}b$ can repress $VEGF_{165}a$-induced endothelial cells proliferation when both variants are mixed in an equimolar amount as shown by ^{3}H-thymidine incorporation. **(F)** $VEGF_{165}b$ has the property to inhibit $VEGF_{165}a$ induced endothelial cells migration in a dose-dependent manner as shown by the decrease percentage of cell migration relative to $VEGF_{165}a$ taken as 100%. **(D E, F)** The statistical significances are reported by comparison to the treatment with $VEGF_{165}a$ alone. ***From (Bates et al., 2002; Hua et al., 2010; Rennel et al., 2008; Woolard et al., 2004).***

This antagonistic effect regarding the pro-angiogenic isoforms of VEGF was also investigated and confirmed *in vivo* in models of pathological neovascularization in the eye (Konopatskaya et al., 2006; Magnussen et al., 2010; Rennel et al., 2009b) and in tumours

(Fig 12B) (Peiris-Pages et al., 2010; Rennel et al., 2008; Rennel et al., 2009a; Rennel et al., 2009b). A small number of publications report however a weak but significant pro-angiogenic effect of $VEGF_{121}b$ and $VEGF_{165}b$ in a matrigel plug assay *in vivo* (Catena et al., 2010), which led to the hypothesis that the inhibitory of effect of $VEGF_{165}b$ would be limited to situations where the pro-angiogenic $VEGF_{xxx}a$ isoforms are highly expressed. It was also shown that $VEGF_{165}b$ is able to increase vascular permeability by signalling through VEGF-R1, although less efficiently than $VEGF_{165}a$ (Glass et al., 2006) that acts mainly through VEGF-R2 (Whittles et al., 2002).

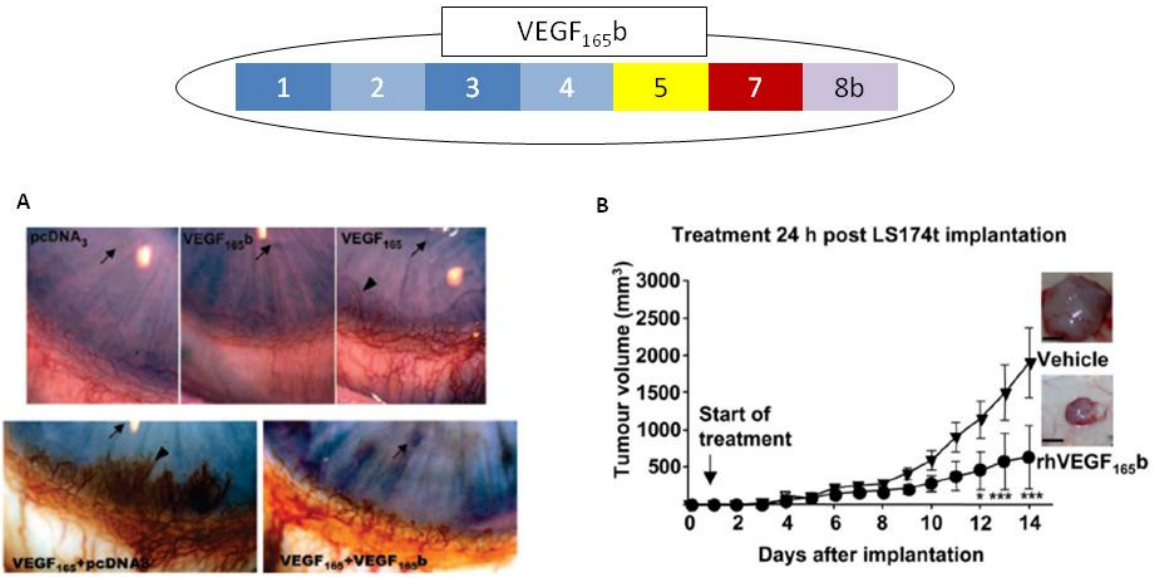


Figure 12 : *In vivo* effect of $VEGF_{165}b$ on angiogenesis

(A) Unlike $VEGF_{165}a$, $VEGF_{165}b$ does not induce ocular vascularization (upper panel). When present in equimolar concentration $VEGF_{165}b$ inhibits the $VEGF_{165}a$-induced effect (lower panel). **(B)** LS174t carcinoma cells express high levels of $VEGF_{165}a$. They form vascularized tumours when grafted in nude mice. By contrast, chronic injection of recombinant $VEGF_{165}b$ reduces tumor growth and vascularisation. ***From (Rennel et al., 2008; Woolard et al., 2004)***

IV-9-2 $VEGF_{xxx}b/VEGF_{xxx}a$ ratio in health and disease

The $VEGF_{xxx}b/VEGF_{xxx}a$ ratio plays a significant role in the maintenance of the physiology of the organ in adulthood (Oltean et al., 2012b). It is also developmentally regulated in ovaries, testes and eyes (Artac et al., 2009; Baba et al., 2012; Baltes-Breitwisch et al., 2010; Caires et al., 2012; McFee et al., 2012). In placenta, where angiogenesis is active, $VEGF_{xxx}b$ represents only 1.5% of the total VEGF (Bevan et al., 2008).

$VEGF_{xxx}b$ isoforms are down-regulated in tumours as compared to healthy tissues in favour of $VEGF_{xxx}a$ (Bates et al., 2002; Bevan et al., 2008; Rennel et al., 2008). $VEGF_{xxx}b$ are also

the main variants expressed in normal eye but their concentration decreases during the proliferative angiogenesis occurring in the vitreous of diabetic patients (Hua et al., 2010; Perrin et al., 2005). By contrast, in glaucoma, a non-angiogenic pathology where VEGF is over-expressed, the $VEGF_{xxx}b$ isoforms are the most abundant (Ergorul et al., 2008). All these information underscore a shift from $VEGF_{xxx}b$ to $VEGF_{xxx}a$ which is responsible for pathological blood vessels formation (Harper and Bates, 2008). This hypothesis is however not supported in breast tumours where up-regulation of $VEGF_{xxx}b$ isoforms has been observed (Catena et al., 2010). A decreased $VEGF_{165}b/VEGF_{165}a$ ratio is also involved in mesangial sclerosis and renal failure in patients suffering from Denis Drash Syndrome (Schumacher et al., 2007). An increased expression of $VEGF_{165}b$ is involved in systemic sclerosis (Manetti et al., 2011), a chronic disease characterized by alterations of angiogenesis and vascular repair, which affects skin and internal organs (Manetti et al., 2010).

IV-9-3 $VEGF_{xxx}b$ in clinic

Bevacizumab is an antibody trapping VEGF-A molecules (see paragraph VII-1). Today, it is the gold-standard treatment for inhibiting angiogenesis. Besides a limited effect, it has also specific disadvantages. It targets both $VEGF_{xxx}a$ and $VEGF_{xxx}b$ isoforms, which potentially reduces its anti-angiogenic properties. Bates and collaborators proposed to use the $VEGF_{xxx}b/VEGF_{xxx}a$ ratio as a predictive biomarker for bevacizumab efficacy in metastatic colorectal cancer (Bates et al., 2012). Using samples of the phase III study (Giantonio et al., 2007), they have demonstrated a significant improvement of the survival of patients with a low $VEGF_{xxx}b$/ $VEGF_{xxx}a$ ratio as compared to the patients with a high ratio.

When used at high doses in the eye for treating macular degeneration, bevacizumab can alter the viability of retinal pigment endothelial cells (Spitzer et al., 2006). Using recombinant $VEGF_{xxx}b$ variants as therapeutic molecules to reduce pathological angiogenesis is an interesting concept because it inhibits angiogenesis while being cytoprotective for retinal pigment endothelial cells, even used at very high concentrations (Bates and Harper, 2005; Harper and Bates, 2008). The most promising effects were observed in *in vivo* models mimicking eyes diseases such macular degeneration and retinopathy of prematurity (ROP). In a choroidal neovascularization model (CNV), $VEGF_{165}b$ limits the excess of abnormal blood vessels induced by laser exposure (Hua et al., 2010). Similarly, a benefit of $VEGF_{121}b$ and $VEGF_{165}b$ treatment on the neovascularization has been reported in several model of oxygen deprivation (Konopatskaya et al., 2006; Magnussen et al., 2010; Rennel et al., 2009b).

$VEGF_{165}b$ has however its own disadvantages, such as a short half-life *in vivo* (Rennel et al., 2008). This could be improved however by increasing its resistance to degradation by endogenous proteases. An alternative would be to induce its synthesis *in vivo* by the host machinery by favouring its expression at the expense of the $VEGF_{xxx}a$ isoforms, a therapeutic goal that could be achieved by regulating the alternative splicing of the VEGF pre-mRNA (Oltean et al., 2012a). For instance, SRPIN340, an antagonist of SRPK1, improves symptoms in a rat model of ROP (Nowak et al., 2010) and knocking down SRPK1 in colorectal tumor cell lines decreases their growth (Amin et al., 2011).

IV-10 Other variants

$VEGF_{148}$ results from the skipping of E6 and of a sequence encompassing E7b to the beginning of E8a, which further changes the reading frame of the 3'-end of the mRNA and leads to a novel carboxy-terminal amino acid sequence (Harper and Bates, 2008; Whittle et al., 1999; Zygalaki et al., 2005). Alternative start codons upstream the canonical ATG codon can also be used, giving rise to isoforms longer than classical VEGF (Tee and Jaffe, 2001). However they do not display activity and their relative importance is still questionable.

Some artificial variants were also created to better understand the role of E6b and E8a as, for example, $VEGF_{138}$ (E1-E5; E6b; E8a), $VEGF_{182}$: E1-E5; E6b; E7; E8a) or $VEGF_{159}$ (E1-E5; E7) (Cebe Suarez et al., 2006; Houck et al., 1991; Lange et al., 2003).

V- Role of the domains encoded by the exons of VEGF

Because of the very active and dynamic mechanism of alternative splicing affecting the VEGF-A pre-mRNA, differences between the VEGF variants directly depends on the presence in the mRNA of specific exon(s) or part of. For this reason, protein domains are usually described in direct relationship with their exonic origin. Their specific functions are briefly summarized here.

Exons 1 and 2

The two first exons code for the ATG start codon and for the 26 hydrophobic amino acids sequence form the signal peptide (Houck et al., 1992; Tischer et al., 1991). This sequence is therefore cleaved during the secretion process and absent from the mature forms.

Exon 3

The binding site for VEGF-R1 is encoded by E3, with a special importance of three amino acids: Asp^{63}, Glu^{64} and Glu^{67} (Keyt et al., 1996b). Two Cys residues (Cys^{51} and Cys^{60}) required for the formation of functional VEGF dimers are also part of the E3-derived sequence (Harper and Bates, 2008; Muller et al., 1997).

Exon 4

The E4-derived sequence contains the binding domain for VEGF-R2, with Arg^{82}, Lys^{84} and His^{86} playing a critical role (Keyt et al., 1996b), and the only glycosylation site (Asn^{75}) present in VEGF-A molecules (Claffey et al., 1995; Neufeld et al., 1994).

Exon 5

The E5-encoded domain contains the main site of cleavage by plasmin (between Arg^{110} and Ala^{111}) (Keyt et al., 1996a; Lauer et al., 2002). The amino-terminal peptide resulting from the digestion possesses 110 amino acids, is still dimerized (Houck et al., 1992) and able to bind to VEGF-R1 and VEGF-R2 (Plouet et al., 1997). It is still active but much less than the intact $VEGF_{165}$a (Mineur et al., 2007). This domain contains also cleavage sites for MMPs (Lee et al., 2005). For instance in human, MMP3 cleaves $VEGF_{165}$a into $VEGF_{114}$ (between Glu^{114} and $Asn^{115)}$. This cleaved form triggers vascular dilatation while a mutated MMPs-resistant $VEGF_{165}$a generates anarchic hyper-branching. This illustrate the importance of taking into consideration the protease micro-environment when studying the effect and properties of VEGF isoforms (Coussens and Werb, 2002).

Exon 6a

The E6a-derived sequence plays a main role in binding to heparin and proteoglycans because of the presence of 12 basic amino acids (Tischer et al., 1991). This domain was also reported to have affinity for NRP1 (Herve et al., 2008; Jia et al., 2001) and NRP2 (Gluzman-Poltorak et al., 2000). It further contains a cleavage site by uPA (urokinase-type Plasminogen Activator) corresponding to its carboxy-terminal sequence (Tyr-Lys-Ser-Trp-Ser-Val), absent in $VEGF_{183}$ (Jingjing et al., 1999).

Exon 6b

$VEGF_{162}$ (E1-5 ; E6a ; E6b ; E8a) possesses significantly less affinity for ECM than $VEGF_{145}$ (E1-5 ; E6a ; E8a) but more than the artificial recombinant $VEGF_{138}$ (E1-5 ; E6b ; E8a) (Lange et al., 2003). This suggests that the E6b-derived sequence has no affinity for ECM components and is even able to limit the affinity of the E6a domain for matrix components.

Exon 7

The E7 domain possesses 3 accessory sites of cleavage by plasmin (Arg^{123}/Arg^{124}, Lys^{125}/His^{126} and Lys^{147}/Ala^{148}) (Lauer et al., 2002) and two additional cleavage sites by MMPs (Ser^{121}/Gln^{122} and Lys^{136}/Cys^{137}) (Lee et al., 2005). An affinity for heparin (Krilleke et al., 2007) and VEGF-R1 (Allain et al., 2012) has been reported. It contains also the first identified binding site for NRP1 (Soker et al., 1997).

Exon 8a

The CDKPRR sequence derived from E8a is currently considered as the main binding site to NRP1 by some authors (Pellet-Many et al., 2008) as suggested by the use of different synthetic peptides (Allain et al., 2012).

Exon 8b

Substitution of the CDKPRR sequence (E8a) by the SLTRKD sequence (E8b) strongly modifies the biological properties of VEGF-A variants (Bates et al., 2002; Catena et al., 2010) by mechanisms that would involve modifications of the 3D-structure of the entire molecule and alteration of pockets formed by positively charged amino acids.

VI- VEGF-receptors and co-receptors

As already briefly mentioned, the regulation of cell phenotype by VEGF-A variants involves 2 transmembrane tyrosine kinase receptors and several co-receptors in charge of modulating their availability and presentation to the receptors.

VI-1 Vascular Endothelial Growth Factor Receptor 1

The Vascular Endothelial Growth Factor Receptor 1 (VEGF-R1, initially described as FLT-1 or Fms-Like Tyrosin Kinase-1) was the first described VEGF receptor. It was identified as an endothelial cell surface protein able to complex with VEGF and possessing similarities with the PDGF receptor (Plouet and Moukadiri, 1990a; Seetharam et al., 1995). Further characterizations showed that VEGFR-1 is a transmembrane receptor containing an extracellular region (7 IgG-like domains), a transmembrane sequence and an intracellular tyrosine kinase domain (Shibuya, 1995). Soluble and truncated versions also exist and are referred to as sVEGF-R1. They are produced both by alternative splicing of the pre-mRNA and by proteolytic processing (Cai et al., 2006; Shibuya, 2001). They play an anti-angiogenic role by sequestrating VEGF-A in the extracellular space, which prevents its binding on

transmembrane receptors present at the cell surface. Like the full-size VEGF-R1, sVEGF-R1 can also act as inhibitor by forming non-functional heterodimer with VEGF-R2 (Cudmore et al., 2012; Kendall and Thomas, 1993; Kendall et al., 1994).

VEGF-R1 binds with high affinity (15 pM) to VEGF-A variants through the IgG loop 2 (Davis-Smyth et al., 1996; Tanaka et al., 1997). The initial binding of VEGF-A on a single VEGF-R1 molecule induces the recruitment of a second VEGF-R1 to form an active dimer. This induces conformational changes allowing trans-phosphorylation of specific tyrosine residues and activation of downstream signalling pathways (Lemmon and Schlessinger, 2010). However, despite the presence of several potential tyrosine phosphorylation sites (Ito et al., 1998), VEGF-R1 is only slightly phosphorylated in endothelial cells upon VEGF-A exposure (de Vries et al., 1992; Meyer et al., 2006). VEGF-R1 is therefore considered as a decoy receptor in charge of repressing excessive angiogenesis. This is illustrated *in vivo* in mouse models. VEGF-R1$^{-/-}$ embryos die *in utero*, due to hyperproliferation of endothelial cells and severe disorganisation and dysfunction of the vascular system (Fong et al., 1995) while the deletion of its tyrosine kinase domain has no effect on angiogenesis (Hiratsuka et al., 1998). In the same context, it has been postulated that preventing interactions between VEGF-A and VEGF-R1 would favour the binding to VEGF-R2 and stimulate angiogenesis (Ho et al., 2012). This could be achieved by PlGF as this VEGF family member binds exclusively and with very high affinity to VEGF-R1 therefore competing with VEGF-A for VEGF-R1, and increasing the amount of VEGF-A available for VEGF-R2 (Park et al., 1994).

Beside the control of angiogenesis, VEGF-R1 plays also a role in the inflammatory response, as shown in cancer, by participating to the recruitment of bone marrow-derived monocytes (Hiratsuka et al., 2001) and macrophage chemotaxis through interactions with PlGF (Clauss et al., 1996; Fischer et al., 2008) but also with VEGF-A isoforms (Barleon et al., 1996; Hiratsuka et al., 1998; Mineur et al., 2007).

In vivo, VEGF-R1 expressed at the surface of macrophages increases their recruitment to wound sites (Lauer et al., 2000) and favours the healing process while sVEGF-R1 seems to delay tissue repair likely by inhibiting inflammatory cells chemotaxis and angiogenesis (Eming et al., 2004).

VI-2 Vascular Endothelial Growth Factor Receptor 2

VEGF-R2, initially identified as KDR (Kinase-insert Domain Receptor) or Flk-1 (Foetal liver kinase-1), is a 210-230 kD glycoprotein with similarities to VEGF-R1 (Koch et al., 2011). It is the main receptor of VEGF in the regulation of angiogenesis (Gille et al., 2001; Terman et al., 1992) as demonstrated by using mutated VEGF variants. An absence of binding to

VEGF-R1 does not affect the pro-angiogenic properties of the mutated VEGF. By contrast, mutations preventing interactions with VEGF-R2 totally abolish the VEGF angiogenic activity (Keyt et al., 1996b). The VEGF-A-VEGF-R2 signalling is central to the regulation of endothelial cell differentiation, proliferation and migration, but also to blood vessel homeostasis and functions (formation, fenestration, maintenance (survival), permeability...) (Fig 13) (Ferrara et al., 2003). The critical function of VEGF-R2 was confirmed *in vivo* since VEGF-R2$^{-/-}$ embryos die at 8.5 - 9.5 dpf, at a development time and with alterations resembling those of VEGF-A$^{-/-}$ embryos (Shalaby et al., 1995).

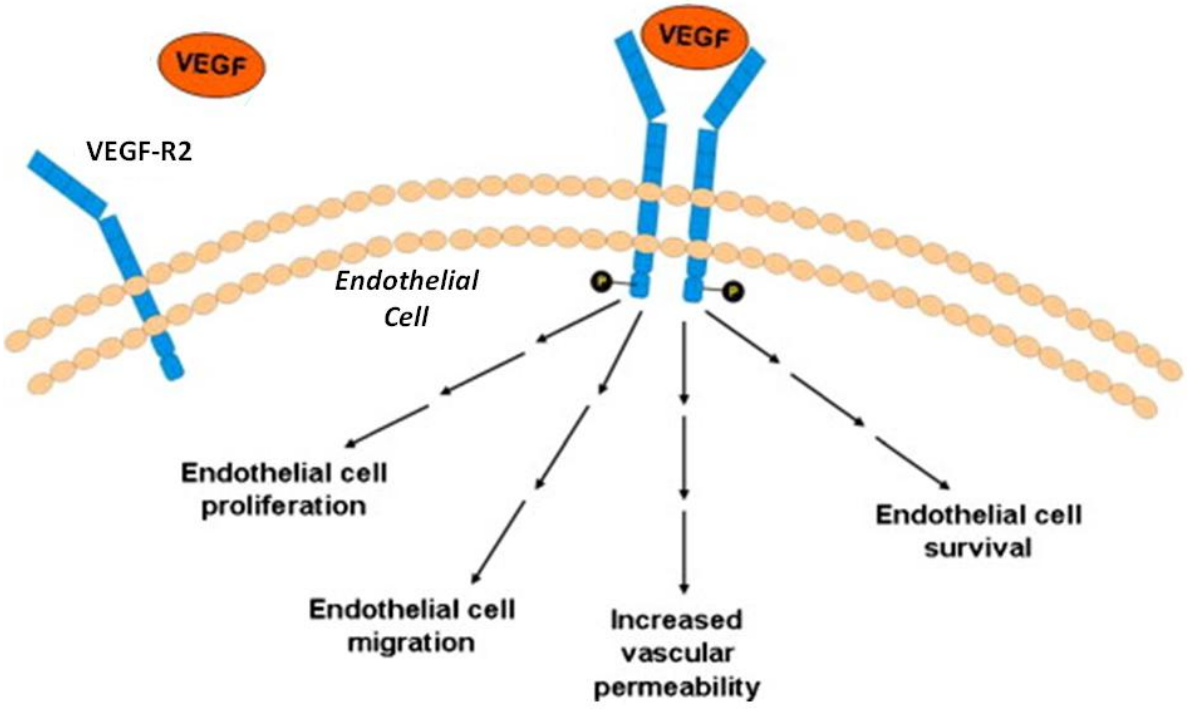


Figure 13 : Effect of VEGF through VEGF-R2 on endothelial cells

The effects of VEGF-A on endothelial cells is mainly mediated by VEGF-R2. VEGF-A binding on one molecule of VEGF-R2 induces the recruitment of a second VEGF-R2 monomer. Their dimerization is a key step for transphosphorylation of specific tyrosines residues and activation of downstream signalling pathways. Integration of these signals is central for regulating endothelial cell phenotype and vessels homeostasis. ***From (Farhat et al., 2012)***

VEGF-R2 is mostly expressed in vascular endothelial cells although it can be found also in lymphatic endothelial cells, neurons, cancer cells and macrophages (Bellon et al., 2010; Dineen et al., 2008; Silva et al., 2011; Smith et al., 2010). Its expression is increased in activated endothelial cells and during sprouting angiogenesis, being more elevated in “tip cells” than in “stalk cells” (Gerhardt et al., 2003). Similarly to VEGF-R1, soluble versions, referred to as sVEGF-R2, have been identified (Ebos et al., 2004; Wen et al., 1998). Beside

potential sequestration of VEGF, these soluble variants seem to regulate mural cells migration and vessels coverage (Lorquet et al., 2010).

Activation

VEGF-R2 interacts with VEGF-A via its IgG-like domains 2 and 3. The reported affinity between the two proteins is high (750 pM) although lower than with VEGF-R1 (15 pM) (Millauer et al., 1993; Terman et al., 1992). The initial interaction between VEGF and VEGF-R2 triggers the recruitment of a second receptor molecule, which allows trans-phosphorylation of several tyrosine residues within the dimer and activation of several downstream signalling pathways (Fig 13) (Stuttfeld and Ballmer-Hofer, 2009). Interestingly, the formation of VEGFR1-VEGFR2 heterodimers have been reported, which could represent another level of regulation by VEGF-R1 of the VEGF-R2 induced effects (Cudmore et al., 2012; Huang et al., 2001). After activation, the VEGF-R2/VEGF complex is rapidly internalized by clathrin-mediated endocytosis, which does not hamper, and even perhaps increases, downstream signalling (Lampugnani et al., 2003). It can be then recycled to the plasma membrane (Zwang and Yarden, 2009) or degraded, depending on the type of endocytosis vesicles involved.

All phosphorylated Tyr are not equivalent as they trigger different signalling cascades (Matsumoto et al., 2005; Takahashi et al., 2001).

Tyr^{1175}

Tyrosine^{1175} in human VEGF-R2 (Tyr^{1173} in mouse) is probably the most important tyrosine for mediating the VEGF-A effects. Its replacement by a phenylalanine residue has been investigated *in vivo*. VEGF-$R2^{Phe1173}$ homozygous embryos die at 9.5 dpf because of defects similar to those observed in VEGF-$R2^{-/-}$ embryos (Sakurai et al., 2005). Tyr^{1175} phosphorylation allows the binding of several intracellular molecules (PLCγ, SHB, Sck) that initiate intracellular signalling (Fig 14) (Cunningham et al., 1997; Holmqvist et al., 2004; Takahashi et al., 2001; Warner et al., 2000). For example, PLCγ (PhospoLipase C gamma) is responsible for the activation of PKC (protein kinase C) and, consequently, $ERK_{1/2}$ (Takahashi et al., 2001). This leads to endothelial cells proliferation. SHB (SH2-domain-containing adaptor protein B) and Sck (Src homology and collagen homology related adaptor protein) induce FAK (Focal Adhesion Kinase) phosphorylation and contribute to cells attachment and migration (Abu-Ghazaleh et al., 2001; Holmqvist et al., 2003). Finally, Sck controls Ras activation and cell proliferation (Meadows et al., 2001).

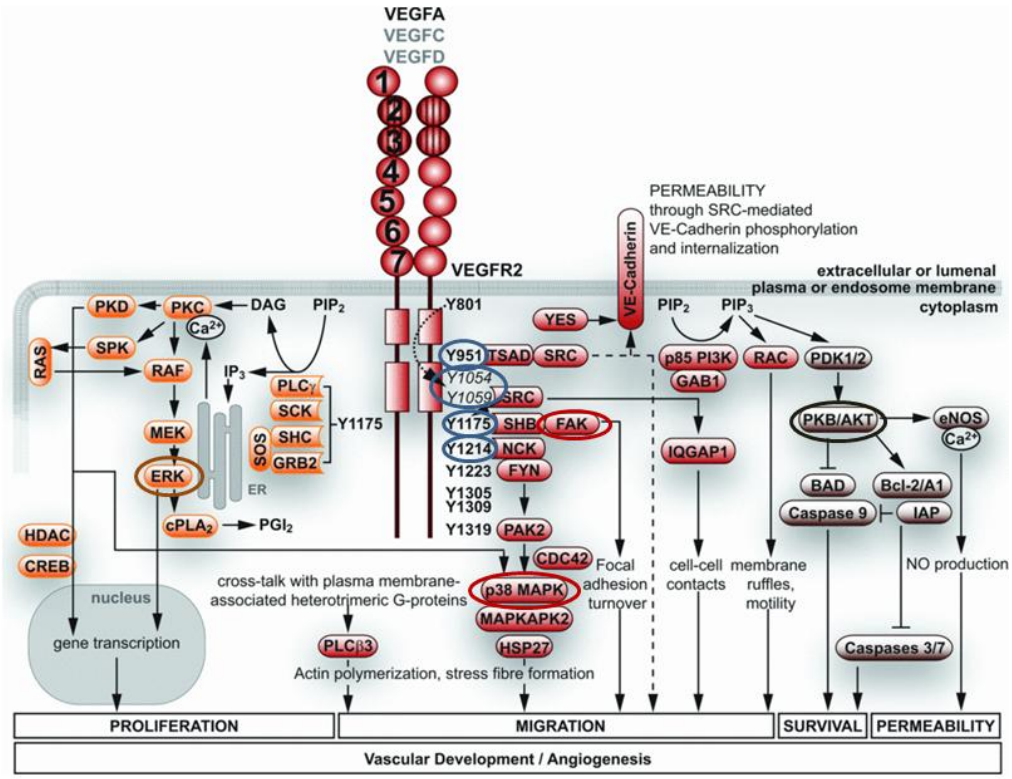


Figure 14 : VEGF-R2 phosphorylation sites, their related signalling pathways and their effects on endothelial cell phenotype and angiogenesis.

Upon interactions with VEGF-A, VEGF-R2 is phosphorylated on several tyrosine residues. Tyr^{1175} is the most important phosphorylation site as it activates, directly or indirectly, several key factors (PLCγ, ERK, FAK, p38...) that regulate endothelial cell proliferation and migration. Tyr^{951}, Tyr^{1054}, Tyr^{1059} and Tyr^{1214} are implicated in vessels migration. It is not completely clear which tyrosine residue(s) is (are) related to Akt pathway. Main tyrosine sites and main downstream molecules studied in the literature are circled. ***From (Koch et al., 2011)***

Tyr^{951}

Tyr^{951} is phosphorylated during angiogenesis, where it plays a role in migration and actin reorganization via activation of TSAd (T-cell-Specific Adapter molecule) (Matsumoto et al., 2005). However, its mutation (into Phe) does not impair mice development showing that this phosphorylation event and its downstream cascades are not critical (Koch et al., 2011).

Tyr^{1054}-Tyr^{1059}

Tyr^{1054} and Tyr^{1059} are also crucial residues for VEGF-R2 activity because they are required for complete receptor internalization and activation (Dougher and Terman, 1999; Kendall et al., 1999). Indeed, Tyr^{1059} allows a maximal phosphorylation of Tyr^{1175} by a positive feedback involving the binding Src and the activation IQGAP1 pathway (IQ-motif-containing GTPase-

activating protein 1). This finally regulates cell-cell contacts, proliferation and migration (Meyer et al., 2008; Yamaoka-Tojo et al., 2006).

Tyr^{1214}

Tyr^{1214} (Tyr^{1212} in mouse), by recruiting the Nck adaptator molecule (non-catalytic region of tyrosine kinase adaptor protein 1) and the Fyn tyrosine kinase, plays a role in the activation of the p38 mitogen-activated protein kinase (MAPK) pathway (Lamalice et al., 2006). However, VEGF-R2^{Phe1212} homozygous mice are viable and fertile (Sakurai et al., 2005). By sharp contrast however, p38$^{-/-}$ mice embryos die at 10.5-11.5 dpf because of vascular abnormalities (Aouadi et al., 2006) and VEGF$_{165}$a efficiently stimulates p38 phosphorylation in endothelial cell expressing only the VEGF-R2^{Phe1214} mutant (Kawamura et al., 2008a). These conflicting data most probably suggest that other phosphorylated tyrosine can also triggers the p38 pathway.

Other regulatory pathways

Although Pl3K (phosphatidylinositol 3-kinase) is a key factor for VEGF-A induced regulation, the mechanisms leading to its activation remain poorly understood (Koch et al., 2011). PI3K regulates endothelial cells migration and survival through Rac and Akt, respectively (Datta et al., 1999). Via eNOS, it regulates also vessels permeability, the first identified role of VEGF-A, which is an essential process for normal tissue homeostasis and during the first steps of angiogenesis (Bates and Harper, 2002). Increased permeability can result from the formation of transendothelial pores and/or from the destabilization of the junctions formed between endothelial cells (Garrido-Urbani et al., 2008). Other cascades involving Src and YES can also regulate cell-cell adhesions through the β-catenin/VE-cadherin pathway (Roura et al., 1999; Wallez and Huber, 2008).

VI-3 Neuropilin 1

Neuropilin 1 (NRP1) is a 130 kD transmembrane protein that was initially identified in neural central system as a receptor for semaphorin 3A/Collapsin1 (Sema3A), a secreted polypeptide playing a role in nerve guidance during development (He and Tessier-Lavigne, 1997; Kolodkin et al., 1997). It can also bind VEGF-A variants at the level of two coagulation factor V/VIII homology domains, referred to as B1 (b1) and B2 (b2), present in the large extracellular region (Fig 15) (Appleton et al., 2007; Pellet-Many et al., 2008). In the recent years, many ligands for NRP1 have been identified (Bagri et al., 2009). The molecule contains also a transmembrane and a small cytoplasmic domain containing a PDZ-binding

motif (SEA domain) but lacking intrinsic catalytic function (Fujisawa et al., 1997). Association between this SEA intracytoplasmic motif and an adaptor molecule named synectin (or GIPC) would allow signalling from NRP1 to intracellular pathways of regulation (Cai and Reed, 1999). Phosphorylations on serine and threonine residues have also been suggested (Shintani et al., 2009) and could participate in the NRP1-induced regulation although it is considered that the main functions of NRP1 rely on its activity as a co-receptor. Soluble variants resulting from AS mechanisms have also been identified and would act as anti-angiogenic factors (Cackowski et al., 2004). It can be also post-translationally modified, in a cell type specific manner, by the addition on Ser^{612} of either a chondroitin sulfate or a heparan sulphate chain, which can modify its affinity for ligand (Pellet-Many et al., 2008; Shintani et al., 2006).

In the specific field of angiogenesis, it has been shown that NRP1 is expressed at the endothelial cells surface, especially in arteries (Herzog et al., 2001; Soker et al., 1998), and in pericytes (Shintani et al., 2006). It is also upregulated in many tumours and transformed cell lines (Bielenberg et al., 2006; Frankel et al., 2008; Jubb et al., 2012b; Klagsbrun et al., 2002), especially in cells with a high metastatic potential (Bachelder et al., 2001; Latil et al., 2000). NRP1 is also present at the surface of immune cells such as naive T-cell and dendritic cells where it plays a role in the regulation of the immune response (Prud'homme and Glinka, 2012; Tordjman et al., 2002).

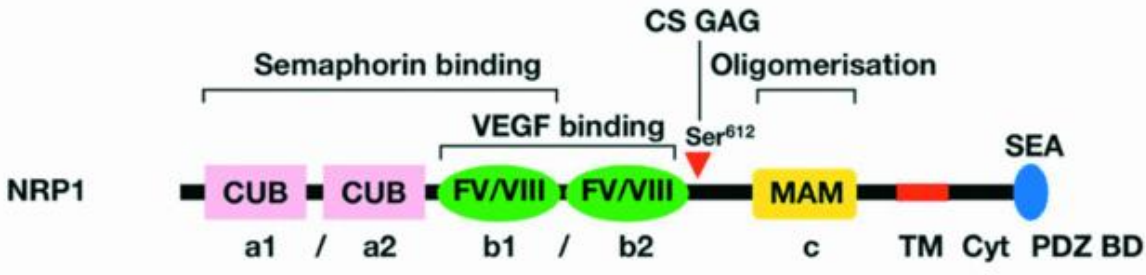


Figure 15 : Schematic structure of Neuropilin 1

Neuropilin 1 is a transmembrane co-receptor containing different domains. An extracellular region contains two CUB domains (a1, a2), two coagulation factor V/VIII homology domains (FV/VIII; b1, b2) and a MAM domain, implicated in oligomerization (c). Domains a1, a2 and b1 are involved in Semaphorin binding while b1 and b2 are involved in VEGF binding. In the extracellular portion, a glycosylation site (CS GAG: Chondroitin Sulfate Glygosaminoglycans) located on Ser^{612} domain is identified. The co-receptor domain is also composed of a transmembrane sequence (TM) and a short cytoplasmic regions (cyt) containing the PDZ binding domain (BD), critical for NRP1 function and formed by the SEA sequence. CUB: Complement protein subcomponents Clr/Cls, Urchin embryonic growth factor and Bone morphogenic protein domain, MAM: Meprin/A5-protein/PTPmu domain. ***From (Pellet-Many et al., 2008)***

NRP1 is crucial during development, as illustrated in NRP1$^{-/-}$ embryos that die at 12.5-13.5 dpf largely because of vascular problems (Kawasaki et al., 1999). Similar alterations were observed in mouse embryos where NRP1 expression was repressed in endothelial cells only and in the zebrafish model by the use of NRP1-specific morpholinos, demonstrating the critical importance of NRP1 for the development of the cardiovascular system (Gu et al., 2003; Lee et al., 2002).

NRP1-NRP2 double KO mice die at 8.5 dpf with even more severe vascular defects (Takashima et al., 2002) while overexpressing NRP1 triggers an excess of capillary growth and anarchic sprouting leading to mortality *in utero* (Kitsukawa et al., 1995).

VI-3-1 NRP1 in angiogenesis and cancer

As already briefly mentioned, NRP1 could potentially have a direct signalling activity upon ligand binding. It is considered however that its role as a co-receptor is probably the most significant, especially in endothelial cells.

Co-receptor activity

Initially NRP1 was considered only as a co-receptor "presenting" VEGF variants to VEGF-R2, as demonstrated for $VEGF_{165}$a and $VEGF_{121}$a for example (Pan et al., 2007b; Shraga-Heled et al., 2007). However, NRP1 is also central to the formation of multi-molecular complexes containing specific VEGF variants (mostly $VEGF_{165}$a and $VEGF_{189}$a), VEGF-R2 and sometimes heparin and HSPG (Fig 16). These associations strengthen the interaction between VEGF and VEGF-R2, which increases the activation of signalling pathways regulating endothelial cell phenotype (Becker et al., 2005; Oh et al., 2002; Soker et al., 2002; Whitaker et al., 2001). As compared to $VEGF_{121}$a, an isoform unable to initiate the formation of such complexes, $VEGF_{165}$a induces a stronger phosphorylation of p38 in endothelial cells, an increased branching of vessels and a most efficient recruitment of pericytes (Cebe-Suarez et al., 2008; Grunstein et al., 2000; Kawamura et al., 2008a). These properties are inhibited by a blocking antibody for NRP1, further confirming its crucial role in modulating angiogenesis (Pan et al., 2007a). In another example, NRP1 downregulation during wound healing delays the repair process by reducing blood vessel formation (Gu et al., 2003; Matthies et al., 2002). Surprisingly, however it seems that VEGF-induced vascular permeability is not influenced by NRP1 (Pan et al., 2007a).

Expression of NRP1 in "tip cells" has been described as a key element controlling sprouting, by regulating filopodia formation and guiding the direction of migration (Gerhardt et al., 2004). It has also been shown that $VEGF_{165}$a can induce the formation of complexes

involving VEGF-R2 and NRP1 molecules expressed on the surface of adjacent cells (VSMC), a process probably required in “tip cell” guidance (Cebe-Suarez et al., 2008; Gerhardt et al., 2003).

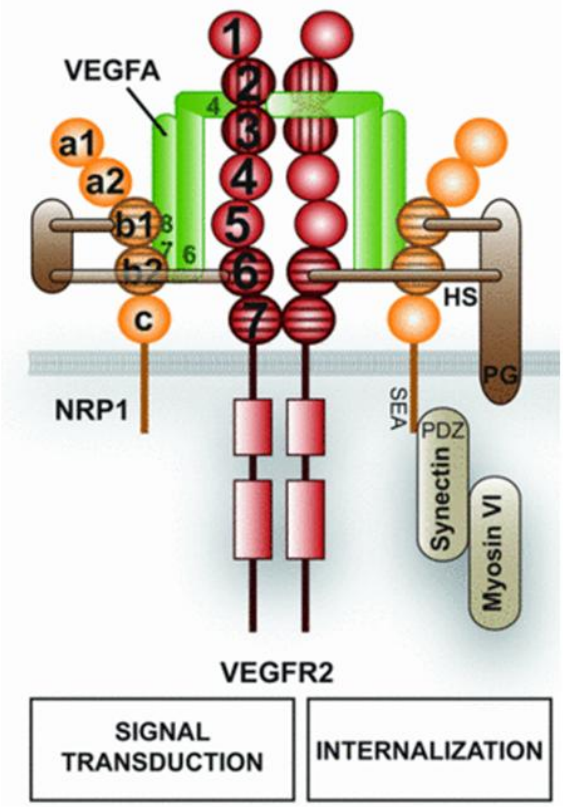


Figure 16 : VEGFR2-VEGF-NRP1-HSPG tetrameric complex

$VEGF_{165}$a or $VEGF_{189}$a (green) can form a complex with VEGF-R2 (red), NRP1 (orange) and HSPG (brown). The E4-encoded domain of VEGF is required for binding to VEGF-R2. The E6, E7 and E8 domains are involved in binding to NRP1 while interactions with HSPG/heparin are mediated by the E6 and E7-derived basic amino acids. This complex strengthens the signalling downstream of VEGF-R2 phosphorylation. Other functions or properties of NRP1 rely on its intracytoplasmic domain through interactions between the SEA sequence and synectin, which would participate in the control of internalization and degradation or recycling of VEGF-R2. ***From (Koch et al., 2011)***

Besides influencing signalling, NRP1 affects also VEGF-R2 internalization, trafficking and degradation (Ballmer-Hofer et al., 2011). When cells are treated with $VEGF_{165}$a, complexes between NRP1 and VEGF-R2 can be recycled to the plasma membrane through Rab11 vesicles. By contrast, $VEGF_{165}$b, which does not bind NRP1, induces internalization in Rab7 vesicles leading to the receptor degradation (Ballmer-Hofer et al., 2011). The SEA domain, by interacting with synectin, plays a critical role in the recycling process (Berger and Ballmer-Hofer, 2011). It has been also suggested that these complexes regulations involving NRP1, VEGF and VEGF-R2 probably operate in other cell types such as pericytes and glioma cells

(Hamerlik et al., 2012; Liu et al., 2005). They are also possibly involved in tube formation by renal epithelial cells (Karihaloo et al., 2005).

Direct signalling activity

It was suggested that NRP1-VEGF binding, without any further interaction with VEGF-R2, could regulate endothelial cells attachment and migration, but not proliferation (Murga et al., 2005; Wang et al., 2003). The SEA-carboxyterminal sequence and its interacting partner, synectin, are essential for these regulations as demonstrated in zebrafish where expression of NRP1 lacking the SEA-motif or, alternatively, the knockdown of synectin, alter blood vessels formation (Wang et al., 2006). p38 is the downstream signalling pathway involved in this process (Ballmer-Hofer et al., 2011; Evans et al., 2011). It has been suggested also that NRP1 could participate in cell adhesion independently of binding to any growth factor (Murga et al., 2005; Shimizu et al., 2000).

NRP1 expression in cancer correlates with poor prognosis, advanced disease state and invasiveness (Hansel et al., 2004; Kawakami et al., 2002; Wang et al., 2012). In absence of VEGF-R2 expression by cancer cells, it was shown that VEGF-NRP1 interactions can regulate however their adhesion, chemotaxis and proliferation *in vitro* and *in vivo* (Bachelder et al., 2001; Herve et al., 2008; Yaqoob et al., 2012). In fibrosarcoma, hypoxia drives NRP1 expression, which stimulates tumour growth through induction of vascular mimicry (Misra et al., 2012). Interestingly, NRP1, in its chondroitin sulphate modified form, could stimulate invasion of human glioblastoma through downstream activation of the $p130^{cas}$ pathway (Frankel et al., 2008).

VI-3-2 Potential use in therapy

Different strategies for inhibiting the VEGF-NRP1 interactions have been tested and evaluated for their efficiency in reducing tumour growth, either directly or indirectly by altering angiogenesis.

- Blocking NRP1 antibodies inhibit angiogenesis *in vivo* and synergizes with an anti-VEGF antibody for reducing tumour growth and blood vessels organization and maturation, particularly by inhibiting pericytes recruitment (Pan et al., 2007a).
- Soluble forms of NRP1 could be used as VEGF trap. Their effects are either direct, by inhibiting cancer cells migration for example (Cackowski et al., 2004; Gagnon et al., 2000), or indirect, by repressing intratumoural angiogenesis (Yamada et al., 2001).
- VEGF mimetic peptides have been also used for the same purpose. A synthetic peptide analogous to the E8a-encoded domain reduces the growth of tumours formed by MDA-

MB231 cells by controlling blood vessel density (Starzec et al., 2006). Other peptides were also evaluated such as those mimetic of the E6a- (Jia et al., 2001) or E7-derived sequences (Soker et al., 1997), or Tufstin (NH_2-Thr-Lys-Pro-Arg-COOH), a peptide with affinity for the b1 domain of NRP1 implicated in VEGF binding (Vander Kooi et al., 2007; von Wronski et al., 2006).

As many aggressive tumour cells are characterized by high levels of NRP1 expression, such peptides conjugated to cytotoxic drugs could be used for targeted therapy, although potential side effects on endothelial cells have to be carefully considered and evaluated (Prud'homme and Glinka, 2012). Promising data have also been observed with peptides coupled to a photosensitizer molecule (Benachour et al., 2012; Tirand et al., 2006).

VI-4 Neuropilin 2

NRP2 displays many similarities with NRP1, such as a 44% sequence identity, high expression levels in the nervous system (Chen et al., 1997; Fujisawa et al., 1997; Kolodkin et al., 1997) and implication, as a co-receptor, in the regulation of angiogenesis (Gluzman-Poltorak et al., 2000; Sulpice et al., 2008). Additionally, NRP2 possesses also a small cytoplasmic domain lacking intrinsic catalytic function (Fujisawa et al., 1997), can be produced as soluble factor (Rossignol et al., 2000) and is upregulated in cancer and is associated with aggressiveness and poor outcomes (Chen et al., 1997; Fakhari et al., 2002; Goel et al., 2012; Handa et al., 2000; Jubb et al., 2012a). It interacts with VEGF-R1 (Gluzman-Poltorak et al., 2001) and induces the formation of NRP2-$VEGF_{165}$a-VEGF-R2 complexes. Their effects on endothelial cells survival and migration have been reported (Favier et al., 2006).

NRP2 possesses however specific properties and function. It is mainly expressed in veins and lymphatics (Herzog et al., 2001; Yuan et al., 2002). $Nrp2^{-/-}$ mice are viable with normal veins and arteries but display a reduced network of small lymphatic vessels and capillaries (Yuan et al., 2002). A reduction of VEGF-induced neo-angiogenesis in $NRP2^{-/-}$ mice has also been reported (Shen et al., 2004). Its b domains can interact with PlGF and VEGF-A variants (Geretti et al., 2007; Gluzman-Poltorak et al., 2000), but with a lower affinity than the corresponding domain of NRP1 because of the absence of critical acidic residues. NRP2 forms also complexes with VEGF-C or VEGF-D and VEGF-R3, which explains its role in lymphangiogenesis (Karpanen et al., 2006). As for NRP1, treatments with NRP2 antibodies or soluble NRP2 reduce lymphangiogenesis, tumour growth and metastasis (Caunt et al., 2008; Geretti et al., 2010).

VI-5 Heparan Sulfate Proteoglycans and heparin

VI-5-1 Structure

The basic proteoglycan unit consists of a "core protein" with one or more glycosaminoglycan (GAG) chain(s) covalently attached on a Ser residue in an appropriate consensus sequence. Proteoglycans can be categorized depending upon the nature of their GAG chains (heparan, chondroitin, dermatan or keratan sulfate), which may be cell type specific. Heparan sulfate is a glycosaminoglycan structurally closely related to heparin. It consists of a variable number of repeating “glucuronic acid - glucosamine” disaccharide units that can be further modified and partially sulfated. It is mostly found at the cell surface and in the ECM (Esko and Selleck, 2002).

VI-5-2 Role in angiogenesis and cancer

VEGF was initially identified as a heparin and heparin sulphate proteoglycan (HSPG)-binding factor (Ferrara and Henzel, 1989; Senger et al., 1983). Later it was demonstrated that VEGF binding to heparin (Cebe Suarez et al., 2006; Houck et al., 1992) and HSPG (Kawamura et al., 2008b) was isoform-specific. These differences in affinities for HSPG are responsible for the variable capacity of the VEGF-A isoforms to be immobilized in the ECM. As an example, $VEGF_{189}$a forms stables complexes with heparin and is efficiently trapped and stored in the ECM and at the cell surface (Park et al., 1993) while $VEGF_{121}$a does not interact with heparin and is therefore more “diffusible”. $VEGF_{165}$a displays intermediate properties (Houck et al., 1992). The simultaneous expression of the three isoforms by tumour cells generates a VEGF gradient that stimulates the formation of a well-organized vascular network (Grunstein et al., 2000). However, a more recent study demonstrates that the binding to heparin is not essential for the localization of the blood vessels in response to VEGF isoforms (Springer et al., 2007).

HSPG have affinity for VEGF-R1, VEGF-R2 and NRP1, but not NRP2 (Park and Lee, 1999; Vander Kooi et al., 2007; Xu et al., 2011a). It was initially reported that HSPG increased the binding of VEGF to its receptors and co-receptor NRP1 (Dougher et al., 1997; Fuh et al., 2000; Gitay-Goren et al., 1992; Tessler et al., 1994), but this was not confirmed in another study for VEGF-R1 and VEGF-R2 (Keyt et al., 1996b). As previously mentioned, HSPG and heparin potentiate VEGF signalling by facilitating the formation of VEGFR2-VEGF-NRP1 complexes at the surface of endothelial cells (Ashikari-Hada et al., 2005; Kawamura et al., 2008a).

VI-6 Other receptors for VEGF

Some VEGF variants, especially the longer variants with affinities for HSPG, present also affinity for other receptors such as integrins, which are transmembrane heterodimers regulating the interactions of cell with the ECM and, therefore, cell adhesion, migration and survival (Chen et al., 2010; Herve et al., 2008; Hutchings et al., 2003). For instance, VEGF can form complex with $\alpha_v\beta_3$, which regulates p38 and FAK pathways activation and affects cell adhesion and migration (Borges et al., 2000; Mahabeleshwar et al., 2006).

VII- Benefits and pitfalls of inhibiting angiogenesis in clinic

The concept of inhibiting angiogenesis for starving tumour and fighting cancer is an old concept (Folkman, 1972). A proof-of-concept study using a blocking antibody targeting VEGF-A was published two decades ago (Kim et al., 1993) and confirmed later in different experimental models (Borgstrom et al., 1998; Borgstrom et al., 1996; Mesiano et al., 1998; Warren et al., 1995).

It rapidly became clear however that using VEGF inhibitors as a monotherapy was efficient only transiently and had only very limited effect on long term outcome. By contrast, treatments combining “classical” chemotherapeutic agents and anti-VEGF molecules currently seem more promising (Ferrara et al., 2007; Garcia et al., 2008; Micha et al., 2007). A concept of vasculature “normalization” explains this synergistic therapeutic effect (Jain, 2005). In tumours, there is an excessive expression of pro-angiogenic molecules, which alters the balance between pro- and anti-angiogenic factors (Fig 17). As a result, the vasculature is poorly organized and vessels remain immature, tortuous and permeable. This has several consequences. The interstitial fluid pressure in the tumour is higher than in the other tissues, which strongly limits the access of chemotherapeutic drugs in the tumour mass. This causes also local hypoxia, which further stimulates pro-angiogenic pathways and contributes to keep abnormal vasculature in the tumour (Fig 17B).

Anti-angiogenic treatments, by restoring a correct balance, tend to destroy preferentially immature vessels and to normalize the vasculature, which reduces intra-tumoural pressure and hypoxia (Fig 17C) and facilitates the delivery of cytotoxic drugs to cancer cells (Jain, 2001). The use of anti-angiogenic molecules requires however a careful dosage because, when use in excess, they can also lead to the destabilization of mature and normal vessels (Fig 17D, upper panel) (Jain and Carmeliet, 2012). In the best case, it may result in tumour regression but usually associated with a reduction of the efficacy of the chemotherapy. In the

worst case, the induced hypoxia could favour metastases and/or stimulate cancer cells to activate alternative pathways for improving their resistance to the treatment. Some of these anti-angiogenic molecules are listed in Table 1 and will be briefly described. They can either target directly VEGF or VEGF-receptors and co-receptors. These compounds can also be used with some success for curing eye diseases due to excessive angiogenesis.

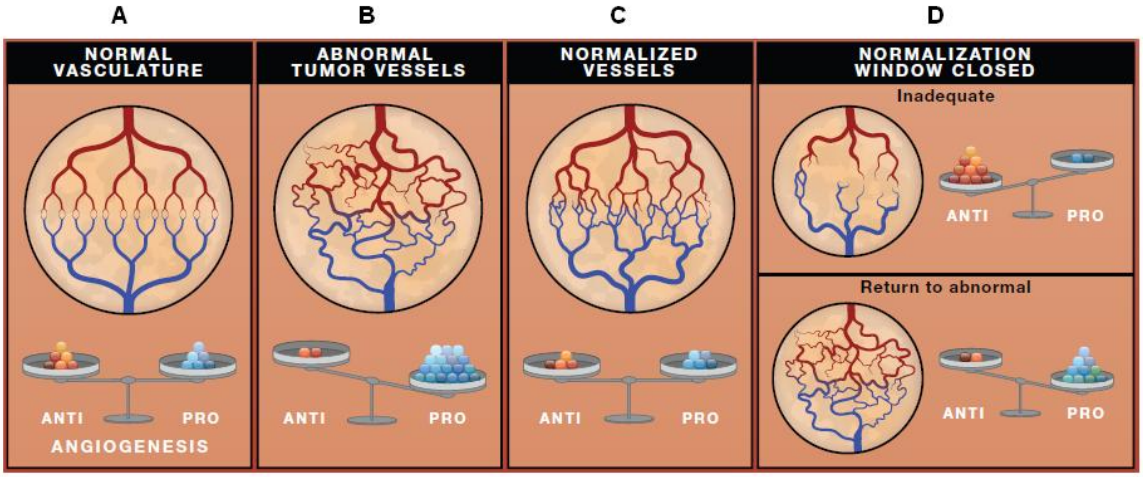


Figure 17 : Blood vessels organization according to the balance between pro- and anti-angiogenic molecules

Normal vasculature homeostasis results from an optimal balance between pro- and anti-angiogenic factors **(A)**. In tumours, blood vessels network is anarchic because of a large excess of pro-angiogenic molecules **(B).** Restoring a correct balance with optimal dosage of anti-VEGF drugs normalizes the blood vessel network **(C)**. Too high concentrations of anti-angiogenic factors strongly affect the vasculature **(D, upper panel)** and stopping the therapy leads to a rapid return to abnormal vessel organization **(D, lower panel)**. ***From (Jain and Carmeliet, 2012)***

VII-1 Bevacizumab (Avastin ®)

Bevacizumab (Avastin®) is a humanized monoclonal IgG1 antibody raised against the E2-E4 encoded sequence of VEGF-A (Presta et al., 1997). Therefore it recognizes both the pro- and anti-angiogenic isoforms as well as the aminoterminal part of cleaved molecules (Gutierrez et al., 2008; Muller et al., 1998; Varey et al., 2008). Bevacizumab is currently the most widely used anti-angiogenic molecule (Jain and Carmeliet, 2012). It can be used as a single agent in second line treatment of glioblastoma but its association with cytotoxic molecules has been approved by the FDA (Food and Drugs Administration of the USA) for treating several cancers, such as advanced colorectal cancer (Escudier et al., 2007; Ferrara et al., 2005; Hurwitz et al., 2004; Rini et al., 2008). As another example, the combination of Avastin and docetaxel was evaluated in metastatic breast cancer. This combined treatment was reported to increase the survival of patients up to 6.0 months as compared to docetaxel alone (Ramaswamy et al., 2006). This synergistic effect seems however to be specific of

some cancer types as the benefit is modest or absent in advanced and metastatic pancreatic cancer (Hayes, 2011; Kindler et al., 2010; Van Cutsem et al., 2009). Furthermore, the initial observation of the positive effect of Avastin for the treatment of metastatic breast cancer has been recently contested (Hayes, 2011)

VII-2 Ranitizumab (Lucentis®)

Ranitizumab, a humanized Fab fragment derived from bevacizumab, is also able to recognize all the VEGF variants (Ferrara et al., 2006). In addition to be much smaller than the parent molecule, it has been designed to have a stronger affinity to VEGF. Its efficiency has been proved in the context of eyes diseases and it is currently the gold reference for the treatment of AMD.

VII-3 VEGF trap (Aflibercept®)

This VEGF decoy is formed by a fusion of extra-cellular domain 2 of VEGF-R1 and domain 3 of VEGF-R2 thanks to the Fc domain of IgG1 (Holash et al., 2002; Teng et al., 2010). It is currently approved as a therapy for AMD and it displays efficacy when combined with chemotherapeutic agents in improving survival of patients with metastatic colorectal cancer (Van Cutsem et al., 2012)

Table 1: Non-exhaustive list of VEGF and VEGF receptors inhibitors

Name	Brand name	Type	Application
Bevacizumab	Avastin	Anti-VEGF Humanized antibody	Cancer
Ranitizumab	Lucentis	Anti-VEGF Humanized antibody fragment	Ocular neo-angiogenic diseases
VEGF-trap	Aflibercept	Extracellular domains of VEGF-R1 and -R2 fused by Fc	Ocular neo-angiogenic diseases, Cancer
Sunitinib	Sutent	Multi-target RTK inhibitor	Cancer
Sorafenib	Nexavar	Multi-target RTK and Raf pathway inhibitor	Cancer
Pazopanib	Votrient	Multi-target RTK inhibitor	Cancer
Axitinib	Inlyta	Multi-target RTK inhibitor	Cancer
Pegaptanib	Macugen	Aptamer anti-$VEGF_{165}$a	Ocular neo-angiogenic diseases
MNRP1685A		NRP1 antibody	Cancer

VII-4 Pegaptanib (Macugen®)

Pegaptanib is an aptamer with a specific affinity for $VEGF_{165}$a. When injected in the eye it represses pathological neovascularisation (Ng et al., 2006). As opposed to bevacizumab (Varey et al., 2008), it has the advantage of not recognizing $VEGF_{165}$b, the variant that permits the survival of endothelial cells but not their proliferation (Magnussen et al., 2010). It is proposed as an alternative to bevacizumab or ranitizumab for the treatment of eyes diseases (Adamis et al., 2006).

VII-5 VEGF-R2 TKI

Several VEGF-R2 tyrosine kinase inhibitors (VEGF-R2 TKI) are currently in development: Sunitinib (Sutent®), Sorafenib (Nexavar®), Pazopanib (Votrient®) or Axitinib (Inlyta®). All of them target VEGF-R2, but some of them can inhibit other tyrosine kinase receptors. They have been proved to display efficacy in cancer therapy by preventing the intracellular phosphorylation of the VEGF-R2 (Escudier et al., 2009; Rini et al., 2009; Sternberg et al., 2010). Amongst them, Sorafenib has the additional advantage to target also downstream signalling pathways (c-Raf, B-Raf) of the VEGF-R2 (Adnane et al., 2006).

VII-6 Resistance and side effects

The use of anti-angiogenic factors in cancer therapy has been so far disappointing for several reasons (Table 2) (Kerbel, 2012; Miller et al., 2005). Resistance to treatment has been reported many times. It could be related to the lack of sensitivity to VEGF of the mature vessels feeding the tumours (Fig 4) (Bergers et al., 2003; Sitohy et al., 2012). The activation of alternative pro-angiogenic pathways, such as the cascade triggered by c-Met, is another mechanism of resistance to current therapies (Casanovas et al., 2005; Huang et al., 2010; Shojaei et al., 2010; You et al., 2011). Another problem relates to dangerous side effects such as thrombo-embolism, hypertension and proteinuria (Kabbinavar et al., 2003; Launay-Vacher and Deray, 2009; Mir et al., 2011). The absence of reliable biomarker that could allow an evaluation of the efficacy of anti-angiogenic drugs and their price, especially for Avastin®, are other features that prevent further development and use. Finally, any interruption in the treatment leads to a rapid reformation of the previous vasculature network because the remaining of dead vessels (channels, ECM including basement membrane, surviving pericytes...) serve as preferential scaffolds for neo-angiogenesis (Mancuso et al., 2006). Other adverse effects such as an increase of metastasis have been observed after a short-term treatment with Sunitinib (Ebos et al., 2009).

Table 2 : Major Problems of Anti-angiogenic therapies	
Disappointing results	Resistance to treatment, limited effect (if any) in Phase III studies
Adverse side effects	Re-growth of tumor after stopping treatment, hypertension, proteinuria
Cost	Excessive price (Avastin)
No biomarker available	Absence of control of the efficacy

From Conference of X. Pivot, Fourth Congress of French Society of Angiogenesis

VII-7 Endocan: a potential biomarker of the expression of VEGF by tumours

The identification of biomarkers allowing the evaluation of the progression of neovascularisation in cancer and during anti-VEGF treatment is needed. Endocan, also referred to as ESM-1 (Endothelial cell-Specific Molecule 1), is a secreted proteoglycan composed of a 165 amino acids core protein covalently linked to one dermatan sulphate side chain (Bechard et al., 2001; Bechard et al., 2000). Its expression is induced by VEGF (Abid et al., 2006; Recchia et al., 2010; Rennel et al., 2007; Strasser et al., 2010) and in cancer, especially during the switch from dormant to fast-growing angiogenic tumours (Almog et al., 2009). Increased endocan concentrations have been measured in serum of patients with lung and renal cancers, as compared to healthy subjects (Leroy et al., 2010; Scherpereel et al., 2003). All these observations suggest that circulating endocan could be a biomarker of neo-angiogenesis and tumour aggressiveness.

VIII- Stimulation of angiogenesis for the treatment of ischemic diseases

While cancers and many eyes diseases could be cured by anti-angiogenic therapies, several clinical conditions are directly or indirectly related to insufficient perfusion, either acute (stroke, heart infarction, tissues grafting) or chronic (angina, skin ulcers...).

Wound healing delay

During tissue repair, hypoxia, pro-inflammatory cytokines and growth factors trigger VEGF secretion by keratinocytes and infiltrating inflammatory cells (Detmar et al., 1995; Frank et al., 1995; Fukumura et al., 1998; Kishimoto et al., 2000). This leads to vascular permeability, local angiogenesis and to the recruitment of additional inflammatory cells and bone-marrow derived endothelial progenitor cells (Brown et al., 1992; Eming and Krieg, 2006; Galiano et al., 2004). Reduced concentrations of VEGF-A delay the healing process. This is illustrated

in a model of normal mice treated with a blocking VEGF antibody (Howdieshell et al., 2001) and in diabetic (db/db) mice where low levels of VEGF in the skin largely participate to the impaired tissue repair. Moreover, treating db/db mice with $VEGF_{165}$a accelerates healing (Corral et al., 1999; Galiano et al., 2004; Romano Di Peppe et al., 2002; Roth et al., 2006). However, this effect is restricted to mice with a delay phenotype as animals overexpressing VEGF do not heal better than their wild-type controls (Hong et al., 2004).

Based on these data it was suggested that VEGF could be used to accelerate the healing process of chronic ulcers that are frequent in diabetic patients and in patients with venous problems. The first experimental evaluations were however disappointing. Several hypotheses have been made to explain this absence of efficacy, such as bad access of VEGF to endothelial cells in the wound bed or degradation of VEGF by the proteases-rich environment (Keyt et al., 1996a; Lauer et al., 2000; Lauer et al., 2002). In this context, the use of variants that are more resistant to proteolysis and more “diffusible” such as $VEGF_{111}$a could help solving these limitations (Lauer et al., 2002; Mineur et al., 2007).

SCIENTIFIC CONTEXT AND AIMS OF THE WORK

Scientific context

VEGF-A, a major regulator of angiogenesis, is involved in physio-pathological processes such as development, menstrual cycle, wound healing, cancer and several eyes diseases. Its expression is regulated at the transcriptional level by activation of its promoter but also by post-translational mechanisms, mainly in hypoxic conditions. Alternative splicing of the pre-mRNA is also a potent regulator of VEGF-A activity as it induces the production of several VEGF variants possessing specific properties. While the role of the constant amino-terminal portion encoded by exons 1 to 4 is currently well defined, the functions of the alternative carboxy-terminal domains have to be deeply characterized in order to better understand why the different VEGF variants have very specific properties regarding the regulation of angiogenesis.

Aims of the work

This work, aiming at better defining the roles and potential cooperation of the domains encoded by the E5 to E8a/E8b terminal exons, can be divided in 4 different but related steps:

- Production and purification of relevant VEGF variants

- Characterization at a biochemical level by determining:
  - their affinity to VEGF receptors and co-receptors
  - their resistance to proteolysis.

- Evaluation of their role on angiogenesis by measuring their effects
  - on various endothelial cell types *in vitro*
  - on tumoural angiogenesis *in vivo.*

- Preliminary characterizations regarding potential clinical applications of our studies are also provided.

MATERIAL AND METHODS

I- Cell culture and transfected cell lines

HEK293 cells were cultured in DMEM (BioWhittaker, Walkersville, MD, USA) containing 10% FBS (Foetal Bovine Serum), penicillin (100 IU/ml) and streptomycin (100 IU/ml) except otherwise indicated. Hygromycin B (100 µg/ml, Roche, Basel, Switzerland) was added to the medium for selection of cells stably transfected with the various expression vectors. Human Umbilical Vascular Endothelial Cells (HUVECs) were cultured on a gelatin coat in M200 medium (Cascade Biologic, Portland, OR, USA) supplemented with LSGS kit (Cascade Biologic) and penicillin. Porcine aortic endothelial cells (PAEC) were cultured in Ham's F-12 (Bio Whittaker) supplemented with 10% FBS, penicillin and fungizone (0.5 µg/ml). For cells overexpressing human VEGF-R1 (PAEC-R1), VEGF-R2 (PAEC-R2) or NRP1 (PAEC-NRP1) (Becker et al., 2005; Waltenberger et al., 1994), G418 (0.5 mg/ml) was used to maintain selection. In order to produce PAEC-R2 further expressing human NRP1, a full length NRP1 cDNA was cloned into the pcDNA4/T0 vector (Invitrogen, Carlsbad, New-Mexico, USA) and the construct was transfected into PAEC-R2 using cationic lipids (GeneJuice Transfection Reagent, Novagen, UK). Cells were then selected in Ham's F-12 containing Zeocin (Invitrogen, Belgium) at 150 µg/ml.

For quantifying the secretion of endocan HUVEC were seeded in M200 supplemented with LSGS on a gelatin-coated 12 wells-plate. At 80% of confluence, cells were starved in M200 containing 0.2% FBS for 4h before the addition of VEGF isoforms (at 1nM). Conditioned media were sampled after 24h and 48h.

II- Chemicals and antibodies

Human plasmin, Evans' blue and formamide were obtained from Sigma-Aldrich (St-Louis, MO, USA). The following antibodies were used for Western blot: anti-ERK 1/2 (rabbit polyclonal, M-5670) and anti-phospho ERK 1/2 (mouse monoclonal, M-8159) were purchased from Sigma-Aldrich; anti-VEGF (rabbit polyclonal, SC-152) was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA); anti-human VEGF-R2 (clone 55B11, rabbit); anti-phospho Tyr1175 of VEGF-R2 (rabbit), anti-Akt (rabbit) and anti-phosphoAkt (rabbit) were purchased from Cell Signalling Technology (Danvers, MA, USA). Goat anti-Rabbit antibody coupled to peroxydase (Cell Signalling Technology) was used for rabbit antibodies. Other secondary antibodies were purchased from Dako (Glostrup, Denmark).

Mimetic peptides A7R (NH_2-Ala-Thr-Trp-Leu-Pro-Pro-Arg-COOH) (Starzec et al., 2006) and R8R (NH_2-Arg-Pro-Lys-Lys-Asp-Arg-Ala-Arg-COOH) were obtained from Polypeptide Group (Strasbourg, France).

III- Cloning, production and purification of VEGF variants recombinant proteins

Cloning of $VEGF_{111}a$, $VEGF_{121}a$ and $VEGF_{165}a$ cDNAs and transfection of HEK293 cells were described elsewhere (Mineur et al., 2007). The coding sequence of $VEGF_{111}b$, $VEGF_{121}b$, $VEGF_{155}a$, $VEGF_{155}b$, $VEGF_{165}b$, $VEGF_{179}a$ and $VEGF_{179}b$ cDNAs were chemically synthesized (Genscript, Piscataway, NJ, USA), subcloned in a pCEP-4 vector and transfected into HEK293 cells using FuGENE 6 transfection reagent (Roche). Hygromycin-resistant transfected cells were cultured up to 90% confluence in medium supplemented with 10% FBS. For VEGF production it was replaced by medium containing 1% FBS and cultures were continued for 2 additional days. VEGF variants were then purified from conditioned media by affinity chromatography using Bevacizumab (Avastin, Roche) coupled to Affi-Gel Hz (Biorad, Hercules, CA, USA) as described by the manufacturer. After sample loading and washing (Tris 0.05 M, NaCl 0.15M, pH 7.5), VEGF was eluted (Glycine 0.1M, pH 3.5) and the recovered fractions were immediately neutralized with 1:10 volume of 1M Tris. Some VEGF variants were also purified by affinity chromatography using heparin-sepharose (CL6B, GE Healthcare). After sample loading and washing ($H_2PO_4^-$-HPO_4^{2-} 0.01 M, NaCl 0.25M, pH 7.3), VEGF were eluted with increasing concentrations of NaCl (0.5M, 1M, 1.5M and 2M). Purity and concentration were evaluated after SDS-PAGE (15% acrylamide) and Sypro Ruby (BioRad) staining (see chapter IV-2). Proteins were dialysed against HBS-EP buffer (GE Healthcare, Little Chalfon, UK) for Surface Plasmon Resonance or diluted in PBS for other purposes.

IV- Analytical procedures

IV-1 Western blot

Western blot analyses were used to evaluate the quality and the purity of the different VEGF variants and to determine the phosphorylation and activation of VEGF-R1, VEGF-R2 and downstream signalling molecules. Proteins were separated by SDS-PAGE (Sodium Dodecyl Sulfate PolyAcrylamide Gel Electrophoresis) and transferred onto polyvinylidene difluoride membranes (Perkin Elmer, Waltham, MA, USA) by electroblotting. Membranes were blocked for 1 hour in PBS-Tween (0.05%) buffer supplemented with 3 % fat free dry milk before probing with the primary antibodies. After washing in PBS-Tween, membranes were

incubated with the appropriate HorseRadish Peroxydase (HRP)-conjugated secondary antibodies. Signals were detected by chemiluminescence using the ECL Western Blotting Analysis Substrate (Thermo, Fischer Scientific Waltham, MA, USA) and x-ray film exposure before quantification using the Fluor-S Multimager and Quantity One software (Bio-Rad Laboratories, Hercules, CA, USA).

IV-2 Sypro Ruby

After SDS-PAGE separation, gels were fixed (30 min) in a solution containing 50% methanol and 7% acetic acid for half an hour, stained in Sypro ruby solution for 4 hours and washed in a solution containing 10% methanol and 7% acetic acid for 30 min. The quantification was performed using the Fluor-S Multimager and Quantity One software. The VEGF concentrations were evaluated by comparison to BSA (Bovine Serum Albumine) samples of defined concentrations run on the same gel.

IV-3 Quantification by ELISA

VEGF concentrations were also evaluated by ELISA using either panVEGF or $VEGF_{165}$b Duoset (R&D systems). Endocan concentration was assessed using JDIYEK ELISA kit (Lunginnov, Lille, France).

IV-4 Mass Spectometry

The amino acids terminal sequence of VEGF variants was analyzed by MALDI-TOF (Matrix-Assisted Laser Desorption/Ionisation and time-of-flight mass spectrometry) process at the GIGA-Proteomic Platform (University of Liège).

V- Binding to VEGF-receptors and co-receptors

V-1 Surface Plasmon Resonance

Surface Plasmon Resonance (SPR) analyses were performed on a Biacore X-100 system (GE Healthcare) as previously described (Fig 18) (Herve et al., 2008). Briefly, Fc chimera human VEGF-R1 or VEGF-R2 and Fc chimera Rat NRP1 or NRP2 (R&D Systems, Minneapolis, MN, USA) were bound to the CM5 Sensorchip (GE Healthcare) using goat polyclonal antibodies against Human Fc (Sigma-Aldrich) fixed by amine binding to the chip. Biotinylated heparin was coupled to a carboxymethylated dextran pre-immobilized with streptavidin (Sensorchip SA, GE Healthcare). For routine evaluation the samples were diluted in HBS-EP buffer and injected for the indicated period of time (100-300 sec) followed by washing in HBS-EP (150-600 sec) in order to evaluate the association and dissociation curves, respectively. For more quantitative measurements (BIAevaluation software 4.1, GE

Healthcare), increasing concentrations of samples (0.4, 1.3, 4.0, 13.0 and 40.0 nM) were successively injected on the chip.

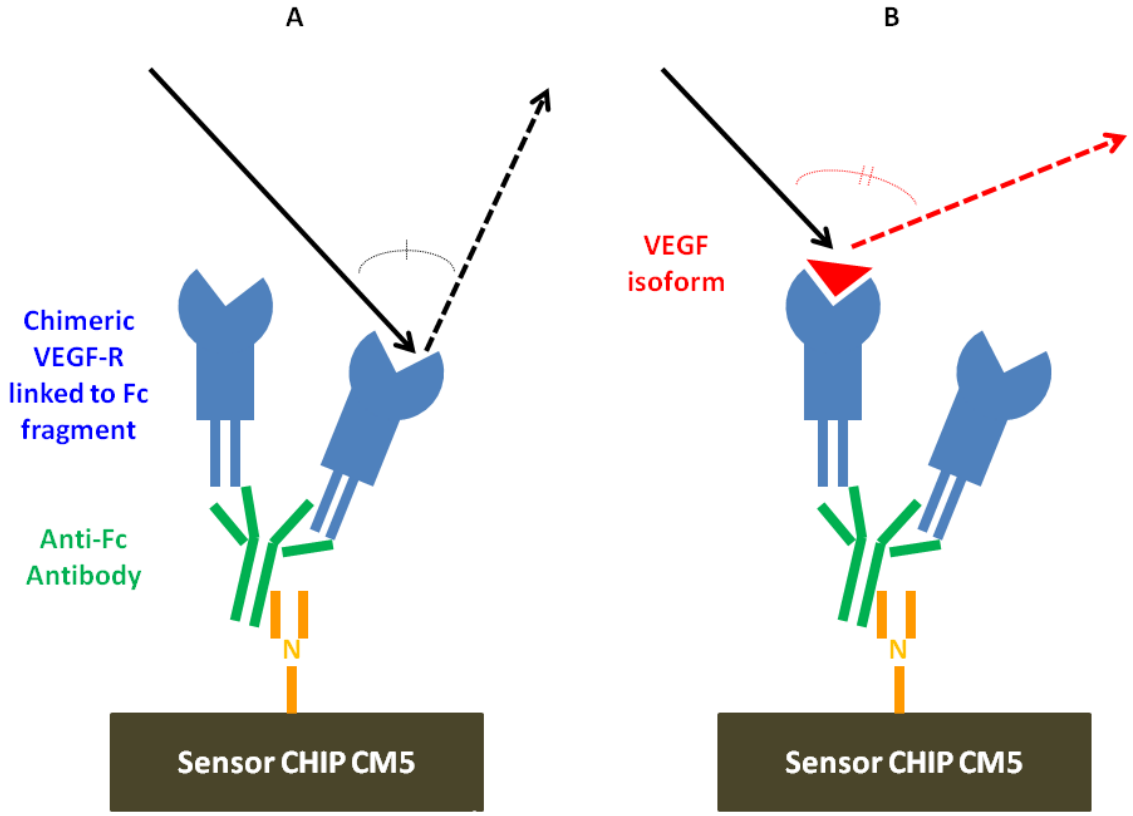


Figure 18 : Surface Plasmon Resonance analyses

VEGF (co-)receptors (blue) are bound to the chip using anti-human Fc antibodies (green) immobilized on the chip (grey) by amine bonds (orange). **(A)** In absence of any ligand an incident light is refracted with specific characteristics, here shown by the black dotted lines. **(B)** After binding of a ligand (VEGF here, red triangle), the angle of the refraction of the light is modified (red dotted line). This modification can be measured, quantified and used to evaluate the association and dissociation parameters. VEGF-R: VEGF-Receptor

V-2 Solid Phase Assay

For direct binding evaluation, solid phase assays were performed by coating 110 ng of VEGF in 50 µl PBS-Tween 0.1% containing BSA 0.1% on a flat bottom polystyrene plate (Maxisorp, Nunc Thermo Scientific) overnight at 4°C. After blocking (PBS-tween-BSA for 24h at 4°C) and washing (PBS-Tween-BSA), VEGF-R2-Fc (0.5µg/ml), NRP1-Fc (0.5µg/ml) + heparin (1µg/ml) or NRP2-Fc (0.5µg/ml) + heparin (1µg/ml) were added in 50µl of buffer for 1 hour at room temperature. Diluted (1/1200) biotinylated antibody against Human Fc (Sigma-Aldrich) was then added and incubated for 30 min at room temperature. Streptavidin-HRP and ABTS substrate (RnD systems) were then added. The Optical Densitiy (OD) was measured at 415 nm after 30min of reaction.

For competition assays, the surface of flat bottom polystyrene wells was treated overnight at 4°C with 100 µl of 2 µg/ml anti-Fc IgG in phosphate buffer saline (PBS, Sigma). After washing and saturation of non-specific interactions with bovine serum albumin (BSA, Sigma), purified recombinant rat NRP1-Fc (20 ng/well in 50 µl PBS-Tween-80 containing BSA 0.5% (PBT), R&D Systems, Abingdon, UK) was added in the wells. Then peptides solution (50 µl in PBT from 0.01 to 300 µM, final concentration) or VEGF (from 5 to 450 nM, final concentration) and biotinylated $VEGF_{165}a$ (1nM, final concentration, R&D Systems) diluted in PBT containing 2 µg/ml of heparin were successively added. After overnight incubation at 4°C, the wells were washed and treated with Streptavidin-HRP and ABTS substrate.

VI- Resistance to degradation

20ng of the different VEGF variants were treated with increasing concentrations of plasmin (0-32 x 10^{-2} IU) or of exudates from skin ulcer and incubated at 37°C for 4, 24 and 48 hours. The products of the reactions were analyzed by western blot as described above.

VII- Angiogenesis *in vitro*

VII-1 Phosphorylation assay

PAEC (Porcine Aortic Endothelial Cells) control, PAEC-R1, PAEC-R2, PAEC-NRP1, PAEC-R2-NRP1 and HUVECs were seeded in 6-well plates and allowed to spread overnight. Cells were then starved for 18 h (PAEC) or 6 h (HUVECs) in appropriate medium deprived of FBS. VEGF variants were added at the indicated concentration and time according to the protocol previously described (Catena et al., 2010; Woolard et al., 2004). Serum-free media and media containing 10% FBS were used as controls. Reactions were stopped by placing cells on ice, immediate washing with ice-cold PBS and lysis in 200 µl of Laemmli buffer. The lysates were sonicated and stored at -20°c until western blot analysis. Quantifications of the signal were performed and the relative phosphorylation indexes (phosphorylated protein/total protein) were calculated. Due to large differences in the levels of phosphorylation, films obtained after different times of exposure were quantified, samples providing intermediate intensities being used as internal comparative calibration signals. To facilitate the comparison between all the samples, the phosphorylation index calculated for $VEGF_{165}a$ was arbitrary set at 1.

VII-2 Proliferation and survival assay

WST-1 and [^{3}H]-thymidine incorporation assays were performed to measure cell proliferation and survival. Different media and time were tested in preliminary experiments for defining the optimal conditions for the different assays.

For the WST-1 assays, 3x10³ HUVECs were seeded overnight in gelatin-coated 96-wells plates (Greiner Bio-One, NC, USA) in M200 supplemented with LSGS. Cells were washed with PBS and incubated in M200 containing 0.5% FBS in the presence or absence of VEGF variants (250 pM). Two days later, 10 µl of WST-1 reagent were added to each well. After 2 hours, the optical density was measured at 450 nm.

For [^{3}H]-thymidine incorporation assays, 1.5x10^4 HUVECs were seeded overnight in gelatin-coated 24-wells plates in M200 supplemented with LSGS. Cells were washed with PBS and further cultured for 24 h in M200 containing 0.5% FBS for the evaluation of proliferation (or 0.1% for the survival assay) supplemented or not with VEGF (250 pM). [^{3}H]-thymidine (1 µM final concentration, 2.5 Ci/mol, Perkin Elmer, Waltham, MA, USA) was then added to the culture medium and the 10 % TCA-precipitable radioactivity was measured 24 h later.

VII-3 Migration assay

The capacity of VEGF variants to induce migration of HUVEC was evaluated using the Boyden Chamber assay. VEGF variants (1nM in 500 µl of M200 supplemented with 0.001% FBS) were placed in the bottom chamber of a 24-wells plate. Inserts with 8 µm diameter pores (Millipore) were coated with attachment factor (Invitrogen) for 1 hour at 37°C and then placed in wells before seeding serum-starved HUVEC in the top chamber (100.000 cells in M200 / 0.001% FBS). After 8 to 16h at 37°C for allowing cells to migrate from the top to the bottom chamber, the inserts were washed twice with PBS and fixed in PFA 4% for 20 min. Using a cotton swab, cells of the upper chamber were removed before staining with Hoechst (Invitrogen) for 5 min. Cells which migrated from top to bottom chamber were counted in 10 fields and the percentage of migration were calculated using the following formula. Percentage of migration = (Cells per Field*1/0.0028637/1x10^5)x10. (0.0028637) represents the area of the insert visible by 1 field of view.

VIII- VEGF Half-life in vivo

Six week-old C57bl6 mice were divided into twelve groups (4-6 mice per group). They received intravenous, intraperitoneal or subcutaneous injections of $VEGF_{111}$a, $VEGF_{111}$b,

$VEGF_{165}a$ and $VEGF_{165}b$ (5 µg in 100µl). Blood samples were collected in the tail using heparitinazed capillaries (Hirschmann Laborgerate, Eberstat, Germany) directly and 0.5, 1, 2, 4 and 8 hours after injection. After centrifugation at 11,7000 rpm for 5 minutes using Autocrit ULTRA-3 centrifuge (BD, Franklin Lakes, NJ, USA), plasma were collected and stored at -20°C before further analysis. VEGF concentrations were evaluated using panVEGF and $VEGF_{165}b$ Duo set ELISA kit (R&D systems). Half-life was evaluated by constructing exponential regression using Excel software (Redmonds, WA, USA), $y=a*exp^{(bx)}$, where "a" and "b" are the maximal concentration and the exponential constant, respectively. Half-life were then calculated according to the following formula : $t_{1/2}=ln(2)/b$.

IX- Angiogenesis *in vivo*

Experimental protocols were approved by the local animal ethical committee (University of Liège, approval document n° 1125). $2x10^6$ HEK293 cells transfected with the empty vector or expressing human VEGF variants were mixed with 200µl of Matrigel depleted in growth factors (Becton Dickinson, Franklin Lakes, NJ, USA) and injected subcutaneously in the flank of nude mice (6-week-old Swiss Nu/Nu, Charles River Laboratories, Wilmington, MA, USA) as described by Mineur and collaborators (Mineur et al., 2007). Four to 19 mice were used per group. Three weeks later, animals were sacrificed and dissected. Tumours and peritumoural tissues were collected and used for RNA purification and RT-PCR amplification, protein analysis and histological and immunohistochemical evaluations. VEGF mRNAs expression in the tumours was evaluated by RT-PCR using panVEGF primers (specific for sequences corresponding to exons 2 and 4, thus common to all isoforms) or $VEGF_{xxx}a$, $VEGF_{111}b$, $VEGF_{121}b$ and $VEGF_{155\text{-}165\text{-}179}b$ specific primer pairs (Table 3). $VEGF_{xxx}b$ protein levels were quantified in protein extracts using $VEGF_{165}b$ specific ELISA (R&D Systems). Angiogenesis was evaluated by measuring CD31 mRNA expression by RT-PCR (Table 3).

For immunohistochemistry analyses, anti-mouse CD31 (rabbit antiserum, at dilution 1:20, Dianova, Hambourg, Germany), anti-collagen IV (in-house developed rabbit anti-serum, at dilution 1:100), anti-α smooth muscle actin (α-SMA, mouse monoclonal, at dilution 1:400, Sigma-Aldrich) and anti-VEGF (1:50) were used. Secondary antibodies were obtained from Dako. The CD31 labelling of vessels in tumoural and peritumoural mouse tissues was quantified as the positive area relative to the total area by using Image J Software (National Institute of Mental Health, Bethesda, MD, USA).

Table 3 : mRNA primers and amplicons size

Primers name	Primers sequences (5' to 3')	Size (bp)	mRNA
P1 (forward) P2 (reverse)	CTG CTC TAC CTC CAC CAT GCC AA AGG GGC ACA CAG GAT GGC TTG AA	197	PanVEGF
P3 (forward) P4 (reverse)	CCT GGT GGA CAT CTT CCA GGA GTA CTC ACC GCC TCG GCT TGT CAC A	245 275 377 407 449	$VEGF_{111}a$ $VEGF_{121}a$ $VEGF_{155}a$ $VEGF_{165}a$ $VEGF_{179}a$
P5 (forward) P6 (reverse)	CCT GGT GGA CAT CTT CCA GGA GTA TCC TGG TGA GAG ATC TGC ATT CAC	237	$VEGF_{111}b$
P7 (forward) P8 (reverse)	CCT GGT GGA CAT CTT CCA GGA GTA GTC TTT CCT GGT GAG AGA GTT TTC TT	273	$VEGF_{121}b$
P9 (forward) P10 (reverse)	CCT GGT GGA CAT CTT CCA GGA GTA CGA TCG TTC TGT ATC AGT CTT TCC T	368 398 440	$VEGF_{155}b$ $VEGF_{165}b$ $VEGF_{179}b$
P11 (forward) P12 (reverse)	GTT CAC CCA CTA ATA GGG AAC GTG A GAT TCT GAC TTA GAG GCG TTC AGT	212 269*	28S
P13 (forward) P14 (reverse)	CAA GGC GAT TGT AGC CAC CTC CA CCA ACA ACT CCC CTT GGT CCA GA	209	CD31

RT-PCR using P3-P4 allows to measure the amplification product of the 5 studied $VEGF_{xxx}a$ variants, which are discriminated according to their size after migration on SDS-PAGE. $VEGF_{111}b$ and $VEGF_{121}b$ are amplified using the P5-P6 and P7-P8 primers, respectively. RT-PCR using P9-P10 allows to measure the amplification product of 3 of the 5 studied $VEGF_{xxx}b$ variants, which are discriminated according to their size after migration on SDS-PAGE. CD31 is amplified using the P13-P14 primers. *internal control. bp: base pairs.

X- Inhibition of Neoangiogenesis

IX-1 Choroidal Neovascularization assay (CNV)

Six week-old C57bl6 mice were anesthetized by intraperitoneal injection of 0.1 ml of a 50:50 mixture of ketamine hypochloride (20 mg/ml) and xylazine hypochloride (100 mg/ml, Phoenix Pharmaceutical, St Joseph, MO, USA). They received 2 to 4 photocoagulation lesions per eyes delivered with a diode green laser (λ - 810nm, 250mV, 75ms, 75μm, 2 to 4 lesions/eye 250mW, IRIDEX Oculight GLX, Moutain View, CA, USA). Eyes were immediately treated with intravitreal (IVT) injection of 2µl PBS containing 10 ng of $VEGF_{111}b$ (15 lesions) or $VEGF_{165}b$ (25 lesions) in one eye and 2µl of PBS (22 lesions) in the other, as negative control (Hua et al., 2010). Injections were performed using a 33-gauge needle (Nanofil, World Precession Instruments, Sarasota, FL, USA) after dilatation of the pupils using 2.5% phenylephrine hydrochloride and 0.8 % tropicamide. Injections were repeated on day 7. On day 14, mice were sacrificed. Choroids were dissected, fixed in 4% PFA and stained with a specific endothelial cell marker (isoloectin B4, 1:100, Vector Laboratories, Burlingame, CA, USA). Areas of pathological neovascularization were measured using Image J software.

IX-2 Oxygen Induced Retinopathy assay (OIR)

For oxygen induced retinopathy (OIR), Sprague Dawley® rats (n=6 per group) were exposed at birth to 7 cycles of atmosphere at 50% O_2 for 24 hours followed by another 24 hours of atmosphere at 10 % oxygen (Penn and Rajaratnam, 2003; Penn et al., 1994). After 14 days animals were placed at room air (21% oxygen). At this time point, they received 10ng of $VEGF_{111}b$ in 2µl PBS in one eye and 2µl PBS in the other as described for CNV. On day 20, pups were sacrificed and retinas were dissected and stained as previously described for CNV (Hua et al., 2010). A clock hour analysis method (Penn et al., 1994) was performed to quantify intravitreal neovascularization (IVNV). Arterial tortuosity, vessels' diameter and avascular areas were analysed using Image J software.

IX-3 Cytoprotection assay

Fresh retinal pigment epithelial cells (RPE) and arising spontaneously transformed retinal pigment epithelial cells (ARPE19) were used to evaluate the cytoprotective effect of VEGF variants against toxic doses of ketocholesterol. $15x10^3$ ARPE19 or $12x10^3$ primary RPE cells were seeded in a 96-wells plate in full DMEM-Glutamax overnight. Cells were starved in absence of FBS and treated with 2.5nM of VEGF isoforms or EGF, used as a positive control (5-wells per condition). 24h later, cells were treated with 14 to 40µM of

Ketocholestrol. After 24h, cell activity was measured by WST-1 reagent (Roche) or LDH (Lactate Deshydrogenase) released in culture media (Promega, Madison, WI).

XI- Permeability *in vivo*

Vascular permeability *in vivo* was evaluated by using a Miles and Miles modified assay (Sounni et al., 2010) using 6 to 8 week-old BALB/C mice. The backs of the animals were shaved 2 days before the experiment. 10µl of VEGF isoforms (50 ng) were injected intradermally in the back of anesthetized mice (n=5 for $VEGF_{121}a$ and $VEGF_{121}b$; n=6 for $VEGF_{111}b$, $VEGF_{155}a$, $VEGF_{155}b$ and $VEGF_{165}b$; n=7 for $VEGF_{111}a$ and $VEGF_{165}a$). Five minutes later, Evans' blue dye was injected intravenously (30 mg/kg in 100 µl PBS). After 30 minutes, mice were perfused intracardiacally with 20 ml of PFA 1%. The skin surrounding the site of injection (~20 mm^2) was then excised, weighed and the Evans' blue dye was then extracted in 1 ml of formamide at 65°c during 48h. The blue staining was quantified (620 nm) and normalized for skin weight.

XII- Effects of VEGF variants on mouse physiology

$VEGF_{111}a$, $VEGF_{111}b$, $VEGF_{165}a$ and $VEGF_{165}b$ (4 µg in 200 µl PBS) and PBS were injected daily subcutaneously in six week-old C57Bl6 mice (n=5 per group). Animals were monitored every day by a general examination controlling animal behaviour, colour of the mucosae and hydration level (pinching test). Mice were also weighed every three days. After 14 days of treatment, the mice were sacrificed and blood was collected. The total blood fractions were used for blood cell counting and characterization (CELL-DYN 3700, Abbott Laboratories, IL, USA). ALT (Alanine Transaminase) and AST (Aspartate Transaminase) hepatic markers were quantified in serum (Department of Biochemistry, Faculty of Veterinary Medicine, University of Liège). Finally isolated cells were used for FACS (Fluorescence Activated Cell Sorting) analysis to evaluate the proportion of specific bone-marrow derived cells population. The following markers were used: GR1 (Granulocytes), CD45b (Lymphocytes), CD11b (Macrophages), TER119 (Erythrocytes) and CD3 (Lymphocytes T). Spleen and liver were collected and weighed while tissues near the site of chronic injections were also recovered, fixed and evaluated for their blood vessels content by immunohistochemistry using a CD31 antibody.

RESULTS

I- Production of purified recombinant VEGF-A variants

Recombinant human VEGF variants ($VEGF_{111}a$, $VEGF_{111}b$, $VEGF_{121}a$, $VEGF_{121}b$, $VEGF_{155}a$, $VEGF_{155}b$, $VEGF_{165}a$ and $VEGF_{165}b$, Fig 19A) were produced using HEK293 cells transfected with pCEP4 vector containing the appropriate cDNA. These cells were selected for their low to undetectable intrinsic expression of VEGF. $VEGF_{111}b$ (E1-E4 & E8b), $VEGF_{155}a$ (E1-E4 & E7-E8a) and $VEGF_{155}b$ (E1-E4 & E7-E8b) are artificial variants never described *in vivo.* All these variants were efficiently produced as described in Material and Methods. After purification, they were analyzed by SDS-PAGE under reducing conditions followed by Sypro Ruby staining or by Western blot analysis (Fig 19B, 19C). $VEGF_{111}a$, $VEGF_{155}a$ and $VEGF_{155}b$ appeared as single products corresponding to the glycosylated monomer while $VEGF_{111}b$, $VEGF_{121}a$, $VEGF_{121}b$, $VEGF_{165}a$ and $VEGF_{165}b$ were expressed as glycosylated and non-glycosylated polypeptides. The increased glycosylation for variants that do not possess the E5-encoded domain was not expected as the single glycosylation site was reported to be in the E4-encoded sequence (Claffey et al., 1995), which is present in all the produced variants. No correlation could be established between the glycosylation rate and the level of VEGF expression (data not shown). Western blots were probed with anti-pan VEGF antibody (Fig 19C, upper panel) or anti-$VEGF_{xxx}b$ antibody (Fig 19C, lower panel), which recognized only the recombinant $VEGF_{xxx}b$ isoforms as expected.

Quantification of the concentration of the VEGF-A isoforms by the panVEGF ELISA was not fully satisfactory because the antibodies used in the ELISA have not an identical affinity for all the variants when quantified in their native form. Indeed, $VEGF_{121}a$ and $VEGF_{165}a$ were efficiently detected while the sensitivity of the assay was reduced for $VEGF_{111}a$, $VEGF_{111}b$, $VEGF_{155}a$ and $VEGF_{165}b$ leading to underestimation of their concentration. The $VEGF_{xxx}b$ ELISA was specific as it allowed quantification of $VEGF_{111}b$ and $VEGF_{165}b$ and did not provide any signal for $VEGF_{xxx}a$ variants.

Gel staining after SDS-PAGE was therefore used for determining the concentration of all the variants. In this assay, we compared in the same run all the VEGF variants to a BSA curve. Depending on the selected cell lines and on the VEGF isoform, the complete production and purification processes allow the recovery of purified recombinant protein at a final concentration ranging from 20 to 150 µg/ml.

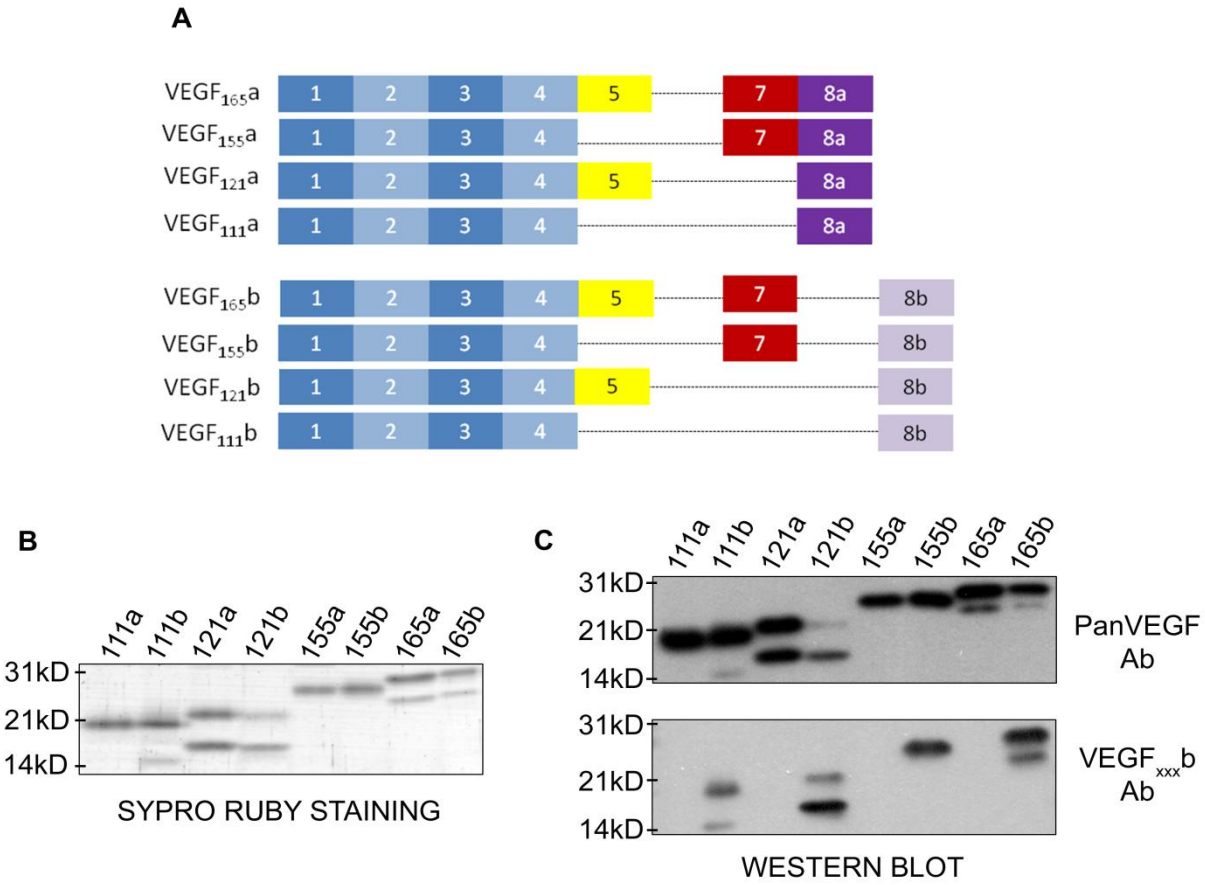


Figure 19 : Structure and analysis of the various recombinant VEGF isoforms

(A) Structure of the human VEGF-A variants used in this part of the work. Exons are represented by boxes and are not drawn to scale. Dotted lines identify the lacking exons in the various isoforms. E6a and E6b are absent from all the isoforms presented here. **(B)** SDS-PAGE analysis of the recombinant VEGF variants after production and purification by affinity chromatography using Avastin-Sepharose. Gel was stained with Sypro Ruby. **(C)** Representative Western blot of the purified VEGF variants using primary antibody against all VEGF isoforms (Pan VEGF, upper panel) and $VEGF_{xxx}b$ (lower panel). Ab: Antibody.

These VEGF preparations were then used for further biochemical and biological characterizations performed in our laboratory or in collaboration with other laboratories. For instance, recombinant $VEGF_{111}a$ was used to improve vascularization of xeno-transplantation of ovarian tissue *in vivo* (Labied et al., 2013) (see annex 2).

II- Receptors binding assay

II-1 Surface Plasmon Resonance

Surface Plasmon Resonance was used to evaluate the ability of the various VEGF variants to bind to VEGF receptors, VEGF-R1 and VEGF-R2, and their co-receptors, NRP1, NRP2 and heparin. This assay also allowed to evaluate the potential formation of complexes between VEGFR-2, VEGF and NRP1 or between NRP1, VEGF and heparin for example.

II-1-1 Binding to VEGF-R1

Increasing concentration of $VEGF_{111}a$, $VEGF_{111}b$, $VEGF_{165}a$ and $VEGF_{165}b$ were injected on VEGF-R1 coated chip. As expected from the presence of E3 encoded domain, which contains the binding site for VEGF-R1 (Fig 20A), all these isoforms displayed rapid association to the receptor followed by a slow dissociation rate (illustated for $VEGF_{111}a$ and $VEGF_{165}b$, Fig 20B, 20C). Dissociation constant (Kd) were quantified. Values were measured within the 1.4 to 4.5 x 10^{-10} range, which is in accordance with published data reporting high affinity of VEGF for VEGF-R1. The addition of soluble heparin (1µg/ml) and/or soluble NRP1 (10nM) did not significantly modified the calculated values.

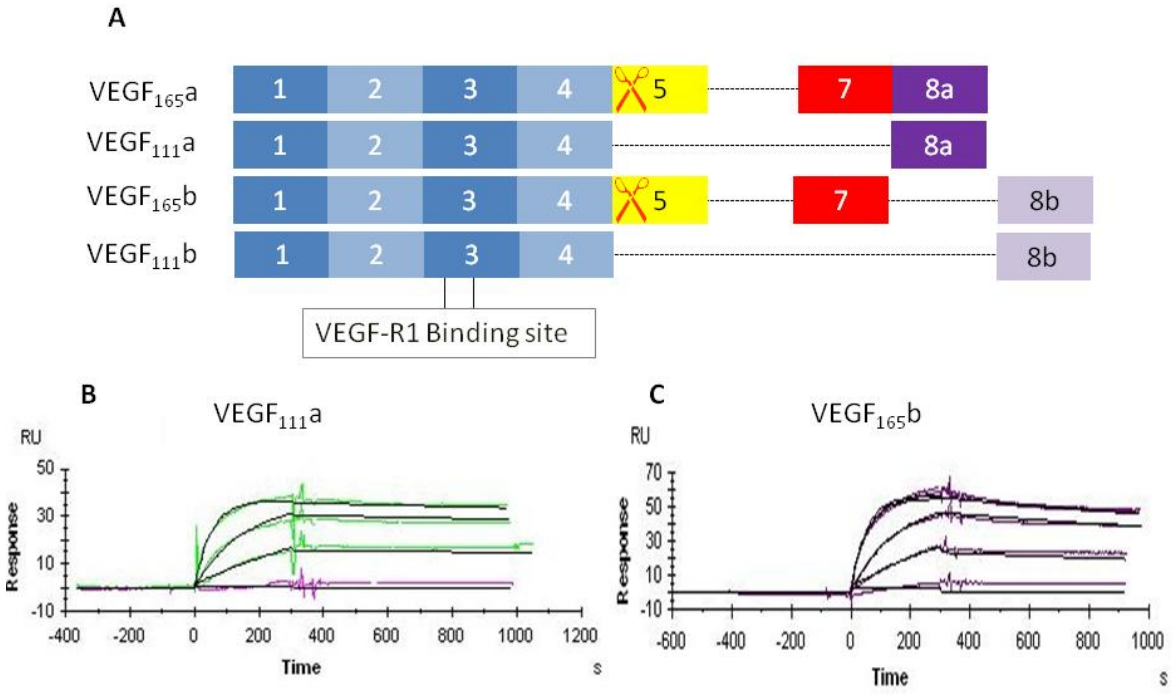


Figure 20 : Binding of VEGF isoforms to VEGF-R1

(A) In VEGF-A, the binding domain for VEGF-R1 is encoded by E3. **(B, C)** Sensorgrams obtained using VEGF-R1 coated chip. 0, 4, 10 and 40 nM of $VEGF_{111}a$ **(B)** or $VEGF_{165}b$ **(C)** were injected for ~250 seconds followed by injection of HBS-EP buffer in order to visualize the dissociation rate. RU: Response in arbitrary units.

II-1-2 Binding to VEGF-R2

$VEGF_{111}a$, $VEGF_{111}b$, $VEGF_{121}a$, $VEGF_{121}b$ $VEGF_{155}a$, $VEGF_{155}b$, $VEGF_{165}a$ and $VEGF_{165}b$ were able to bind to VEGF-R2 with similar Kd within a range from $1x10^{-10}$ to $1x10^{-11}$ M (illustrated for $VEGF_{111}a$ and $VEGF_{165}b$, Fig 21B, C). These results were in accordance with published data (Hua et al., 2010; Waltenberger et al., 1994). Interestingly, we reproducibly observed a higher association rate for $VEGF_{111}a$ ($Ka=2.6x10^{11}$) as compared to $VEGF_{165}a$ ($Ka=0.15x10^{11}$).

Addition of heparin (1µg/ml) to $VEGF_{165}a$ (10nM) did not modify the affinity of the ligand for its receptor. However, when $VEGF_{165}a$ was used at a lesser concentration (4nM), heparin increased the response by a factor of about 2 (data not shown).

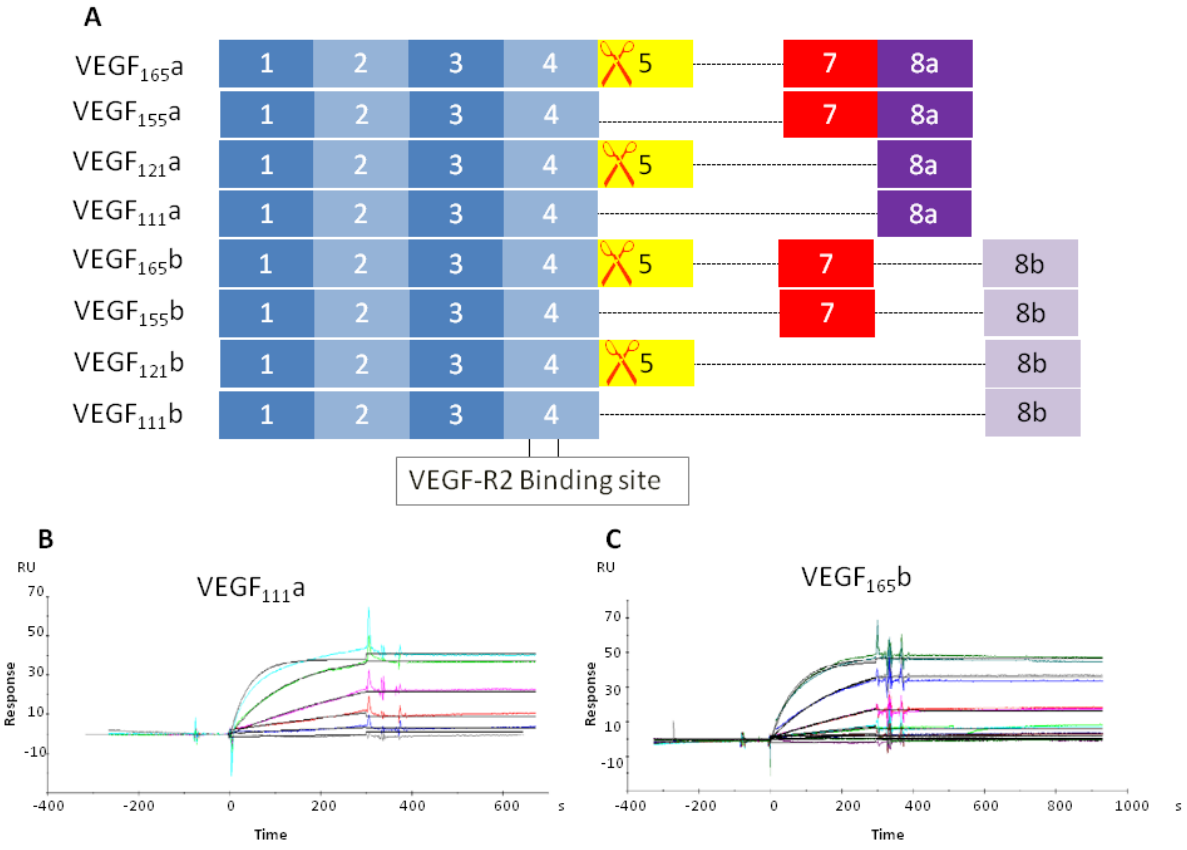


Figure 21 : Binding of VEGF the various isoforms to VEGF-R2 depends on the presence of E4-encoded domain

(A) The VEGF-R2 binding site is located in the E4-encoded domain. **(B, C)** Sensorgrams obtained using VEGF-R2 coated chip. 0, 1, 4, 10 and 40 nM of $VEGF_{111}a$ **(B)** and $VEGF_{165}b$ **(C)** were injected for ~250 seconds followed by injection of HBS-EP buffer in order to visualize the dissociation rate. RU: Response in arbitrary units

II-1-3 Binding to NRP1

When NRP1 was immobilized on the chip, only $VEGF_{165}$a and $VEGF_{121}$a bound efficiently (Fig 22C, 22E). $VEGF_{155}$a was also able to bind but with a reduced association rate as visualized by the flattened slope of the curve (Fig 22D). The absence of interaction of $VEGF_{xxx}$b (illustrated only for $VEGF_{165}$b in Fig 22G) confirmed previous data describing the implication of the E8a-encoded sequence in binding to NRP1 (Cebe Suarez et al., 2006; Pan et al., 2007b). By contrast, the absence of interaction of $VEGF_{111}$a (Fig 22B) suggests that the E8a domain is not sufficient for binding. Since the presence of the entire E8a-encoded sequence was confirmed by mass spectrometry analysis of $VEGF_{111}$a (data not shown), it suggests that the E8a-domain must cooperate with another domain for efficient binding. Because the only difference between $VEGF_{111}$a (non-binder) and $VEGF_{121}$a (binder) is the presence of the domain encoded by exon 5, its contribution to the binding capacity to NRP1 is likely.

The binding of VEGF to NRP1 coated sensorchips was stimulated by heparin only for $VEGF_{155}$a (1.5-fold) and $VEGF_{165}$a (4.5-fold) (not illustrated) suggesting a role for the E7 domain in the formation of multimolecular complexes. A role for the E5-encoded basic domain is also suggested when considering the marked difference between SPR responses obtained with $VEGF_{155}$a and $VEGF_{165}$a.

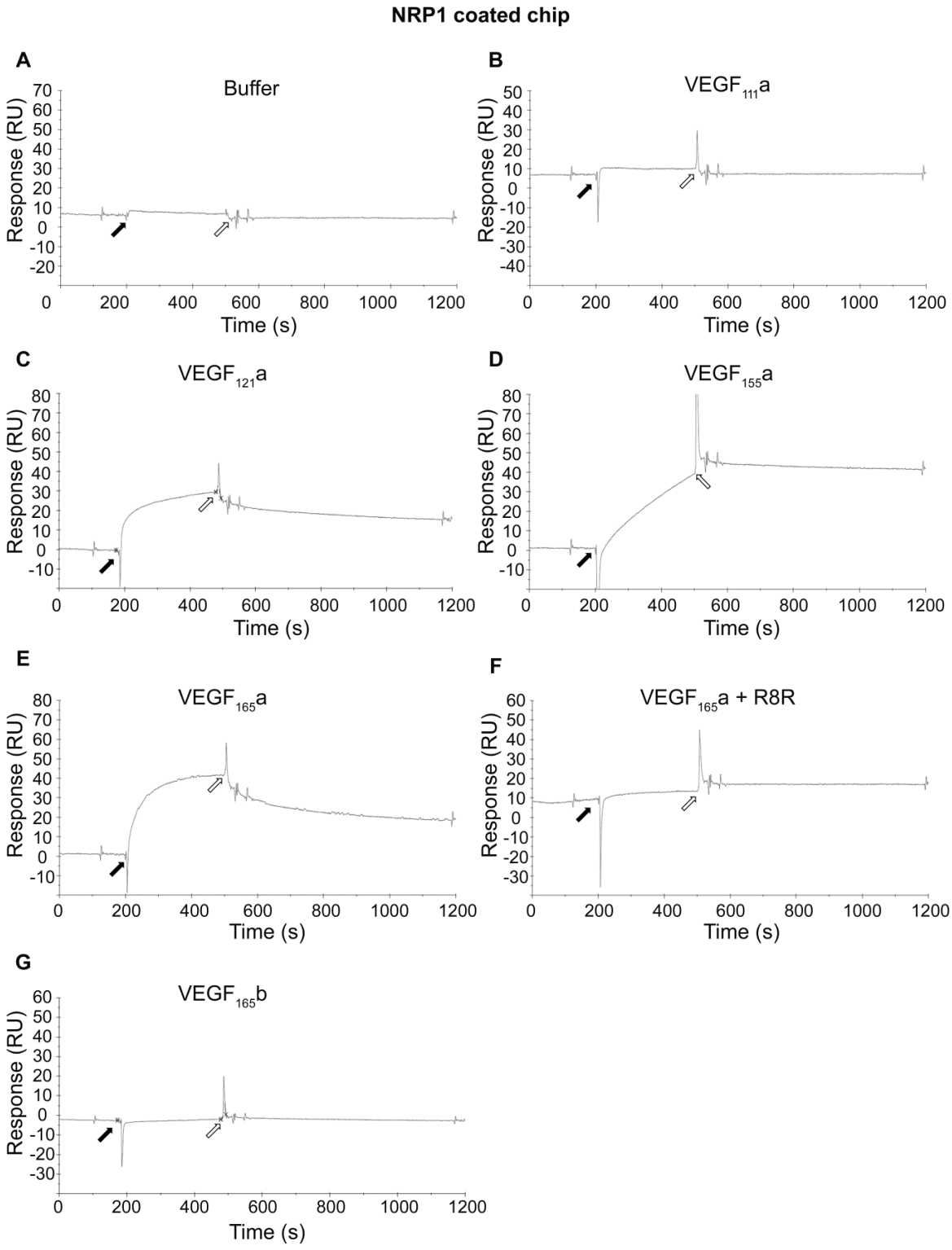


Figure 22 : Binding of VEGF variants to NRP1

Binding of VEGF variants (40nM) to NRP1 was measured by Surface Plasmon Resonance. **(A)** HBS-EP control buffer, **(B)** $VEGF_{111}a$, **(C)** $VEGF_{121}a$, **(D)** $VEGF_{155}a$, **(E)** $VEGF_{165}a$ alone or **(F)** in combination with the R8R peptide (300µM, mimicking the E5 encoded sequence) and **(G)** $VEGF_{165}b$ were injected (black arrow) for ~250 seconds followed by injection of HBS-EP buffer (open arrow) in order to visualize the dissociation rate. RU: Response in arbitrary units.

II-1-4 Binding to NRP2

None of the tested VEGF-A variants ($VEGF_{111}a$, $VEGF_{111}b$, $VEGF_{165}a$ and $VEGF_{165}b$) had a significant direct affinity for NRP2. When heparin (1µg/ml) was co-injected, only $VEGF_{165}a$ was able to form stables complexes (illustrated for $VEGF_{111}a$ and $VEGF_{165}a$ in Fig 23A and 23B).

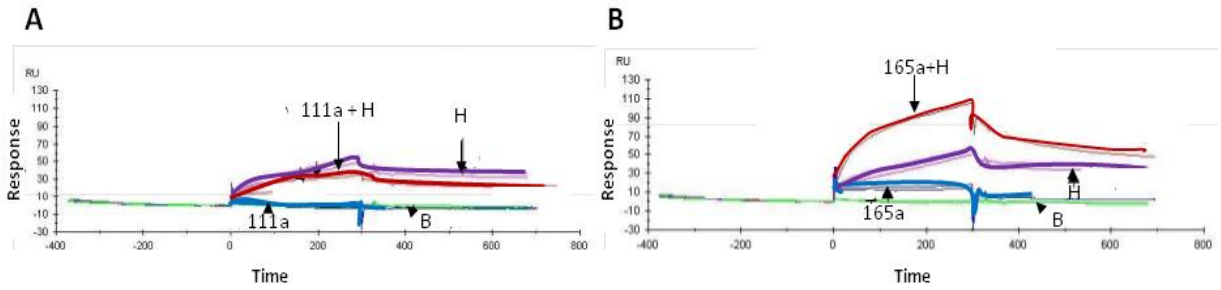


Figure 23 : **Binding of VEGF variants to NRP2**

The binding of $VEGF_{111}a$ **(A)** and $VEGF_{165}a$ **(B)** (both at 40 nM) to NRP2 was evaluated in absence or presence of heparin (1µg/ml). HBS-EP control buffer (green), VEGF with (red) or without heparin (blue), or heparin alone (violet) were injected for ~250 seconds followed by injection of HBS-EP buffer in order to visualize the dissociation rate. RU: Response in arbitrary units; B: Buffer ; H: Heparin ; 111a: $VEGF_{111}a$; 165a: $VEGF_{165}a$.

II-1-5 Binding to heparin

Some VEGF variants have been reported to interact with heparan sulfate proteoglycans through their E7-encoded domain (Kawamura et al., 2008b). SPR experiments indicated that the injection of $VEGF_{xxx}a$ variants to biotinylated heparin coupled to a streptavidin-coated sensorchip resulted in a significant binding of $VEGF_{165}a$ and $VEGF_{155}a$, although at a considerably lesser extent, while the other variants failed to bind, which confirms the importance of the sequence encoded by exon 7 (Fig 24). However, $VEGF_{165}b$ and $VEGF_{155}b$ (illustrated for $VEGF_{165}b$ in Fig 24F), two variants possessing the sequence encoded by exon 7, also failed to bind to heparin, demonstrating a direct implication of the E8a domain or an inhibitory function of the E8b sequence on this interaction. Moreover, the important difference between $VEGF_{155}a$ and $VEGF_{165}a$ suggests a role for the E5-encoded domain. These data were confirmed using heparin-sepharose affinity chromatography (data not shown).

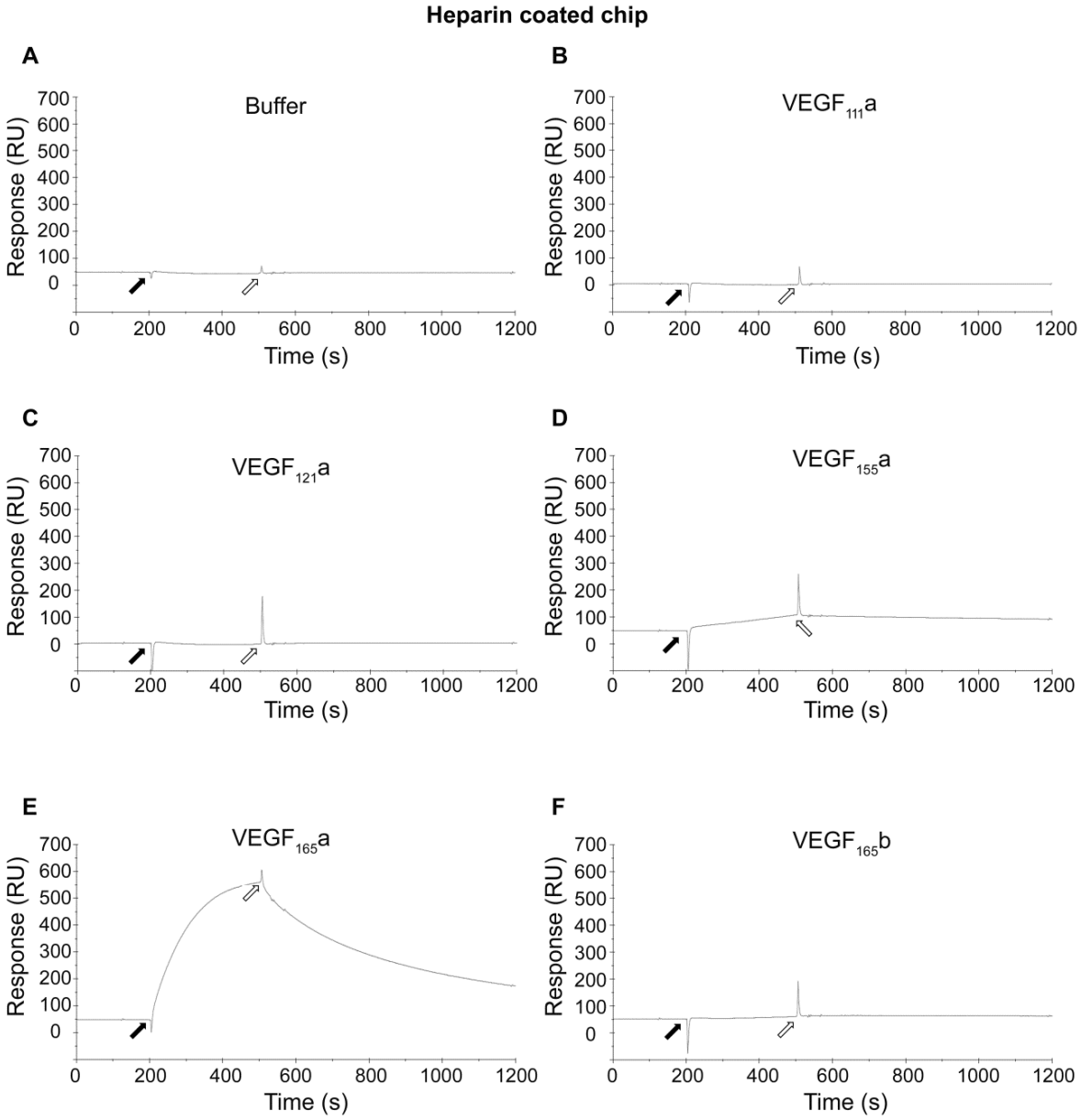


Figure 24 : Binding of VEGF variants to heparin

Binding of VEGF variants (200 nM) to heparin was measured by Surface Plasmon Resonance. **(A)** HBS-EP buffer, **(B)** $VEGF_{111}a$, **(C)** $VEGF_{121}a$, **(D)** $VEGF_{155}a$, **(E)** $VEGF_{165}a$ and **(F)** $VEGF_{165}b$ were injected (black arrow) for ~250 seconds followed by injection of HBS-EP buffer (open arrow) in order to visualize the dissociation rate. RU: Response in arbitrary units.

Simultaneous injection of $VEGF_{165}a$ (40nM) and soluble NRP1 (15nM) on the heparin chip (Fig 25) strongly improves the binding (566 RU) as compared to $VEGF_{165}a$ (132 RU) and NRP1 (131 RU) alone. Such synergy was not observed when soluble NRP1 was injected concomitantly with $VEGF_{121}a$, confirming data previously observed with the NRP1 chip.

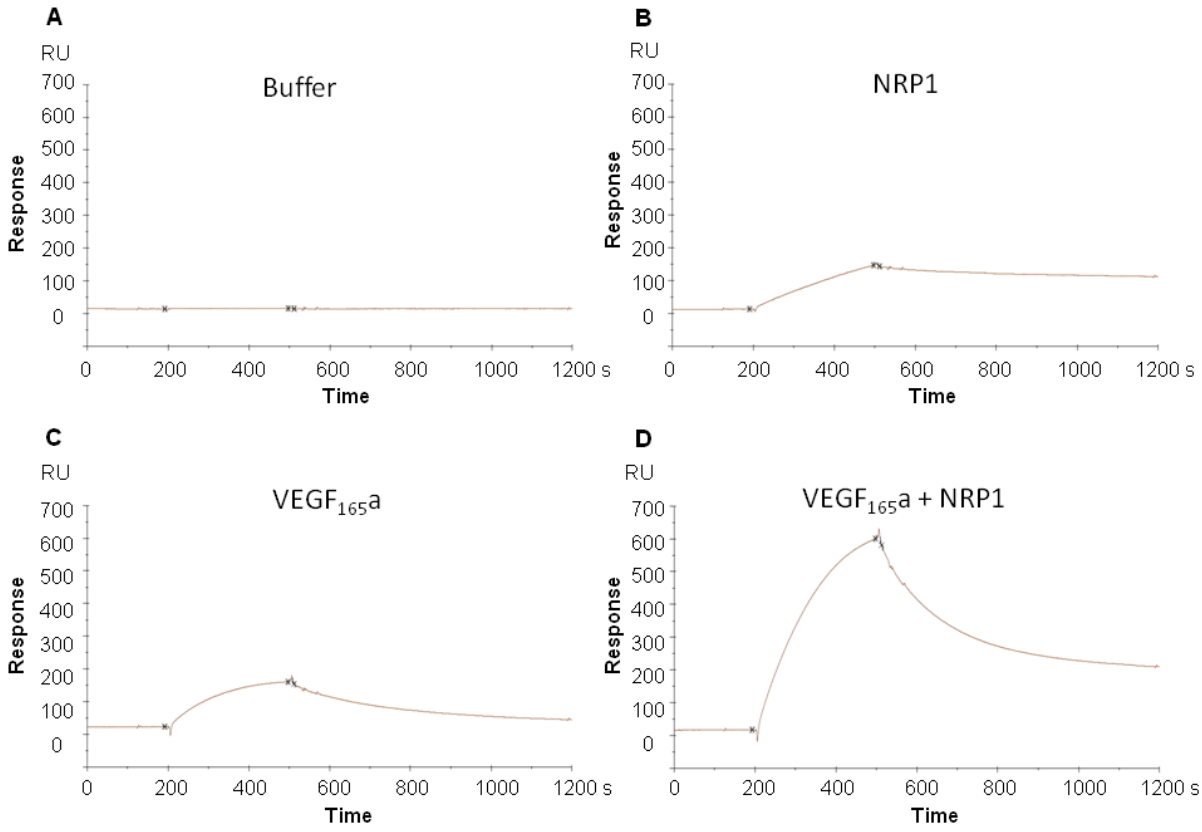


Figure 25 : Reinforcement of binding to heparin in presence of sNRP1

Binding of $VEGF_{165}a$ (40 nM) and soluble NRP1 (15nM), separately or concomitantly, to heparin was measured by Surface Plasmon Resonance. **(A)** HBS-EP buffer, **(B)** soluble NRP1, **(C)** $VEGF_{165}a$ and **(D)** mixture of $VEGF_{165}a$ and soluble NRP1 were injected for ~250 seconds followed by injection of HBS-EP buffer in order to visualize the dissociation rate. RU: Response in arbitrary units.

II-1-6 VEGFR2-VEGF multimolecular complexes

The formation of multimolecular complexes between VEGF-R2, NRP1, heparin (H) and VEGF variants was also evaluated. The different VEGF variants (20 nM) and NRP1 (20 nM) were sequentially injected in the absence or presence of heparin (1 µg/ml) on a VEGF-R2 sensorchip (Fig 26). As expected, the initial injection of VEGF induced a response on the sensorgram (illustrated here for $VEGF_{165}$a (Fig 26A), $VEGF_{121}$a (Fig 26D) and $VEGF_{165}$b (Fig 26H) for example). When $VEGF_{165}$a is already bound on VEGF-R2, subsequent injection of NRP1 alone (Fig 26A) had only a limited effect but resulted in a sharp additional response in the presence of heparin (compare Fig 26B to Fig 26A). When $VEGF_{165}$a was omitted the response to NRP1 + H was much more modest (compare Fig 26C to Fig 26B), indicating weaker or less numerous protein interactions and underscoring the importance of $VEGF_{165}$a in the formation of large complexes with VEGF-R2. $VEGF_{155}$a, the only other tested isoform possessing both the E7 and E8a-derived sequences, was also able to induce the formation of complexes with NRP1 in presence of heparin (not shown). By sharp contrast, none of the other isoforms was able to significantly mediate the interactions between VEGF-R2 and NRP1 as shown by a similar response to NRP1+H in presence or absence of VEGF as illustrated for $VEGF_{121}$a and $VEGF_{165}$b (compare Fig 26D and Fig 26H to Fig 26C).

To address the question of a potential competition between the various VEGF variants, successive injections on VEGF-R2 chips were performed. As an example, a saturating amount of $VEGF_{165}$a (80 nM) was injected first followed by sequential injections of $VEGF_{165}$b (20nM) and NRP1+heparin. In these conditions, $VEGF_{165}$a could efficiently bind to VEGF-R2 and induce the formation of VEGFR2-VEGF-NRP1-heparin complexes (Fig 26E). By contrast, when $VEGF_{165}$b (80nM) was injected first only a limited amount of $VEGF_{165}$a could be immobilized and the formation of the complex was strongly inhibited (Fig 26F). Similar data were obtained when $VEGF_{121}$a (Fig 26G) or $VEGF_{155}$b (not shown) were used in place of $VEGF_{165}$b for the first injection, again demonstrating a competition of these variants with $VEGF_{165}$a for binding to VEGF-R2 and further confirming that $VEGF_{165}$a is able to promote the formation of complexes while $VEGF_{155}$b, $VEGF_{165}$b and $VEGF_{121}$a are not efficient (summarized in Fig 26I).

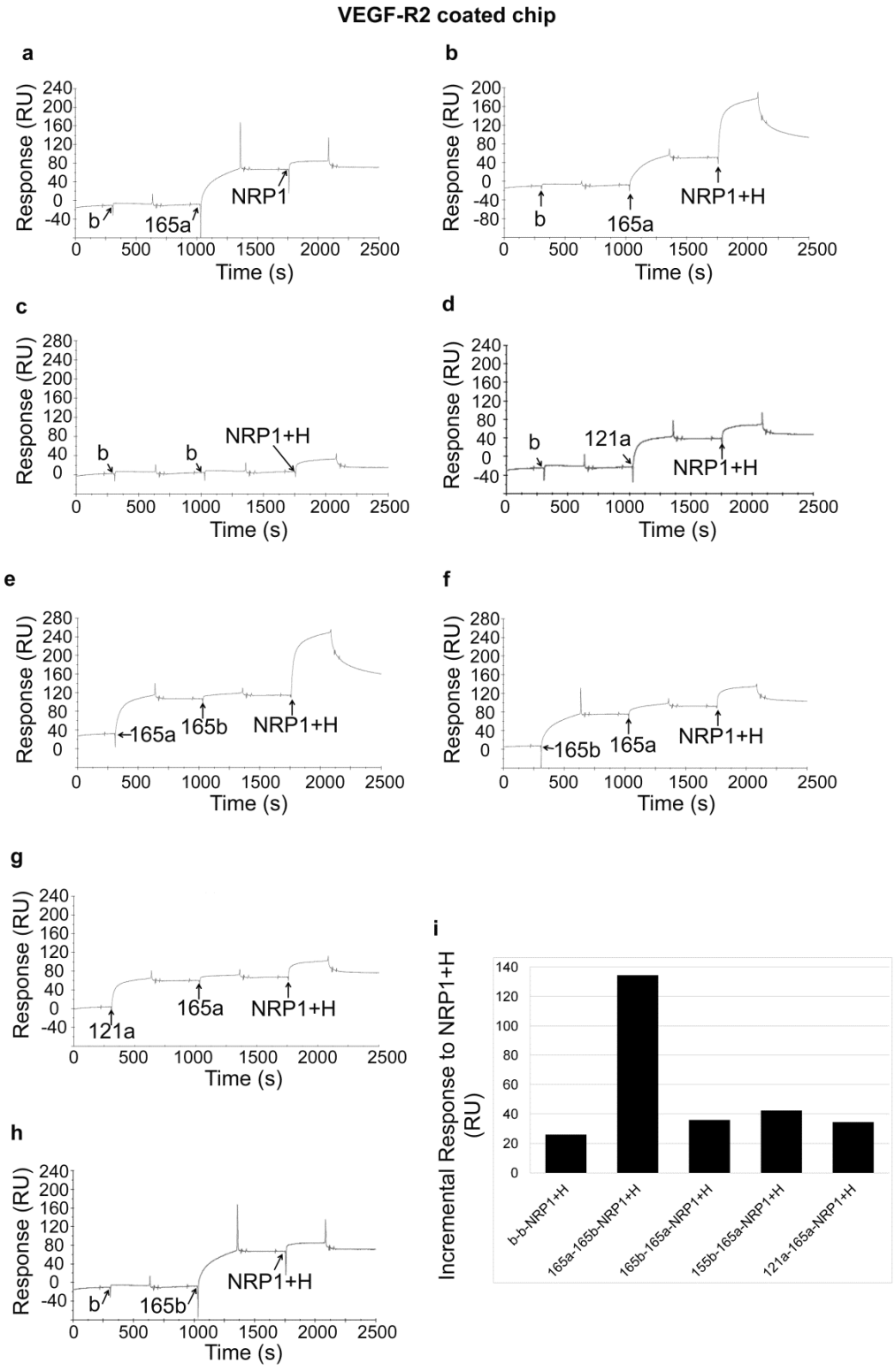


Figure 26 : Ability of VEGF variants to form VEGFR2-VEGF-NRP1-heparin complexes

(A-H) The formation of the VEGFR2-VEGF-NRP1-heparin complexes was measured by Surface Plasmon Resonance. VEGF-R2 was coated on the sensorchip and successive injections were made as indicated on the drawings. **(I)** Increment of response induced by addition of NRP1+heparin after injection of various VEGF variants. b: HBS-EP buffer; 121a: $VEGF_{121}a$; 165a: $VEGF_{165}a$; 165b: $VEGF_{165}b$; NRP1: neuropilin-1; H: heparin.

The effect of PlGF on the formation of VEGF-R2-NRP1-heparin complexes was also investigated. Surprisingly, despite an absence of direct binding of PlGF to VEGF-R2, it reduced the binding of NRP1 to VEGF-R2 (Fig 27). All the data obtained by Surface Plasmon Resonance are summarized in Table 4.

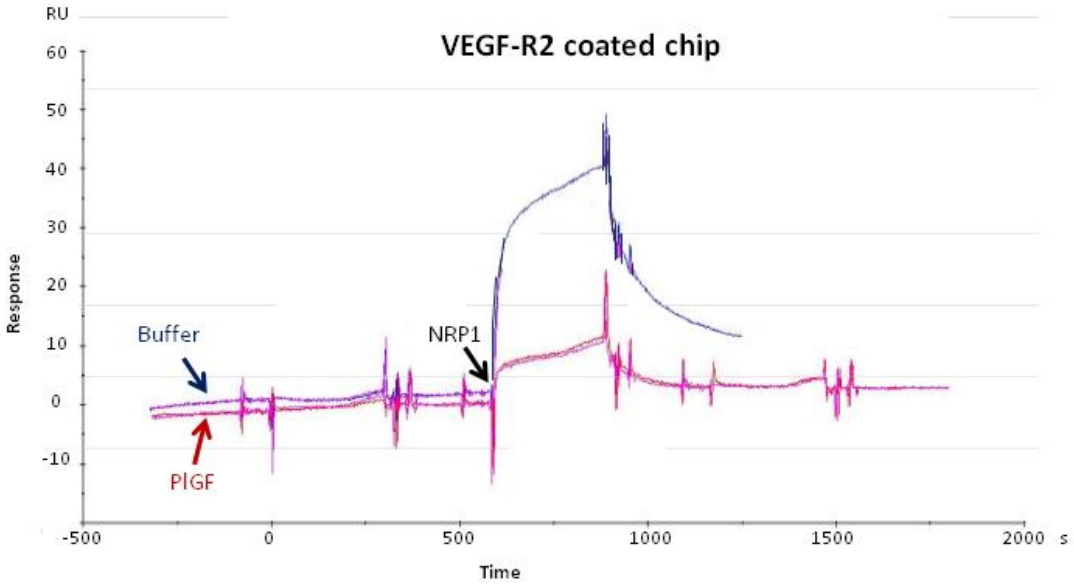


Figure 27 : PlGF inhibits direct binding of NRP1 to VEGF-R2

The influence of PlGF on formation of the VEGFR2-NRP1-heparin complexes was measured in duplicate by Surface Plasmon Resonance on VEGF-R2 coated sensorchips. Successive injections consisted in either HBS-EP Buffer followed by NRP1 + heparin (blue) or PlGF and then NRP1 + heparin (red).

Table 4 : Summary of the ability of the VEGF variants to bind to VEGF receptors and co-receptors

	Presence of the relevant exon-encoded domain	Direct binding on					Formation of complexes including:		
		VEGF-R1	VEGF-R2	NRP1	NRP2	H	NRP2+H	NRP1+H	VEGFR2+NRP1+H
$VEGF_{111}a$	E1-4 ; E8a	Yes	Yes	No	No	No	No	No	No
$VEGF_{111}b$	E1-4 ; E8b	Yes	Yes	No	No	No	No	No	No
$VEGF_{121}a$	E1-4 ; E5 ; E8a	nd	Yes	Yes	nd	No	nd	Yes	No
$VEGF_{121}b$	E1-4 ; E5 ; E8b	nd	Yes	No	nd	No	nd	No	No
$VEGF_{155}a$	E1-4 ; E7 ; E8a	nd	Yes	Yes	nd	Yes	nd	Yes	Yes
$VEGF_{155}b$	E1-4 ; E7 ; E8b	nd	Yes	No	nd	No	nd	No	No
$VEGF_{165}a$	E1-4 ; E5 ; E7 ; E8a	Yes	Yes	Yes	No	Yes	Yes	Yes	Yes
$VEGF_{165}b$	E1-4 ; E5 ; E7 ; E8b	Yes	Yes	No	No	No	No	No	No

nd: not determined; E: exon; H: heparin

II-2 Solid phase assay

Data obtained in the SPR model were further validated in a solid phase ELISA assay using $VEGF_{111}a$, $VEGF_{111}b$, $VEGF_{165}a$, $VEGF_{165}b$ and PlGF-coated plates. VEGF-R2 bound efficiently to all variants while only $VEGF_{165}a$ was able to interact with NRP1 or NRP2 in presence of heparin, as expected from the SPR data (Fig 28). As expected, PlGF bound to NRP1 but not to VEGF-R2 and NRP2. In another experimental setting, a solid phase assay was also used for evaluating the competition for immobilized NRP1 between fixed concentration of biotinylated $VEGF_{165}a$ and increasing amount (from 5 to 450 nM) of other VEGF variants. As a control, we first verified that $VEGF_{165}a$ was able to completely inhibit the binding of biotinylated $VEGF_{165}a$. By contrast, none of the other variant was able to compete with $VEGF_{165}a$ for NRP1, except for a slight effect of $VEGF_{155}a$ at 40 nM, again in agreement with the SPR results (not illustrated).

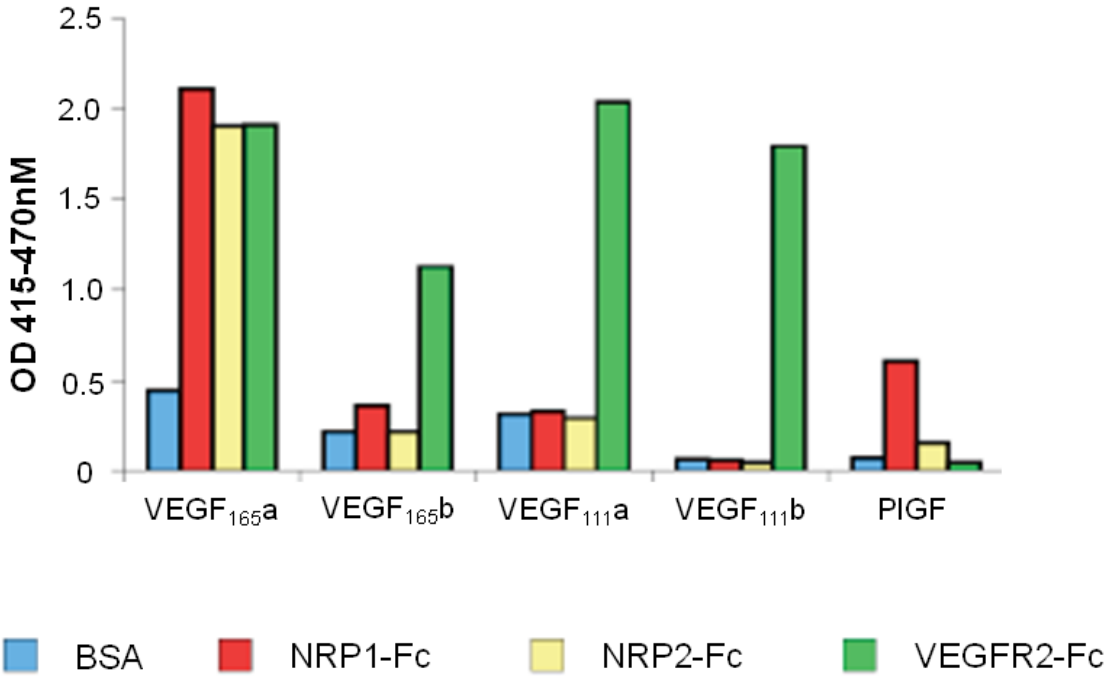


Figure 28 : Binding of VEGF variants to VEGF-R2, NRP1 and NRP2 as determined in a solid phase assay

96-wells plates were coated with PlGF, $VEGF_{111}a$, $VEGF_{111}b$, $VEGF_{165}a$ or $VEGF_{165}b$, washed and incubated with solution containing BSA (negative control) or Fc-coupled VEGF-R2 (VEGF-R2-Fc), NRP1 (NRP1-Fc) or NRP2 (NRP2-Fc) in presence of heparin. After washing and incubation with HRP-coupled anti-Fc antibody, the relative amounts of bound protein were detected by quantifying the HRP activity immobilized in the wells. PlGF: Placental Growth Factor, BSA: Bovine Serum Albumine, OD: Optical Density.

II-3 Effect of mimetic peptides of E5 and E8a encoded domain on VEGF binding to NRP1

The study of the various VEGF isoforms by Surface Plasmon Resonance and solid phase assay suggested the implication of the E5- and E8a-encoded domains for NRP1 binding. This hypothesis was further investigated by using synthetic peptides. Peptides mimicking the E8a domain, A7R, (Starzec et al., 2007; Starzec et al., 2006) or the E5 domain (R8R) were used. They fully inhibited the binding of $VEGF_{165}a$ on NRP1 immobilized on SPR sensorchip (illustrated for R8R, compare Fig 22F to Fig 22E), confirming previous data. They also prevented the formation of the super-complexes involving VEGF-R2, NRP1, $VEGF_{165}a$ and heparin (Compare Fig 29A to 29B, for effect of A7R and 29A to 29C for effect of R8R), confirming that these two domains are required.

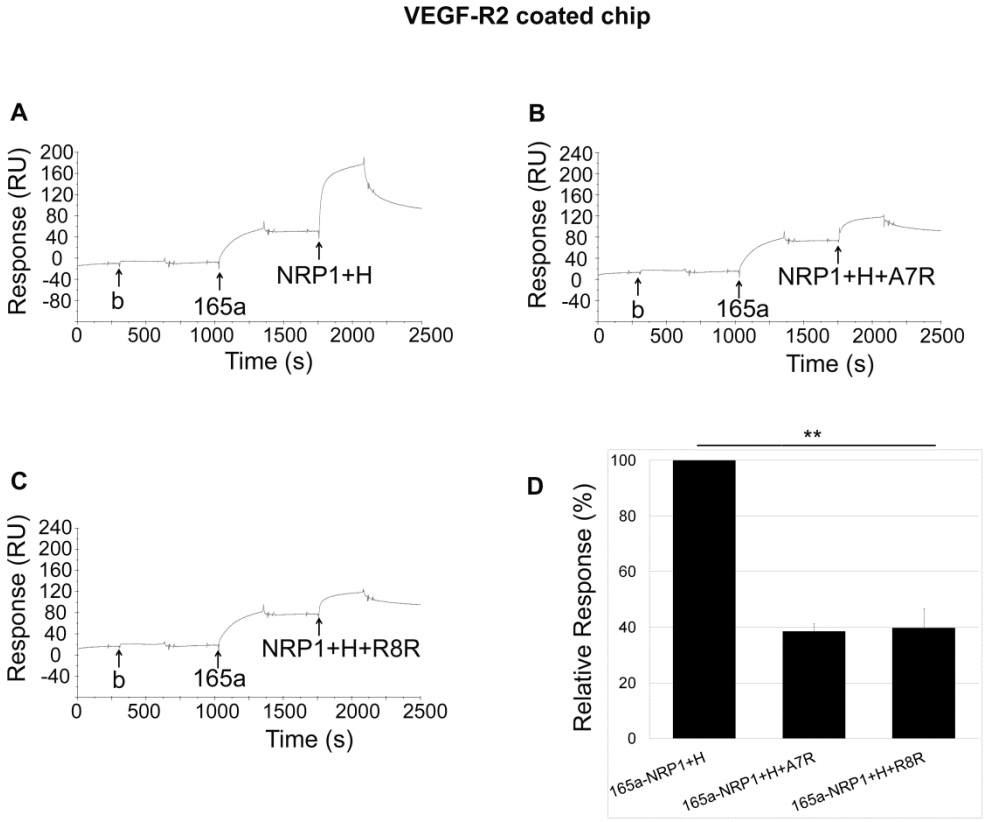


Figure 29 : **Effect of peptides mimicking the E5- (R8R) or the E8a- (A7R) derived domains on the formation of VEGFR2-$VEGF_{165}a$-NRP1-heparin super-complexes**

(A-C) The formation of the VEGFR2-$VEGF_{165}a$-NRP1-heparin super-complexes was assessed by Surface Plasmon Resonance. VEGF-R2 was coated on the sensorchip and successive injections were made as indicated on the drawings. **(D)** Quantification of the response induced by the addition of NRP1+H in the absence or presence of mimetic peptides (A7R and R8R). Results are expressed in percentage of values recorded in absence of peptide. b: HBS-EP buffer; H: heparin.

These properties were further investigated in a solid phase assay where NRP1 was immobilized in the well. Pre-treatment with A7R, R8R or a peptide with the sequence encoded by exon 8a totally inhibited the binding of biotinylated $VEGF_{165}a$ on immobilized NRP1 (Fig 30) whereas a peptide with the sequence encoded by E8b had no effect. Altogether these results clearly demonstrated the importance of the E5- and E8a-encoded domains for VEGF binding to NRP1, the E8b sequence being not involved.

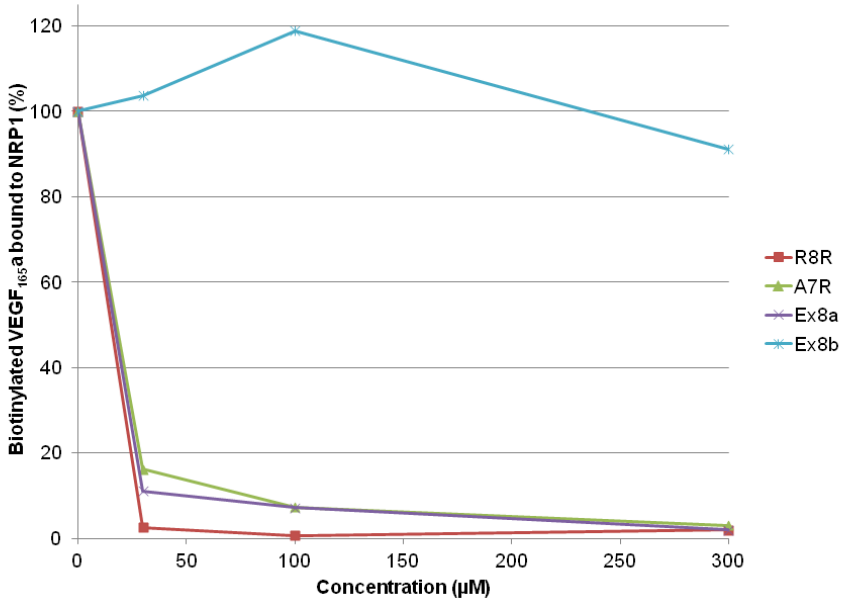


Figure 30 : Effect of mimetic peptides on the binding of $VEGF_{165}a$ on NRP1

Biotinylated $VEGF_{165}a$ was incubated in 96-wells plates previously coated with NRP1 and pre-treated with increasing concentrations(0 to 300 nM) of peptides mimetic of the domain encoded by exon 8a (Ex8a, A7R), exon 8b (Ex8b) or exon 5 (R8R). After washing and incubation with HRP-coupled streptavidin, quantification of the immobilized HRP activity was used to evaluate the relative level of $VEGF_{165}a$ bound to NRP1. Data are expressed comparatively to the values obtained in absence of peptides, taken as 100%.

III- Resistance of VEGF isoforms to proteolysis

As we have shown in chapter II, the C-terminal domains of VEGF are required for the formation of complexes with NRP1 and heparin while the VEGF-R1 and VEGF-R2 binding sites are located more NH2-terminally. As a result, any proteolytic cleavage in the middle of the VEGF molecule should strongly affect its overall biological activity, underscoring the importance of evaluating the sensitivity to proteases of the different variants. The various VEGF isoforms were incubated for increasing times with increasing concentrations of plasmin and analyzed by Western blot using an antibody specific for a N-terminal epitope (Fig 31A). Both glycosylated and non-glycosylated forms of $VEGF_{165}a$ and $VEGF_{165}b$ were rapidly converted into smaller products, likely corresponding to the $VEGF_{110}$ fragment obtained after cleavage at a previously described site in the E5-derived sequence (Arg^{110}/Ala^{111}, (Keyt et al., 1996a)). As expected from the absence of this sensitive region, $VEGF_{111}a$ and $VEGF_{111}b$ were resistant to degradation, even at the highest concentrations and the longest incubation times (Fig 31A), and $VEGF_{111}b$ fully kept its E8b-encoded terminal domain, as shown by using a specific anti-E8b domain antibody (Fig 31B). In spite of the lack of the E5-sequence, cleavage products of $VEGF_{155}a$ and $VEGF_{155}b$ already appeared with 2-4 x 10^{-2} IU of plasmin. The size of the two degradation products observed was compatible with a cleavage occurring in the sequence encoded by E7. This was further supported by the loss of immunoreactivity to the $VEGF_{xxx}b$ antibody of the plasmin-treated $VEGF_{155}b$ (Fig 31C), suggesting that the E7-encoded domain might become more exposed to plasmin in absence of the E5-sequence. The situation for the $VEGF_{121}$ isoforms was more complex since $VEGF_{121}a$ was cleaved by plasmin while $VEGF_{121}b$ was not although it did possess the E5-derived domain. This suggests that the alternative use of the sequence encoded by E8a or E8b affects the three-dimensional structure of the molecules and the accessibility to the proteolytic enzymes.

$VEGF_{111}a$, $VEGF_{111}b$, $VEGF_{165}a$ and $VEGF_{165}b$ were also incubated with fluids collected from non-healing wound of three patients with a chronic skin ulcer (Fig 31D) as these exudate are known to be rich in various proteases including plasmin (Palolahti et al., 1993) and metalloproteinases (Yager et al., 1996). After 24h of treatment, $VEGF_{111}a$ and $VEGF_{111}b$ remained intact whereas $VEGF_{165}a$ and $VEGF_{165}b$ were degraded likely into $VEGF_{110}$.

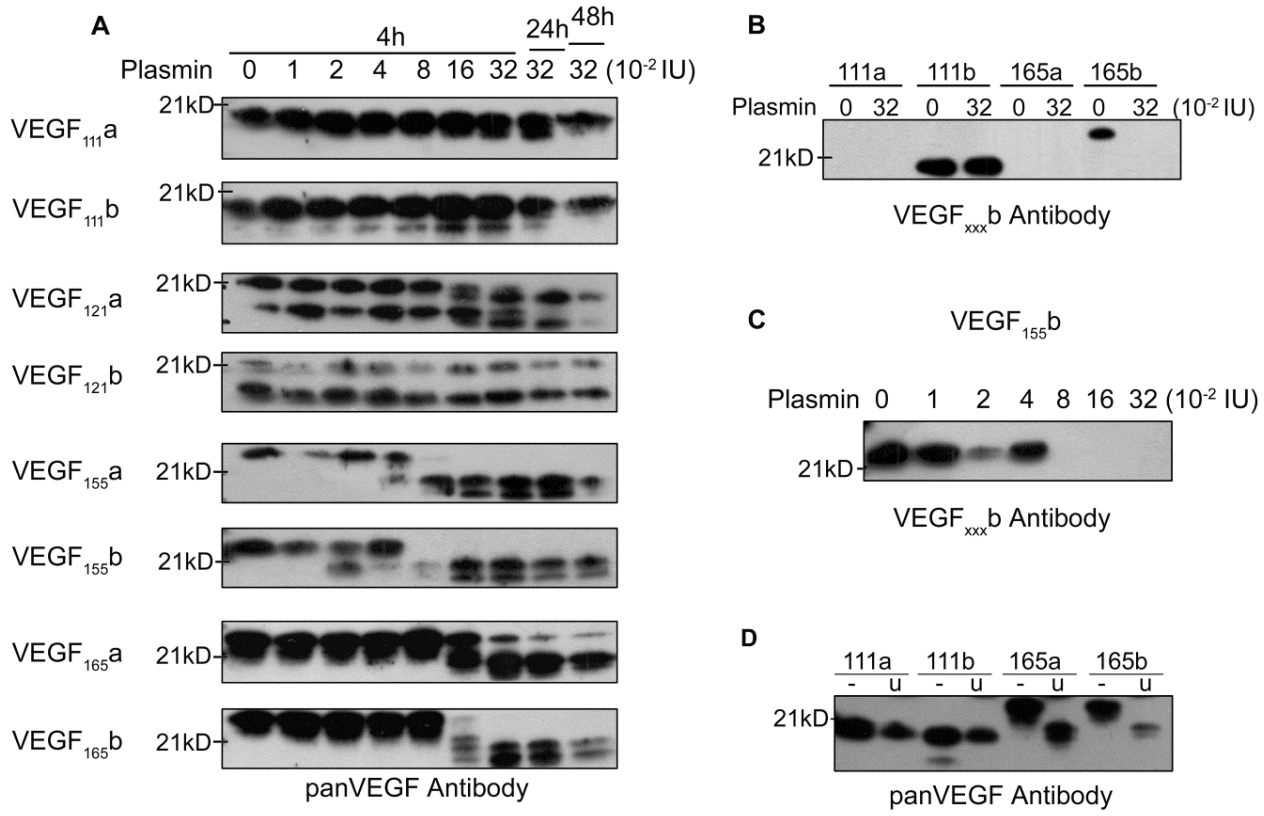


Figure 31 : Effect of treatment by plasmin and exudate from skin ulcer on VEGF variants

(A) 20ng of VEGF variants were treated with increasing amounts of plasmin (expressed in 10^{-2} IU) for the indicated times at 37°C. The products of the reactions were analyzed by Western blot using a panVEGF antibody. **(B, C)** VEGF variants were treated for 4 h with the indicated concentrations of plasmin before Western blot analysis using an anti-$VEGF_{xxx}b$ antibody. **(D)** VEGF variants were incubated for 4 h in PBS (-) or in exudate from skin ulcer (u) before Western blot analysis using a panVEGF antibody. 111a: $VEGF_{111}a$, 111b: $VEGF_{111}b$, 165a: $VEGF_{165}a$, 165b: $VEGF_{165}b$, IU: International Unit.

IV- *In vitro* assay

IV-1 Effect of VEGF variants on VEGF signalling

The effects of the VEGF variants were extensively characterized in various culture conditions. Only some data will be presented here to illustrate specific features and findings. Two cell models were used for evaluating angiogenesis in culture. Porcine Aortic Endothelial Cells (PAEC) do not express VEGF receptors and co-receptors at their surface. They were stably transfected with an empty vector (PAEC control) or with expression vectors for human NRP1 (PAEC-NRP1), VEGF-R1 (PAEC-R1), VEGF-R2 (PAEC-R2) or VEGF-R2 and NRP1 (PAEC-R2-NRP1) (Becker et al., 2005; Miao et al., 1999; Waltenberger et al., 1994). The second model used HUVEC, primary human endothelial cells that express VEGF-R1, VEGF-R2, NRP1 and HSPG at their surface.

IV-1-1 VEGF variants do not regulate endothelial cells through direct interactions with NRP1

For evaluating any potential direct effect of VEGF variants, PAEC cells, either control or expressing NRP1, were treated with VEGF isoforms. The activation of ERK1/2 was measured as it is one of the main pathways regulating endothelial cells proliferation. An increased phosphorylation was induced by FBS, used as a positive control independent of NRP1 signalling. None of the VEGF variants was active, confirming that these cells are not responsive and that no direct effect can be mediated by NRP1 (Fig 32).

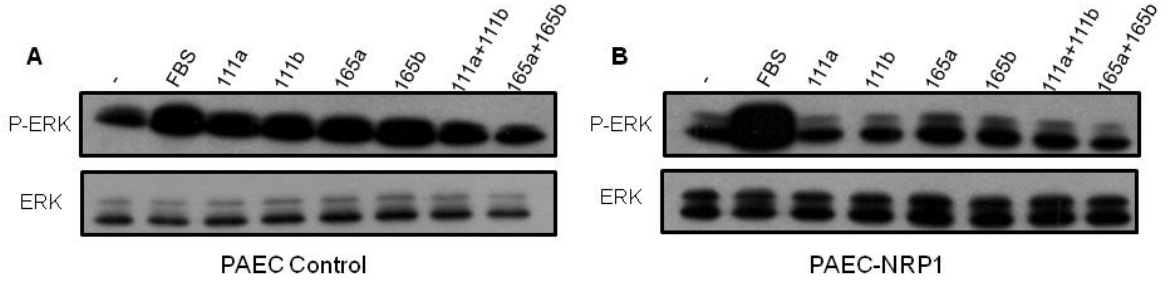


Figure 32 : Evaluation of the effect of VEGF variants on ERK phosphorylation in PAEC either control or expressing NRP1

(A) PAEC-Control and **(B)** PAEC-NRP1 were treated by addition of FBS or with 1 nM of VEGF variants for 10min in serum free medium. Protein extracts were then analyzed by Western blot using antibodies against ERK1/2 (ERK) or phospho-ERK1/2 (P-ERK). -: Control medium; FBS: medium containing Foetal Bovine Serum; 111a: $VEGF_{111}a$; 111b: $VEGF_{111}b$; 165a: $VEGF_{165}a$; 165b: $VEGF_{165}b$; 111a+111b: $VEGF_{111}a$ and $VEGF_{111}b$ added both at 1nM; 165a + 165b: $VEGF_{165}a$ and $VEGF_{165}b$ added both at 1nM.

IV-1-2 VEGF variants act through VEGF-R2 but not through VEGF-R1

The presence of VEGF-R1 at the cell surface of PAEC did not result in any marked specific response triggered by VEGF variants (Fig 33A). By contrast, the presence of VEGF in the culture medium induced a rapid phosphorylation of VEGF-R2 in PAEC-R2 (Fig 33B). Most interestingly, the level of phosphorylation was highly variant-specific and was correlated to the level of ERK1/2 and Akt phosphorylation. Co-treatment of $VEGF_{111}a$ and $VEGF_{111}b$ or of $VEGF_{165}a$ and $VEGF_{165}b$ did not result in any synergistic or antagonistic effects.

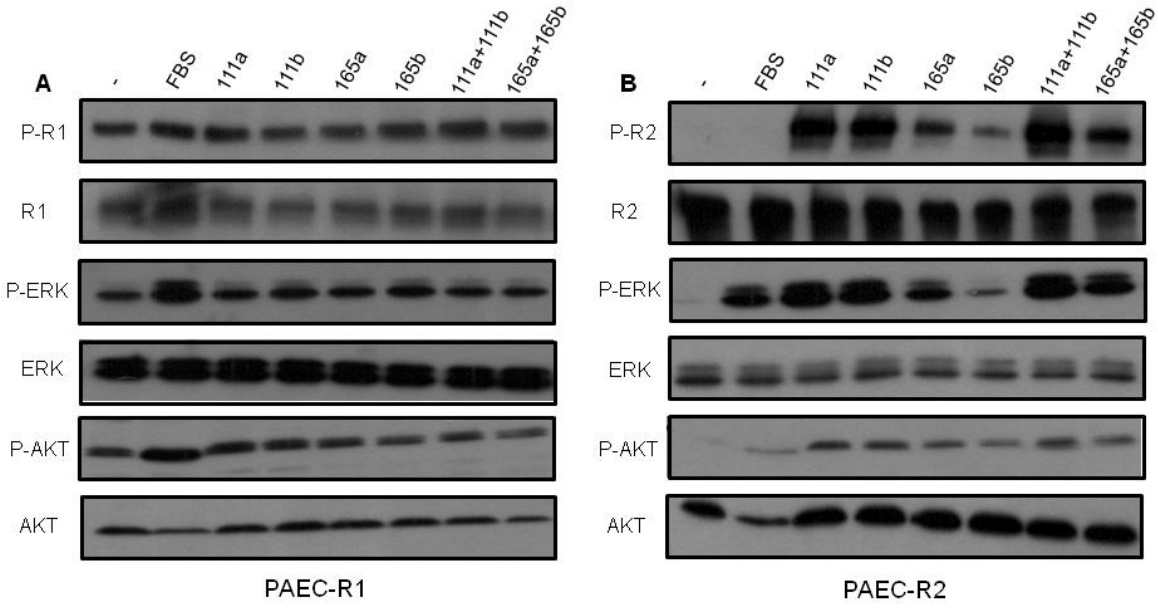


Figure 33 : Effect of VEGF variants on VEGF-R1/R2 phosphorylation and signalling pathways activation in PAEC-R1 and PAEC-R2

PAEC-R1 **(A)** and PAEC- R2 **(B)** were treated with FBS or with 1 nM of VEGF variants for 10 min in serum free medium. Protein extracts were analyzed by Western blot using **(A)** antibodies against VEGF-R1 (R), phospho-VEGFR1 (P-R1), ERK1/2 (ERK), phospho-ERK1/2 (P-ERK), AKT or phospho-AKT (P-AKT) and **(B)** VEGF-R2 (R2), phospho-VEGFR2 (Tyr1175, P-R2), ERK1/2, phospho-ERK1/2, AKT or phospho-AKT. -: Control medium; FBS: medium containing Foetal Bovine Serum; 111a: $VEGF_{111}a$; 111b: $VEGF_{111}b$; 165a: $VEGF_{165}a$; 165b: $VEGF_{165}b$; 111a+111b: $VEGF_{111}a$ and $VEGF_{111}b$ (both at 1nM); 165a + 165b: $VEGF_{165}a$ and $VEGF_{165}b$ (both at 1nM).

IV-1-3 NRP1 potentiates the VEGF-R2 phosphorylation induced by $VEGF_{165}a$

The effects of the 8 VEGF variants were also studied in parallel on PAEC-R2 and PAEC-R2-NRP1 for evaluating the activity of NRP1 as a co-receptor stimulating VEGF-R2 phosphorylation and activation of downstream signalling factors. The shortest VEGF isoforms, whatever the composition of the terminal exon ($VEGF_{111}a/b$ and $VEGF_{121}a/b$), were the strongest inducers of VEGF-R2 phosphorylation in PAEC-R2, while $VEGF_{165}a$ was active but to a lesser extent (Fig 34A). $VEGF_{155}a$, $VEGF_{155}b$ and $VEGF_{165}b$ were barely active, although they possess the VEGF-R2 binding sequence. A similar pattern of phosphorylation/activation was observed for ERK1/2 (Fig 34A, 34C). The results obtained in PAEC-R2-NRP1 cells (Fig 34B, 34D) were similar to those in PAEC-R2, with the significant exception that $VEGF_{165}a$ was as active in these cells as the short VEGF isoforms.

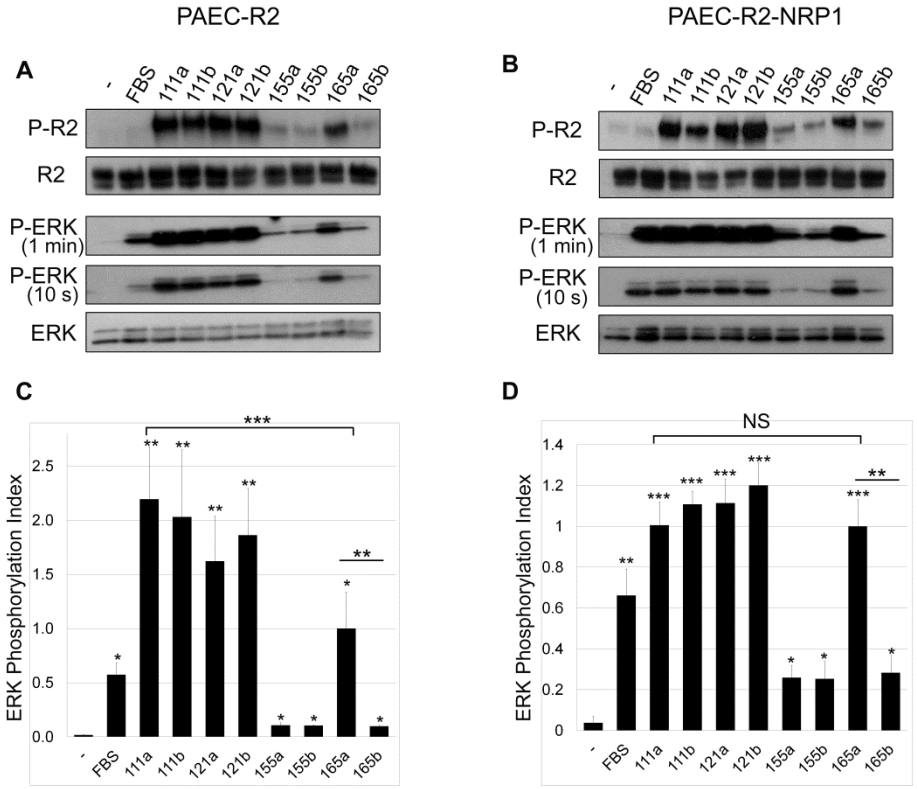


Figure 34 : Effect of VEGF variants on PAEC-R2 and PAEC-R2-NRP1

(A) PAEC-R2 and **(B)** PAEC-R2-NRP1 were treated with 1 nM of VEGF variants for 10 min in serum free medium and analyzed by Western blot using antibodies against VEGF-R2 (R2), phospho-VEGFR2 (Tyr1175, P-R2), ERK1/2 (ERK) or phospho-ERK1/2 (P-ERK). For P-ERK two different film exposures are shown (10 s and 1 min). **(C, D)** The phosphorylation indexes (P-ERK/ERK) were calculated and compared to the index measured for $VEGF_{165}a$ (arbitrary set at 1) in each cell line. FBS: Foetal Bovine Serum; 111a: $VEGF_{111}a$; 111b: $VEGF_{111}b$; 121a: $VEGF_{121}a$; 121b: $VEGF_{121}b$; 155a: $VEGF_{155}a$; 155b: $VEGF_{155}b$; 165a: $VEGF_{165}a$; 165b: $VEGF_{165}b$. *p<0.05, **p<0.01, ***p<0.001 (t-test of Student, sample versus control or between two specific conditions indicated by the bar).

IV-1-4 VEGF$_{165}$a is the most active isoform in HUVEC

HUVEC are characterized by the simultaneous expression at their surface of a relatively physiological number of VEGF-R1, VEGF-R2 and NRP1 (5,000 – 20,000 compared to 150,000 in the PAEC-R2 or PAEC-R2-NRP1 cells) (Herve et al., 2008; Waltenberger et al., 1994). This did not modify however their response to VEGF except for an increased relative stimulation of the VEGF-R2 signalling cascade specifically in presence of VEGF$_{165}$a (Fig 35A, 35B). This makes this isoform the most active in HUVEC and confirms the SPR and solide phase assay data showing that VEGF$_{165}$a is the only isoform able to interact significantly with NRP1 and to induce the formation of complexes with VEGFR2 and NRP1. This also suggests that in physiological situations where VEGF-R2 and NRP1 are less abundant than in transfected cells, VEGF$_{165}$a is more active than the other isoforms.

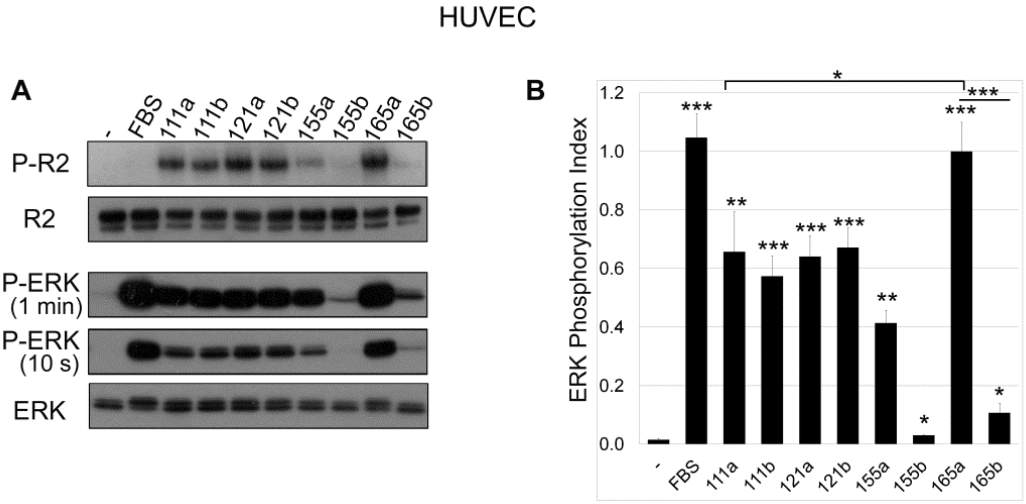


Figure 35 : Effect of VEGF variants on HUVEC

(A) HUVEC were treated with 1nM of VEGF variants for 10min in serum free medium and were analyzed by Western blot using antibodies against VEGF-R2 (R2), phospho-VEGFR2 (Tyr1175, P-R2), ERK1/2 (ERK) or phospho-ERK1/2 (P-ERK). For P-ERK two different film exposures are shown (10 s and 1 min). **(B)** The phosphorylation indexes (P-ERK/ERK) were calculated and compared to the index measured for VEGF$_{165}$a (arbitrary set at 1). FBS: Foetal Bovine Serum; 111a: VEGF$_{111}$a; 111b: VEGF$_{111}$b; 121a: VEGF$_{121}$a; 121b: VEGF$_{121}$b; 155a: VEGF$_{155}$a; 155b: VEGF$_{155}$b; 165a: VEGF$_{165}$a; 165b: VEGF$_{165}$b. *p<0.05, **p<0.01, ***p<0.001 (t-test of Student, sample versus control or between two specific conditions indicated by the bar).

To further characterize the specific activity of the different isoforms, HUVEC and PAE-R2 were also treated during increasing periods of time or with various concentrations of VEGF. Time-course experiments showed a maximal ERK1/2 phosphorylation 5 to 15 min after addition of any VEGF variant (Fig 36A, 36B). No effect was observed at 1 min and the phosphorylation was highly reduced at 30 min whatever the VEGF isoforms. Using various concentrations (0.1 to 3nM), we also confirmed that treatment with 1 nM of VEGF was the most appropriate concentration for comparing the effects of all the variants (data not shown). Indeed stimulation of phosphorylation was already detected after treatments at 0.1 nM in PAEC-R2 or at 0.3 nM in HUVEC while 3 nM was already a saturating concentration for both cell lines.

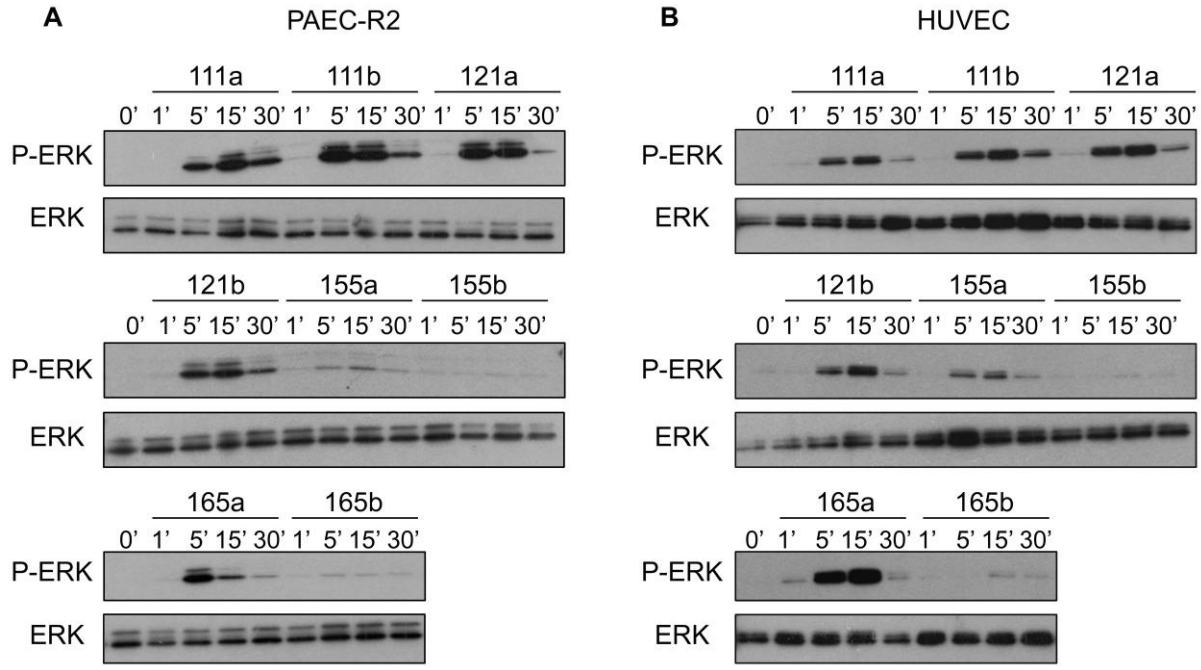


Figure 36 : Time-course experiments evaluating the effects of VEGF variants on ERK1/2 phosphorylation on endothelial cells

(A) PAEC-R2 and **(B)** HUVEC were treated with 1nM of VEGF variants for 0, 1, 5, 15 or 30 min in serum free medium. Protein extracts were then analyzed by Western blot using ERK1/2 (ERK) and phospho-ERK1/2 (P-ERK) specific antibodies

IV-1-5 VEGF$_{165}$b does not affect VEGF$_{165}$a-induced phosphorylation

VEGF$_{165}$b has been reported in the literature to be able to inhibit the VEGF$_{165}$a-induced activation of the VEGF-R2 signalling pathway (Woolard et al., 2004). Such inhibitory effect was however not observed in the previous experimental settings used with PAEC-R2 (Fig 33B). This was further investigated here in PAEC-R2 and HUVEC, using various types of treatments and different concentrations of VEGF$_{165}$a and VEGF$_{165}$b (Fig 37). Inhibition of the VEGF$_{165}$a-induced activation was never observed, even with cells pre-treated with VEGF$_{165}$b before the addition of VEGF$_{165}$a (Fig 37C, 37D).

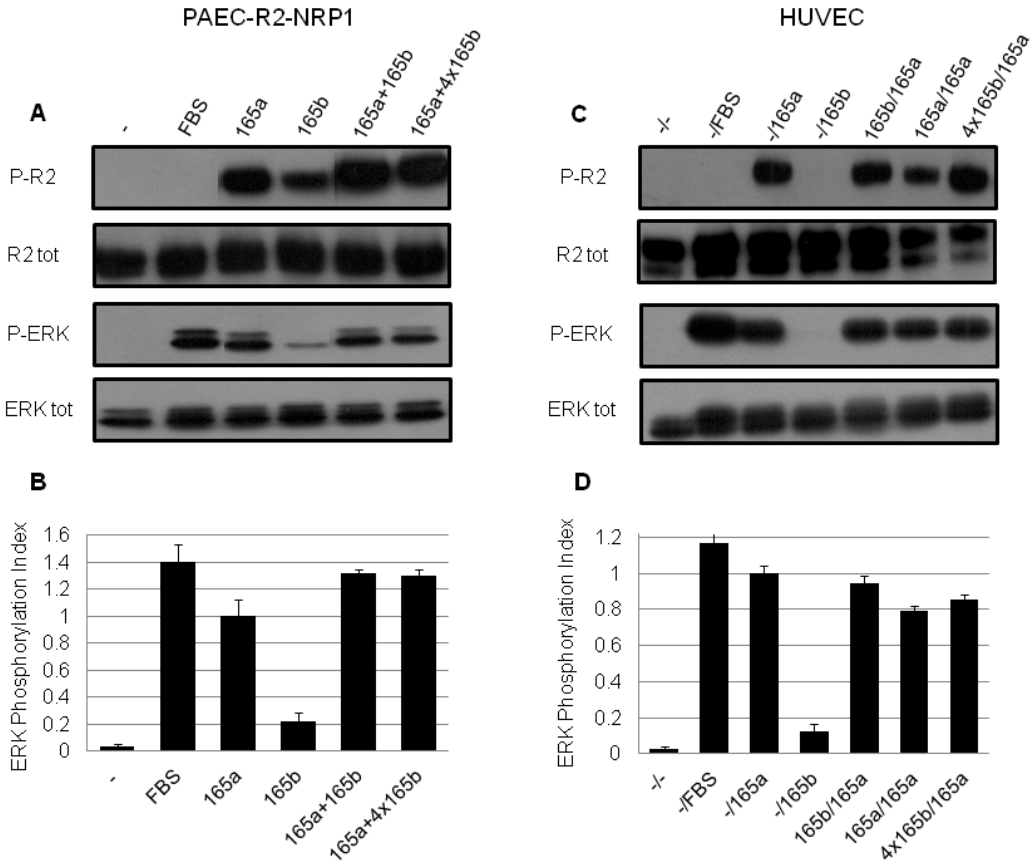


Figure 37 : Effect of VEGF$_{165}$b on VEGF$_{165}$a-induced phosphorylation of VEGF-R2 and ERK1/2

(A) PAEC-R2-NRP1 were treated with 1nM of VEGF$_{165}$a and VEGF$_{165}$b, added alone or together for 10min in serum free medium. **(C)** HUVEC were pre-treated without or with VEGF$_{165}$a or VEGF$_{165}$b for 15 min, before addition of VEGF$_{165}$a or VEGF$_{165}$b for 10 min. Cells were lysed and analyzed by Western blot using antibodies against VEGF-R2 (R2), phospho-VEGFR2 (Tyr1175, P-R2), ERK1/2 (ERK) or phospho-ERK1/2 (P-ERK). **(B, D)** The phosphorylation indexes (P-ERK/ERK) were calculated and compared to the index measured for VEGF$_{165}$a alone (arbitrary set at 1). FBS: Foetal Bovine Serum, 165a: 1nM VEGF$_{165}$a, 165b: 1nM VEGF$_{165}$b, 4x165b: 4nM VEGF$_{165}$b, 165b/165a: pre-treatment with VEGF$_{165}$b during 15' then addition of VEGF$_{165}$a.

IV-2 Effect of VEGF variants on endothelial cells proliferation and survival

Several preliminary experiments were performed to determine the best experimental conditions to evaluate the effects of VEGF on HUVEC survival and proliferation. Different conditions of medium and times of treatment were tested but the most critical parameter was the serum concentration. A concentration of 0.1% FBS for 48h was chosen for the survival assays while for the proliferation assays 0.5% for 48h was the best compromise, being high enough to prevent any side effect related to survival issues but too low to stimulate proliferation in absence of VEGF.

Using a WST-1 assay, we demonstrated that the proliferation rate was drastically increased by $VEGF_{165}a$ and to a lesser extent by $VEGF_{111}a$, $VEGF_{111}b$, $VEGF_{121}a$, $VEGF_{121}b$ and $VEGF_{155}a$ while $VEGF_{155}b$ and $VEGF_{165}b$ had no stimulatory effect (Fig 38A). The effect of combining $VEGF_{165}a$ (250 pM) with either $VEGF_{165}b$ or $VEGF_{155}b$ (1 nM) was also evaluated. Surprinsingly, the effect of $VEGF_{165}a$ was suppressed by the two $VEGF_{xxx}b$ variants and the proliferation rate returned to control levels. These results were confirmed by using a [^{3}H]-thymidine incorporation assay (data not shown).

It was previously suggested that $VEGF_{165}b$ favours endothelial cells survival despite its reduced effect on VEGF-R2 signalling pathway (Woolard et al., 2004). This was clearly confirmed here as it had the same activity as $VEGF_{165}a$ and $VEGF_{111}a$ used as positive controls. $VEGF_{111}b$ was also active (Fig 38B).

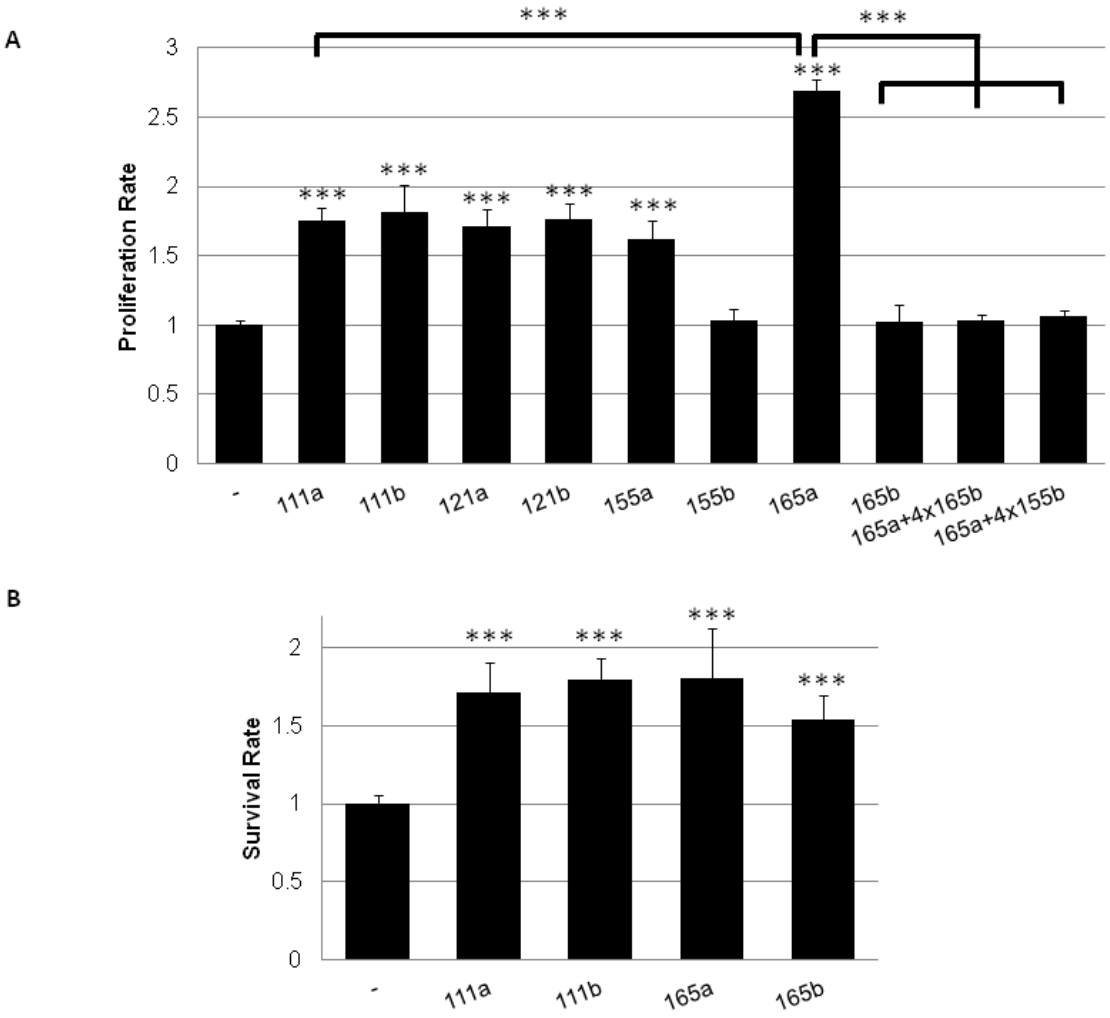


Figure 38 : Effect of VEGF variants on HUVEC proliferation and survival

HUVEC were seeded in complete medium during 16h. They were then washed before being cultured for 48h: **(A)** in a medium containing 0.5% FBS or **(B)** in a medium containing 0.1% FBS with or without 250pM of VEGF variants in order to evaluate **(A)** their proliferation or **(B)** their survival, respectively. -: Control medium; H: 1µg/ml heparin; FBS: Foetal Bovine Serum; 111a: $VEGF_{111}a$; 111b: $VEGF_{111}b$; 121a: $VEGF_{121}a$; 121b: $VEGF_{121}b$; 155a: $VEGF_{155}a$; 155b: $VEGF_{155}b$; 165a: $VEGF_{165}a$; 165b: $VEGF_{165}b$; 165a + 4x 155b: 250pM $VEGF_{165}a$ mixed with 1nM $VEGF_{165}b$; 165a + 4x 165b: 250pM $VEGF_{165}a$ mixed with 1nM $VEGF_{155}b$; *p<0.05, **p<0.01, ***p<0.001 (t-test of Student, sample versus control or between two specific conditions indicated by the bar).

IV-3 Effect of VEGF variants on endothelial cells migration

Various VEGF isoforms, alone or in combination, were evaluated for their capacity to induce the migration of HUVEC in Boyden chambers assay. A strong activity was observed for $VEGF_{111}a$, $VEGF_{111}b$ and $VEGF_{165}a$ (Fig 39). By contrast, $VEGF_{165}b$ was not chemotactic and was further able to inhibit the $VEGF_{165}a$-induced migration. The addition of an antibody specific for the $VEGF_{xxx}b$ isoforms inhibited the chemotactic activity of $VEGF_{111}b$ and the inhibitory function of $VEGF_{165}b$, further strengthening the data obtained in cell proliferation assay.

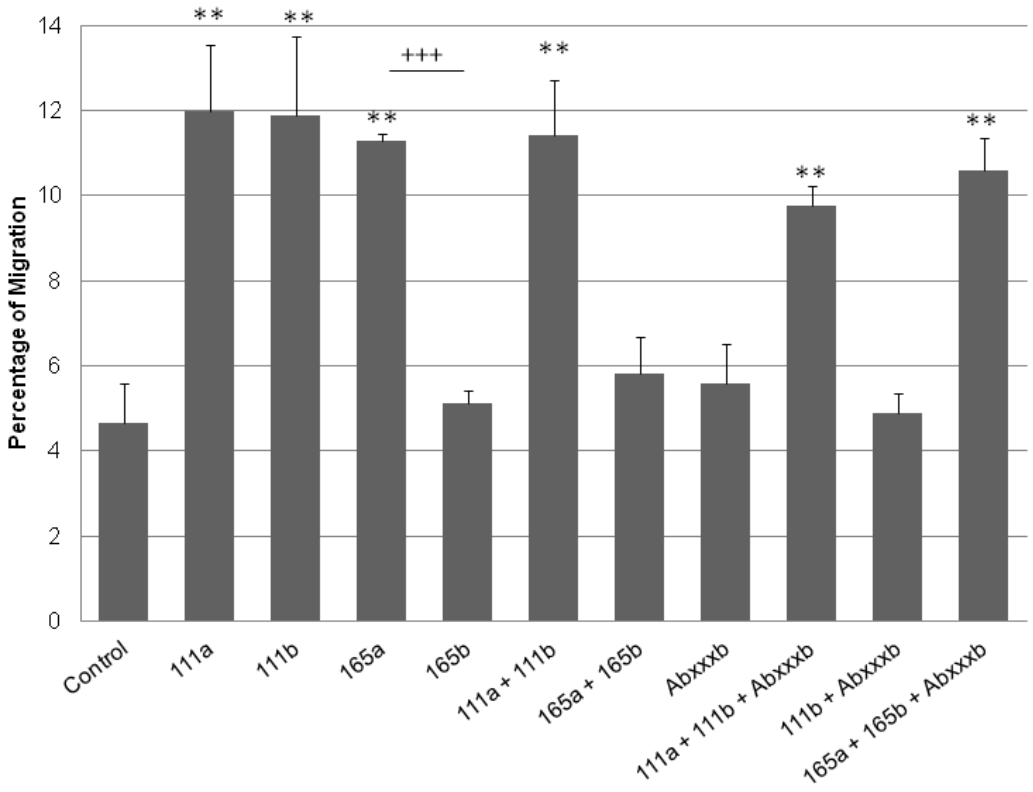


Figure 39 : Effect of VEGF variants on HUVEC migration

The chemotactic effect of VEGF variants were assessed in a Boyden chambers assay. VEGF isoforms were used alone (1 nM) or in association (1 nM each). In some conditions the $VEGF_{xxx}b$ antibody was added (1 nM). The percentage of migrating cells was calculated as described in Material and Method. 111a: $VEGF_{111}a$, 111b: $VEGF_{111}b$, 165a: $VEGF_{165}a$, 165b: $VEGF_{165}b$, Abxxxb: Specific antibody directed against $VEGF_{xxx}b$ isoforms. ** $p<0.01$ (t-test of Student, sample versus control), +++ $p<0.001$ (t-test of Student, between two specific conditions indicated by the bar).

IV-4 Role of exon 5 binding to NRP1 in VEGF effects *in vitro*

In VEGF-A, the E5-encoded domain is involved in binding to NRP1 and HSPG (Fig 22, 24, 28-30). Its role as an inducer of the VEGF-R2 signalling cascade was investigated in culture by the use of the R8R mimetic peptide. Endothelial cells were pre-treated with the R8R peptide before supplementing the medium with $VEGF_{165}$a. In PAEC-R2, the peptide did not modify the $VEGF_{165}$a-induced phosphorylation of VEGF-R2 (Fig 40A, 40C), which was expected as these cells do not express NRP1. By contrast, in similar conditions the peptide markedly reduced the VEGF-R2 activation in PAEC-R2-NRP1 (Fig 40B, 40D). Most importantly, R8R was also able to inhibit the $VEGF_{165}$a-induced proliferation of HUVEC (Fig 40E) to a rate similar to that observed with $VEGF_{111}$a, confirming the antagonistic effect of the E5-mimetic peptide and the implication of the E5-encoded domain on the NRP1 function.

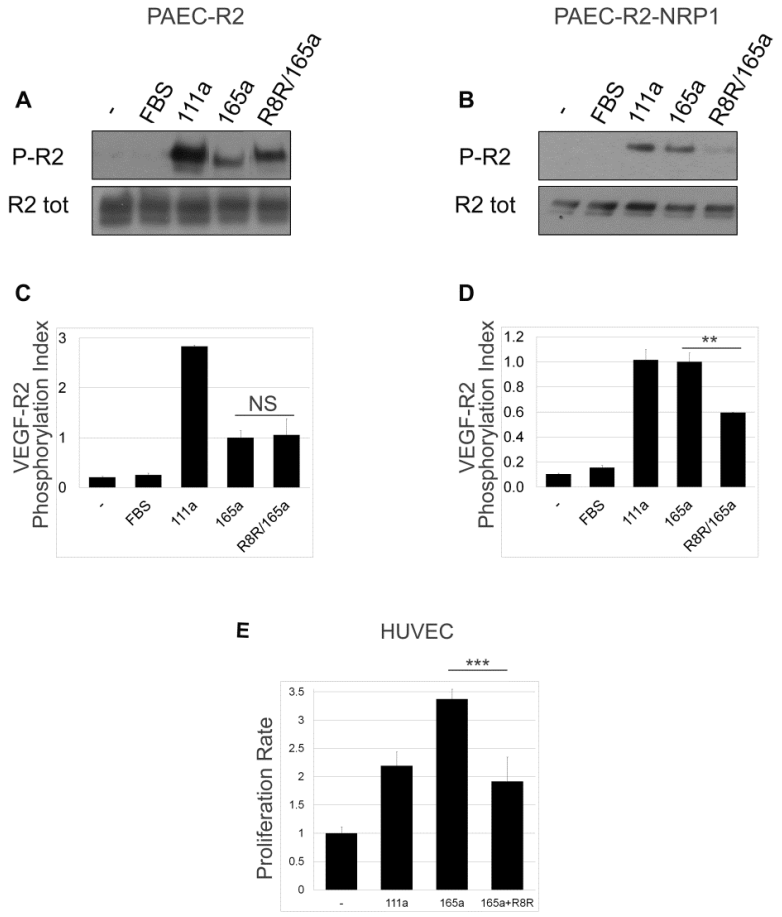


Figure 40 Effect of R8R on VEGF-induced signalling in vitro

(A) PAEC-R2 and **(B)** PAEC-R2-NRP1 were treated for 10 min at 37°c with 1 nM of $VEGF_{111}$a or 1nM of $VEGF_{165}$a in the absence or presence of R8R (300µM). Protein extracts were then analyzed by Western blot using VEGF-R2 (R2 tot) and phospho-VEGFR2 (P-R2) specific antibodies **(C, D).** Calculated P-R2/R2 ratio in **(C)** PAEC-R2 and **(D)** PAEC-R2-NRP1. **(E)** HUVEC proliferation was measured by WST-1 assay in absence of VEGF, in the presence of $VEGF_{111}$a (250 pM), in the presence of $VEGF_{165}$a (250pM), or in the presence of both $VEGF_{165}$a (250 pM) and R8R (300µM). Only statistics related to the effects of R8R are reported. **p<0.01, ***p<0.001 (t-test of Student).

IV-5 Cytoprotective effect of VEGF isoforms on RPE cells

$VEGF_{165}b$ was reported to be cytoprotective in retina (Magnussen et al., 2010) as it protects retinal pigment epithelial cells (RPE cells) from apoptosis induced by toxic doses of ketocholesterol. This previously published protocol was used to assess the effect of 2.5nM of $VEGF_{111}a$, $VEGF_{111}b$, $VEGF_{165}a$ or $VEGF_{165}b$.

In immortalized retinal ARPE19 cells, the addition of all isoforms, including $VEGF_{165}b$, induced a 1.5-fold increase of cell viability after ketocholestrerol treatment, which is similar to the effect observed with EGF used as a positive control (Fig 41A). The protective effect was also indirectly confirmed by LDH release in the culture medium (Fig 41B). A similar trend was observed with primary RPE cells, although VEGF isoforms were slightly less active than EGF (Fig 41C). Altogether these data suggest that $VEGF_{111}a$, $VEGF_{111}b$, $VEGF_{165}a$ and $VEGF_{165}b$ conferred a survival advantage to the retinal epithelial cells, which could benefit to therapy of eyes diseases.

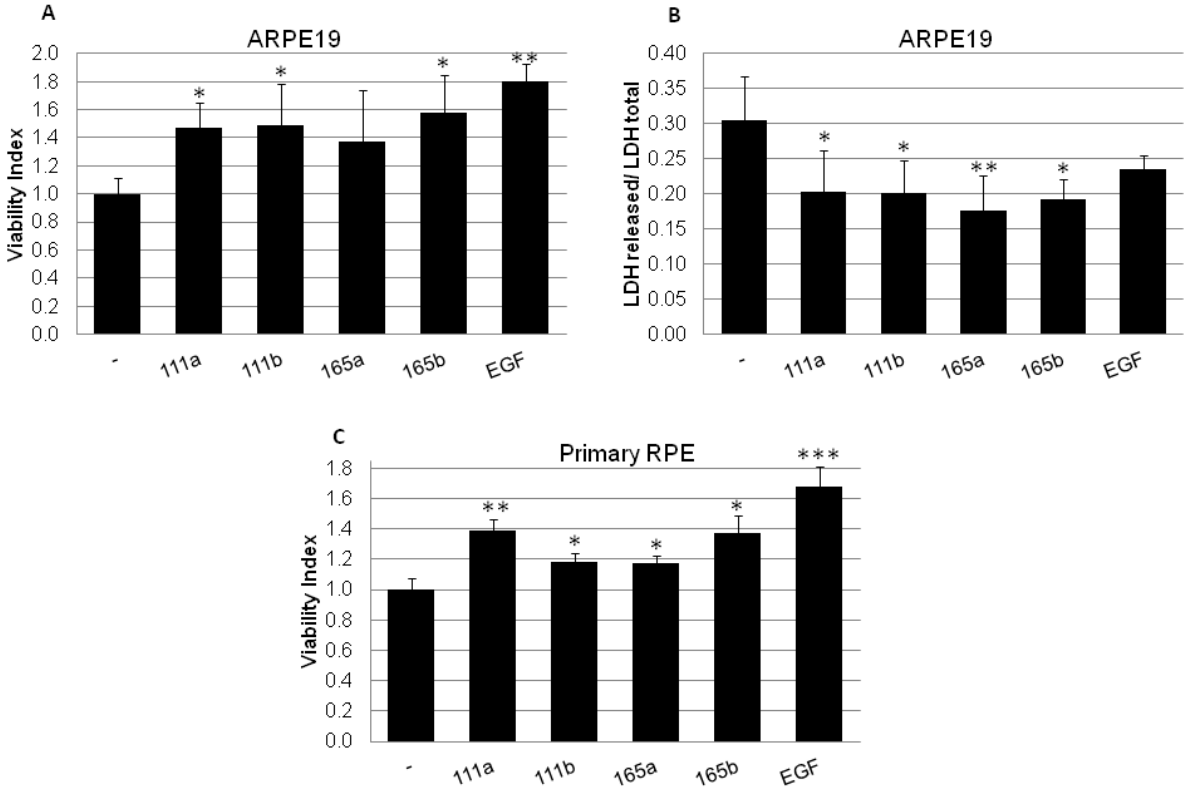
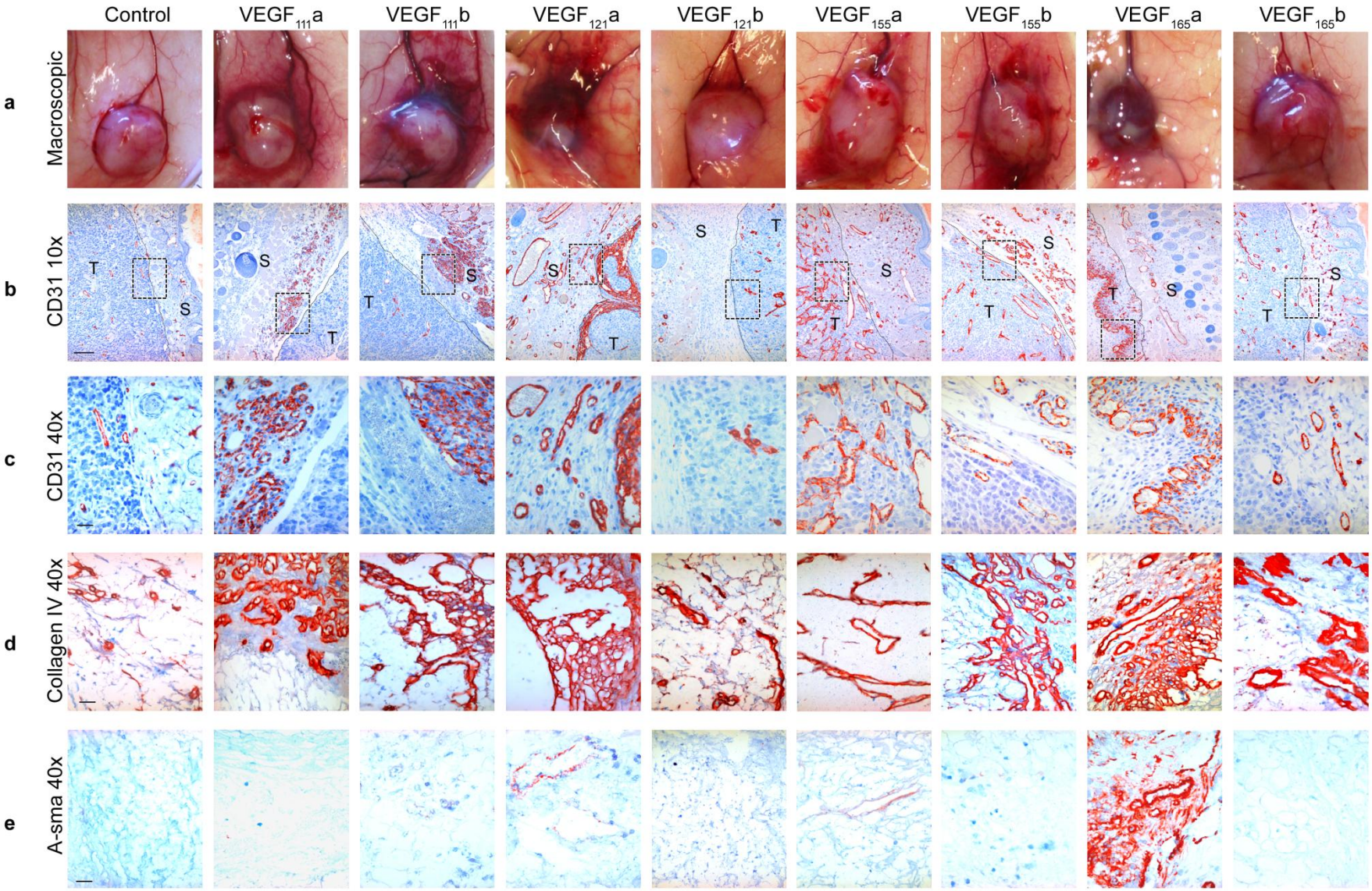


Figure 41 Effect of VEGF variants on cytoprotection of retinal epithelial cells

(A, B) ARPE19 and **(C)** RPE cells were treated with a toxic concentration of ketocholesterol (18 µM) and VEGF variants or EGF as described in Material and Methods. Cells viability was evaluated using **(A, C)** The WST-1 colorimetric method and **(B)** measurement of the LDH released in the medium. ARPE19: Spontaneously Arising Retinal Pigment Epithelial Cells; Primary RPE: Primary Retinal Pigment Epithelial Cells, -: control, 111a: $VEGF_{111}a$, 111b: $VEGF_{111}b$, 165a: $VEGF_{165}a$, 165b: $VEGF_{165}b$, EGF: Epidermal Growth Factor. Statistical difference between control and treated cells: *p<0.05, ** p<0.01, ***p<0.001, paired t-test.

V- VEGF isoforms generate different patterns of angiogenesis *in vivo*.

HEK293 cells transfected with the empty vector or expressing the various VEGF isoforms at a similar level *in vitro* were used to locally deliver recombinant VEGF variants in nude mice. Animals were weighed during the entire experiment. No significant weight variation was noted among the different groups, as it was previously observed for $VEGF_{111}a$, $VEGF_{121}a$ and $VEGF_{165}a$ (Mineur et al., 2007). After three weeks, mice were sacrificed and tumours were dissected. The overall level of human VEGF produced *in vivo* by HEK293 cells was measured in the tumours at the mRNA level. Primers (P1-P2) enabling amplification of sequence between E2 to E4 of the human but not the mouse transcripts were used (Table 3). No significant difference of VEGF mRNA levels was observed among the various groups injected with VEGF-expressing HEK293 cells while no signal was detected in the group injected with control cells (Fig 43A). Immunodetection using a VEGF antibody showed an intense and similar labelling in the tumour tissue for all the VEGF isoforms (data not shown) confirming the RT-PCR quantifications. By using primers specific for each of the $VEGF_{xxx}a$ (P3-P4) or $VEGF_{xxx}b$ subfamily (P5-P6, P7-P8 and P9-P10) (Table 3), we validated also the identity of the expressed VEGF variants (not illustrated).


Control
VEGF111a
VEGF111b
VEGF121a
VEGF121b
VEGF155a
VEGF155b
VEGF165a
VEGF165b
a
Macroscopic
b
CD31 10x
c
CD31 40x
d
Collagen IV 40x
e
A-sma 40x
T
S

Tumours and surrounding tissues were examined macroscopically. The most obvious differences were the intense reddish colour of the $VEGF_{165}a$ and $VEGF_{121}a$ tumours compared to the others, and the formation of a dense network of small capillaries in the dermis close to tumours, especially those expressing $VEGF_{111}a$ or $VEGF_{111}b$ (Fig 42A). The number of vessels, their size and their partition between the tumours and surrounding tissues were also evaluated by immunolabelling using anti-mouse CD31 antibodies (Fig 42B, 42C). Control tumours contained a low number of small blood vessels that were however functional based on the presence of red blood cells (not shown). As expected from macroscopic observation, vessels density was high within the tumour in the $VEGF_{165}a$ and $VEGF_{121}a$ mice, while it was scarce in $VEGF_{111}a$, $VEGF_{111}b$ and $VEGF_{121}b$ tumours. Slightly higher levels were induced by $VEGF_{155}b$ and $VEGF_{165}b$, and intermediate levels were seen in $VEGF_{155}a$ expressing tumours. To substantiate these observations, the surface of positive CD31 labelling was measured and expressed in the percentage of the total surface of the tumoural tissue present in the sections. It was significantly higher in $VEGF_{121}a$ and $VEGF_{165}a$, and to a lesser extent in $VEGF_{155}a$ tumours as compared to the control tumours (Fig 43B). $VEGF_{165}b$ and $VEGF_{155}b$ were active, although markedly less than their $VEGF_{165}a$ and $VEGF_{155}a$ counterparts, while $VEGF_{111}a$, $VEGF_{111}b$ and $VEGF_{121}b$ expression did not significantly increase the intratumoural CD31-positive surface, as compared to controls. By contrast, the ranking for the number of vessels in the skin adjacent to the tumours was $VEGF_{111}a = VEGF_{111}b > VEGF_{121}a > VEGF_{155}b = VEGF_{165}a = VEGF_{165}b = VEGF_{155}a > VEGF_{121}b$ = control (Fig 42B, 42C and Fig 43C).

Figure 42 : Effects of VEGF variants on *in vivo* tumour angiogenesis using HEK293 cells expressing the various VEGF isoforms

Two millions HEK293 cells, transfected with the empty vector (control) or expressing the VEGF variants, were mixed with Matrigel and injected in the flanks of nude mice. **(A)** Macroscopic view of the tumours formed in each group. **(B)** CD31 immunostaining showing the presence of blood vessels in the tumour (T) and the adjacent skin (S) (scale bar = 100µm). **(C)** Enlarged view of the region delineated by the rectangle in each picture shown in **(B)** (scale bar = 25 µm). Staining of **(D)** type IV collagen and **(E)** α-SMA in regions where angiogenesis was the highest (scale bar = 25 µm). (Page 111)

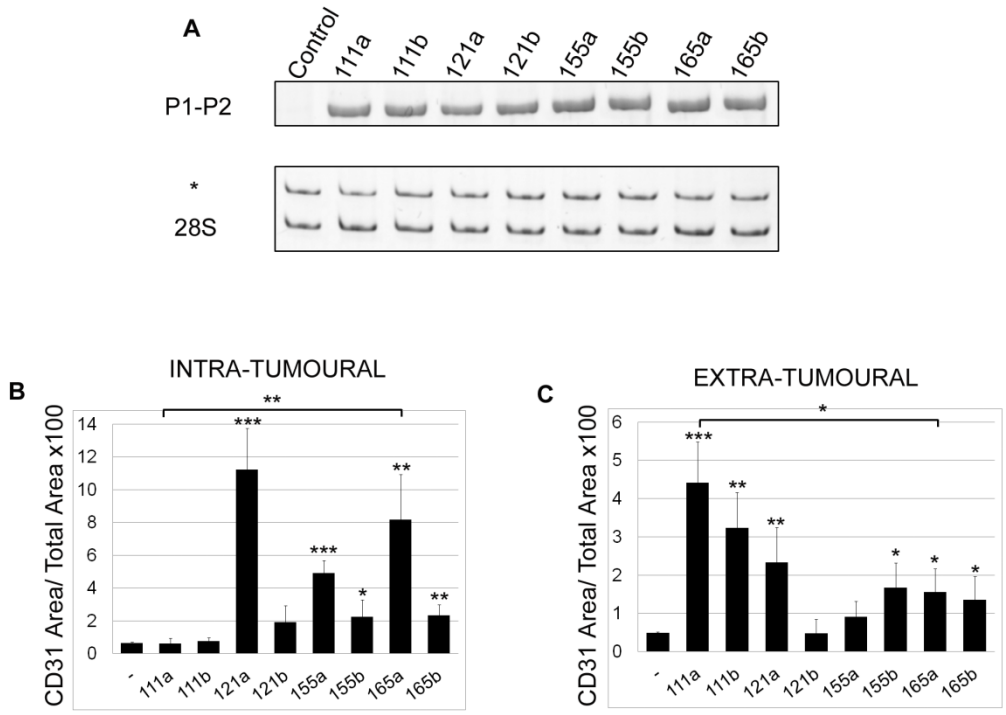


Figure 43 : Quantification of the effects of VEGF variants in tumour angiogenesis using HEK293 cells expressing the various VEGF isoforms

Two millions HEK293 cells, either control or expressing VEGF variants, were mixed with Matrigel and injected in the flank of nude mice. **(A)** The VEGF mRNA levels (upper panel) were measured by RT-PCR using primers P1 and P2 (see Table 1) that enable the amplification of all the VEGF variants with production of a single amplicon. The 28S rRNA was measured in parallel to normalize the quantities of RNA input in the reactions. * indicates the amplicon formed from an internal standard co-amplified with 28S rRNA to take into account potential variations of the PCR efficiency. Percentages of the CD31 stained surfaces in **(B)** the tumour or **(C)** the adjacent skin were quantified using Image J software. * $p<0.05$, ** $p<0.01$, *** $p<0.001$ (unpaired t-test).

Maturation of the blood vessels was also investigated by immunostaining of the basement membrane (type IV collagen) and α-SMA positive pericytes. Whatever the conditions, the majority of the formed vessels were delineated by a basement membrane (Fig 42D). Striking differences were however observed by staining of α-SMA (Fig 42E). Positive cells were found mainly in the vessels growing in or around the tumours expressing $VEGF_{165}a$. They were also found although at a much reduced level, in the presence of $VEGF_{155}a$ and $VEGF_{121}a$. They were almost totally absent in other samples, notably those expressing $VEGF_{165}b$.

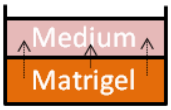
Effect of tumour expressing VEGF variants on mice physiology

Toxicity triggered by HEK cells, either control or expressing $VEGF_{111}a$, $VEGF_{111}b$, $VEGF_{165}a$ or $VEGF_{165}b$, on nude mice (4 mice per group) was evaluated using different parameters. The weekly monitoring showed healthy animals with a normal general examination, pink

mucosae, normal hydration and absence of ascite. After death, control of organs did not identify any hepatomegaly or splenomegaly in mice bearing VEGF tumours except for $VEGF_{111}a$ mice which displayed a slightly higher spleen weight (1.20 ± 0.04 time the weight of the control spleen). Hepatic enzymes as well as complete blood counts were similar between the groups (not illustrated).

Diffusibility of VEGF variants in Matrigel *ex vivo*

The formation of dense network of small capillaries in healthy tissues close to the tumor and the absence of significant intra-tumoural vascularisation are highly specific to HEK293 cells expressing either $VEGF_{111}a$ or $VEGF_{111}b$. This particularity is probably related to an increased "diffusibilty" of these variants. As HEK293 cells forming tumours in mice are injected in suspension in Matrigel, we further evaluated the diffusion of VEGF variants out of Matrigel matrix. Solutions of Matrigel containing the different VEGF isoforms (1 nM) were poured in 12-wells plate and polymerized at 37°c. Serum free culture medium was then added in the wells and the VEGF progressively released from Matrigel into the medium was quantified by ELISA (Fig 44). Maximal concentrations, indicative of an equilibrium between the VEGF present in the Matrigel and in the medium, were found within 2-4 hours for $VEGF_{111}a$ and later (8h-20h) for other tested isoforms. Although indirect, this semi-quantitative evaluation shows again an increased diffusion for $VEGF_{111}a$, $VEGF_{111}b$ having not been evaluated in this assay.



	Peak of VEGF in the medium
$VEGF_{111}a$	2h-4h
$VEGF_{121}a$	8h-20h
$VEGF_{155}a$	8h-20h
$VEGF_{165}a$	8h-20h
$VEGF_{165}b$	8h-20h

Figure 44 : Diffusion of VEGF from Matrigel *in vitro*

Matrigel solution (200 µl) containing VEGF variants (1nM) were polymerized in 12-wells plate and covered by serum free medium (200µl). VEGF released in the culture medium was quantified by ELISA after increasing incubation times (2 to 20 hours). Maximum concentration in the conditioned medium was observed already after 2-4 hours for $VEGF_{111}a$ and at later time points for the other isoforms.

Effect of the combination of $VEGF_{xxx}a$ and $VEGF_{xxx}b$ on angiogenesis *in vivo*

Based on our observations that $VEGF_{165}b$ was able to significantly inhibit $VEGF_{165}a$-induced proliferation and migration *in vitro* (Fig 38A and Fig 39), we evaluated if a similar repression also occurred *in vivo*. A total number of 2 x 10^6 HEK293 cells expressing $VEGF_{165}a$ and $VEGF_{165}b$ were injected at ratios of 1:1 or 1:4 (Fig 45). The observed patterns of angiogenesis were intermediate to those observed when $VEGF_{165}a$ or $VEGF_{165}b$ were expressed alone and seemed to result from the actual abundance of each isoform rather than to inhibition of the effect of $VEGF_{165}a$ by $VEGF_{165}b$. The observations were similar for $VEGF_{121}a$ and $VEGF_{121}b$.

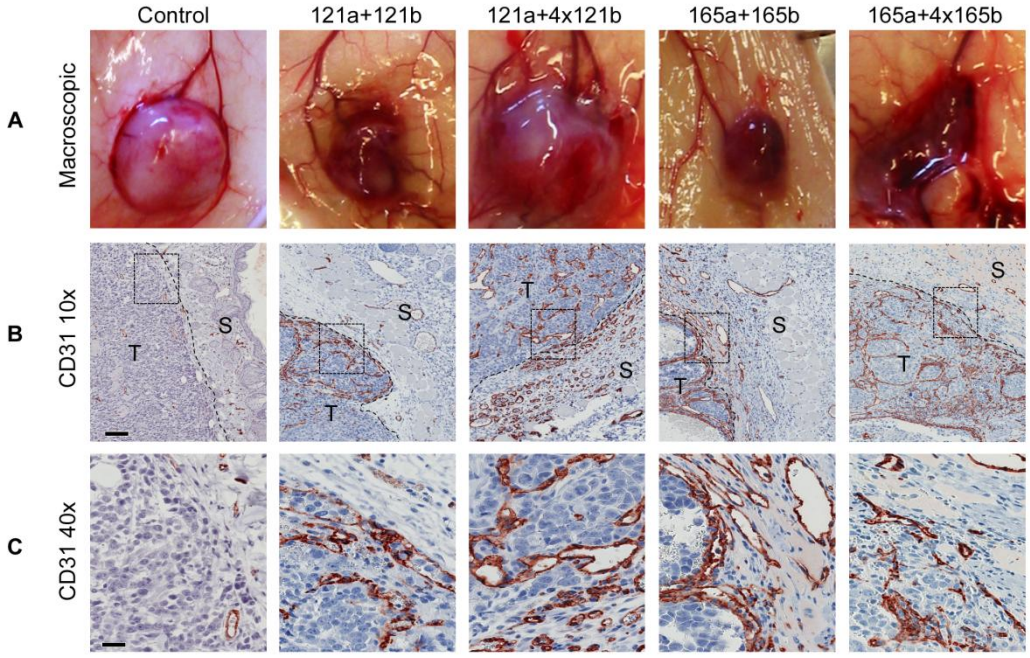


Figure 45 : Effects on tumour angiogenesis of co-expressing $VEGF_{165}a$ and $VEGF_{165}b$ or $VEGF_{121}a$ and $VEGF_{121}b$

Two millions HEK293 cells were mixed with Matrigel and injected in the flank of nude mice. In addition to control cells, mixture of HEK293 cells expressing $VEGF_{165}a$ and $VEGF_{165}b$ were evaluated, either 1 x 10^6 each (165a + 165b) or 4 x 10^5 expressing $VEGF_{165}a$ and 1.6 x 10^6 expressing $VEGF_{165}b$ (165a + 4x165b). See Fig 40 for comparison with cells expressing only $VEGF_{165}a$ or $VEGF_{165}b$. The same experiment was performed for $VEGF_{121}a$ and $VEGF_{121}b$ **(A)** Macroscopic view of the tumours obtained in each group. Control (n=5), 121a+121b (n=5), 121a+4x121b (n=5), 165a+165b (n=4), 165a+4x165b (n=5) **(B)** CD31 immunostaining on paraffin sections showing the tumour (T) and the adjacent skin (S) (scale bar = 100 µm). Black lined rectangles delineate the areas of the sections represented in **(C)** at higher magnification (scale bar = 25µm).

VI- As Opposed to $VEGF_{165}b$, $VEGF_{111}b$ does not inhibit pathologically induced neovascularization in the eyes.

As the pro-angiogenic effect of a $VEGF_{xxx}b$ isoform was not initially expected, we further evaluated $VEGF_{111}b$ in other *in vivo* models that previously allowed to demonstrate the specific properties of $VEGF_{165}b$ in reducing neovascularization in ocular models (Hua et al., 2010; Magnussen et al., 2010).

VI-1 Choroidal Neovascularization

Choroidal neovascularization (CNV), a model of Age-related Macular Degeneration (AMD), was induced by LASER-photocoagulation in mice. The damaged eyes were then treated by injection of PBS, $VEGF_{111}b$ or $VEGF_{165}b$. $VEGF_{165}b$ injection significantly inhibited neovascularization as compared with PBS ($0.97 \pm 0.34 \times 10^4$ µm² vs $2.62 \pm 0.50 \times 10^4$ µm²) (Fig 46A, 46B). By contrast, $VEGF_{111}b$ did not inhibit neovascularisation and even induced a slight, although not statistically significant, increase of the neovascularized area ($4.76 \pm 1.28 \times 10^4$ µm²) as compared to control eyes.

VI-2 Oxygen Induced Retinopathy

Oxygen Induced Retinopathy (OIR) in rat pups is a model of Retinopathy of Prematurity. In this assay, $VEGF_{165}b$ reduced pathological angiogenesis similarly to what was observed in the CNV model (Magnussen et al., 2010) (Gammons et al., unpublished data). By contrast, treatment with $VEGF_{111}b$ stimulated neo-vascularization as compared to PBS-treated eyes (Fig 46C). The diameter and the tortuosity of the vessels were however not affected.

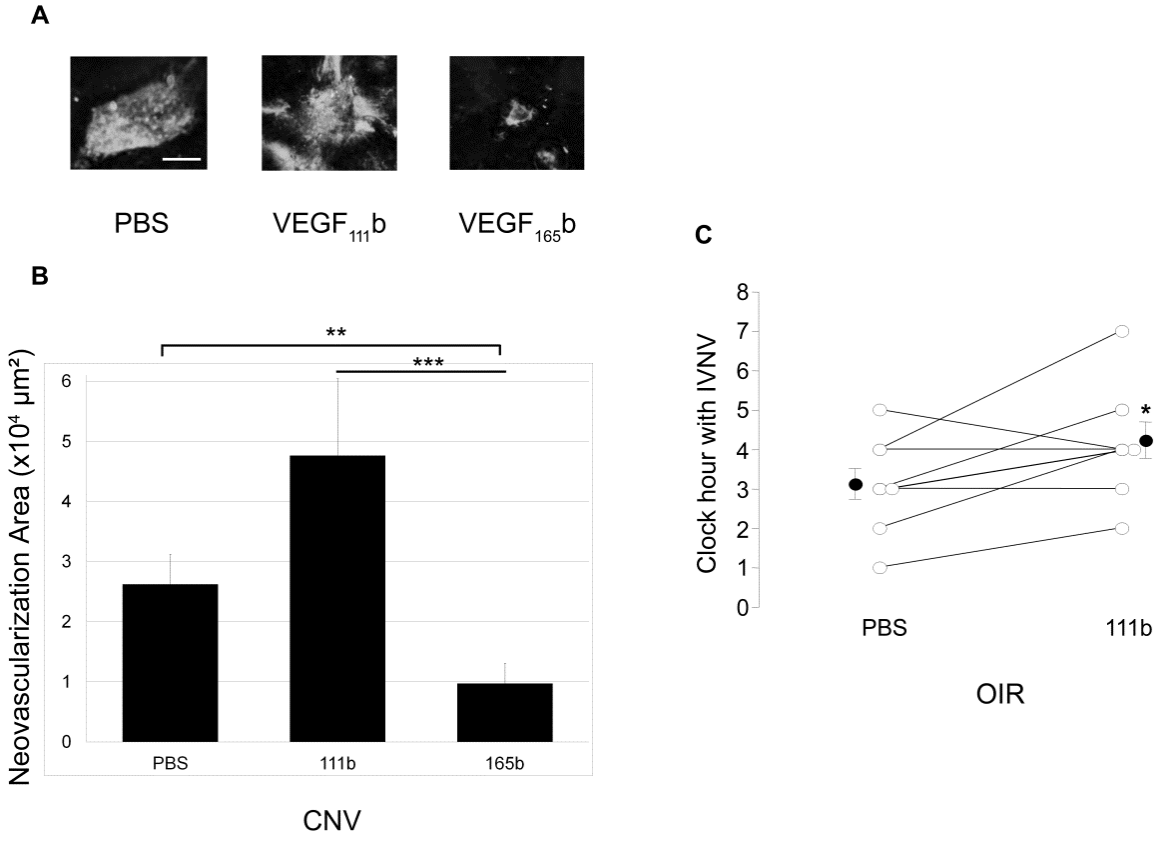


Figure 46 : Effect of $VEGF_{111}b$ and $VEGF_{165}b$ on pathologically induced neovascularization in eye models

(A, B) Choroidal Neovascularization was induced in mice by LASER impacts and the eyes were injected with PBS, $VEGF_{111}b$ or $VEGF_{165}b$ immediately and 7 days later. **(A)** Representative pictures of the choroid after isolectin B4 immunofluorescence staining (scale bar = 50 µm). **(B)** Quantification of the area of neovascularization. **(C)** Oxygen Induced Retinopathy was induced in rats. For each rat, one eye was injected with PBS and the other with 10 ng of $VEGF_{111}b$, allowing paired comparisons (represented by lines). Quantification of the number of Intravitral Neovascularization (IVNV) per clock hour. Open circles represent quantifications in individual samples and black circles the mean. * $p<0.05$, (OIR, paired t-test of Student). ** $p<0.01$; *** $p<0.001$ (CNV, t-test of Student). CNV: Choroidal Neovascularization; OIR: Oxygen Induced Retinopathy; 111b: $VEGF_{111}b$; 165b: $VEGF_{165}b$.

VII- Effects of the different isoforms on vascular permeability *in vivo*

Based on data of a preliminary dose-response experiment using $VEGF_{165}a$ (not shown), 50 ng of the different VEGF isoforms were injected intradermally followed by an intravenous delivery of Evans' blue dye. As compared to PBS controls, an increased permeability close to the site of injection was observed for $VEGF_{111}a$, $VEGF_{111}b$, $VEGF_{121}a$, $VEGF_{155}a$ and $VEGF_{165}a$ while the effect of the other variants was not statistically significant (Fig 47A). The total surface of skin displaying an increased permeability is also an interesting parameter. Although hard to precisely quantify because of the difficulties to define the exact border between affected and non-affected skin, it clearly appeared that $VEGF_{111}a$ and $VEGF_{111}b$ were active at a longer distance than the other isoforms as shown in a representative example for $VEGF_{111}a$ and $VEGF_{165}a$ (Fig 47B), suggesting higher diffusibility.

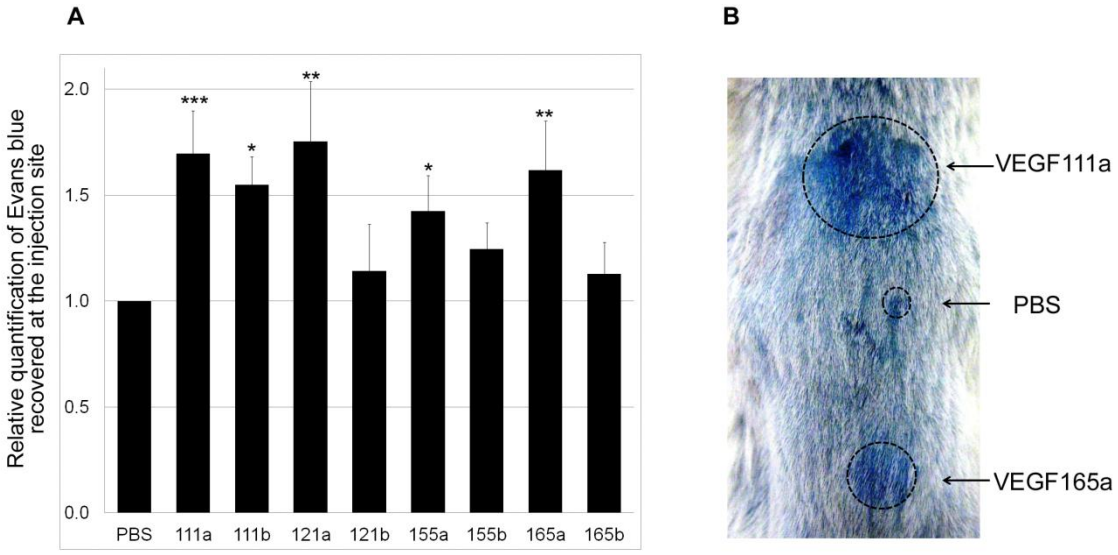


Figure 47: *In vivo* vascular permeability

A Miles and Miles modified assay was used to evaluate vascular permeability induced by the VEGF variants. **(A)** Quantification of the Evans' blue dye in the skin surrounding the site of injection (~ 20 mm^2). The results were normalized for the weight of the skin samples and compared to the PBS control in each mouse. **(B)** Picture of the back of a mouse injected with $VEGF_{111}a$, $VEGF_{165}a$ and PBS. Areas of increased vessel permeability were delineated with a black dotted line. 111a: $VEGF_{111}a$, 111b: $VEGF_{111}b$, 121a: $VEGF_{121}a$, 121b: $VEGF_{121}b$, 155a: $VEGF_{155}a$, 155b: $VEGF_{155}b$, 165a: $VEGF_{165}a$, 165b: $VEGF_{165}b$. * $p<0.05$, ** $p<0.01$ and *** $p<0.001$, (t-test of Student).

VIII- Pharmacokinetic and toxicology of VEGF variants *in vivo*

VIII- 1 Half-life of VEGF variants *in vivo*

The half-life *in vivo* of $VEGF_{111}a$, $VEGF_{111}b$, $VEGF_{165}a$ and $VEGF_{165}b$ was evaluated after intra-venous (IV), intra-peritoneal (IP) or subcutaneous (SC) injections in mice. Their plasmatic concentration was quantified using an ELISA specific for human VEGF (Table 5A). After IV injection, half-life of $VEGF_{165}b$ (12 ± 1 min) was the shortest and similar to the previously published values (12 min (Rennel et al., 2008); 13.2 min (Zhu et al., 2012)). The half-lifes of the other isoforms were slightly higher, ranging between 20 and 30 min.

After IP injections, the isoforms with the longest and the shortest half-life were $VEGF_{111}a$ and $VEGF_{165}b$, respectively, while after SC administration the ranking was $VEGF_{111}a$ > $VEGF_{111}b$ > $VEGF_{165}a$ > $VEGF_{165}b$. Altogether, these data show that the variants possessing the 6 amino acids encoded by exon 8b have a reduced half-life as compared to the equivalent variants possessing the alternative E8a-derived sequence. Moreover, these results highlight that the lack of the sequence encoded by E5 and E7 in $VEGF_{111}a$ confers an increased half-life *in vivo,* likely related to the resistance to degradation or to reduced interactions with the ECM.

Table 5 : Variations in the plasmatic concentrations of VEGF variants after different types of administration

A

Half-life in blood stream (min)			
	Method of injection		
	IV	IP	SC
$VEGF_{111}a$	21±2	113±39	100±13
$VEGF_{111}b$	29±8	38±5	72±19
$VEGF_{165}a$	28±4	48±5	52±12
$VEGF_{165}b$	12±1	14±5	25±12

B

Maximal Plasmatic concentration (ng/ml) after non-systemic injection		
	Method of injection	
	IP	SC
$VEGF_{111}a$	35.0±8.1	37.2±11.8
$VEGF_{111}b$	31.7±10.1	36.6±6.1
$VEGF_{165}a$	9.6±3.1	8.0±3.7
$VEGF_{165}b$	11.5±4.2	4.7±1.7

IV: Intravenous, IP: Intraperitoneal, SC: Subcutaneous.

The release from the site of injection into the blood stream is another critical parameter to be considered for evaluating the potential effects of VEGF variants *in vivo.* The maximal plasmatic concentrations were detected after 1 to 2 hours for all the variants. Marked differences were however observed when considering the actual concentration at the peak (Table 5B). Data clearly demonstrate that $VEGF_{111}a$ and $VEGF_{111}b$ can diffuse more efficiently than $VEGF_{165}a$ and $VEGF_{165}b$.

The ability of $VEGF_{111}a$ and $VEGF_{165}a$ to diffuse *in vivo* was investigated in another model. The purified VEGF were injected under the skin of mice. After 8 hours tissues around the site of injection were recovered, total proteins were extracted and VEGF concentrations were determined. As compared to $VEGF_{165}a$, the local concentration of $VEGF_{111}a$ was reduced confirming its increased diffusibility (Fig 48).

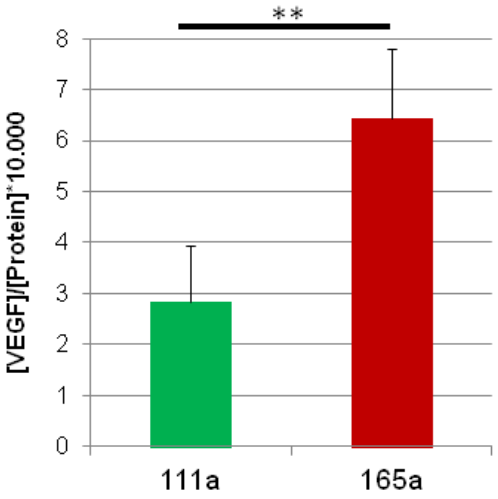


Figure 48 : $VEGF_{111}a$ and $VEGF_{165}a$ diffusion in the skin *in vivo*

$VEGF_{111}a$ and $VEGF_{165}a$ were injected subcutaneously. After 8h, proteins were extracted from the site of injection and the VEGF concentrations were measured. The results are reported as the ratio to total protein concentrations. ** p<0.01, t-test of Student.

VIII-2 Pathophysiological effects of VEGF variants *in vivo*

Xue and collaborators reported that daily subcutaneous injections of 4 µg of $VEGF_{165}a$ for 14 days induced splenomegaly and hepatomegaly (Xue et al., 2008). Later, they observed the similar effects in mice bearing tumor overexpressing $VEGF_{165}a$ together with increased hepatic parameters, ascite, anemia and bone-marrow defects (Xue et al., 2009). As we never saw such phenotype in our models of tumour angiogenesis, we decided to further characterize the effects of chronic injection of VEGF.

4 µg of VEGF ($VEGF_{111}a$, $VEGF_{111}b$, $VEGF_{165}a$, $VEGF_{165}b$) or PBS were daily injected during 14 days in the neck of C57Bl6 mice. Every day monitoring of animals showed that they were healthy (normal general aspect, pink mucosae, absence of ascite, normal

hydration, for example). No death was recorded during the experiment. At the end of the experiment, blood samples were collected and mice were sacrificed and dissected. Spleen and liver had a similar weight in all groups. Different organs and tissues were fixed and embedded in paraffin. Hematoxylin-eosin staining did not reveal any striking difference among the mice. Blood cell populations were similar in all the conditions and hepatic markers (ALT and AST) did not show any liver alteration induced by VEGF treatment. Altogether these data demonstrate that a prolonged treatment with VEGF injected at a dose more than 100 times higher than the physiological concentration did not induced anemia, inflammation, liver failure or any other obvious side effect.

Subcutaneous tissues close to the site of injection were also analyzed. They contained more blood vessels, as determined by CD31 immunostaining, for mice treated with $VEGF_{111}a$, $VEGF_{111}b$ and $VEGF_{165}a$ than in the $VEGF_{165}b$ and PBS groups (not illustrated).

IX- Effect of the combination of $VEGF_{111}a$ and $VEGF_{165}a$ on angiogenesis *in vivo*

To evaluate any potential synergistic effects between $VEGF_{111}a$ and $VEGF_{165}a$ on angiogenesis *in vivo*, HEK293 cells expressing $VEGF_{111}a$ or $VEGF_{165}a$ were injected either simultaneously or alone as a control in nude mice. Tumours expressing both $VEGF_{111}a$ and $VEGF_{165}a$ were reddish and the adjacent skin invaded by numerous blood vessels (Fig 49A). An enlargement of the lateral thoracic vein was also seen, but at a lesser extent than with $VEGF_{111}a$ alone. This is probably due to the number of cells expressing $VEGF_{111}a$: $2x10^6$ in $VEGF_{111}a$ group alone as compared to $1x10^6$ in "$VEGF_{111}a$+$VEGF_{165}a$" group. Microscopically, invasion of small and immature vessels (CD31+, α-SMA-) in the peritumoural tissue and formation of large vessels surrounded by pericytes inside or close to the tumour (CD31+, α-SMA+) were observed which was indicative of an addition of the effects mediated by the two VEGF isoforms (Fig 49B, 49C).

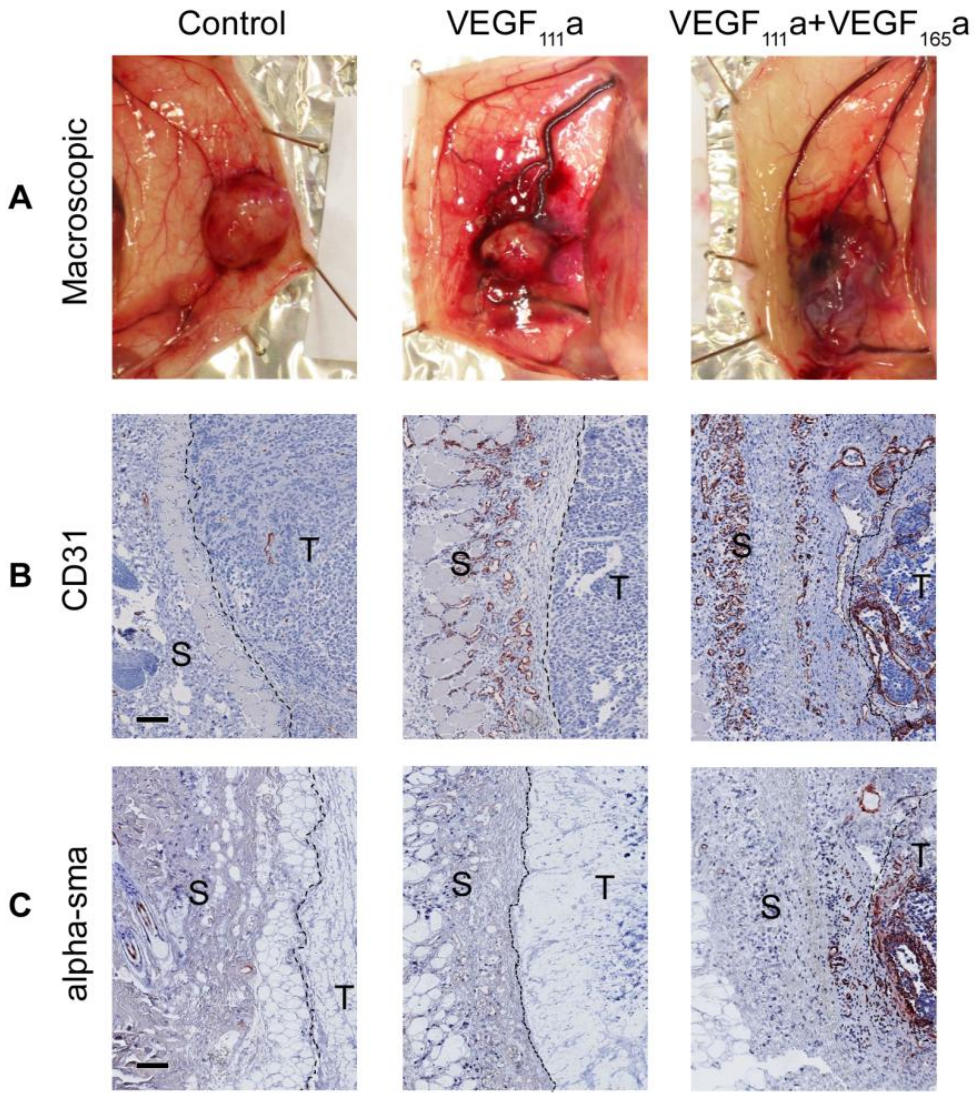


Figure 49 : Tumour angiogenesis by cells co-expressing $VEGF_{111}a$ and $VEGF_{165}a$

Two millions HEK293 cells, transfected with the empty vector (control) or expressing one or both VEGF variants, were mixed with Matrigel and injected in the flanks of nude mice. **(A)** Macroscopic view of the tumours obtained in some groups. Control (n=4), $VEGF_{111}a$ (n=4), $VEGF_{111}a$ + $VEGF_{165}a$ ($1x10^6$ HEK cells expressing $VEGF_{111}a$ and $1x10^6$ HEK cells expressing $VEGF_{165}a$, n=6). **(B)** CD31 immunostaining of paraffin sections showing the tumour (T) and the adjacent skin (S) (scale bar = 100 µm). **(C)** α-SMA immunostaining showing the presence of pericytes (scale bar = 100µm).

X- Development of new VEGF variants

The development of efficient pro-angiogenic therapies would probably benefit from the use of VEGF variants possessing complementary specific properties. As an example the use of variants displaying different affinities for the ECM should favour the formation of VEGF gradients for recruiting and guiding vessel sprouts. The development of a VEGF variant able to stimulate the VEGF-R2 signalling cascade while displaying no affinity for VEGF-R1 would also be worth testing as VEGF-R1 is believed to be a competitor for VEGF-R2 when expressed as a soluble form or at the endothelial cell surface.

IX-1 $VEGF_{179}a$ and $VEGF_{179}b$

$VEGF_{189}a$ is mainly characterized by its high affinity for matrix components, due to the presence of the E6a-encoded domain (Houck et al., 1992; Park et al., 1993). This is a key property for forming gradient especially when co-expressed with more diffusible variants. It is however sensitive to proteolysis (Plouet et al., 1997). As $VEGF_{111}a$ and $VEGF_{111}b$, are resistant to degradation presumably because of the absence of the E5-encoded domain, we produced $VEGF_{179}a$ and $VEGF_{179}b$ variants that are identical to $VEGF_{189}a$ and $VEGF_{189}b$, excepted for the absence of the 10 amino acids coded by E5 (Fig 50A).

Their characterization is ongoing. As expected, both isoforms are mainly found associated with the cell layer in normal culture conditions but they can be released in the culture medium in the continuous presence of heparin (Fig 50B). For each variant 2 to 3 major products were identified by Western Blot: the full size glycosylated product (≈ 31 kD) and lower molecular weight bands (≈ 22 kD and 20 kD) likely corresponding to cleaved products. The size of these products together with the fact that only the native form of $VEGF_{179}b$ is recognized by $VEGF_{xxx}b$ antibody (Fig 50C) suggest that cleavages occur in the E6a-derived basic domain. We are currently developing a procedure, combining Avastin-Sepharose and Heparin-Sepharose Chromatography, for purifying the full-size $VEGF_{179}a$ and $VEGF_{179}b$ as illustrated in Fig 50D.

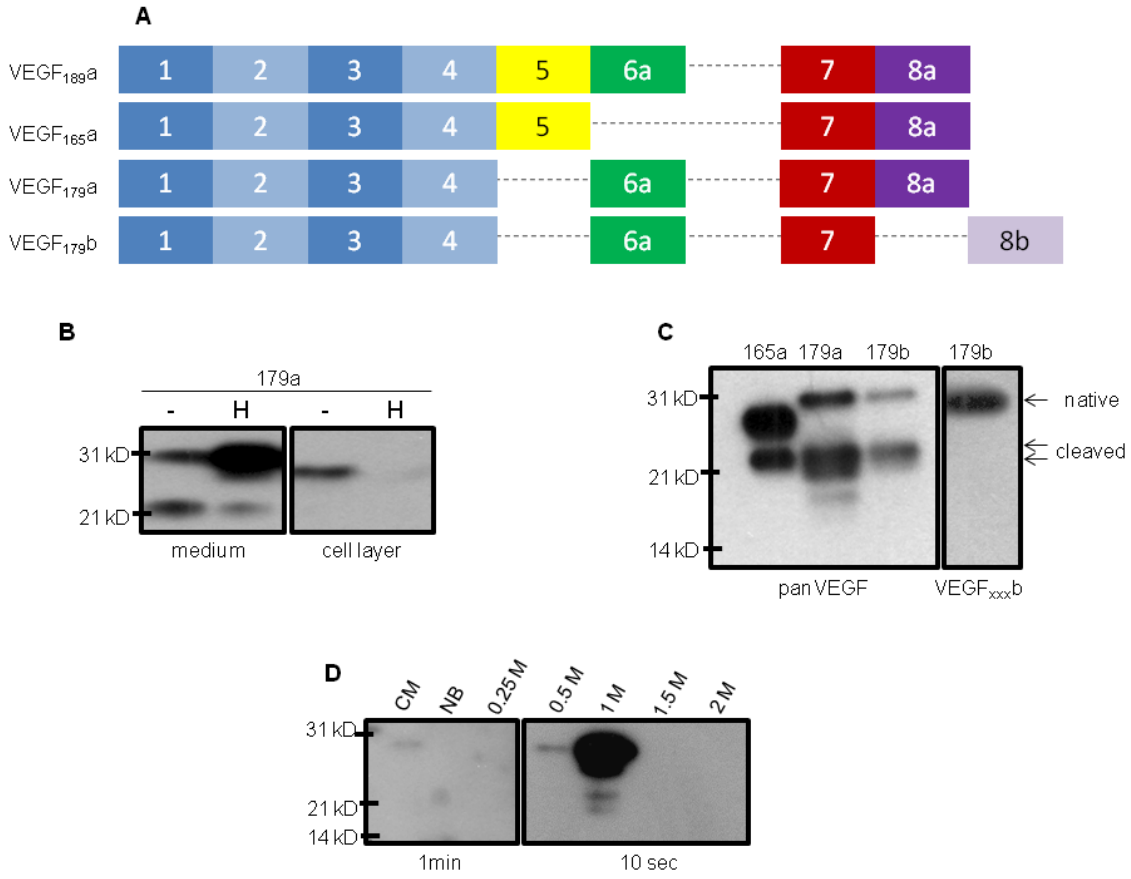


Figure 50 : Production and purification of $VEGF_{179}a$ and $VEGF_{179}b$

(A) $VEGF_{179}a$ and $VEGF_{179}b$ can be considered as $VEGF_{189}$ variants lacking the E5-encoded sequence. **(B)** Western blot analyses (panVEGF antibody) of conditioned medium **(left panel)** and cell layer **(right panel)** of HEK cells expressing $VEGF_{179}a$ cultured in presence (H) or absence (-) of heparin (50µg/ml). **(C)** Western blot analyses of conditioned medium of HEK cells expressing $VEGF_{165}a$, $VEGF_{179}a$ or $VEGF_{179}b$ using panVEGF antibody **(left panel)** and $VEGF_{xxx}b$ antibody **(right panel)**. $VEGF_{179}a$ and $VEGF_{179}b$ are both detected as a full size glycosylated product (≈30 kD) and smaller products probably resulting from proteolytic cleavage in the E6a-encoded domain (≈22 kD and 20 kD). **(D)** Western-blot (panVEGF antibody) of the different fractions collected during a Heparin Sepharose Chromatography of the conditioned medium from HEK293 expressing $VEGF_{179}a$. Two exposures times (10 sec and 1 min) are shown. The cleaved products did not bind while the full size $VEGF_{179}a$ was eluted at 1 M NaCl. 165a: $VEGF_{165}a$, 179a: $VEGF_{179}a$, 179b: $VEGF_{179}b$, CM: Conditioned Medium, NB: Not Bound fraction.

Effect of $VEGF_{179}a$ and $VEGF_{179}b$ in vivo

Besides the still ongoing *in vitro* characterizations of $VEGF_{179}a$ and $VEGF_{179}b$, they were also used *in vivo* in the model of HEK293 cells implanted in nude mice. After 3 weeks, tumours expressing $VEGF_{179}a$ or $VEGF_{179}b$ had the same general appearance as tumours expressing $VEGF_{165}a$ and $VEGF_{165}b$, respectively (Fig 51A). Immunostaining of endothelial cells (CD31, Fig 51B) or smooth muscle cells /pericytes (α-SMA, Fig 51C) was in agreement with the macroscopic observations. The vascular network consisted in enlarged vessels

covered by α-SMA positive cells for $VEGF_{179}a$ and $VEGF_{165}a$ while vessels were much smaller and immature for $VEGF_{179}b$ as also observed for $VEGF_{165}b$.

Altogether these data show that the presence of the E6a-encoded domain does not modify the VEGF-induced angiogenesis, at least in this model.

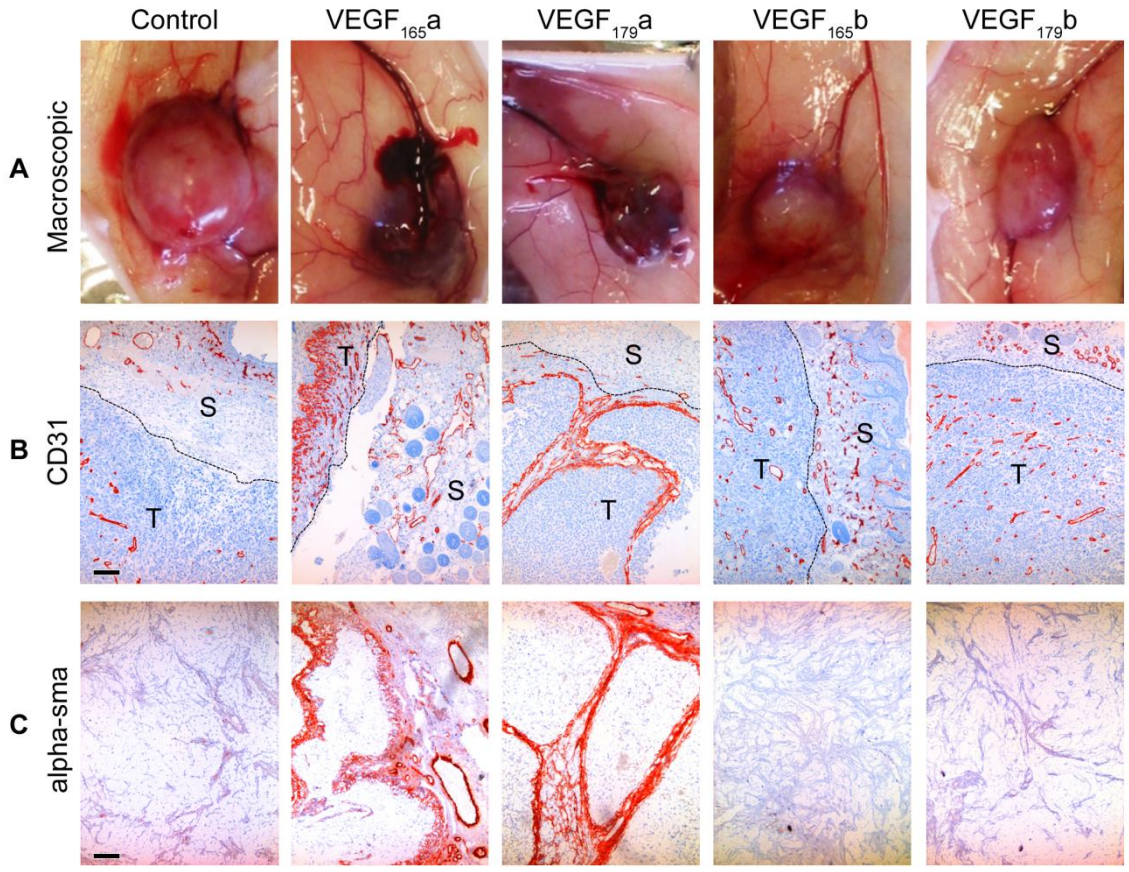


Figure 51 : *In vivo* tumour angiogenesis induced by $VEGF_{179}a$ and $VEGF_{179}b$

Two millions HEK293 cells, transfected with the empty vector (control) or expressing different VEGF variants, were mixed with Matrigel and injected in the flanks of nude mice. In this experiment, tumours expressing $VEGF_{165}a$ or $VEGF_{165}b$ were used as reference controls for $VEGF_{179}a$ and $VEGF_{179}b$, respectively **(A)** Macroscopic view of the tumours obtained in each group. Control (n=5), $VEGF_{165}a$ (n=6), $VEGF_{165}b$ (n=6), $VEGF_{179}a$ (n=4), and $VEGF_{179}b$ (n=6). **(B)** CD31 immunostaining showing the presence of blood vessels in the tumour (T) and the adjacent skin (S). **(C)** α-SMA immunostaining showing the presence of pericytes (scale bar = 100µm).

IX-2 $VEGF_{111}a$-R1m

As VEGF-R1 is considered as a decoy receptor for VEGF at endothelial cell surface and, consequently, as an inhibitor of VEGF signalling in angiogenesis, we designed, produced and purified $VEGF_{111}a$-R1m, a $VEGF_{111}a$ mutated at the three critical sites for VEGF-R1 binding (Asp^{63} mutated in Ala, Glu^{64} in Ala and Glu^{67} in Ala). In a preliminary experiment, $VEGF_{111}a$-R1m significantly triggered VEGF-R2 and ERK1/2 phosphorylation as compared

to a negative control (not illustrated). This showed that the variant was active on angiogenesis at least *in vitro*. The effects of $VEGF_{111}$a-R1m and $VEGF_{111}$a were also compared in the HEK293 tumour model. $VEGF_{111}$a-R1m displayed the same pattern of vascularization as the native $VEGF_{111}$a with the formation in the adjacent skin of the tumour of a dense network of small blood vessels (Fig 52), not covered by pericytes as highlighted by an absence of staining with an anti-α-SMA immunostaining (not illustrated).

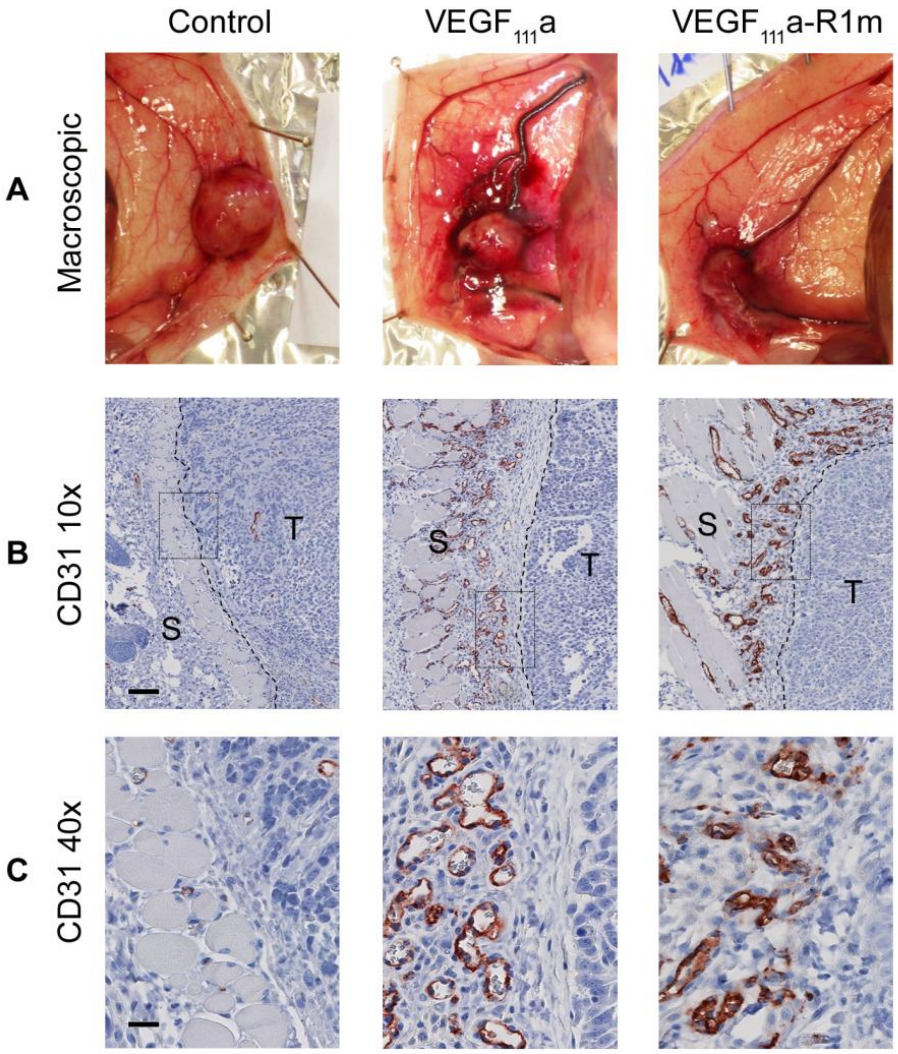


Figure 52 : *In vivo* tumour angiogenesis induced by $VEGF_{111}$a-R1m

Two millions HEK293 cells, transfected with the empty vector (control) or expressing the VEGF variants, were mixed with Matrigel and injected in the flanks of nude mice. **(A)** Macroscopic views of the tumours obtained in each group. Control (n=4), $VEGF_{111}$a (n=4), $VEGF_{111}$a-R1m (n=6). **(B)** CD31 immunostaining in the tumour (T) and the adjacent skin (S) (scale bar = 100 µm). Black lined rectangles delineate the areas of the sections represented in **(C)** at higher magnification (scale bar = 25µm).

XI- Endocan as a marker of the activity of the VEGF variants

Although the VEGF variants display specific effects *in vitro* and *in vivo*, it is not clear yet how regulations operating through a single receptor (VEGF-R2) can be responsible for such diverse responses. Endocan is a dermatan sulphate proteoglycan that promotes the activity of HGF (Sarrazin et al., 2006). It has been reported to be induced by VEGF in endothelial cells (Rennel et al., 2007). As a proteoglycan, endocan could also influence the cell response to VEGF by functioning as a co-receptor displaying preferential affinities for some specific VEGF variants. We therefore evaluated the induction of endocan secretion by our variants as an additional tool for comparing their biological activity. Its affinity towards VEGF receptors and co-receptors is currently under investigation.

XI-1 Stimulation of endocan synthesis by VEGF

HUVEC were treated with 1nM of VEGF variants for 24h or 48h. Endocan concentrations in the conditioned medium were quantified by ELISA (Fig 53). $VEGF_{165}b$ did not stimulate endocan secretion while $VEGF_{165}a$ was the most active (2.6-fold stimulation). Lower but still significant stimulations were observed for other variants (2-fold stimulation). Similar trends were observed after 48 hours (not shown) and were in good correlation with the proliferation assay data (Fig 38).

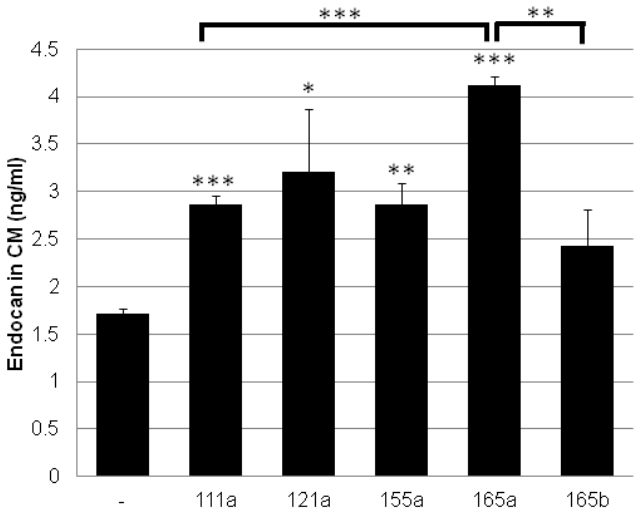


Figure 53 : Secretion of endocan from endothelial cells treated with VEGF variants

HUVEC were treated for 24h with 1nM of VEGF variants. Endocan secreted in the conditioned medium (CM) was quantified by ELISA. 111a: $VEGF_{111}a$, 121a: $VEGF_{121}a$, 155a: $VEGF_{155}a$, 165a: $VEGF_{165}a$, 165b: $VEGF_{165}b$. * $p<0.05$, ** $p<0.01$ and *** $p<0.001$ (t-test of Student).

XI-2 Binding of Endocan to VEGF-receptors and co-receptors

By SPR analyses, endocan was found to bind with high affinity to heparin (Fig 54) but not to VEGF-R2 or NRP1 (not illustrated). Binding was never observed when the two constituents of endocan, the protein core and the dermatan sulphate side chain, were injected separately. Future studies will evaluate the binding of VEGF variants on endocan and the effect of endocan on the formation of large complexes involving heparin, VEGF-R2 and NRP1.

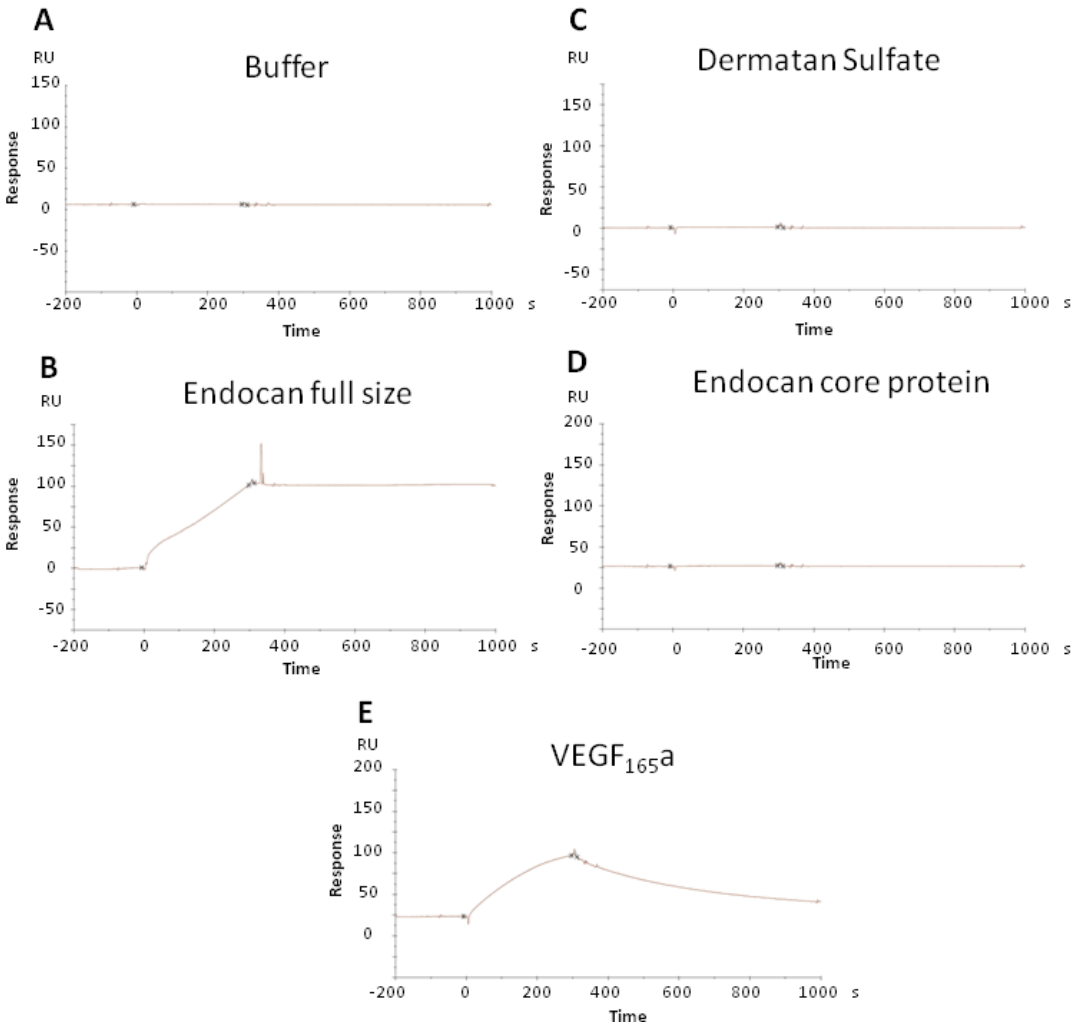


Figure 54 : Binding of endocan to heparin

Binding of endocan (40 nM) to heparin was measured by Surface Plasmon Resonance. **(A)** HBS-EP buffer, **(B)** Endocan full size, **(C)** dermatan sulfate side chain alone, **(D)** endocan core protein alone **(E)** or $VEGF_{165}a$ (40nM), used as a positive control, were injected for ~250 seconds followed by injection of HBS-EP buffer in order to visualize the dissociation rate. RU: Response in arbitrary units.

GENERAL DISCUSSION

VEGF is considered as a promising molecule for treating ischemic diseases whereas inhibiting its activity has found beneficial therapeutic applications in ocular diseases and cancers. VEGF is produced as several different isoforms resulting from a dynamic mechanism of alternative splicing of the primary transcript. The roles of the amino-terminal domains encoded by the exons 1 to 4 are well-established. They contain the signal peptide, dimerization and glycosylation sites as well as the binding domains to VEGF-R1 and VEGF-R2. The functions of the C-terminal domains are also becoming more characterized. For example, a limited proteolytic processing (mainly in the E5-encoded sequence) is required for the release and the bioavailability of the VEGF variants firmly immobilized in the ECM by their E6a-encoded domain (Keyt et al., 1996a; Lee et al., 2005; Plouet et al., 1997). The C-terminal part of VEGF is also required for interactions with NRP1. A role for the 6 amino acids encoded by E8a has been demonstrated but potential participation of other domains are still controversial (Cebe-Suarez et al., 2008; Cebe Suarez et al., 2006). An additional, but possibly related, controversy concerns the activity of the VEGF isoforms produced by differential splicing of E8 into E8a or E8b, the latter resulting in a VEGF subfamily referred to as $VEGF_{xxx}b$ (Bates et al., 2002; Harper and Bates, 2008). Although these variants were largely reported as anti-angiogenic (Bates et al., 2002; Hua et al., 2010; Rennel et al., 2009b; Woolard et al., 2004), a recent publication reports a weak pro-angiogenic effect of $VEGF_{121}b$ and $VEGF_{165}b$ (Catena et al., 2010).

To explore the functions of the C-terminal part of the VEGF-A protein, we generated and characterized the properties of a set of variants translated from mRNA containing exons 1-4, a combination of exons 5 to 7 and exon 8a or exon 8b: $VEGF_{111}a$, $VEGF_{111}b$, $VEGF_{121}a$, $VEGF_{121}b$, $VEGF_{155}a$, $VEGF_{155}b$, $VEGF_{165}a$, $VEGF_{165}b$, $VEGF_{179}a$ and $VEGF_{179}b$. $VEGF_{165}a$ and $VEGF_{121}a$ are the two most abundant pro-angiogenic isoforms (Ferrara, 2010) while $VEGF_{165}b$ and $VEGF_{121}b$ are the two most largely described endogenous $VEGF_{xxx}b$ isoforms. $VEGF_{111}a$ is the shortest isoform produced by alternative splicing that still contains the binding sites for VEGF-R1 and VEGF-R2 (Mineur et al., 2007). The other variants ($VEGF_{111}b$, $VEGF_{155}a$, $VEGF_{155}b$, $VEGF_{179}a$ and $VEGF_{179}b$) have not been observed endogenously but were designed here as tools to investigate the functions of the different domains and to evaluate their potential therapeutic interest. All these variants possess the Cys residues required for the dimerization of VEGF and the Cystine knots that participate in the correct folding of the protein (Iyer and Acharya, 2011; Muller et al., 2002). To reduce the possibility of inappropriate folding and/or glycosylation, two features that could alter the properties of our recombinant growth factors, the different VEGF isoforms were produced in human cells. The data obtained during this work have been summarized (table 8) for

facilitating the understanding of the following discussion. $VEGF_{111}a$, $VEGF_{111}b$, $VEGF_{121}a$, $VEGF_{121}b$, $VEGF_{155}a$, $VEGF_{155}b$, $VEGF_{165}a$ and $VEGF_{165}b$ were systematically compared for their physical interactions with receptors and co-receptors and their biological effect, in cell culture and *in vivo*. $VEGF_{179}a$ and $VEGF_{179}b$ were produced more recently, which explain why their characterization is only partial. The discussion will address more specifically new insights provided by our work concerning the function of the C-terminal domain of VEGF-A isoforms.

I- The domain encoded by E8a cooperates with upstream sequences for NRP1 and heparin binding

NRP1 is a co-receptor for several factors, including VEGF. The formation of a complex between VEGF-R2, VEGF and NRP1 strengthens VEGF-R2 phosphorylation, downstream signalling pathways and angiogenesis (Soker et al., 2002). Amongst the most abundant isoforms, $VEGF_{165}a$ (E1-4, 5, 7, 8a) has been reproducibly shown to bind to NRP1 while $VEGF_{121}a$ (E1-4, 5, 8a) was initially described as unable to stably interact (Herve et al., 2008). This apparent lack of high affinity binding has been later attributed to partial degradation of the E8a-encoded 6 amino acids sequence (CDKPRR:Cys-Asp-Lys-Pro-Arg-Arg) (Cebe-Suarez et al., 2008; Pan et al., 2007a; Parker et al., 2012).

By Surface Plasmon Resonance (SPR) we showed here a binding of $VEGF_{165}a$ (E1-5, 7, 8a), $VEGF_{121}a$ (E1-5, 8a) and $VEGF_{155}a$ (E1-4, 7, 8a) on NRP1 and the absence of affinity of all the $VEGF_{xxx}b$ isoforms. As the synthetic $VEGF_{159}$ variant (exons 1-5, 7) is also unable to bind (Cebe Suarez et al., 2006), it strongly suggests that the absence of affinity of the $VEGF_{xxx}b$ isoforms for NRP1 is due to the absence of the CDKPRR sequence (E8a) and not caused by the presence of the E8b-encoded SLTRKD (Ser-Leu-Thr-Arg-Lys-Asp) sequence. Interestingly, $VEGF_{111}a$ (E1-4, 8a) has no affinity for NRP1, although it possess the CDKPRR terminal sequence, demonstrating that the E8a-encoded sequence alone is not sufficient and must cooperate with another domain for efficient binding. The E5-encoded sequence is probably involved as this 10 amino acids domain represents the only difference between $VEGF_{111}a$ and $VEGF_{121}a$. In support of this hypothesis, we demonstrated that a peptide mimicking the E5-encoded sequence (R8R) blocks the binding of $VEGF_{165}a$ to NRP1, reduces the activation of VEGFR2-ERK1/2 pathway in PAEC-R2-NRP1 to a level similar to that observed in PAEC-R2 and partly inhibits the $VEGF_{165}a$-induced proliferation of HUVEC. By contrast, $VEGF_{155}a$ does not possess this domain but can bind NRP1, although with a slower association rate as compared to $VEGF_{165}a$ and $VEGF_{121}a$, suggesting a further cooperation between E8a and E7-encoded sequences.

Table 6 : Summary of the properties of the different VEGF isoforms

	Exons	Binding to			Forma-tion of complex	Resis-tance to plasmin	Signaling			Prolife-ration	In vivo angiogenesis		Vessel permeability	
		VEGF-R2	NRP1	Heparin			PAEC-R2	PAEC-R2-NRP1	HUVEC	HUVEC	Intra-tumoral	Extra-tumoral	Local	Distant
$VEGF_{111}a$	E1-4; E8a	Yes	-	-	-	Yes	+++	+++	++	++	-	+++	+++	+++
$VEGF_{111}b$	E1-4; E8b	Yes	-	-	-	Yes	+++	+++	++	++	-	+++	+++	+++
$VEGF_{121}a$	E1-4; E5; E8a	Yes	Yes	-	-	-	+++	+++	++	++	+++	++	+++	-
$VEGF_{121}b$	E1-4; E5; E8b	Yes	-	-	-	Yes	+++	+++	++	++	-	-	-	-
$VEGF_{155}a$	E1-4; E7-8a	Yes	Yes	Yes	Yes	-	+/-	+	+	++	++	+/-	++	-
$VEGF_{155}b$	E1-4; E7-8b	Yes	-	-	-	-	+/-	+/-	+/-	-	+	+	-	-
$VEGF_{165}a$	E1-4; E5; E7-8a	Yes	Yes	Yes	Yes	-	++	+++	+++	+++	+++	+	+++	-
$VEGF_{165}b$	E1-4; E5; E7-8b	Yes	-	-	-	-	+/-	+/-	+/-	-	+	+	-	-
$VEGF_{179}a$	E1-4; E6a; E7-8a	nd	nd	Yes	nd	nd	nd	nd	nd	nd	+++	+	nd	nd
$VEGF_{179}a$	E1-4; E6a; E7-8b	nd	nd	nd	nd	nd	nd	nd	nd	nd	+	+	nd	nd

E: Exon ; - : Negative ; +/- : Low ; + : Medium ; ++ : High ; +++ : Very High; nd: not determined

NMR studies have shown that an intramolecular disulfide bridge is formed between Cys^{160} (encoded by E8a) and Cys^{146} (encoded by E7) in $VEGF_{165}a$, causing a specific folding that brings the KPRR basic C-terminal sequence close to other basic residues encoded by E7 (Grunewald et al., 2010). A recent publication (Parker et al., 2012) reports that the E8a encoded C-terminal R is crucial for NRP1 binding and that the E7-derived sequence allows additional interactions that are however not essential since $VEGF_{121}a$ can bind NRP1. In the light of our results, we suggest that the maximal direct interaction between VEGF and NRP1 involves three domains. The E8a-encoded 6 amino acids are crucial but need to cooperate with the E5 and/or the E7 sequences (Fig 52) to adopt an optimal 3D-structure. Such cooperation between these three domains are likely to participate in the specificity of the effects induced by the different VEGF isoforms.

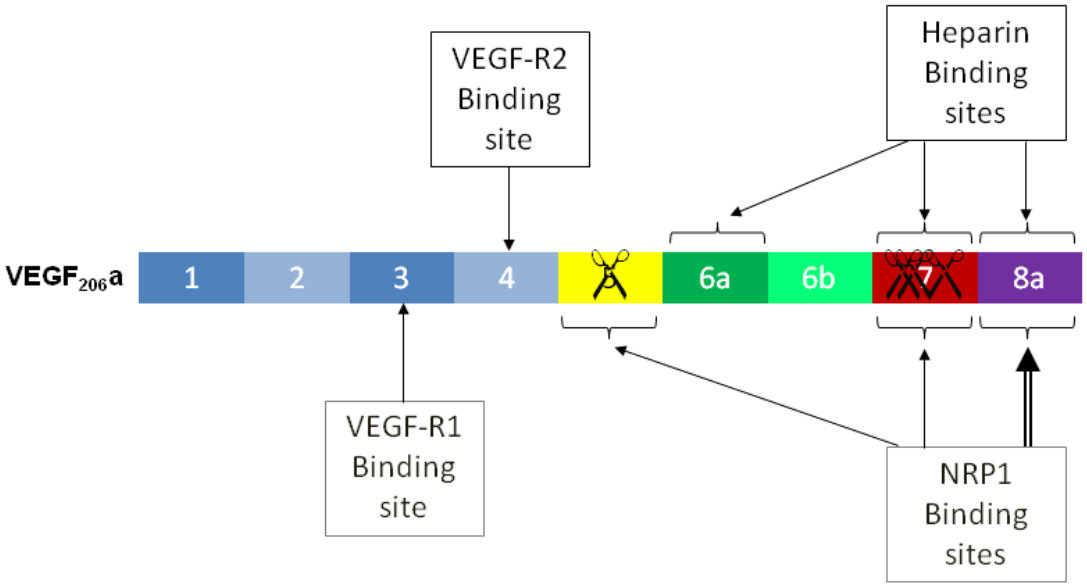


Figure 55 : Roles of the exon-encoded domains of VEGF for (co-)receptors binding and susceptibility to cleavage by plasmin

This drawing is based on previous publications and on SPR studies performed during this work. More specifically, we have demonstrated that the E5-encoded sequence is crucial for efficient binding to NRP1 and that maximal interactions require the presence of the domains encoded by E5, E7 and E8a. Similar cooperation have been demonstrated for efficient heparin binding. It also shows preferential sites of proteolytic degradation (black scissors). This work confirmed that plasmin cleaves VEGF in E5-encoded domain and highlights that in absence of this sequence, additional sites of cleavage can be found in E7 encoded sequence.

The use of heparin *in vitro* allows mimicking the effect of HSPG found in the extracellular matrix. A strong binding of $VEGF_{189}a$ and $VEGF_{206}a$ to heparin and HSPG occurs through the highly basic E6a-encoded domain (Houck et al., 1991). Such strong affinity was also observed here for $VEGF_{179}a$ for example. As this domain is not present in the 8 variants extensively characterized in this work, this gave us the opportunity to investigate the potential role of domains with lower affinities. We showed that only variants possessing the E7+E8a domains ($VEGF_{165}a$ and $VEGF_{155}a$) are able to bind to heparin (Fig 52). This is in agreement with a lack of retention of $VEGF_{165}b$ on heparin-Sepharose (Cebe Suarez et al., 2006) or on HSPG coated nitrocellulose filters (Kawamura et al., 2008b). These data indicate that E7 and E8a both contribute to heparin binding of VEGF. A possible explanation relies on the formation of the disulfide bridge between Cys^{146} and Cys^{160} and on the proper positioning of basic residues present in E7 and E8a. Alternatively, the SLTRKD sequence from E8b, especially the acidic aspartic acid terminal residue, might prevent the interaction between heparin and the basic residues oriented at the surface of the E7 encoded domain.

In the SPR assays, injection of $VEGF_{165}a$ in presence of heparin drastically increased the binding to NRP1 as compared to injection of $VEGF_{165}a$ alone. Conversely injection of $VEGF_{165}a$ in presence of soluble NRP1 drastically enhanced the binding to heparin as compared to injection of $VEGF_{165}a$ alone. These results are in agreement with a model describing two types of $VEGF_{165}a$-NRP1 interaction, one being direct and the other bridged by heparin (Vander Kooi et al., 2007). Both of them implicate the E7-E8a domain and may synergize. Since the effect of heparin on the binding of $VEGF_{155}a$ to NRP1 is much more modest than the effect observed for $VEGF_{165}a$, it strongly suggests that the highly basic E5-derived domain is also involved. It remains to determine however whether this domain interacts with NRP1 independently or in synergy with the E7/E8a encoded sequence for forming a large negatively charged pocket.

II- VEGF variants display diverse relative activities in different models due to dynamic interactions with receptors and co-receptors

The specific effects of the different VEGF variants were extensively explored at a cellular level in HUVEC and Porcine Aortic Endothelial Cells (PAEC) expressing VEGF-R1 only (PAEC-R1), VEGF-R2 only (PAEC-R2), NRP1 only (PAEC-NR1) or both VEGF-R2 and NRP1 (PAEC-R2-NRP1). PAEC-R1 and PAEC-NRP1 do not respond significantly to VEGF. When only VEGF-R2 is expressed (PAEC-R2), short variants ($VEGF_{111}a/b$ and $VEGF_{121}a/b$)

are systematically and significantly more active than $VEGF_{165}$a. This difference no longer exists in PAEC-R2-NRP1, illustrating the importance of NRP1 in the full activity of $VEGF_{165}$a. In HUVEC, $VEGF_{165}$a is the most active in terms of intracellular signalling and cell proliferation.

These apparently surprising observations can be explained by considering the dynamic interactions between the VEGF variants and their receptors and co-receptors. For clarity reasons, $VEGF_{111}$a and $VEGF_{165}$a will be only considered here although the described mechanisms would affect similarly the activity of the other VEGF variants. As predicted by computational models (Mac Gabhann et al., 2006; Mac Gabhann and Popel, 2005, 2007a, b), the overexpression of recombinant VEGF receptors at the PAEC membrane induces a "Dynamic Pre-Dimerization" process in absence of any ligand (Fig 53A), while in more physiological situations most of these receptors are monomeric (Mac Gabhann and Popel, 2007a).

In PAEC-R2 (Fig 53A), the higher activity of $VEGF_{111}$a as compared to $VEGF_{165}$a probably results from two different features. In absence of the E5-E7 encoded regions, $VEGF_{111}$a has no affinity for the ECM, which explains higher diffusibility and bioavailability. Moreover, its small size should improve its accessibility in the binding pockets present in the pre-dimerized receptors. This is supported by SPR data evaluating the binding of VEGF variants on VEGF-R2. In this model where VEGF-R2 are pre-dimerized because of the Fc fragments, $VEGF_{111}$a has a higher association rate as compared to $VEGF_{165}$a while the Kd are similar. This strongly suggests that a limiting step is accessibility to the binding pocket within the dimer.

In PAEC-R2-NRP1 (Fig 53B), VEGF-R2 is expected to be also pre-dimerized as in PAEC-R2. However the presence of NRP1 increases the relative activity of $VEGF_{165}$a by acting as a co-receptor (Becker et al., 2005; Soker et al., 2002) while it does not affect the signals triggered by $VEGF_{111}$a, explaining why the signalling by both variants is now similar.

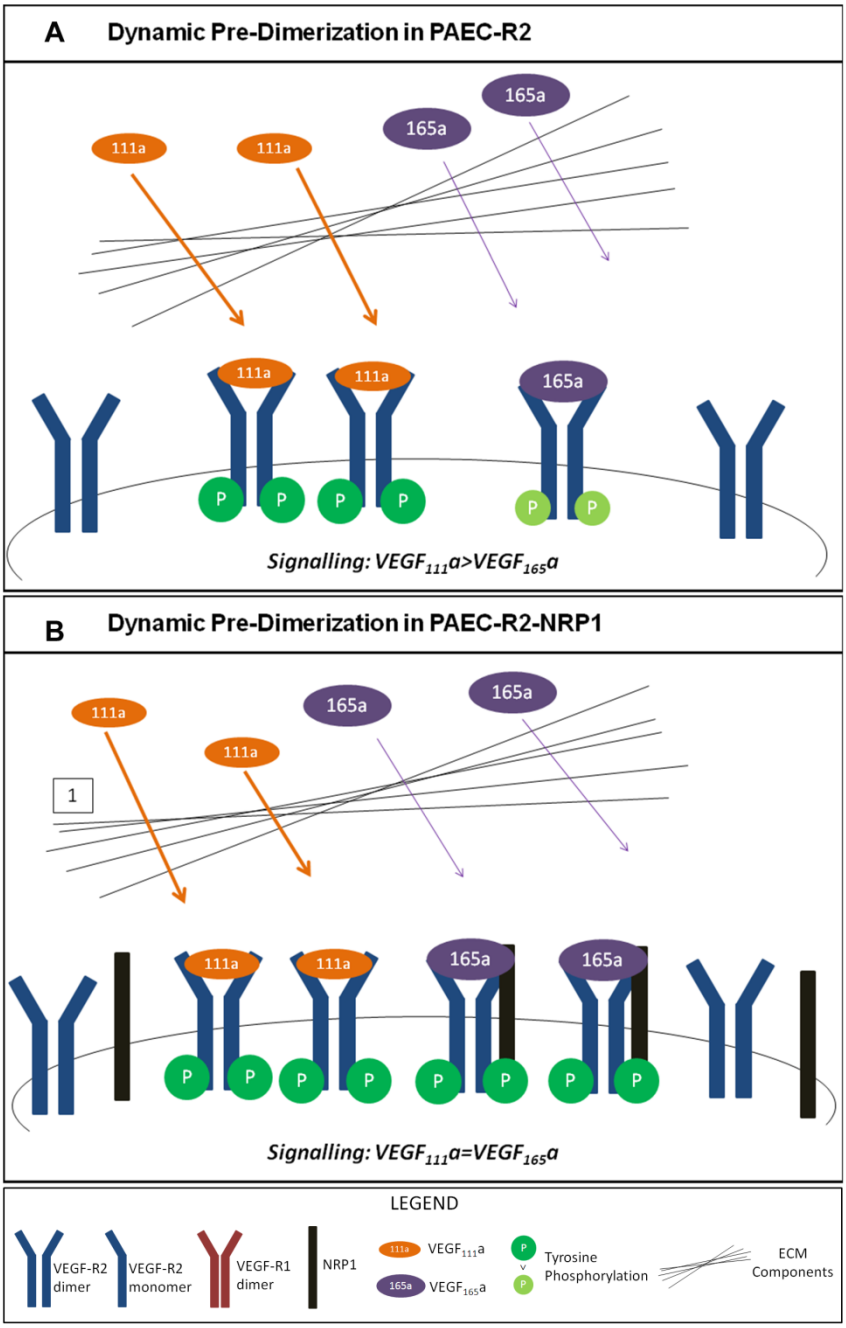


Figure 56 : Modelisation of VEGF-R2 activation by $VEGF_{111}a$ and $VEGF_{165}a$ in PAEC-R2 (A) and PAEC-R2-NRP1 (B)

According to *in silico* models, a high number of VEGF-R2, as found at the surface of PAEC-R2 and PAEC-R2-NRP1, should result in a "Dynamic Pre-Dimerization" process. As $VEGF_{111}a$ has much more limited interactions with the ECM or the cell surface components than $VEGF_{165}a$, it can bind more rapidly to pre-dimerized VEGF-R2. Its small size could also favour its interactions with the pre-dimerized binding pockets. These two features would explain the high activity of $VEGF_{111}a$ in PAEC-R2. **(B)** The additional presence of NRP1 is not expected to modify the pre-dimerized process. However, it should favour the "presentation" of $VEGF_{165}a$, but not of $VEGF_{111}a$, to VEGF-R2, explaining the increased activity of $VEGF_{165}a$ only.

The situation is completely different in HUVEC (Fig 54) because several receptors and co-receptors (VEGF-R1, VEGF-R2, NRP1, NRP2 and HSPG) are expressed at the cell surface, all of them at more physiological levels than when expressed as recombinant proteins (Herve et al., 2008). In these cells, VEGF-R1 and VEGF-R2 are not pre-dimerized, which should strongly reduce problems related to accessibility, mainly for the longer isoforms. The presence of NRP1, and potentially NRP2, at the cell surface has several consequences, either clearly demonstrated or suggested by our data and other publications (Favier et al., 2006; Soker et al., 1998). NRP1 favours the binding of $VEGF_{165}a$ on VEGF-R2 monomers, which induces dimer formation and downstream signalling (Fig 54A). Such mechanism is not possible for VEGF variants that do not bind NRP1, such as $VEGF_{111}a$. As another potential mechanism, it was also reported that VEGFR2-$VEGF_{165}a$-NRP1 complexes are internalized in specific endosomes (characterized by the presence of Rab 11) where they continue to activate downstream pathways (Ballmer-Hofer et al., 2011). In absence of NRP1, the endosomal trafficking is modified and degradation occurs more rapidly, which suppresses the VEGF-induced signalling.

The presence of VEGF-R1 might have also important consequences (Fig 54B). Although it has high affinity for all the tested VEGF variants (SPR data) it does not induce a significant downstream signalling upon VEGF binding (PAEC-R1 in culture). At the surface of HUVEC, it sequesters VEGF isoforms, reducing their bioavailability for VEGF-R2. While it should affect similarly the effect of the different VEGF variants, this is not the case because of the presence of NRP1. It has been shown indeed that NRP1 can decrease the binding to VEGF-R1 of $VEGF_{165}a$ but not of $VEGF_{121}a$ and probably not of $VEGF_{111}a$ (Fuh et al., 2000). It could also favour the release of $VEGF_{165}a$ already bound to VEGF-R1, which would increase its availability for VEGF-R2. Although not tested specifically here, it is most likely that the release from VEGF-R1 by NRP1 is directly related to the affinity of VEGF variants for NRP1. These dynamic and finely tuned interactions involving several molecules would explain some apparent discrepancies between data obtained in different types of models, such as short term VEGF-R2 phosphorylation and cell proliferation for example. For *in vivo* analyses, especially regarding clinical applications, it underlines also the critical need of studying the expression and the effect of individual variants rather than considering them globally as VEGF-A factors having identical properties.

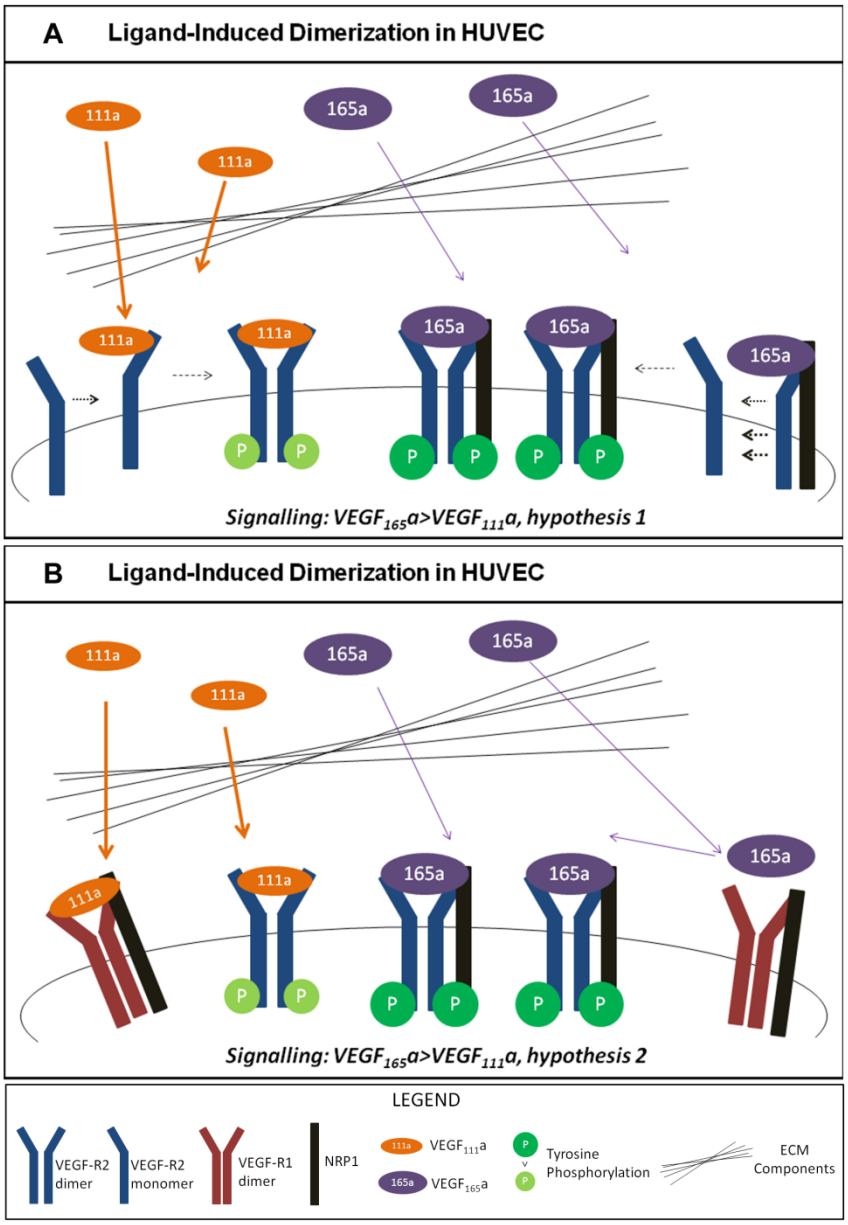


Figure 57 : Modelisation of VEGF-R2 activation by $VEGF_{111}a$ and $VEGF_{165}a$ in HUVEC

As compared to the different PAEC models, HUVEC express at their surface a more physiological number of VEGF-R1, VEGF-R2 or NRP1. The expression of NRP2 and of endothelial cell specific proteoglycans is not illustrated here although it is expected to fine tune the response of HUVEC to VEGF. Several non mutually exclusive mechanisms could explain the stronger effects of $VEGF_{165}a$ in these cells as compared to other isoforms, including $VEGF_{111}a$. Two are illustrated here. **(A)** As VEGF-R2 is monomeric, the small size of $VEGF_{111}a$ is no longer an advantage for binding. Moreover, the presence of NRP1 should favour the binding of $VEGF_{165}a$ on VEGF-R2, which should stimulate its dimerization and trans-phosphorylation, and finally downstream signalling pathway. **(B)** It has been reported that NRP1 could reduce interactions between VEGF-R1 and $VEGF_{165}a$, making it more available for VEGF-R2 binding and signalling. This mechanism is unlikely for $VEGF_{111}a$ as it does not bind to NRP1.

III- The E8b-encoded sequence is not sufficient to determine the anti-angiogenic activity of $VEGF_{xxx}b$ variants

The effect of substituting the E8a-encoded sequence by its corresponding E8b counterpart was carefully investigated. The activity of the longest isoforms ($VEGF_{155}b$ and $VEGF_{165}b$) was strongly affected by the substitution, whatever the cell model and independently of the presence of NRP1. These two VEGF variants were also able to prevent the $VEGF_{165}a$-induced proliferation of HUVEC, probably by a competition for VEGF-R2 as suggested by SPR data. It has been suggested that $VEGF_{165}b$ induces an incorrect relative positioning/orientation of the two VEGF-R2 molecules to form a functional dimer, which would prevent their efficient transphosphorylation (Kawamura et al., 2008b). This hypothesis is confirmed here since $VEGF_{165}b$ binds to VEGF-R2 (SPR) but is unable to induce a robust phosphorylation of the VEGF-R2 and the downstream intracellular signalling factors.

In sharp contrast, $VEGF_{121}b$ and $VEGF_{111}b$ were pro-angiogenic *in vitro*, demonstrating that the presence of the E8b domain alone is not sufficient to convert an endothelial activating VEGF variant into an inhibitory factor. This observation does not correlate with previous data describing the anti-angiogenic properties of $VEGF_{121}b$ (Rennel et al., 2009b), but it is worth mentioning that these experiments were essentially performed in other models, including cell migration or tumour formation by cells expressing $VEGF_{121}b$. Our *in vitro* data were validated *in vivo* in the two models of induced pathological angiogenesis in the eye that were previously used for the demonstration of the anti-angiogenic properties of $VEGF_{165}b$ (Hua et al., 2010; Magnussen et al., 2010). In contrast to $VEGF_{165}b$, injection of $VEGF_{111}b$ did not inhibit, and even slightly stimulated, ocular angiogenesis, confirming our *in vitro* data. It is therefore unlikely that the SLTRKD sequence encoded by E8b is by itself sufficient for the specific properties of $VEGF_{165}b$. This hypothesis is further strengthened by data showing that soluble SLTRKD peptide is not anti-angiogenic (unpublished observations, Rennel and Bates). Our data showing that $VEGF_{155}b$ and $VEGF_{165}b$ (and potentially $VEGF_{179}b$, as described below) have the same properties suggest that the folding of the domain formed by the E7 and E8b-encoded sequences would induce steric hindrance and lead to modification of the conformation of the VEGF dimer, which would ultimately prevent perfect VEGF-R2 dimerization and autophosphorylation (Harper and Bates, 2008). This might also explain why $VEGF_{111}b$, although possessing the SLTRKD terminal sequence, is pro-angiogenic due to the lack of the E7-derived sequence and because its size is not sufficient to interfere with receptor dimerization.

IV- Combinations of the E5, E7 and E8a/E8b-encoded domains determine vessel density and patterning *in vivo*

Subcutaneous injection of HEK293 cells provides an attractive model to evaluate the long term effects of the continuous expression of specific VEGF variants on angiogenesis (Mineur et al., 2007). Indeed, these cells express low levels of endogenous VEGF and form a tumour-like mass containing few blood vessels and growing independently of VEGF expression, which facilitates data analyses by dissociating vascularization from other features. The expression of $VEGF_{165}a$, $VEGF_{155}a$ or $VEGF_{121}a$ induced a dense vascularization of the tumor and led to the formation of enlarged blood vessels while $VEGF_{111}a$ or $VEGF_{111}b$ resulted in the presence of hundreds of small vessels but only in the adjacent dermis. Reasons for such differences remain to be firmly identified. It is not related to variability in protein production and receptor binding as all the variants were expressed in the tumour at similar levels and had comparable affinity for VEGF-R2. Different retention times in the Matrigel plug embedding the cells were also considered as potentially regulating the available VEGF concentration. This was evaluated *in vitro* by measuring the release of purified VEGF from a Matrigel plug immersed in culture medium. Diffusion rate was maximal for $VEGF_{111}a$, the equilibrium between plug and medium concentrations being observed after 2 to 4 hours. All the other tested isoforms reached equilibrium within 8-20 hours. Although the differences are significant, they are not expected to play a major role in our model of tumour angiogenesis as the duration of the entire experiment is much longer (3 weeks) and because the Matrigel plug is rapidly degraded in growing tumours. These data suggest however that $VEGF_{111}a$ and $VEGF_{111}b$ are able to diffuse more freely than the other isoforms and therefore to induce angiogenesis at longer distance from their site of production. This hypothesis was further evaluated *in vivo* in complementary models.

In the Miles and Miles' modified assay $VEGF_{165}a$, $VEGF_{111}a$ and $VEGF_{111}b$ induce a similar vascular permeability close to the site of injection. However the total surface of affected skin was much larger for $VEGF_{111}a$ and $VEGF_{111}b$ which demonstrates their capacity to diffuse at long distance in tissues *in vivo.* In another model, VEGF variants were injected intraperitoneously and subcutaneously and their blood concentrations were evaluated as a function of time. $VEGF_{111}a$ was found at higher concentration and with a longer half-life as compared to $VEGF_{165}a$ for example, which again illustrates its high capacity to diffuse and its reduced interactions with the extracellular environment.

The similar properties of $VEGF_{111}a$ and $VEGF_{111}b$ *in vivo* were expected from *in vitro* data. They further confirmed that the E8b domain alone is not sufficient to repress angiogenesis. By contrast, the weak pro-angiogenic activities of $VEGF_{155}b$ and $VEGF_{165}b$ in the tumoural

model were surprising as they were inactive or even inhibitors in other models. Proteolytic processing in the particular tumour environment, which is usually not observed in culture medium and in the eye (Fig 46A) (Magnussen et al., 2010), could account for this apparent discrepancy. The cleavage in the E5/E7 domains would shorten $VEGF_{165}b$, prevent the steric hindrance induced by the substitution of E8a by the E8b sequence and thus convert an anti-angiogenic variant into moderately pro-angiogenic processed forms (Fig 55B). The strongly limited effect of $VEGF_{121}b$ *in vivo*, while it was pro-angiogenic in cell culture is not yet understood. Dose-response and time-course experiments performed *in vitro* do not show marked differences between $VEGF_{121}b$ and $VEGF_{121}a$, $VEGF_{111}a$ or $VEGF_{111}b$. Its remarkable resistance to degradation could potentially be implicated but the involved mechanisms still remain elusive.

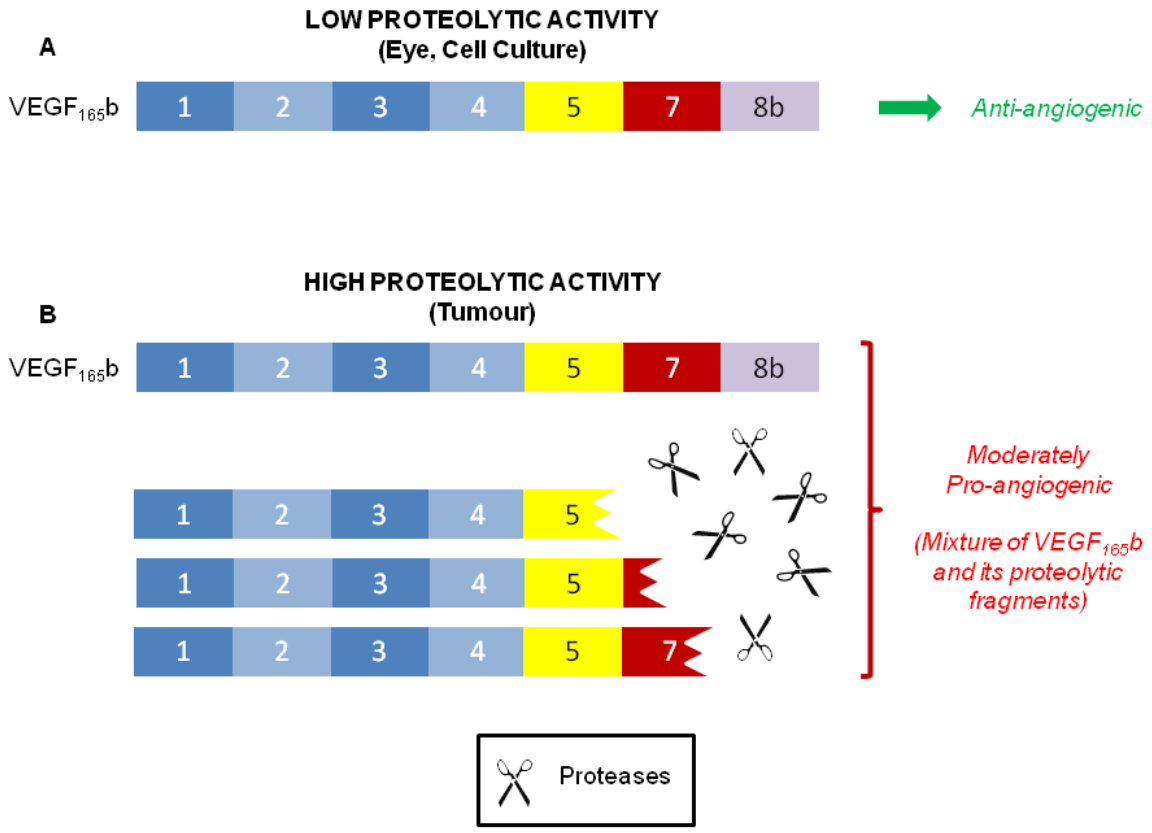


Figure 58 : The activity of $VEGF_{165}b$ may be regulated by proteolytic processing

(A) In environments characterized by low protease activity, $VEGF_{165}b$ is not cleaved, which preserved its anti-angiogenic function. **(B)** In presence of active proteases, such as plasmin or MMP, most of the $VEGF_{165}b$ is processed (in the E5- and E7- derived domains) into smaller moderately pro-angiogenic fragments.

In all the experimental conditions, the blood vessels formed in or close to the tumours were lined by a continuous basement membrane and were functional as evidenced by the presence of red blood cells. However, only $VEGF_{165}$a and, to a much lesser extent, $VEGF_{121}$a and $VEGF_{155}$a promoted the recruitment of pericytes/alpha-SMA positive cells around the vessels. These three variants are able to bind NRP1, suggesting a differential and more sustained regulation of endothelial cells that would subsequently recruit pericytes likely by secreting PDGF-B (Hellstrom et al., 1999; Lindahl et al., 1997). It would be interesting to evaluate the PDGF-B secretion by HUVEC treated by our VEGF variants. Alternatively, a direct recruitment of pericytes by some VEGF variants might be hypothesized since these cells have been suggested to respond to $VEGF_{165}$a by a NRP1-VEGFR2 axis (Liu et al., 2005) and/or by a NRP1-VEGFR1-PI3K axis (Banerjee et al., 2008).

CONCLUSIONS AND PERSPECTIVES

Our work aimed at a better understanding of the functions and cooperation between the C-terminal domains of VEGF-A. Here, we provide new data demonstrating that the E5-encoded domain regulates the VEGF properties by modifying its resistance to degradation, its capacity to diffuse and its binding to NRP1. We confirmed some previously reported effects of $VEGF_{xxx}b$ isoforms but we further showed that their anti-angiogenic properties strongly depend upon the domain organization of the VEGF molecule itself and upon the cell and tissue environment. The therapeutic use of $VEGF_{xxx}b$ isoforms in clinic as anti-angiogenic molecules should therefore be considered in the context of isoforms resistant to degradation which could improve their effect. We have demonstrated that some VEGF variants induce the formation of small and poorly matured vessels at distance of their site of secretion while other isoforms trigger locally the formation of large vessels covered by pericytes. According to these data, we suggest also that the treatment of ischemic diseases by VEGF should probably benefit from using a combination of different isoforms synergizing for inducing a more robust angiogenesis. Among the different perspectives resulting from this work, some related fields of research seem more promising and would merit further studies.

I- Regulation of "tip"/"stalk" cells phenotype by VEGF variants

The specific vascular patterns observed *in vivo* during our work demonstrate that different VEGF variants affect differentially sprouting angiogenesis and blood vessel maturation. Today the Notch receptor/ Notch ligand pathway is identified as one of the most critical cascade regulating the "tip"/ "stalk" fate during angiogenesis.

In a preliminary experiment, we observed that $VEGF_{165}a$, but not $VEGF_{111}a$, stimulates the expression of Dll4 (a notch ligand). This could explain why $VEGF_{165}a$ favours the formation of mature and enlarged vessels ("stalk" cell phenotype) and why $VEGF_{111}a$ induces only poorly the Notch pathway which would shift the balance to "tips" cell phenotype and abundant sprouting. These observations should be confirmed and strengthened in other models. All the members of this regulating cascade (Notch 1 and 2; Jagged 1 and 2; Dll1 and 4) should be also evaluated simultaneously as some of them have opposing effects on angiogenesis and compete for an identical receptor, as shown for Jagged 1 and Dll4 for example (Kume, 2012). Identifying the entire regulatory pathway, from VEGF binding at the cell surface to transcriptomic modifications, responsible for such marked differences in the regulation of endothelial cell fate would be most interesting for future clinical applications. In the same context, endocan could be used as a helpful marker, as it has been reported to be more specifically produced by tip cells (Abid et al., 2006; Recchia et al., 2010; Sarrazin et al.,

2006; Strasser et al., 2010) and to be induced more efficiently by $VEGF_{165}a$ than by the other isoforms.

II- Regulation of VSMC/pericytes by VEGF variants

In our work we have shown that maturation of blood vessels by recruitment of smooth muscle cells/pericytes is much faster and efficient with $VEGF_{165}a$. Several hypotheses could explain this specific effect and should be verified. As a first example, endothelial cells treated with $VEGF_{165}a$ could express a specific pattern of growth factors (as PDGF-B) and cytokines that would, in a second step, influence the phenotype of the pericytes. As a second example, $VEGF_{165}a$ could have a more direct effect on pericytes, either by direct signalling through NRP1 or by facilitating the formation of larges complexes containing VEGF-R1 and NRP1 for instance. These hypotheses could be first evaluated by transcriptomic analyses of endothelial cells and pericytes, cultured alone or in co-culture models and treated with different VEGF variants. Production and evaluation of the effects of $VEGF_{165}a$ mutated at the binding site for VEGF-R2 would also be useful for better defining VEGF functions related to the unique binding to NRP1, both in endothelial cells and VSMC/Pericytes.

III- Development of new VEGF variants for future clinical applications

Based on our data new types of variants with specific or improved properties could be envisaged. As a first example, modified versions of $VEGF_{165}b$ that would be more resistant to proteolysis and/or that would possess a C-terminal domain of increasing length for interfering with VEGF-R2 dimerization would be worth to be evaluated as anti-angiogenic factors.

We have shown that $VEGF_{111}a$ and $VEGF_{111}b$ are pro-angiogenic and resistant to proteolysis. They are also freely diffusible which is an advantage for systemic or long distance effects. It limits however their use for local applications. Replacement of the E8a- or E8b-derived sequence by a short domain possessing affinity for cell surface or ECM components could help to solve this problem.

Besides the development of new molecules, the effects of the association of already available variants should be more extensively characterized as their complementary properties could possibly improve the treatment of ischemic diseases. Experiment using $VEGF_{111}a$ only have been performed in several models including skin wound healing, heart infarction (in collaboration with FATH and CARD, Université Catholique de Louvain) and

ovary fragments grafting (Labied et al., 2013) (see annex 2). Some of these studies could be repeated for evaluating the benefits of using simultaneously several VEGF isoforms.

BIBLIOGRAPHY

Abid, M.R., Yi, X., Yano, K., Shih, S.C., and Aird, W.C. (2006). Vascular endocan is preferentially expressed in tumor endothelium. Microvascular research *72*, 136-145.

Abu-Ghazaleh, R., Kabir, J., Jia, H., Lobo, M., and Zachary, I. (2001). Src mediates stimulation by vascular endothelial growth factor of the phosphorylation of focal adhesion kinase at tyrosine 861, and migration and anti-apoptosis in endothelial cells. The Biochemical journal *360*, 255-264.

Adamis, A.P., Altaweel, M., Bressler, N.M., Cunningham, E.T., Jr., Davis, M.D., Goldbaum, M., Gonzales, C., Guyer, D.R., Barrett, K., and Patel, M. (2006). Changes in retinal neovascularization after pegaptanib (Macugen) therapy in diabetic individuals. Ophthalmology *113*, 23-28.

Adams, R.H., and Alitalo, K. (2007). Molecular regulation of angiogenesis and lymphangiogenesis. Nature reviews Molecular cell biology *8*, 464-478.

Adams, R.H., and Eichmann, A. (2010). Axon guidance molecules in vascular patterning. Cold Spring Harbor perspectives in biology *2*, a001875.

Adnane, L., Trail, P.A., Taylor, I., and Wilhelm, S.M. (2006). Sorafenib (BAY 43-9006, Nexavar), a dual-action inhibitor that targets RAF/MEK/ERK pathway in tumor cells and tyrosine kinases VEGFR/PDGFR in tumor vasculature. Methods in enzymology *407*, 597-612.

Aiello, L.P., Avery, R.L., Arrigg, P.G., Keyt, B.A., Jampel, H.D., Shah, S.T., Pasquale, L.R., Thieme, H., Iwamoto, M.A., Park, J.E.*, et al.* (1994). Vascular endothelial growth factor in ocular fluid of patients with diabetic retinopathy and other retinal disorders. The New England journal of medicine *331*, 1480-1487.

Allain, B., Jarray, R., Borriello, L., Leforban, B., Dufour, S., Liu, W.Q., Pamonsinlapatham, P., Bianco, S., Larghero, J., Hadj-Slimane, R.*, et al.* (2012). Neuropilin-1 regulates a new VEGF-induced gene, Phactr-1, which controls tubulogenesis and modulates lamellipodial dynamics in human endothelial cells. Cellular signalling *24*, 214-223.

Allt, G., and Lawrenson, J.G. (2001). Pericytes: cell biology and pathology. Cells, tissues, organs *169*, 1-11.

Almog, N., Ma, L., Raychowdhury, R., Schwager, C., Erber, R., Short, S., Hlatky, L., Vajkoczy, P., Huber, P.E., Folkman, J.*, et al.* (2009). Transcriptional switch of dormant tumors to fast-growing angiogenic phenotype. Cancer research *69*, 836-844.

Amin, E.M., Oltean, S., Hua, J., Gammons, M.V., Hamdollah-Zadeh, M., Welsh, G.I., Cheung, M.K., Ni, L., Kase, S., Rennel, E.S.*, et al.* (2011). WT1 mutants reveal SRPK1 to be a downstream angiogenesis target by altering VEGF splicing. Cancer cell *20*, 768-780.

Ancelin, M., Buteau-Lozano, H., Meduri, G., Osborne-Pellegrin, M., Sordello, S., Plouet, J., and Perrot-Applanat, M. (2002). A dynamic shift of VEGF isoforms with a transient and selective progesterone-induced expression of VEGF189 regulates angiogenesis and vascular permeability in human uterus. Proceedings of the National Academy of Sciences of the United States of America *99*, 6023-6028.

Aouadi, M., Binetruy, B., Caron, L., Le Marchand-Brustel, Y., and Bost, F. (2006). Role of MAPKs in development and differentiation: lessons from knockout mice. Biochimie *88*, 1091-1098.

Appleton, B.A., Wu, P., Maloney, J., Yin, J., Liang, W.C., Stawicki, S., Mortara, K., Bowman, K.K., Elliott, J.M., Desmarais, W.*, et al.* (2007). Structural studies of neuropilin/antibody complexes provide insights into semaphorin and VEGF binding. The EMBO journal *26*, 4902-4912.

Artac, R.A., McFee, R.M., Smith, R.A., Baltes-Breitwisch, M.M., Clopton, D.T., and Cupp, A.S. (2009). Neutralization of vascular endothelial growth factor antiangiogenic isoforms is more effective than treatment with proangiogenic isoforms in stimulating vascular development and follicle progression in the perinatal rat ovary. Biology of reproduction *81*, 978-988.

Ashikari-Hada, S., Habuchi, H., Kariya, Y., and Kimata, K. (2005). Heparin regulates vascular endothelial growth factor165-dependent mitogenic activity, tube formation, and its receptor phosphorylation of human endothelial cells. Comparison of the effects of heparin and modified heparins. The Journal of biological chemistry *280*, 31508-31515.

Baba, T., McLeod, D.S., Edwards, M.M., Merges, C., Sen, T., Sinha, D., and Lutty, G.A. (2012). VEGF 165 b in the developing vasculatures of the fetal human eye. Developmental dynamics : an official publication of the American Association of Anatomists *241*, 595-607.

Bachelder, R.E., Crago, A., Chung, J., Wendt, M.A., Shaw, L.M., Robinson, G., and Mercurio, A.M. (2001). Vascular endothelial growth factor is an autocrine survival factor for neuropilin-expressing breast carcinoma cells. Cancer research *61*, 5736-5740.

Bacic, M., Edwards, N.A., and Merrill, M.J. (1995). Differential expression of vascular endothelial growth factor (vascular permeability factor) forms in rat tissues. Growth Factors *12*, 11-15.

Bagri, A., Tessier-Lavigne, M., and Watts, R.J. (2009). Neuropilins in tumor biology. Clinical cancer research : an official journal of the American Association for Cancer Research *15*, 1860-1864.

Ballmer-Hofer, K., Andersson, A.E., Ratcliffe, L.E., and Berger, P. (2011). Neuropilin-1 promotes VEGFR-2 trafficking through Rab11 vesicles thereby specifying signal output. Blood *118*, 816-826.

Baltes-Breitwisch, M.M., Artac, R.A., Bott, R.C., McFee, R.M., Kerl, J.G., Clopton, D.T., and Cupp, A.S. (2010). Neutralization of vascular endothelial growth factor antiangiogenic isoforms or administration of proangiogenic isoforms stimulates vascular development in the rat testis. Reproduction *140*, 319-329.

Banerjee, S., Mehta, S., Haque, I., Sengupta, K., Dhar, K., Kambhampati, S., Van Veldhuizen, P.J., and Banerjee, S.K. (2008). VEGF-A165 induces human aortic smooth muscle cell migration by activating neuropilin-1-VEGFR1-PI3K axis. Biochemistry *47*, 3345-3351.

Barleon, B., Sozzani, S., Zhou, D., Weich, H.A., Mantovani, A., and Marme, D. (1996). Migration of human monocytes in response to vascular endothelial growth factor (VEGF) is mediated via the VEGF receptor flt-1. Blood *87*, 3336-3343.

Bates, D.O., Catalano, P.J., Symonds, K.E., Varey, A.H., Ramani, P., O'Dwyer, P.J., Giantonio, B.J., Meropol, N.J., Benson, A.B., and Harper, S.J. (2012). Association between VEGF Splice Isoforms

and Progression-Free Survival in Metastatic Colorectal Cancer Patients Treated with Bevacizumab. Clinical cancer research : an official journal of the American Association for Cancer Research.

Bates, D.O., Cui, T.G., Doughty, J.M., Winkler, M., Sugiono, M., Shields, J.D., Peat, D., Gillatt, D., and Harper, S.J. (2002). VEGF165b, an inhibitory splice variant of vascular endothelial growth factor, is down-regulated in renal cell carcinoma. Cancer research *62*, 4123-4131.

Bates, D.O., and Harper, S.J. (2002). Regulation of vascular permeability by vascular endothelial growth factors. Vascular pharmacology *39*, 225-237.

Bates, D.O., and Harper, S.J. (2005). Therapeutic potential of inhibitory VEGF splice variants. Future Oncol *1*, 467-473.

Bates, D.O., and Jones, R.O. (2003). The role of vascular endothelial growth factor in wound healing. The international journal of lower extremity wounds *2*, 107-120.

Bechard, D., Gentina, T., Delehedde, M., Scherpereel, A., Lyon, M., Aumercier, M., Vazeux, R., Richet, C., Degand, P., Jude, B.*, et al.* (2001). Endocan is a novel chondroitin sulfate/dermatan sulfate proteoglycan that promotes hepatocyte growth factor/scatter factor mitogenic activity. The Journal of biological chemistry *276*, 48341-48349.

Bechard, D., Meignin, V., Scherpereel, A., Oudin, S., Kervoaze, G., Bertheau, P., Janin, A., Tonnel, A., and Lassalle, P. (2000). Characterization of the secreted form of endothelial-cell-specific molecule 1 by specific monoclonal antibodies. Journal of vascular research *37*, 417-425.

Becker, P.M., Waltenberger, J., Yachechko, R., Mirzapoiazova, T., Sham, J.S., Lee, C.G., Elias, J.A., and Verin, A.D. (2005). Neuropilin-1 regulates vascular endothelial growth factor-mediated endothelial permeability. Circulation research *96*, 1257-1265.

Bellon, A., Luchino, J., Haigh, K., Rougon, G., Haigh, J., Chauvet, S., and Mann, F. (2010). VEGFR2 (KDR/Flk1) signaling mediates axon growth in response to semaphorin 3E in the developing brain. Neuron *66*, 205-219.

Benachour, H., Seve, A., Bastogne, T., Frochot, C., Vanderesse, R., Jasniewski, J., Miladi, I., Billotey, C., Tillement, O., Lux, F.*, et al.* (2012). Multifunctional Peptide-conjugated hybrid silica nanoparticles for photodynamic therapy and MRI. Theranostics *2*, 889-904.

Benjamin, L.E., Hemo, I., and Keshet, E. (1998). A plasticity window for blood vessel remodelling is defined by pericyte coverage of the preformed endothelial network and is regulated by PDGF-B and VEGF. Development *125*, 1591-1598.

Berger, P., and Ballmer-Hofer, K. (2011). The reception and the party after: how vascular endothelial growth factor receptor 2 explores cytoplasmic space. Swiss medical weekly *141*, w13318.

Bergers, G., Song, S., Meyer-Morse, N., Bergsland, E., and Hanahan, D. (2003). Benefits of targeting both pericytes and endothelial cells in the tumor vasculature with kinase inhibitors. The Journal of clinical investigation *111*, 1287-1295.

Berget, S.M., Moore, C., and Sharp, P.A. (1977). Spliced segments at the 5' terminus of adenovirus 2 late mRNA. Proceedings of the National Academy of Sciences of the United States of America *74*, 3171-3175.

Berse, B., Brown, L.F., Van de Water, L., Dvorak, H.F., and Senger, D.R. (1992). Vascular permeability factor (vascular endothelial growth factor) gene is expressed differentially in normal tissues, macrophages, and tumors. Molecular biology of the cell *3*, 211-220.

Bevan, H.S., van den Akker, N.M., Qiu, Y., Polman, J.A., Foster, R.R., Yem, J., Nishikawa, A., Satchell, S.C., Harper, S.J., Gittenberger-de Groot, A.C.*, et al.* (2008). The alternatively spliced anti-angiogenic family of VEGF isoforms VEGFxxxb in human kidney development. Nephron Physiology *110*, p57-67.

Bielenberg, D.R., Pettaway, C.A., Takashima, S., and Klagsbrun, M. (2006). Neuropilins in neoplasms: expression, regulation, and function. Experimental cell research *312*, 584-593.

Bills, V.L., Varet, J., Millar, A., Harper, S.J., Soothill, P.W., and Bates, D.O. (2009). Failure to up-regulate VEGF165b in maternal plasma is a first trimester predictive marker for pre-eclampsia. Clin Sci (Lond) *116*, 265-272.

Blaustein, M., Pelisch, F., Tanos, T., Munoz, M.J., Wengier, D., Quadrana, L., Sanford, J.R., Muschietti, J.P., Kornblihtt, A.R., Caceres, J.F.*, et al.* (2005). Concerted regulation of nuclear and cytoplasmic activities of SR proteins by AKT. Nature structural & molecular biology *12*, 1037-1044.

Borges, E., Jan, Y., and Ruoslahti, E. (2000). Platelet-derived growth factor receptor beta and vascular endothelial growth factor receptor 2 bind to the beta 3 integrin through its extracellular domain. The Journal of biological chemistry *275*, 39867-39873.

Borgstrom, P., Bourdon, M.A., Hillan, K.J., Sriramarao, P., and Ferrara, N. (1998). Neutralizing anti-vascular endothelial growth factor antibody completely inhibits angiogenesis and growth of human prostate carcinoma micro tumors in vivo. The Prostate *35*, 1-10.

Borgstrom, P., Hillan, K.J., Sriramarao, P., and Ferrara, N. (1996). Complete inhibition of angiogenesis and growth of microtumors by anti-vascular endothelial growth factor neutralizing antibody: novel concepts of angiostatic therapy from intravital videomicroscopy. Cancer research *56*, 4032-4039.

Brown, L.F., Yeo, K.T., Berse, B., Yeo, T.K., Senger, D.R., Dvorak, H.F., and van de Water, L. (1992). Expression of vascular permeability factor (vascular endothelial growth factor) by epidermal keratinocytes during wound healing. The Journal of experimental medicine *176*, 1375-1379.

Burchardt, M., Burchardt, T., Chen, M.W., Shabsigh, A., de la Taille, A., Buttyan, R., and Shabsigh, R. (1999). Expression of messenger ribonucleic acid splice variants for vascular endothelial growth factor in the penis of adult rats and humans. Biology of reproduction *60*, 398-404.

Burri, P.H., Hlushchuk, R., and Djonov, V. (2004). Intussusceptive angiogenesis: its emergence, its characteristics, and its significance. Developmental dynamics : an official publication of the American Association of Anatomists *231*, 474-488.

Cackowski, F.C., Xu, L., Hu, B., and Cheng, S.Y. (2004). Identification of two novel alternatively spliced Neuropilin-1 isoforms. Genomics *84*, 82-94.

Cai, H., and Reed, R.R. (1999). Cloning and characterization of neuropilin-1-interacting protein: a PSD-95/Dlg/ZO-1 domain-containing protein that interacts with the cytoplasmic domain of neuropilin-1. The Journal of neuroscience : the official journal of the Society for Neuroscience *19*, 6519-6527.

Cai, J., Jiang, W.G., Grant, M.B., and Boulton, M. (2006). Pigment epithelium-derived factor inhibits angiogenesis via regulated intracellular proteolysis of vascular endothelial growth factor receptor 1. The Journal of biological chemistry *281*, 3604-3613.

Caires, K.C., de Avila, J.M., Cupp, A.S., and McLean, D.J. (2012). VEGFA family isoforms regulate spermatogonial stem cell homeostasis in vivo. Endocrinology *153*, 887-900.

Carmeliet, P. (2000). Mechanisms of angiogenesis and arteriogenesis. Nature medicine *6*, 389-395.

Carmeliet, P. (2005). Angiogenesis in life, disease and medicine. Nature *438*, 932-936.

Carmeliet, P., Ferreira, V., Breier, G., Pollefeyt, S., Kieckens, L., Gertsenstein, M., Fahrig, M., Vandenhoeck, A., Harpal, K., Eberhardt, C. *et al.* (1996). Abnormal blood vessel development and lethality in embryos lacking a single VEGF allele. Nature *380*, 435-439.

Carmeliet, P., and Jain, R.K. (2011). Molecular mechanisms and clinical applications of angiogenesis. Nature *473*, 298-307.

Carmeliet, P., Ng, Y.S., Nuyens, D., Theilmeier, G., Brusselmans, K., Cornelissen, I., Ehler, E., Kakkar, V.V., Stalmans, I., Mattot, V. *et al.* (1999). Impaired myocardial angiogenesis and ischemic cardiomyopathy in mice lacking the vascular endothelial growth factor isoforms VEGF164 and VEGF188. Nature medicine *5*, 495-502.

Casanovas, O., Hicklin, D.J., Bergers, G., and Hanahan, D. (2005). Drug resistance by evasion of antiangiogenic targeting of VEGF signaling in late-stage pancreatic islet tumors. Cancer cell *8*, 299-309.

Catena, R., Larzabal, L., Larrayoz, M., Molina, E., Hermida, J., Agorreta, J., Montes, R., Pio, R., Montuenga, L.M., and Calvo, A. (2010). VEGFb and VEGFb are weakly angiogenic isoforms of VEGF-A. Molecular cancer *9*, 320.

Caunt, M., Mak, J., Liang, W.C., Stawicki, S., Pan, Q., Tong, R.K., Kowalski, J., Ho, C., Reslan, H.B., Ross, J. *et al.* (2008). Blocking neuropilin-2 function inhibits tumor cell metastasis. Cancer cell *13*, 331-342.

Cebe-Suarez, S., Grunewald, F.S., Jaussi, R., Li, X., Claesson-Welsh, L., Spillmann, D., Mercer, A.A., Prota, A.E., and Ballmer-Hofer, K. (2008). Orf virus VEGF-E NZ2 promotes paracellular NRP-1/VEGFR-2 coreceptor assembly via the peptide RPPR. The FASEB journal : official publication of the Federation of American Societies for Experimental Biology *22*, 3078-3086.

Cebe Suarez, S., Pieren, M., Cariolato, L., Arn, S., Hoffmann, U., Bogucki, A., Manlius, C., Wood, J., and Ballmer-Hofer, K. (2006). A VEGF-A splice variant defective for heparan sulfate and neuropilin-1 binding shows attenuated signaling through VEGFR-2. Cellular and molecular life sciences : CMLS *63*, 2067-2077.

Charnock-Jones, D.S., Sharkey, A.M., Rajput-Williams, J., Burch, D., Schofield, J.P., Fountain, S.A., Boocock, C.A., and Smith, S.K. (1993). Identification and localization of alternately spliced mRNAs for vascular endothelial growth factor in human uterus and estrogen regulation in endometrial carcinoma cell lines. Biology of reproduction *48*, 1120-1128.

Chen, H., Chedotal, A., He, Z., Goodman, C.S., and Tessier-Lavigne, M. (1997). Neuropilin-2, a novel member of the neuropilin family, is a high affinity receptor for the semaphorins Sema E and Sema IV but not Sema III. Neuron *19*, 547-559.

Chen, T.T., Luque, A., Lee, S., Anderson, S.M., Segura, T., and Iruela-Arispe, M.L. (2010). Anchorage of VEGF to the extracellular matrix conveys differential signaling responses to endothelial cells. The Journal of cell biology *188*, 595-609.

Cheung, C.Y., Singh, M., Ebaugh, M.J., and Brace, R.A. (1995). Vascular endothelial growth factor gene expression in ovine placenta and fetal membranes. American journal of obstetrics and gynecology *173*, 753-759.

Chow, L.T., Gelinas, R.E., Broker, T.R., and Roberts, R.J. (1977). An amazing sequence arrangement at the 5' ends of adenovirus 2 messenger RNA. Cell *12*, 1-8.

Chung, A.S., and Ferrara, N. (2011). Developmental and pathological angiogenesis. Annual review of cell and developmental biology *27*, 563-584.

Chung, A.S., Lee, J., and Ferrara, N. (2010). Targeting the tumour vasculature: insights from physiological angiogenesis. Nature reviews Cancer *10*, 505-514.

Claffey, K.P., Senger, D.R., and Spiegelman, B.M. (1995). Structural requirements for dimerization, glycosylation, secretion, and biological function of VPF/VEGF. Biochimica et biophysica acta *1246*, 1-9.

Clauss, M., Weich, H., Breier, G., Knies, U., Rockl, W., Waltenberger, J., and 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. The Journal of biological chemistry *271*, 17629-17634.

Cohen, T., Gitay-Goren, H., Sharon, R., Shibuya, M., Halaban, R., Levi, B.Z., and Neufeld, G. (1995). VEGF121, a vascular endothelial growth factor (VEGF) isoform lacking heparin binding ability, requires cell-surface heparan sulfates for efficient binding to the VEGF receptors of human melanoma cells. The Journal of biological chemistry *270*, 11322-11326.

Corral, C.J., Siddiqui, A., Wu, L., Farrell, C.L., Lyons, D., and Mustoe, T.A. (1999). Vascular endothelial growth factor is more important than basic fibroblastic growth factor during ischemic wound healing. Arch Surg *134*, 200-205.

Coussens, L.M., and Werb, Z. (2002). Inflammation and cancer. Nature *420*, 860-867.

Cudmore, M.J., Hewett, P.W., Ahmad, S., Wang, K.Q., Cai, M., Al-Ani, B., Fujisawa, T., Ma, B., Sissaoui, S., Ramma, W*., et al.* (2012). The role of heterodimerization between VEGFR-1 and VEGFR-2 in the regulation of endothelial cell homeostasis. Nature communications *3*, 972.

Cui, T.G., Foster, R.R., Saleem, M., Mathieson, P.W., Gillatt, D.A., Bates, D.O., and Harper, S.J. (2004). Differentiated human podocytes endogenously express an inhibitory isoform of vascular endothelial growth factor (VEGF165b) mRNA and protein. American journal of physiology Renal physiology *286*, F767-773.

Cunningham, S.A., Arrate, M.P., Brock, T.A., and Waxham, M.N. (1997). Interactions of FLT-1 and KDR with phospholipase C gamma: identification of the phosphotyrosine binding sites. Biochemical and biophysical research communications *240*, 635-639.

Damert, A., Machein, M., Breier, G., Fujita, M.Q., Hanahan, D., Risau, W., and Plate, K.H. (1997). Up-regulation of vascular endothelial growth factor expression in a rat glioma is conferred by two distinct hypoxia-driven mechanisms. Cancer research *57*, 3860-3864.

Datta, S.R., Brunet, A., and Greenberg, M.E. (1999). Cellular survival: a play in three Akts. Genes & development *13*, 2905-2927.

Davis-Smyth, T., Chen, H., Park, J., Presta, L.G., and Ferrara, N. (1996). The second immunoglobulin-like domain of the VEGF tyrosine kinase receptor Flt-1 determines ligand binding and may initiate a signal transduction cascade. The EMBO journal *15*, 4919-4927.

de Vries, C., Escobedo, J.A., Ueno, H., Houck, K., Ferrara, N., and Williams, L.T. (1992). The fms-like tyrosine kinase, a receptor for vascular endothelial growth factor. Science *255*, 989-991.

Dejana, E., Tournier-Lasserve, E., and Weinstein, B.M. (2009). The control of vascular integrity by endothelial cell junctions: molecular basis and pathological implications. Developmental cell *16*, 209-221.

Detmar, M., Yeo, K.T., Nagy, J.A., Van de Water, L., Brown, L.F., Berse, B., Elicker, B.M., Ledbetter, S., and Dvorak, H.F. (1995). Keratinocyte-derived vascular permeability factor (vascular endothelial growth factor) is a potent mitogen for dermal microvascular endothelial cells. The Journal of investigative dermatology *105*, 44-50.

Diaz, R., Pena, C., Silva, J., Lorenzo, Y., Garcia, V., Garcia, J.M., Sanchez, A., Espinosa, P., Yuste, R., Bonilla, F*., et al.* (2008). p73 Isoforms affect VEGF, VEGF165b and PEDF expression in human colorectal tumors: VEGF165b downregulation as a marker of poor prognosis. International journal of cancer Journal international du cancer *123*, 1060-1067.

Dineen, S.P., Lynn, K.D., Holloway, S.E., Miller, A.F., Sullivan, J.P., Shames, D.S., Beck, A.W., Barnett, C.C., Fleming, J.B., and Brekken, R.A. (2008). Vascular endothelial growth factor receptor 2 mediates macrophage infiltration into orthotopic pancreatic tumors in mice. Cancer research *68*, 4340-4346.

DiSalvo, J., Bayne, M.L., Conn, G., Kwok, P.W., Trivedi, P.G., Soderman, D.D., Palisi, T.M., Sullivan, K.A., and Thomas, K.A. (1995). Purification and characterization of a naturally occurring vascular endothelial growth factor.placenta growth factor heterodimer. The Journal of biological chemistry *270*, 7717-7723.

Dor, Y., Djonov, V., Abramovitch, R., Itin, A., Fishman, G.I., Carmeliet, P., Goelman, G., and Keshet, E. (2002). Conditional switching of VEGF provides new insights into adult neovascularization and pro-angiogenic therapy. The EMBO journal *21*, 1939-1947.

Dor, Y., Porat, R., and Keshet, E. (2001). Vascular endothelial growth factor and vascular adjustments to perturbations in oxygen homeostasis. American journal of physiology Cell physiology *280*, C1367-1374.

Dougher, A.M., Wasserstrom, H., Torley, L., Shridaran, L., Westdock, P., Hileman, R.E., Fromm, J.R., Anderberg, R., Lyman, S., Linhardt, R.J.*, et al.* (1997). Identification of a heparin binding peptide on the extracellular domain of the KDR VEGF receptor. Growth Factors *14*, 257-268.

Dougher, M., and Terman, B.I. (1999). Autophosphorylation of KDR in the kinase domain is required for maximal VEGF-stimulated kinase activity and receptor internalization. Oncogene *18*, 1619-1627.

Ebos, J.M., Bocci, G., Man, S., Thorpe, P.E., Hicklin, D.J., Zhou, D., Jia, X., and Kerbel, R.S. (2004). A naturally occurring soluble form of vascular endothelial growth factor receptor 2 detected in mouse and human plasma. Molecular cancer research : MCR *2*, 315-326.

Ebos, J.M., Lee, C.R., Cruz-Munoz, W., Bjarnason, G.A., Christensen, J.G., and Kerbel, R.S. (2009). Accelerated metastasis after short-term treatment with a potent inhibitor of tumor angiogenesis. Cancer cell *15*, 232-239.

Eichmann, A., Corbel, C., Nataf, V., Vaigot, P., Breant, C., and Le Douarin, N.M. (1997). Ligand-dependent development of the endothelial and hemopoietic lineages from embryonic mesodermal cells expressing vascular endothelial growth factor receptor 2. Proceedings of the National Academy of Sciences of the United States of America *94*, 5141-5146.

Eilken, H.M., and Adams, R.H. (2010). Dynamics of endothelial cell behavior in sprouting angiogenesis. Current opinion in cell biology *22*, 617-625.

Eming, S.A., and Krieg, T. (2006). Molecular mechanisms of VEGF-A action during tissue repair. The journal of investigative dermatology Symposium proceedings / the Society for Investigative Dermatology, Inc [and] European Society for Dermatological Research *11*, 79-86.

Eming, S.A., Lauer, G., Cole, M., Jurk, S., Christ, H., Hornig, C., Krieg, T., and Weich, H.A. (2004). Increased levels of the soluble variant of the vascular endothelial growth factor receptor VEGFR-1 are associated with a poor prognosis in wound healing. The Journal of investigative dermatology *123*, 799-802.

Ergorul, C., Ray, A., Huang, W., Darland, D., Luo, Z.K., and Grosskreutz, C.L. (2008). Levels of vascular endothelial growth factor-A165b (VEGF-A165b) are elevated in experimental glaucoma. Molecular vision *14*, 1517-1524.

Escudier, B., Eisen, T., Stadler, W.M., Szczylik, C., Oudard, S., Staehler, M., Negrier, S., Chevreau, C., Desai, A.A., Rolland, F.*, et al.* (2009). Sorafenib for treatment of renal cell carcinoma: Final efficacy and safety results of the phase III treatment approaches in renal cancer global evaluation trial. Journal of clinical oncology : official journal of the American Society of Clinical Oncology *27*, 3312-3318.

Escudier, B., Pluzanska, A., Koralewski, P., Ravaud, A., Bracarda, S., Szczylik, C., Chevreau, C., Filipek, M., Melichar, B., Bajetta, E.*, et al.* (2007). Bevacizumab plus interferon alfa-2a for treatment of metastatic renal cell carcinoma: a randomised, double-blind phase III trial. Lancet *370*, 2103-2111.

Esko, J.D., and Selleck, S.B. (2002). Order out of chaos: assembly of ligand binding sites in heparan sulfate. Annual review of biochemistry *71*, 435-471.

Evans, I.M., Yamaji, M., Britton, G., Pellet-Many, C., Lockie, C., Zachary, I.C., and Frankel, P. (2011). Neuropilin-1 signaling through p130Cas tyrosine phosphorylation is essential for growth factor-dependent migration of glioma and endothelial cells. Molecular and cellular biology *31*, 1174-1185.

Fakhari, M., Pullirsch, D., Abraham, D., Paya, K., Hofbauer, R., Holzfeind, P., Hofmann, M., and Aharinejad, S. (2002). Selective upregulation of vascular endothelial growth factor receptors neuropilin-1 and -2 in human neuroblastoma. Cancer *94*, 258-263.

Farhat, F.S., Tfayli, A., Fakhruddin, N., Mahfouz, R., Otrock, Z.K., Alameddine, R.S., Awada, A.H., and Shamseddine, A. (2012). Expression, prognostic and predictive impact of VEGF and bFGF in non-small cell lung cancer. Critical reviews in oncology/hematology *84*, 149-160.

Favier, B., Alam, A., Barron, P., Bonnin, J., Laboudie, P., Fons, P., Mandron, M., Herault, J.P., Neufeld, G., Savi, P.*, et al.* (2006). Neuropilin-2 interacts with VEGFR-2 and VEGFR-3 and promotes human endothelial cell survival and migration. Blood *108*, 1243-1250.

Ferrara, N. (2004). Vascular endothelial growth factor as a target for anticancer therapy. The oncologist *9 Suppl 1*, 2-10.

Ferrara, N. (2010). Binding to the extracellular matrix and proteolytic processing: two key mechanisms regulating vascular endothelial growth factor action. Molecular biology of the cell *21*, 687-690.

Ferrara, N., and Alitalo, K. (1999). Clinical applications of angiogenic growth factors and their inhibitors. Nature medicine *5*, 1359-1364.

Ferrara, N., Carver-Moore, K., Chen, H., Dowd, M., Lu, L., O'Shea, K.S., Powell-Braxton, L., Hillan, K.J., and Moore, M.W. (1996). Heterozygous embryonic lethality induced by targeted inactivation of the VEGF gene. Nature *380*, 439-442.

Ferrara, N., Damico, L., Shams, N., Lowman, H., and Kim, R. (2006). Development of ranibizumab, an anti-vascular endothelial growth factor antigen binding fragment, as therapy for neovascular age-related macular degeneration. Retina *26*, 859-870.

Ferrara, N., and Davis-Smyth, T. (1997). The biology of vascular endothelial growth factor. Endocrine reviews *18*, 4-25.

Ferrara, N., Gerber, H.P., and LeCouter, J. (2003). The biology of VEGF and its receptors. Nature medicine *9*, 669-676.

Ferrara, N., and Henzel, W.J. (1989). Pituitary follicular cells secrete a novel heparin-binding growth factor specific for vascular endothelial cells. Biochemical and biophysical research communications *161*, 851-858.

Ferrara, N., Hillan, K.J., and Novotny, W. (2005). Bevacizumab (Avastin), a humanized anti-VEGF monoclonal antibody for cancer therapy. Biochemical and biophysical research communications *333*, 328-335.

Ferrara, N., Houck, K., Jakeman, L., and Leung, D.W. (1992). Molecular and biological properties of the vascular endothelial growth factor family of proteins. Endocrine reviews *13*, 18-32.

Ferrara, N., Houck, K.A., Jakeman, L.B., Winer, J., and Leung, D.W. (1991a). The vascular endothelial growth factor family of polypeptides. Journal of cellular biochemistry *47*, 211-218.

Ferrara, N., and Kerbel, R.S. (2005). Angiogenesis as a therapeutic target. Nature *438*, 967-974.

Ferrara, N., Mass, R.D., Campa, C., and Kim, R. (2007). Targeting VEGF-A to treat cancer and age-related macular degeneration. Annual review of medicine *58*, 491-504.

Ferrara, N., Winer, J., and Burton, T. (1991b). Aortic smooth muscle cells express and secrete vascular endothelial growth factor. Growth Factors *5*, 141-148.

Fischer, C., Mazzone, M., Jonckx, B., and Carmeliet, P. (2008). FLT1 and its ligands VEGFB and PlGF: drug targets for anti-angiogenic therapy? Nature reviews Cancer *8*, 942-956.

Folkman, J. (1972). Anti-angiogenesis: new concept for therapy of solid tumors. Annals of surgery *175*, 409-416.

Folkman, J. (1995). Angiogenesis in cancer, vascular, rheumatoid and other disease. Nature medicine *1*, 27-31.

Fong, G.H., Rossant, J., Gertsenstein, M., and Breitman, M.L. (1995). Role of the Flt-1 receptor tyrosine kinase in regulating the assembly of vascular endothelium. Nature *376*, 66-70.

Forsythe, J.A., Jiang, B.H., Iyer, N.V., Agani, F., Leung, S.W., Koos, R.D., and Semenza, G.L. (1996). Activation of vascular endothelial growth factor gene transcription by hypoxia-inducible factor 1. Molecular and cellular biology *16*, 4604-4613.

Francois, B., Sternberg, E.M., and Fee, E. (2012). The Lourdes Medical Cures Revisited. Journal of the history of medicine and allied sciences.

Frank, S., Hubner, G., Breier, G., Longaker, M.T., Greenhalgh, D.G., and Werner, S. (1995). Regulation of vascular endothelial growth factor expression in cultured keratinocytes. Implications for normal and impaired wound healing. The Journal of biological chemistry *270*, 12607-12613.

Frankel, P., Pellet-Many, C., Lehtolainen, P., D'Abaco, G.M., Tickner, M.L., Cheng, L., and Zachary, I.C. (2008). Chondroitin sulphate-modified neuropilin 1 is expressed in human tumour cells and modulates 3D invasion in the U87MG human glioblastoma cell line through a p130Cas-mediated pathway. EMBO reports *9*, 983-989.

Fuh, G., Garcia, K.C., and de Vos, A.M. (2000). The interaction of neuropilin-1 with vascular endothelial growth factor and its receptor flt-1. The Journal of biological chemistry *275*, 26690-26695.

Fujisawa, H., Kitsukawa, T., Kawakami, A., Takagi, S., Shimizu, M., and Hirata, T. (1997). Roles of a neuronal cell-surface molecule, neuropilin, in nerve fiber fasciculation and guidance. Cell and tissue research *290*, 465-470.

Fukumura, D., Xavier, R., Sugiura, T., Chen, Y., Park, E.C., Lu, N., Selig, M., Nielsen, G., Taksir, T., Jain, R.K.*, et al.* (1998). Tumor induction of VEGF promoter activity in stromal cells. Cell *94*, 715-725.

Gagnon, M.L., Bielenberg, D.R., Gechtman, Z., Miao, H.Q., Takashima, S., Soker, S., and Klagsbrun, M. (2000). Identification of a natural soluble neuropilin-1 that binds vascular endothelial growth factor: In vivo expression and antitumor activity. Proceedings of the National Academy of Sciences of the United States of America *97*, 2573-2578.

Galiano, R.D., Tepper, O.M., Pelo, C.R., Bhatt, K.A., Callaghan, M., Bastidas, N., Bunting, S., Steinmetz, H.G., and Gurtner, G.C. (2004). Topical vascular endothelial growth factor accelerates diabetic wound healing through increased angiogenesis and by mobilizing and recruiting bone marrow-derived cells. The American journal of pathology *164*, 1935-1947.

Garcia, A.A., Hirte, H., Fleming, G., Yang, D., Tsao-Wei, D.D., Roman, L., Groshen, S., Swenson, S., Markland, F., Gandara, D.*, et al.* (2008). Phase II clinical trial of bevacizumab and low-dose metronomic oral cyclophosphamide in recurrent ovarian cancer: a trial of the California, Chicago, and Princess Margaret Hospital phase II consortia. Journal of clinical oncology : official journal of the American Society of Clinical Oncology *26*, 76-82.

Garrido-Urbani, S., Bradfield, P.F., Lee, B.P., and Imhof, B.A. (2008). Vascular and epithelial junctions: a barrier for leucocyte migration. Biochemical Society transactions *36*, 203-211.

Gerber, H.P., Hillan, K.J., Ryan, A.M., Kowalski, J., Keller, G.A., Rangell, L., Wright, B.D., Radtke, F., Aguet, M., and Ferrara, N. (1999). VEGF is required for growth and survival in neonatal mice. Development *126*, 1149-1159.

Geretti, E., Shimizu, A., Kurschat, P., and Klagsbrun, M. (2007). Site-directed mutagenesis in the B-neuropilin-2 domain selectively enhances its affinity to VEGF165, but not to semaphorin 3F. The Journal of biological chemistry *282*, 25698-25707.

Geretti, E., van Meeteren, L.A., Shimizu, A., Dudley, A.C., Claesson-Welsh, L., and Klagsbrun, M. (2010). A mutated soluble neuropilin-2 B domain antagonizes vascular endothelial growth factor bioactivity and inhibits tumor progression. Molecular cancer research : MCR *8*, 1063-1073.

Gerhardt, H., and Betsholtz, C. (2003). Endothelial-pericyte interactions in angiogenesis. Cell and tissue research *314*, 15-23.

Gerhardt, H., Golding, M., Fruttiger, M., Ruhrberg, C., Lundkvist, A., Abramsson, A., Jeltsch, M., Mitchell, C., Alitalo, K., Shima, D.*, et al.* (2003). VEGF guides angiogenic sprouting utilizing endothelial tip cell filopodia. The Journal of cell biology *161*, 1163-1177.

Gerhardt, H., Ruhrberg, C., Abramsson, A., Fujisawa, H., Shima, D., and Betsholtz, C. (2004). Neuropilin-1 is required for endothelial tip cell guidance in the developing central nervous system. Developmental dynamics : an official publication of the American Association of Anatomists *231*, 503-509.

Giantonio, B.J., Catalano, P.J., Meropol, N.J., O'Dwyer, P.J., Mitchell, E.P., Alberts, S.R., Schwartz, M.A., and Benson, A.B., 3rd (2007). Bevacizumab in combination with oxaliplatin, fluorouracil, and leucovorin (FOLFOX4) for previously treated metastatic colorectal cancer: results from the Eastern Cooperative Oncology Group Study E3200. Journal of clinical oncology : official journal of the American Society of Clinical Oncology *25*, 1539-1544.

Gille, H., Kowalski, J., Li, B., LeCouter, J., Moffat, B., Zioncheck, T.F., Pelletier, N., and Ferrara, N. (2001). Analysis of biological effects and signaling properties of Flt-1 (VEGFR-1) and KDR (VEGFR-2). A reassessment using novel receptor-specific vascular endothelial growth factor mutants. The Journal of biological chemistry *276*, 3222-3230.

Gitay-Goren, H., Soker, S., Vlodavsky, I., and Neufeld, G. (1992). The binding of vascular endothelial growth factor to its receptors is dependent on cell surface-associated heparin-like molecules. The Journal of biological chemistry *267*, 6093-6098.

Glass, C.A., Harper, S.J., and Bates, D.O. (2006). The anti-angiogenic VEGF isoform VEGF165b transiently increases hydraulic conductivity, probably through VEGF receptor 1 in vivo. The Journal of physiology *572*, 243-257.

Gluzman-Poltorak, Z., Cohen, T., Herzog, Y., and Neufeld, G. (2000). Neuropilin-2 is a receptor for the vascular endothelial growth factor (VEGF) forms VEGF-145 and VEGF-165 [corrected]. The Journal of biological chemistry *275*, 18040-18045.

Gluzman-Poltorak, Z., Cohen, T., Shibuya, M., and Neufeld, G. (2001). Vascular endothelial growth factor receptor-1 and neuropilin-2 form complexes. The Journal of biological chemistry *276*, 18688-18694.

Goel, H.L., Chang, C., Pursell, B., Leav, I., Lyle, S., Xi, H.S., Hsieh, C.C., Adisetiyo, H., Roy-Burman, P., Coleman, I.M.*, et al.* (2012). VEGF/Neuropilin-2 Regulation of Bmi-1 and Consequent Repression of IGF-IR Define a Novel Mechanism of Aggressive Prostate Cancer. Cancer discovery *2*, 906-921.

Gospodarowicz, D., Abraham, J.A., and Schilling, J. (1989). Isolation and characterization of a vascular endothelial cell mitogen produced by pituitary-derived folliculo stellate cells. Proceedings of the National Academy of Sciences of the United States of America *86*, 7311-7315.

Gout, S., Brambilla, E., Boudria, A., Drissi, R., Lantuejoul, S., Gazzeri, S., and Eymin, B. (2012). Abnormal expression of the pre-mRNA splicing regulators SRSF1, SRSF2, SRPK1 and SRPK2 in non small cell lung carcinoma. PloS one *7*, e46539.

Grunewald, F.S., Prota, A.E., Giese, A., and Ballmer-Hofer, K. (2010). Structure-function analysis of VEGF receptor activation and the role of coreceptors in angiogenic signaling. Biochimica et biophysica acta *1804*, 567-580.

Grunstein, J., Masbad, J.J., Hickey, R., Giordano, F., and Johnson, R.S. (2000). Isoforms of vascular endothelial growth factor act in a coordinate fashion To recruit and expand tumor vasculature. Molecular and cellular biology *20*, 7282-7291.

Gu, C., Rodriguez, E.R., Reimert, D.V., Shu, T., Fritzsch, B., Richards, L.J., Kolodkin, A.L., and Ginty, D.D. (2003). Neuropilin-1 conveys semaphorin and VEGF signaling during neural and cardiovascular development. Developmental cell *5*, 45-57.

Gutierrez, J., Konecny, G.E., Hong, K., Burges, A., Henry, T.D., Lambiase, P.D., Lee Wong, W., and Meng, Y.G. (2008). A new ELISA for use in a 3-ELISA system to assess concentrations of VEGF splice variants and VEGF(110) in ovarian cancer tumors. Clinical chemistry *54*, 597-601.

Hamerlik, P., Lathia, J.D., Rasmussen, R., Wu, Q., Bartkova, J., Lee, M., Moudry, P., Bartek, J., Jr., Fischer, W., Lukas, J*., et al.* (2012). Autocrine VEGF-VEGFR2-Neuropilin-1 signaling promotes glioma stem-like cell viability and tumor growth. The Journal of experimental medicine *209*, 507-520.

Handa, A., Tokunaga, T., Tsuchida, T., Lee, Y.H., Kijima, H., Yamazaki, H., Ueyama, Y., Fukuda, H., and Nakamura, M. (2000). Neuropilin-2 expression affects the increased vascularization and is a prognostic factor in osteosarcoma. International journal of oncology *17*, 291-295.

Hansel, D.E., Wilentz, R.E., Yeo, C.J., Schulick, R.D., Montgomery, E., and Maitra, A. (2004). Expression of neuropilin-1 in high-grade dysplasia, invasive cancer, and metastases of the human gastrointestinal tract. The American journal of surgical pathology *28*, 347-356.

Harper, S.J., and Bates, D.O. (2008). VEGF-A splicing: the key to anti-angiogenic therapeutics? Nature reviews Cancer *8*, 880-887.

Harris, S., Craze, M., Newton, J., Fisher, M., Shima, D.T., Tozer, G.M., and Kanthou, C. (2012). Do Anti-Angiogenic VEGF (VEGFxxxb) Isoforms Exist? A Cautionary Tale. PloS one *7*, e35231.

Hayes, D.F. (2011). Bevacizumab treatment for solid tumors: boon or bust? JAMA : the journal of the American Medical Association *305*, 506-508.

He, Z., and Tessier-Lavigne, M. (1997). Neuropilin is a receptor for the axonal chemorepellent Semaphorin III. Cell *90*, 739-751.

Hellstrom, M., Kalen, M., Lindahl, P., Abramsson, A., and Betsholtz, C. (1999). Role of PDGF-B and PDGFR-beta in recruitment of vascular smooth muscle cells and pericytes during embryonic blood vessel formation in the mouse. Development *126*, 3047-3055.

Hellstrom, M., Phng, L.K., Hofmann, J.J., Wallgard, E., Coultas, L., Lindblom, P., Alva, J., Nilsson, A.K., Karlsson, L., Gaiano, N.*, et al.* (2007). Dll4 signalling through Notch1 regulates formation of tip cells during angiogenesis. Nature *445*, 776-780.

Herbert, S.P., and Stainier, D.Y. (2011). Molecular control of endothelial cell behaviour during blood vessel morphogenesis. Nature reviews Molecular cell biology *12*, 551-564.

Herve, M.A., Buteau-Lozano, H., Mourah, S., Calvo, F., and Perrot-Applanat, M. (2005). VEGF189 stimulates endothelial cells proliferation and migration in vitro and up-regulates the expression of Flk-1/KDR mRNA. Experimental cell research *309*, 24-31.

Herve, M.A., Buteau-Lozano, H., Vassy, R., Bieche, I., Velasco, G., Pla, M., Perret, G., Mourah, S., and Perrot-Applanat, M. (2008). Overexpression of vascular endothelial growth factor 189 in breast cancer cells leads to delayed tumor uptake with dilated intratumoral vessels. The American journal of pathology *172*, 167-178.

Herzog, Y., Kalcheim, C., Kahane, N., Reshef, R., and Neufeld, G. (2001). Differential expression of neuropilin-1 and neuropilin-2 in arteries and veins. Mechanisms of development *109*, 115-119.

Hilmi, C., Guyot, M., and Pages, G. (2012). VEGF spliced variants: possible role of anti-angiogenesis therapy. Journal of nucleic acids *2012*, 162692.

Hiratsuka, S., Maru, Y., Okada, A., Seiki, M., Noda, T., and Shibuya, M. (2001). Involvement of Flt-1 tyrosine kinase (vascular endothelial growth factor receptor-1) in pathological angiogenesis. Cancer research *61*, 1207-1213.

Hiratsuka, S., Minowa, O., Kuno, J., Noda, T., and Shibuya, M. (1998). Flt-1 lacking the tyrosine kinase domain is sufficient for normal development and angiogenesis in mice. Proceedings of the National Academy of Sciences of the United States of America *95*, 9349-9354.

Ho, V.C., Duan, L.J., Cronin, C., Liang, B.T., and Fong, G.H. (2012). Elevated VEGF Receptor-2 Abundance Contributes to Increased Angiogenesis in VEGF Receptor-1 Deficient Mice. Circulation.

Holash, J., Davis, S., Papadopoulos, N., Croll, S.D., Ho, L., Russell, M., Boland, P., Leidich, R., Hylton, D., Burova, E.*, et al.* (2002). VEGF-Trap: a VEGF blocker with potent antitumor effects. Proceedings of the National Academy of Sciences of the United States of America *99*, 11393-11398.

Holash, J., Wiegand, S.J., and Yancopoulos, G.D. (1999). New model of tumor angiogenesis: dynamic balance between vessel regression and growth mediated by angiopoietins and VEGF. Oncogene *18*, 5356-5362.

Holmqvist, K., Cross, M., Riley, D., and Welsh, M. (2003). The Shb adaptor protein causes Src-dependent cell spreading and activation of focal adhesion kinase in murine brain endothelial cells. Cellular signalling *15*, 171-179.

Holmqvist, K., Cross, M.J., Rolny, C., Hagerkvist, R., Rahimi, N., Matsumoto, T., Claesson-Welsh, L., and Welsh, M. (2004). The adaptor protein shb binds to tyrosine 1175 in vascular endothelial growth factor (VEGF) receptor-2 and regulates VEGF-dependent cellular migration. The Journal of biological chemistry *279*, 22267-22275.

Hong, Y.K., Lange-Asschenfeldt, B., Velasco, P., Hirakawa, S., Kunstfeld, R., Brown, L.F., Bohlen, P., Senger, D.R., and Detmar, M. (2004). VEGF-A promotes tissue repair-associated lymphatic vessel formation via VEGFR-2 and the alpha1beta1 and alpha2beta1 integrins. The FASEB journal : official publication of the Federation of American Societies for Experimental Biology *18*, 1111-1113.

Houck, K.A., Ferrara, N., Winer, J., Cachianes, G., Li, B., and Leung, D.W. (1991). The vascular endothelial growth factor family: identification of a fourth molecular species and characterization of alternative splicing of RNA. Mol Endocrinol *5*, 1806-1814.

Houck, K.A., Leung, D.W., Rowland, A.M., Winer, J., and Ferrara, N. (1992). Dual regulation of vascular endothelial growth factor bioavailability by genetic and proteolytic mechanisms. The Journal of biological chemistry *267*, 26031-26037.

Howdieshell, T.R., Callaway, D., Webb, W.L., Gaines, M.D., Procter, C.D., Jr., Sathyanarayana, Pollock, J.S., Brock, T.L., and McNeil, P.L. (2001). Antibody neutralization of vascular endothelial growth factor inhibits wound granulation tissue formation. The Journal of surgical research *96*, 173-182.

Hua, J., Spee, C., Kase, S., Rennel, E.S., Magnussen, A.L., Qiu, Y., Varey, A., Dhayade, S., Churchill, A.J., Harper, S.J.*, et al.* (2010). Recombinant human VEGF165b inhibits experimental choroidal neovascularization. Investigative ophthalmology & visual science *51*, 4282-4288.

Huang, D., Ding, Y., Zhou, M., Rini, B.I., Petillo, D., Qian, C.N., Kahnoski, R., Futreal, P.A., Furge, K.A., and Teh, B.T. (2010). Interleukin-8 mediates resistance to antiangiogenic agent sunitinib in renal cell carcinoma. Cancer research *70*, 1063-1071.

Huang, K., Andersson, C., Roomans, G.M., Ito, N., and Claesson-Welsh, L. (2001). Signaling properties of VEGF receptor-1 and -2 homo- and heterodimers. The international journal of biochemistry & cell biology *33*, 315-324.

Hurwitz, H., Fehrenbacher, L., Novotny, W., Cartwright, T., Hainsworth, J., Heim, W., Berlin, J., Baron, A., Griffing, S., Holmgren, E.*, et al.* (2004). Bevacizumab plus irinotecan, fluorouracil, and leucovorin for metastatic colorectal cancer. The New England journal of medicine *350*, 2335-2342.

Hutchings, H., Ortega, N., and Plouet, J. (2003). Extracellular matrix-bound vascular endothelial growth factor promotes endothelial cell adhesion, migration, and survival through integrin ligation. The FASEB journal : official publication of the Federation of American Societies for Experimental Biology *17*, 1520-1522.

Ikeda, E., Achen, M.G., Breier, G., and Risau, W. (1995). Hypoxia-induced transcriptional activation and increased mRNA stability of vascular endothelial growth factor in C6 glioma cells. The Journal of biological chemistry *270*, 19761-19766.

Isner, J.M., and Asahara, T. (1999). Angiogenesis and vasculogenesis as therapeutic strategies for postnatal neovascularization. The Journal of clinical investigation *103*, 1231-1236.

Ito, N., Wernstedt, C., Engstrom, U., and Claesson-Welsh, L. (1998). Identification of vascular endothelial growth factor receptor-1 tyrosine phosphorylation sites and binding of SH2 domain-containing molecules. The Journal of biological chemistry *273*, 23410-23418.

Iyer, N.V., Kotch, L.E., Agani, F., Leung, S.W., Laughner, E., Wenger, R.H., Gassmann, M., Gearhart, J.D., Lawler, A.M., Yu, A.Y.*, et al.* (1998). Cellular and developmental control of O2 homeostasis by hypoxia-inducible factor 1 alpha. Genes & development *12*, 149-162.

Iyer, S., and Acharya, K.R. (2011). Tying the knot: the cystine signature and molecular-recognition processes of the vascular endothelial growth factor family of angiogenic cytokines. The FEBS journal *278*, 4304-4322.

Jain, R.K. (2001). Normalizing tumor vasculature with anti-angiogenic therapy: a new paradigm for combination therapy. Nature medicine *7*, 987-989.

Jain, R.K. (2005). Normalization of tumor vasculature: an emerging concept in antiangiogenic therapy. Science *307*, 58-62.

Jain, R.K., and Carmeliet, P. (2012). SnapShot: Tumor angiogenesis. Cell *149*, 1408-1408 e1401.

Jakeman, L.B., Armanini, M., Phillips, H.S., and Ferrara, N. (1993). Developmental expression of binding sites and messenger ribonucleic acid for vascular endothelial growth factor suggests a role for this protein in vasculogenesis and angiogenesis. Endocrinology *133*, 848-859.

Jia, H., Jezequel, S., Lohr, M., Shaikh, S., Davis, D., Soker, S., Selwood, D., and Zachary, I. (2001). Peptides encoded by exon 6 of VEGF inhibit endothelial cell biological responses and angiogenesis induced by VEGF. Biochemical and biophysical research communications *283*, 164-173.

Jingjing, L., Srinivasan, B., Bian, X., Downey, H.F., and Roque, R.S. (2000). Vascular endothelial growth factor is increased following coronary artery occlusion in the dog heart. Molecular and cellular biochemistry *214*, 23-30.

Jingjing, L., Srinivasan, B., and Roque, R.S. (2001). Ectodomain shedding of VEGF183, a novel isoform of vascular endothelial growth factor, promotes its mitogenic activity in vitro. Angiogenesis *4*, 103-112.

Jingjing, L., Xue, Y., Agarwal, N., and Roque, R.S. (1999). Human Muller cells express VEGF183, a novel spliced variant of vascular endothelial growth factor. Investigative ophthalmology & visual science *40*, 752-759.

Jubb, A.M., Sa, S.M., Ratti, N., Strickland, L.A., Schmidt, M., Callahan, C.A., and Koeppen, H. (2012a). Neuropilin-2 expression in cancer. Histopathology *61*, 340-349.

Jubb, A.M., Strickland, L.A., Liu, S.D., Mak, J., Schmidt, M., and Koeppen, H. (2012b). Neuropilin-1 expression in cancer and development. The Journal of pathology *226*, 50-60.

Kabbinavar, F., Hurwitz, H.I., Fehrenbacher, L., Meropol, N.J., Novotny, W.F., Lieberman, G., Griffing, S., and Bergsland, E. (2003). Phase II, randomized trial comparing bevacizumab plus fluorouracil (FU)/leucovorin (LV) with FU/LV alone in patients with metastatic colorectal cancer. Journal of clinical oncology : official journal of the American Society of Clinical Oncology *21*, 60-65.

Kaplan, J.B., Sridharan, L., Zaccardi, J.A., Dougher-Vermazen, M., and Terman, B.I. (1997). Characterization of a soluble vascular endothelial growth factor receptor-immunoglobulin chimera. Growth Factors *14*, 243-256.

Karamysheva, A.F. (2008). Mechanisms of angiogenesis. Biochemistry Biokhimiia *73*, 751-762.

Karihaloo, A., Karumanchi, S.A., Cantley, W.L., Venkatesha, S., Cantley, L.G., and Kale, S. (2005). Vascular endothelial growth factor induces branching morphogenesis/tubulogenesis in renal epithelial cells in a neuropilin-dependent fashion. Molecular and cellular biology *25*, 7441-7448.

Karpanen, T., Heckman, C.A., Keskitalo, S., Jeltsch, M., Ollila, H., Neufeld, G., Tamagnone, L., and Alitalo, K. (2006). Functional interaction of VEGF-C and VEGF-D with neuropilin receptors. The FASEB journal : official publication of the Federation of American Societies for Experimental Biology *20*, 1462-1472.

Kawakami, T., Tokunaga, T., Hatanaka, H., Kijima, H., Yamazaki, H., Abe, Y., Osamura, Y., Inoue, H., Ueyama, Y., and Nakamura, M. (2002). Neuropilin 1 and neuropilin 2 co-expression is significantly correlated with increased vascularity and poor prognosis in nonsmall cell lung carcinoma. Cancer *95*, 2196-2201.

Kawamura, H., Li, X., Goishi, K., van Meeteren, L.A., Jakobsson, L., Cebe-Suarez, S., Shimizu, A., Edholm, D., Ballmer-Hofer, K., Kjellen, L*., et al.* (2008a). Neuropilin-1 in regulation of VEGF-induced activation of p38MAPK and endothelial cell organization. Blood *112*, 3638-3649.

Kawamura, H., Li, X., Harper, S.J., Bates, D.O., and Claesson-Welsh, L. (2008b). Vascular endothelial growth factor (VEGF)-A165b is a weak in vitro agonist for VEGF receptor-2 due to lack of coreceptor binding and deficient regulation of kinase activity. Cancer research *68*, 4683-4692.

Kawasaki, T., Kitsukawa, T., Bekku, Y., Matsuda, Y., Sanbo, M., Yagi, T., and Fujisawa, H. (1999). A requirement for neuropilin-1 in embryonic vessel formation. Development *126*, 4895-4902.

Keck, P.J., Hauser, S.D., Krivi, G., Sanzo, K., Warren, T., Feder, J., and Connolly, D.T. (1989). Vascular permeability factor, an endothelial cell mitogen related to PDGF. Science *246*, 1309-1312.

Kelly, B.D., Hackett, S.F., Hirota, K., Oshima, Y., Cai, Z., Berg-Dixon, S., Rowan, A., Yan, Z., Campochiaro, P.A., and Semenza, G.L. (2003). Cell type-specific regulation of angiogenic growth factor gene expression and induction of angiogenesis in nonischemic tissue by a constitutively active form of hypoxia-inducible factor 1. Circulation research *93*, 1074-1081.

Kendall, R.L., Rutledge, R.Z., Mao, X., Tebben, A.J., Hungate, R.W., and Thomas, K.A. (1999). Vascular endothelial growth factor receptor KDR tyrosine kinase activity is increased by autophosphorylation of two activation loop tyrosine residues. The Journal of biological chemistry *274*, 6453-6460.

Kendall, R.L., and Thomas, K.A. (1993). Inhibition of vascular endothelial cell growth factor activity by an endogenously encoded soluble receptor. Proceedings of the National Academy of Sciences of the United States of America *90*, 10705-10709.

Kendall, R.L., Wang, G., DiSalvo, J., and Thomas, K.A. (1994). Specificity of vascular endothelial cell growth factor receptor ligand binding domains. Biochemical and biophysical research communications *201*, 326-330.

Kerbel, R.S. (2012). Strategies for Improving the Clinical Benefit of Antiangiogenic Drug Based Therapies for Breast Cancer. Journal of mammary gland biology and neoplasia.

Keyt, B.A., Berleau, L.T., Nguyen, H.V., Chen, H., Heinsohn, H., Vandlen, R., and Ferrara, N. (1996a). The carboxyl-terminal domain (111-165) of vascular endothelial growth factor is critical for its mitogenic potency. The Journal of biological chemistry *271*, 7788-7795.

Keyt, B.A., Nguyen, H.V., Berleau, L.T., Duarte, C.M., Park, J., Chen, H., and Ferrara, N. (1996b). Identification of vascular endothelial growth factor determinants for binding KDR and FLT-1 receptors. Generation of receptor-selective VEGF variants by site-directed mutagenesis. The Journal of biological chemistry *271*, 5638-5646.

Kim, K.J., Li, B., Winer, J., Armanini, M., Gillett, N., Phillips, H.S., and Ferrara, N. (1993). Inhibition of vascular endothelial growth factor-induced angiogenesis suppresses tumour growth in vivo. Nature *362*, 841-844.

Kindler, H.L., Niedzwiecki, D., Hollis, D., Sutherland, S., Schrag, D., Hurwitz, H., Innocenti, F., Mulcahy, M.F., O'Reilly, E., Wozniak, T.F.*, et al.* (2010). Gemcitabine plus bevacizumab compared with gemcitabine plus placebo in patients with advanced pancreatic cancer: phase III trial of the Cancer and Leukemia Group B (CALGB 80303). Journal of clinical oncology : official journal of the American Society of Clinical Oncology *28*, 3617-3622.

Kirton, J.P., and Xu, Q. (2010). Endothelial precursors in vascular repair. Microvascular research *79*, 193-199.

Kishimoto, J., Ehama, R., Ge, Y., Kobayashi, T., Nishiyama, T., Detmar, M., and Burgeson, R.E. (2000). In vivo detection of human vascular endothelial growth factor promoter activity in transgenic mouse skin. The American journal of pathology *157*, 103-110.

Kitsukawa, T., Shimono, A., Kawakami, A., Kondoh, H., and Fujisawa, H. (1995). Overexpression of a membrane protein, neuropilin, in chimeric mice causes anomalies in the cardiovascular system, nervous system and limbs. Development *121*, 4309-4318.

Klagsbrun, M., Takashima, S., and Mamluk, R. (2002). The role of neuropilin in vascular and tumor biology. Advances in experimental medicine and biology *515*, 33-48.

Koch, S., Tugues, S., Li, X., Gualandi, L., and Claesson-Welsh, L. (2011). Signal transduction by vascular endothelial growth factor receptors. The Biochemical journal *437*, 169-183.

Kolodkin, A.L., Levengood, D.V., Rowe, E.G., Tai, Y.T., Giger, R.J., and Ginty, D.D. (1997). Neuropilin is a semaphorin III receptor. Cell *90*, 753-762.

Konopatskaya, O., Churchill, A.J., Harper, S.J., Bates, D.O., and Gardiner, T.A. (2006). VEGF165b, an endogenous C-terminal splice variant of VEGF, inhibits retinal neovascularization in mice. Molecular vision *12*, 626-632.

Krilleke, D., DeErkenez, A., Schubert, W., Giri, I., Robinson, G.S., Ng, Y.S., and Shima, D.T. (2007). Molecular mapping and functional characterization of the VEGF164 heparin-binding domain. The Journal of biological chemistry *282*, 28045-28056.

Kume, T. (2012). Ligand-dependent Notch signaling in vascular formation. Advances in experimental medicine and biology *727*, 210-222.

Kurz, H., Burri, P.H., and Djonov, V.G. (2003). Angiogenesis and vascular remodeling by intussusception: from form to function. News in physiological sciences : an international journal of physiology produced jointly by the International Union of Physiological Sciences and the American Physiological Society *18*, 65-70.

Kurz, H., Wilting, J., Sandau, K., and Christ, B. (1998). Automated evaluation of angiogenic effects mediated by VEGF and PlGF homo- and heterodimers. Microvascular research *55*, 92-102.

Kvanta, A., Algvere, P.V., Berglin, L., and Seregard, S. (1996). Subfoveal fibrovascular membranes in age-related macular degeneration express vascular endothelial growth factor. Investigative ophthalmology & visual science *37*, 1929-1934.

Labied, S., Delforge, Y., Munaut, C., Blacher, S., Colige, A., Delcombel, R., Henry, L., Fransolet, M., Jouan, C., d'Hauterive, S.P.*, et al.* (2013). Isoform 111 of Vascular Endothelial Growth Factor (VEGF111) Improves Angiogenesis of Ovarian Tissue Xenotransplantation. Transplantation *95*, 426-433.

Lamalice, L., Houle, F., and Huot, J. (2006). Phosphorylation of Tyr1214 within VEGFR-2 triggers the recruitment of Nck and activation of Fyn leading to SAPK2/p38 activation and endothelial cell migration in response to VEGF. The Journal of biological chemistry *281*, 34009-34020.

Lambert, C.A., Mineur, P., and Nusgens, B.V. (2008). [VEGF111: Dr Jekyll and Mr Hyde?]. Medecine sciences : M/S *24*, 579-580.

Lampugnani, L., Salvetti, C., and Tsalev, D.L. (2003). Hydride generation atomic absorption spectrometry with different flow systems and in-atomizer trapping for determination of cadmium in water and urine-overview of existing data on cadmium vapour generation and evaluation of critical parameters. Talanta *61*, 683-698.

Lange, T., Guttmann-Raviv, N., Baruch, L., Machluf, M., and Neufeld, G. (2003). VEGF162, a new heparin-binding vascular endothelial growth factor splice form that is expressed in transformed human cells. The Journal of biological chemistry *278*, 17164-17169.

Latil, A., Bieche, I., Pesche, S., Valeri, A., Fournier, G., Cussenot, O., and Lidereau, R. (2000). VEGF overexpression in clinically localized prostate tumors and neuropilin-1 overexpression in metastatic forms. International journal of cancer Journal international du cancer *89*, 167-171.

Lauer, G., Sollberg, S., Cole, M., Flamme, I., Sturzebecher, J., Mann, K., Krieg, T., and Eming, S.A. (2000). Expression and proteolysis of vascular endothelial growth factor is increased in chronic wounds. The Journal of investigative dermatology *115*, 12-18.

Lauer, G., Sollberg, S., Cole, M., Krieg, T., and Eming, S.A. (2002). Generation of a novel proteolysis resistant vascular endothelial growth factor165 variant by a site-directed mutation at the plasmin sensitive cleavage site. FEBS letters *531*, 309-313.

Launay-Vacher, V., and Deray, G. (2009). Hypertension and proteinuria: a class-effect of antiangiogenic therapies. Anti-cancer drugs *20*, 81-82.

Lee, P., Goishi, K., Davidson, A.J., Mannix, R., Zon, L., and Klagsbrun, M. (2002). Neuropilin-1 is required for vascular development and is a mediator of VEGF-dependent angiogenesis in zebrafish. Proceedings of the National Academy of Sciences of the United States of America *99*, 10470-10475.

Lee, S., Jilani, S.M., Nikolova, G.V., Carpizo, D., and Iruela-Arispe, M.L. (2005). Processing of VEGF-A by matrix metalloproteinases regulates bioavailability and vascular patterning in tumors. The Journal of cell biology *169*, 681-691.

Lei, J., Jiang, A., and Pei, D. (1998). Identification and characterization of a new splicing variant of vascular endothelial growth factor: VEGF183. Biochimica et biophysica acta *1443*, 400-406.

Lemmon, M.A., and Schlessinger, J. (2010). Cell signaling by receptor tyrosine kinases. Cell *141*, 1117-1134.

Leroy, X., Aubert, S., Zini, L., Franquet, H., Kervoaze, G., Villers, A., Delehedde, M., Copin, M.C., and Lassalle, P. (2010). Vascular endocan (ESM-1) is markedly overexpressed in clear cell renal cell carcinoma. Histopathology *56*, 180-187.

Leung, D.W., Cachianes, G., Kuang, W.J., Goeddel, D.V., and Ferrara, N. (1989). Vascular endothelial growth factor is a secreted angiogenic mitogen. Science *246*, 1306-1309.

Levy, A.P., Levy, N.S., Wegner, S., and Goldberg, M.A. (1995). Transcriptional regulation of the rat vascular endothelial growth factor gene by hypoxia. The Journal of biological chemistry *270*, 13333-13340.

Li, Z.Y., Zhu, F., Hu, J.L., Peng, G., Chen, J., Zhang, S., Chen, X., Zhang, R.G., Chen, L.J., Liu, P.*, et al.* (2011). Sp1 inhibition-mediated upregulation of VEGF 165 b induced by rh-endostatin enhances antiangiogenic and anticancer effect of rh-endostatin in A549. Tumour biology : the journal of the International Society for Oncodevelopmental Biology and Medicine *32*, 677-687.

Lindahl, P., Johansson, B.R., Leveen, P., and Betsholtz, C. (1997). Pericyte loss and microaneurysm formation in PDGF-B-deficient mice. Science *277*, 242-245.

Liu, W., Parikh, A.A., Stoeltzing, O., Fan, F., McCarty, M.F., Wey, J., Hicklin, D.J., and Ellis, L.M. (2005). Upregulation of neuropilin-1 by basic fibroblast growth factor enhances vascular smooth muscle cell migration in response to VEGF. Cytokine *32*, 206-212.

Liu, Z.J., Shirakawa, T., Li, Y., Soma, A., Oka, M., Dotto, G.P., Fairman, R.M., Velazquez, O.C., and Herlyn, M. (2003). Regulation of Notch1 and Dll4 by vascular endothelial growth factor in arterial endothelial cells: implications for modulating arteriogenesis and angiogenesis. Molecular and cellular biology *23*, 14-25.

Lorquet, S., Berndt, S., Blacher, S., Gengoux, E., Peulen, O., Maquoi, E., Noel, A., Foidart, J.M., Munaut, C., and Pequeux, C. (2010). Soluble forms of VEGF receptor-1 and -2 promote vascular maturation via mural cell recruitment. The FASEB journal : official publication of the Federation of American Societies for Experimental Biology *24*, 3782-3795.

Mac Gabhann, F., Ji, J.W., and Popel, A.S. (2006). Computational model of vascular endothelial growth factor spatial distribution in muscle and pro-angiogenic cell therapy. PLoS computational biology *2*, e127.

Mac Gabhann, F., and Popel, A.S. (2005). Differential binding of VEGF isoforms to VEGF receptor 2 in the presence of neuropilin-1: a computational model. American journal of physiology Heart and circulatory physiology *288*, H2851-2860.

Mac Gabhann, F., and Popel, A.S. (2007a). Dimerization of VEGF receptors and implications for signal transduction: a computational study. Biophysical chemistry *128*, 125-139.

Mac Gabhann, F., and Popel, A.S. (2007b). Interactions of VEGF isoforms with VEGFR-1, VEGFR-2, and neuropilin in vivo: a computational model of human skeletal muscle. American journal of physiology Heart and circulatory physiology *292*, H459-474.

Maes, C., Stockmans, I., Moermans, K., Van Looveren, R., Smets, N., Carmeliet, P., Bouillon, R., and Carmeliet, G. (2004). Soluble VEGF isoforms are essential for establishing epiphyseal vascularization and regulating chondrocyte development and survival. The Journal of clinical investigation *113*, 188-199.

Magnussen, A.L., Rennel, E.S., Hua, J., Bevan, H.S., Beazley Long, N., Lehrling, C., Gammons, M., Floege, J., Harper, S.J., Agostini, H.T.*, et al.* (2010). VEGF-A165b is cytoprotective and antiangiogenic in the retina. Investigative ophthalmology & visual science *51*, 4273-4281.

Mahabeleshwar, G.H., Feng, W., Phillips, D.R., and Byzova, T.V. (2006). Integrin signaling is critical for pathological angiogenesis. The Journal of experimental medicine *203*, 2495-2507.

Mancuso, M.R., Davis, R., Norberg, S.M., O'Brien, S., Sennino, B., Nakahara, T., Yao, V.J., Inai, T., Brooks, P., Freimark, B.*, et al.* (2006). Rapid vascular regrowth in tumors after reversal of VEGF inhibition. The Journal of clinical investigation *116*, 2610-2621.

Manetti, M., Guiducci, S., Ibba-Manneschi, L., and Matucci-Cerinic, M. (2010). Mechanisms in the loss of capillaries in systemic sclerosis: angiogenesis versus vasculogenesis. Journal of cellular and molecular medicine *14*, 1241-1254.

Manetti, M., Guiducci, S., Romano, E., Ceccarelli, C., Bellando-Randone, S., Conforti, M.L., Ibba-Manneschi, L., and Matucci-Cerinic, M. (2011). Overexpression of VEGF165b, an inhibitory splice variant of vascular endothelial growth factor, leads to insufficient angiogenesis in patients with systemic sclerosis. Circulation research *109*, e14-26.

Matsumoto, T., Bohman, S., Dixelius, J., Berge, T., Dimberg, A., Magnusson, P., Wang, L., Wikner, C., Qi, J.H., Wernstedt, C.*, et al.* (2005). VEGF receptor-2 Y951 signaling and a role for the adapter molecule TSAd in tumor angiogenesis. The EMBO journal *24*, 2342-2353.

Matthies, A.M., Low, Q.E., Lingen, M.W., and DiPietro, L.A. (2002). Neuropilin-1 participates in wound angiogenesis. The American journal of pathology *160*, 289-296.

McFee, R.M., Rozell, T.G., and Cupp, A.S. (2012). The balance of proangiogenic and antiangiogenic VEGFA isoforms regulate follicle development. Cell and tissue research *349*, 635-647.

Meadows, K.N., Bryant, P., and Pumiglia, K. (2001). Vascular endothelial growth factor induction of the angiogenic phenotype requires Ras activation. The Journal of biological chemistry *276*, 49289-49298.

Mehta, D., and Malik, A.B. (2006). Signaling mechanisms regulating endothelial permeability. Physiological reviews *86*, 279-367.

Merdzhanova, G., Edmond, V., De Seranno, S., Van den Broeck, A., Corcos, L., Brambilla, C., Brambilla, E., Gazzeri, S., and Eymin, B. (2008). E2F1 controls alternative splicing pattern of genes involved in apoptosis through upregulation of the splicing factor SC35. Cell death and differentiation *15*, 1815-1823.

Merdzhanova, G., Gout, S., Keramidas, M., Edmond, V., Coll, J.L., Brambilla, C., Brambilla, E., Gazzeri, S., and Eymin, B. (2010). The transcription factor E2F1 and the SR protein SC35 control the ratio of pro-angiogenic versus antiangiogenic isoforms of vascular endothelial growth factor-A to inhibit neovascularization in vivo. Oncogene *29*, 5392-5403.

Mesiano, S., Ferrara, N., and Jaffe, R.B. (1998). Role of vascular endothelial growth factor in ovarian cancer: inhibition of ascites formation by immunoneutralization. The American journal of pathology *153*, 1249-1256.

Meyer, R.D., Mohammadi, M., and Rahimi, N. (2006). A single amino acid substitution in the activation loop defines the decoy characteristic of VEGFR-1/FLT-1. The Journal of biological chemistry *281*, 867-875.

Meyer, R.D., Sacks, D.B., and Rahimi, N. (2008). IQGAP1-dependent signaling pathway regulates endothelial cell proliferation and angiogenesis. PloS one *3*, e3848.

Miao, H.Q., Lee, P., Lin, H., Soker, S., and Klagsbrun, M. (2000). Neuropilin-1 expression by tumor cells promotes tumor angiogenesis and progression. The FASEB journal : official publication of the Federation of American Societies for Experimental Biology *14*, 2532-2539.

Miao, H.Q., Soker, S., Feiner, L., Alonso, J.L., Raper, J.A., and Klagsbrun, M. (1999). Neuropilin-1 mediates collapsin-1/semaphorin III inhibition of endothelial cell motility: functional competition of collapsin-1 and vascular endothelial growth factor-165. The Journal of cell biology *146*, 233-242.

Micha, J.P., Goldstein, B.H., Rettenmaier, M.A., Genesen, M., Graham, C., Bader, K., Lopez, K.L., Nickle, M., and Brown, J.V., 3rd (2007). A phase II study of outpatient first-line paclitaxel, carboplatin, and bevacizumab for advanced-stage epithelial ovarian, peritoneal, and fallopian tube cancer. International journal of gynecological cancer : official journal of the International Gynecological Cancer Society *17*, 771-776.

Milkiewicz, M., Ispanovic, E., Doyle, J.L., and Haas, T.L. (2006). Regulators of angiogenesis and strategies for their therapeutic manipulation. The international journal of biochemistry & cell biology *38*, 333-357.

Millauer, B., Wizigmann-Voos, S., Schnurch, H., Martinez, R., Moller, N.P., Risau, W., and Ullrich, A. (1993). High affinity VEGF binding and developmental expression suggest Flk-1 as a major regulator of vasculogenesis and angiogenesis. Cell *72*, 835-846.

Miller-Kasprzak, E., and Jagodzinski, P.P. (2008). 5-Aza-2'-deoxycytidine increases the expression of anti-angiogenic vascular endothelial growth factor 189b variant in human lung microvascular endothelial cells. Biomedicine & pharmacotherapy = Biomedecine & pharmacotherapie *62*, 158-163.

Miller, K.D., Chap, L.I., Holmes, F.A., Cobleigh, M.A., Marcom, P.K., Fehrenbacher, L., Dickler, M., Overmoyer, B.A., Reimann, J.D., Sing, A.P.*, et al.* (2005). Randomized phase III trial of capecitabine compared with bevacizumab plus capecitabine in patients with previously treated metastatic breast cancer. Journal of clinical oncology : official journal of the American Society of Clinical Oncology *23*, 792-799.

Mineur, P., Colige, A.C., Deroanne, C.F., Dubail, J., Kesteloot, F., Habraken, Y., Noel, A., Voo, S., Waltenberger, J., Lapiere, C.M.*, et al.* (2007). Newly identified biologically active and proteolysis-resistant VEGF-A isoform VEGF111 is induced by genotoxic agents. J Cell Biol *179*, 1261-1273.

Mir, O., Coriat, R., Cabanes, L., Ropert, S., Billemont, B., Alexandre, J., Durand, J.P., Treluyer, J.M., Knebelmann, B., and Goldwasser, F. (2011). An observational study of bevacizumab-induced hypertension as a clinical biomarker of antitumor activity. The oncologist *16*, 1325-1332.

Misra, R.M., Bajaj, M.S., and Kale, V.P. (2012). Vasculogenic Mimicry of HT1080 Tumour Cells In Vivo: Critical Role of HIF-1alpha-Neuropilin-1 Axis. PloS one *7*, e50153.

Miura, K., Fujibuchi, W., and Unno, M. (2012). Splice isoforms as therapeutic targets for colorectal cancer. Carcinogenesis.

Morbidelli, L., Birkenhaeger, R., Roeckl, W., Granger, H.J., Kaerst, U., Weich, H.A., and Ziche, M. (1997). Distinct capillary density and progression promoted by vascular endothelial growth factor-A homodimers and heterodimers. Angiogenesis *1*, 117-130.

Muller, Y.A., Chen, Y., Christinger, H.W., Li, B., Cunningham, B.C., Lowman, H.B., and de Vos, A.M. (1998). VEGF and the Fab fragment of a humanized neutralizing antibody: crystal structure of the complex at 2.4 A resolution and mutational analysis of the interface. Structure *6*, 1153-1167.

Muller, Y.A., Heiring, C., Misselwitz, R., Welfle, K., and Welfle, H. (2002). The cystine knot promotes folding and not thermodynamic stability in vascular endothelial growth factor. The Journal of biological chemistry *277*, 43410-43416.

Muller, Y.A., Li, B., Christinger, H.W., Wells, J.A., Cunningham, B.C., and de Vos, A.M. (1997). Vascular endothelial growth factor: crystal structure and functional mapping of the kinase domain receptor binding site. Proceedings of the National Academy of Sciences of the United States of America *94*, 7192-7197.

Munaut, C., Colige, A.C., and Lambert, C.A. (2010). Alternative splicing: a promising target for pharmaceutical inhibition of pathological angiogenesis? Current pharmaceutical design *16*, 3864-3876.

Murga, M., Fernandez-Capetillo, O., and Tosato, G. (2005). Neuropilin-1 regulates attachment in human endothelial cells independently of vascular endothelial growth factor receptor-2. Blood *105*, 1992-1999.

Nagy, J.A., Chang, S.H., Shih, S.C., Dvorak, A.M., and Dvorak, H.F. (2010). Heterogeneity of the tumor vasculature. Seminars in thrombosis and hemostasis *36*, 321-331.

Nagy, J.A., Dvorak, A.M., and Dvorak, H.F. (2007). VEGF-A and the induction of pathological angiogenesis. Annual review of pathology *2*, 251-275.

Neufeld, G., Cohen, T., Gengrinovitch, S., and Poltorak, Z. (1999). Vascular endothelial growth factor (VEGF) and its receptors. The FASEB journal : official publication of the Federation of American Societies for Experimental Biology *13*, 9-22.

Neufeld, G., Cohen, T., Shraga, N., Lange, T., Kessler, O., and Herzog, Y. (2002). The neuropilins: multifunctional semaphorin and VEGF receptors that modulate axon guidance and angiogenesis. Trends in cardiovascular medicine *12*, 13-19.

Neufeld, G., Tessler, S., Gitay-Goren, H., Cohen, T., and Levi, B.Z. (1994). Vascular endothelial growth factor and its receptors. Progress in growth factor research *5*, 89-97.

Ng, E.W., Shima, D.T., Calias, P., Cunningham, E.T., Jr., Guyer, D.R., and Adamis, A.P. (2006). Pegaptanib, a targeted anti-VEGF aptamer for ocular vascular disease. Nature reviews Drug discovery *5*, 123-132.

Ng, Y.S., Rohan, R., Sunday, M.E., Demello, D.E., and D'Amore, P.A. (2001). Differential expression of VEGF isoforms in mouse during development and in the adult. Developmental dynamics : an official publication of the American Association of Anatomists *220*, 112-121.

Nowak, D.G., Amin, E.M., Rennel, E.S., Hoareau-Aveilla, C., Gammons, M., Damodoran, G., Hagiwara, M., Harper, S.J., Woolard, J., Ladomery, M.R.*, et al.* (2010). Regulation of vascular endothelial growth factor (VEGF) splicing from pro-angiogenic to anti-angiogenic isoforms: a novel therapeutic strategy for angiogenesis. The Journal of biological chemistry *285*, 5532-5540.

Nowak, D.G., Woolard, J., Amin, E.M., Konopatskaya, O., Saleem, M.A., Churchill, A.J., Ladomery, M.R., Harper, S.J., and Bates, D.O. (2008). Expression of pro- and anti-angiogenic isoforms of VEGF is differentially regulated by splicing and growth factors. Journal of cell science *121*, 3487-3495.

Ogawa, S., Oku, A., Sawano, A., Yamaguchi, S., Yazaki, Y., and Shibuya, M. (1998). A novel type of vascular endothelial growth factor, VEGF-E (NZ-7 VEGF), preferentially utilizes KDR/Flk-1 receptor and carries a potent mitotic activity without heparin-binding domain. The Journal of biological chemistry *273*, 31273-31282.

Oh, H., Takagi, H., Otani, A., Koyama, S., Kemmochi, S., Uemura, A., and Honda, Y. (2002). Selective induction of neuropilin-1 by vascular endothelial growth factor (VEGF): a mechanism

contributing to VEGF-induced angiogenesis. Proceedings of the National Academy of Sciences of the United States of America *99*, 383-388.

Okada, F., Rak, J.W., Croix, B.S., Lieubeau, B., Kaya, M., Roncari, L., Shirasawa, S., Sasazuki, T., and Kerbel, R.S. (1998). Impact of oncogenes in tumor angiogenesis: mutant K-ras up-regulation of vascular endothelial growth factor/vascular permeability factor is necessary, but not sufficient for tumorigenicity of human colorectal carcinoma cells. Proceedings of the National Academy of Sciences of the United States of America *95*, 3609-3614.

Olofsson, B., Pajusola, K., Kaipainen, A., von Euler, G., Joukov, V., Saksela, O., Orpana, A., Pettersson, R.F., Alitalo, K., and Eriksson, U. (1996). Vascular endothelial growth factor B, a novel growth factor for endothelial cells. Proceedings of the National Academy of Sciences of the United States of America *93*, 2576-2581.

Oltean, S., Gammons, M., Hulse, R., Hamdollah-Zadeh, M., Mavrou, A., Donaldson, L., Salmon, A.H., Harper, S.J., Ladomery, M.R., and Bates, D.O. (2012a). SRPK1 inhibition in vivo: modulation of VEGF splicing and potential treatment for multiple diseases. Biochemical Society transactions *40*, 831-835.

Oltean, S., Neal, C.R., Mavrou, A., Patel, P., Ahad, T., Alsop, C., Lee, T., Sison, K., Qiu, Y., Harper, S.J.*, et al.* (2012b). VEGF165b overexpression restores normal glomerular water permeability in VEGF164-overexpressing adult mice. American journal of physiology Renal physiology.

Oshika, Y., Nakamura, M., Tokunaga, T., Ohnishi, Y., Abe, Y., Tsuchida, T., Tomii, Y., Kijima, H., Yamazaki, H., Ozeki, Y.*, et al.* (2000). Ribozyme approach to downregulate vascular endothelial growth factor (VEGF) 189 expression in non-small cell lung cancer (NSCLC). Eur J Cancer *36*, 2390-2396.

Palolahti, M., Lauharanta, J., Stephens, R.W., Kuusela, P., and Vaheri, A. (1993). Proteolytic activity in leg ulcer exudate. Experimental dermatology *2*, 29-37.

Pan, Q., Chanthery, Y., Liang, W.C., Stawicki, S., Mak, J., Rathore, N., Tong, R.K., Kowalski, J., Yee, S.F., Pacheco, G.*, et al.* (2007a). Blocking neuropilin-1 function has an additive effect with anti-VEGF to inhibit tumor growth. Cancer cell *11*, 53-67.

Pan, Q., Chathery, Y., Wu, Y., Rathore, N., Tong, R.K., Peale, F., Bagri, A., Tessier-Lavigne, M., Koch, A.W., and Watts, R.J. (2007b). Neuropilin-1 binds to VEGF121 and regulates endothelial cell migration and sprouting. The Journal of biological chemistry *282*, 24049-24056.

Park, J.E., Chen, H.H., Winer, J., Houck, K.A., and 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. The Journal of biological chemistry *269*, 25646-25654.

Park, J.E., Keller, G.A., and Ferrara, N. (1993). The vascular endothelial growth factor (VEGF) isoforms: differential deposition into the subepithelial extracellular matrix and bioactivity of extracellular matrix-bound VEGF. Molecular biology of the cell *4*, 1317-1326.

Park, M., and Lee, S.T. (1999). The fourth immunoglobulin-like loop in the extracellular domain of FLT-1, a VEGF receptor, includes a major heparin-binding site. Biochemical and biophysical research communications *264*, 730-734.

Parker, M.W., Xu, P., Li, X., and Vander Kooi, C.W. (2012). Structural basis for the selective vascular endothelial growth factor-A (VEGF-A) binding to neuropilin-1. The Journal of biological chemistry.

Peiris-Pages, M. (2012). The role of VEGF 165b in pathophysiology. Cell adhesion & migration *6*.

Peiris-Pages, M., Harper, S.J., Bates, D.O., and Ramani, P. (2010). Balance of pro- versus anti-angiogenic splice isoforms of vascular endothelial growth factor as a regulator of neuroblastoma growth. The Journal of pathology *222*, 138-147.

Pellet-Many, C., Frankel, P., Jia, H., and Zachary, I. (2008). Neuropilins: structure, function and role in disease. The Biochemical journal *411*, 211-226.

Penn, J.S., and Rajaratnam, V.S. (2003). Inhibition of retinal neovascularization by intravitreal injection of human rPAI-1 in a rat model of retinopathy of prematurity. Investigative ophthalmology & visual science *44*, 5423-5429.

Penn, J.S., Tolman, B.L., and Henry, M.M. (1994). Oxygen-induced retinopathy in the rat: relationship of retinal nonperfusion to subsequent neovascularization. Investigative ophthalmology & visual science *35*, 3429-3435.

Perrin, R.M., Konopatskaya, O., Qiu, Y., Harper, S., Bates, D.O., and Churchill, A.J. (2005). Diabetic retinopathy is associated with a switch in splicing from anti- to pro-angiogenic isoforms of vascular endothelial growth factor. Diabetologia *48*, 2422-2427.

Pieren, M., Prota, A.E., Ruch, C., Kostrewa, D., Wagner, A., Biedermann, K., Winkler, F.K., and Ballmer-Hofer, K. (2006). Crystal structure of the Orf virus NZ2 variant of vascular endothelial growth factor-E. Implications for receptor specificity. The Journal of biological chemistry *281*, 19578-19587.

Plouet, J., Moro, F., Bertagnolli, S., Coldeboeuf, N., Mazarguil, H., Clamens, S., and Bayard, F. (1997). Extracellular cleavage of the vascular endothelial growth factor 189-amino acid form by urokinase is required for its mitogenic effect. The Journal of biological chemistry *272*, 13390-13396.

Plouet, J., and Moukadiri, H. (1990a). Characterization of the receptor to vasculotropin on bovine adrenal cortex-derived capillary endothelial cells. The Journal of biological chemistry *265*, 22071-22074.

Plouet, J., and Moukadiri, H. (1990b). Specific binding of vasculotropin to bovine brain capillary endothelial cells. Biochimie *72*, 51-55.

Plouet, J., Schilling, J., and Gospodarowicz, D. (1989). Isolation and characterization of a newly identified endothelial cell mitogen produced by AtT-20 cells. The EMBO journal *8*, 3801-3806.

Poltorak, Z., Cohen, T., and Neufeld, G. (2000). The VEGF splice variants: properties, receptors, and usage for the treatment of ischemic diseases. Herz *25*, 126-129.

Poltorak, Z., Cohen, T., Sivan, R., Kandelis, Y., Spira, G., Vlodavsky, I., Keshet, E., and Neufeld, G. (1997). VEGF145, a secreted vascular endothelial growth factor isoform that binds to extracellular matrix. The Journal of biological chemistry *272*, 7151-7158.

Potente, M., Gerhardt, H., and Carmeliet, P. (2011). Basic and therapeutic aspects of angiogenesis. Cell *146*, 873-887.

Presta, L.G., Chen, H., O'Connor, S.J., Chisholm, V., Meng, Y.G., Krummen, L., Winkler, M., and Ferrara, N. (1997). Humanization of an anti-vascular endothelial growth factor monoclonal antibody for the therapy of solid tumors and other disorders. Cancer research *57*, 4593-4599.

Pritchard-Jones, R.O., Dunn, D.B., Qiu, Y., Varey, A.H., Orlando, A., Rigby, H., Harper, S.J., and Bates, D.O. (2007). Expression of VEGF(xxx)b, the inhibitory isoforms of VEGF, in malignant melanoma. British journal of cancer *97*, 223-230.

Prud'homme, G.J., and Glinka, Y. (2012). Neuropilins are multifunctional coreceptors involved in tumor initiation, growth, metastasis and immunity. Oncotarget *3*, 921-939.

Rak, J., Mitsuhashi, Y., Bayko, L., Filmus, J., Shirasawa, S., Sasazuki, T., and Kerbel, R.S. (1995). Mutant ras oncogenes upregulate VEGF/VPF expression: implications for induction and inhibition of tumor angiogenesis. Cancer research *55*, 4575-4580.

Ramaswamy, B., Elias, A.D., Kelbick, N.T., Dodley, A., Morrow, M., Hauger, M., Allen, J., Rhoades, C., Kendra, K., Chen, H.X.*, et al.* (2006). Phase II trial of bevacizumab in combination with weekly docetaxel in metastatic breast cancer patients. Clinical cancer research : an official journal of the American Association for Cancer Research *12*, 3124-3129.

Ramsauer, M., and D'Amore, P.A. (2002). Getting Tie(2)d up in angiogenesis. The Journal of clinical investigation *110*, 1615-1617.

Recchia, F.M., Xu, L., Penn, J.S., Boone, B., and Dexheimer, P.J. (2010). Identification of genes and pathways involved in retinal neovascularization by microarray analysis of two animal models of retinal angiogenesis. Investigative ophthalmology & visual science *51*, 1098-1105.

Rennel, E., Mellberg, S., Dimberg, A., Petersson, L., Botling, J., Ameur, A., Westholm, J.O., Komorowski, J., Lassalle, P., Cross, M.J.*, et al.* (2007). Endocan is a VEGF-A and PI3K regulated gene with increased expression in human renal cancer. Experimental cell research *313*, 1285-1294.

Rennel, E.S., Hamdollah-Zadeh, M.A., Wheatley, E.R., Magnussen, A., Schuler, Y., Kelly, S.P., Finucane, C., Ellison, D., Cebe-Suarez, S., Ballmer-Hofer, K.*, et al.* (2008). Recombinant human VEGF165b protein is an effective anti-cancer agent in mice. Eur J Cancer *44*, 1883-1894.

Rennel, E.S., Harper, S.J., and Bates, D.O. (2009a). Therapeutic potential of manipulating VEGF splice isoforms in oncology. Future Oncol *5*, 703-712.

Rennel, E.S., Varey, A.H., Churchill, A.J., Wheatley, E.R., Stewart, L., Mather, S., Bates, D.O., and Harper, S.J. (2009b). VEGF(121)b, a new member of the VEGF(xxx)b family of VEGF-A splice isoforms, inhibits neovascularisation and tumour growth in vivo. British journal of cancer *101*, 1183-1193.

Rini, B.I., Halabi, S., Rosenberg, J.E., Stadler, W.M., Vaena, D.A., Ou, S.S., Archer, L., Atkins, J.N., Picus, J., Czaykowski, P.*, et al.* (2008). Bevacizumab plus interferon alfa compared with interferon alfa monotherapy in patients with metastatic renal cell carcinoma: CALGB 90206. Journal of clinical oncology : official journal of the American Society of Clinical Oncology *26*, 5422-5428.

Rini, B.I., Wilding, G., Hudes, G., Stadler, W.M., Kim, S., Tarazi, J., Rosbrook, B., Trask, P.C., Wood, L., and Dutcher, J.P. (2009). Phase II study of axitinib in sorafenib-refractory metastatic renal cell carcinoma. Journal of clinical oncology : official journal of the American Society of Clinical Oncology *27*, 4462-4468.

Romano Di Peppe, S., Mangoni, A., Zambruno, G., Spinetti, G., Melillo, G., Napolitano, M., and Capogrossi, M.C. (2002). Adenovirus-mediated VEGF(165) gene transfer enhances wound healing by promoting angiogenesis in CD1 diabetic mice. Gene therapy *9*, 1271-1277.

Rossignol, M., Gagnon, M.L., and Klagsbrun, M. (2000). Genomic organization of human neuropilin-1 and neuropilin-2 genes: identification and distribution of splice variants and soluble isoforms. Genomics *70*, 211-222.

Roth, D., Piekarek, M., Paulsson, M., Christ, H., Bloch, W., Krieg, T., Davidson, J.M., and Eming, S.A. (2006). Plasmin modulates vascular endothelial growth factor-A-mediated angiogenesis during wound repair. The American journal of pathology *168*, 670-684.

Roura, S., Miravet, S., Piedra, J., Garcia de Herreros, A., and Dunach, M. (1999). Regulation of E-cadherin/Catenin association by tyrosine phosphorylation. The Journal of biological chemistry *274*, 36734-36740.

Ruhrberg, C., Gerhardt, H., Golding, M., Watson, R., Ioannidou, S., Fujisawa, H., Betsholtz, C., and Shima, D.T. (2002). Spatially restricted patterning cues provided by heparin-binding VEGF-A control blood vessel branching morphogenesis. Genes & development *16*, 2684-2698.

Sainson, R.C., Aoto, J., Nakatsu, M.N., Holderfield, M., Conn, E., Koller, E., and Hughes, C.C. (2005). Cell-autonomous notch signaling regulates endothelial cell branching and proliferation during vascular tubulogenesis. The FASEB journal : official publication of the Federation of American Societies for Experimental Biology *19*, 1027-1029.

Sakurai, Y., Ohgimoto, K., Kataoka, Y., Yoshida, N., and Shibuya, M. (2005). Essential role of Flk-1 (VEGF receptor 2) tyrosine residue 1173 in vasculogenesis in mice. Proceedings of the National Academy of Sciences of the United States of America *102*, 1076-1081.

Sarrazin, S., Adam, E., Lyon, M., Depontieu, F., Motte, V., Landolfi, C., Lortat-Jacob, H., Bechard, D., Lassalle, P., and Delehedde, M. (2006). Endocan or endothelial cell specific molecule-1 (ESM-1): a potential novel endothelial cell marker and a new target for cancer therapy. Biochimica et biophysica acta *1765*, 25-37.

Scherpereel, A., Gentina, T., Grigoriu, B., Senechal, S., Janin, A., Tsicopoulos, A., Plenat, F., Bechard, D., Tonnel, A.B., and Lassalle, P. (2003). Overexpression of endocan induces tumor formation. Cancer research *63*, 6084-6089.

Schumacher, V.A., Jeruschke, S., Eitner, F., Becker, J.U., Pitschke, G., Ince, Y., Miner, J.H., Leuschner, I., Engers, R., Everding, A.S.*, et al.* (2007). Impaired glomerular maturation and lack of VEGF165b in Denys-Drash syndrome. Journal of the American Society of Nephrology : JASN *18*, 719-729.

Seetharam, L., Gotoh, N., Maru, Y., Neufeld, G., Yamaguchi, S., and Shibuya, M. (1995). A unique signal transduction from FLT tyrosine kinase, a receptor for vascular endothelial growth factor VEGF. Oncogene *10*, 135-147.

Senger, D.R., Galli, S.J., Dvorak, A.M., Perruzzi, C.A., Harvey, V.S., and Dvorak, H.F. (1983). Tumor cells secrete a vascular permeability factor that promotes accumulation of ascites fluid. Science *219*, 983-985.

Shalaby, F., Rossant, J., Yamaguchi, T.P., Gertsenstein, M., Wu, X.F., Breitman, M.L., and Schuh, A.C. (1995). Failure of blood-island formation and vasculogenesis in Flk-1-deficient mice. Nature *376*, 62-66.

Shen, J., Samul, R., Zimmer, J., Liu, H., Liang, X., Hackett, S., and Campochiaro, P.A. (2004). Deficiency of neuropilin 2 suppresses VEGF-induced retinal neovascularization. Mol Med *10*, 12-18.

Shibuya, M. (1995). Role of VEGF-flt receptor system in normal and tumor angiogenesis. Advances in cancer research *67*, 281-316.

Shibuya, M. (2001). Structure and dual function of vascular endothelial growth factor receptor-1 (Flt-1). The international journal of biochemistry & cell biology *33*, 409-420.

Shimizu, M., Murakami, Y., Suto, F., and Fujisawa, H. (2000). Determination of cell adhesion sites of neuropilin-1. The Journal of cell biology *148*, 1283-1293.

Shintani, Y., Takashima, S., Asano, Y., Kato, H., Liao, Y., Yamazaki, S., Tsukamoto, O., Seguchi, O., Yamamoto, H., Fukushima, T.*, et al.* (2006). Glycosaminoglycan modification of neuropilin-1 modulates VEGFR2 signaling. The EMBO journal *25*, 3045-3055.

Shintani, Y., Takashima, S., Kato, H., Komamura, K., and Kitakaze, M. (2009). Extracellular protein kinase CK2 is a novel associating protein of neuropilin-1. Biochemical and biophysical research communications *385*, 618-623.

Shojaei, F., Lee, J.H., Simmons, B.H., Wong, A., Esparza, C.O., Plumlee, P.A., Feng, J., Stewart, A.E., Hu-Lowe, D.D., and Christensen, J.G. (2010). HGF/c-Met acts as an alternative angiogenic pathway in sunitinib-resistant tumors. Cancer research *70*, 10090-10100.

Shraga-Heled, N., Kessler, O., Prahst, C., Kroll, J., Augustin, H., and Neufeld, G. (2007). Neuropilin-1 and neuropilin-2 enhance VEGF121 stimulated signal transduction by the VEGFR-2 receptor. The FASEB journal : official publication of the Federation of American Societies for Experimental Biology *21*, 915-926.

Shweiki, D., Itin, A., Soffer, D., and Keshet, E. (1992). Vascular endothelial growth factor induced by hypoxia may mediate hypoxia-initiated angiogenesis. Nature *359*, 843-845.

Silva, S.R., Bowen, K.A., Rychahou, P.G., Jackson, L.N., Weiss, H.L., Lee, E.Y., Townsend, C.M., Jr., and Evers, B.M. (2011). VEGFR-2 expression in carcinoid cancer cells and its role in tumor growth and metastasis. International journal of cancer Journal international du cancer *128*, 1045-1056.

Sims, D.E. (1986). The pericyte--a review. Tissue & cell *18*, 153-174.

Sims, D.E. (1991). Recent advances in pericyte biology--implications for health and disease. The Canadian journal of cardiology *7*, 431-443.

Sitohy, B., Nagy, J.A., and Dvorak, H.F. (2012). Anti-VEGF/VEGFR Therapy for Cancer: Reassessing the Target. Cancer research *72*, 1909-1914.

Skotheim, R.I., and Nees, M. (2007). Alternative splicing in cancer: noise, functional, or systematic? The international journal of biochemistry & cell biology *39*, 1432-1449.

Smith, N.R., Baker, D., James, N.H., Ratcliffe, K., Jenkins, M., Ashton, S.E., Sproat, G., Swann, R., Gray, N., Ryan, A.*, et al.* (2010). Vascular endothelial growth factor receptors VEGFR-2 and VEGFR-3 are localized primarily to the vasculature in human primary solid cancers. Clinical cancer research : an official journal of the American Association for Cancer Research *16*, 3548-3561.

Soker, S., Fidder, H., Neufeld, G., and Klagsbrun, M. (1996). Characterization of novel vascular endothelial growth factor (VEGF) receptors on tumor cells that bind VEGF165 via its exon 7-encoded domain. The Journal of biological chemistry *271*, 5761-5767.

Soker, S., Gollamudi-Payne, S., Fidder, H., Charmahelli, H., and Klagsbrun, M. (1997). Inhibition of vascular endothelial growth factor (VEGF)-induced endothelial cell proliferation by a peptide corresponding to the exon 7-encoded domain of VEGF165. The Journal of biological chemistry *272*, 31582-31588.

Soker, S., Miao, H.Q., Nomi, M., Takashima, S., and Klagsbrun, M. (2002). VEGF165 mediates formation of complexes containing VEGFR-2 and neuropilin-1 that enhance VEGF165-receptor binding. Journal of cellular biochemistry *85*, 357-368.

Soker, S., Takashima, S., Miao, H.Q., Neufeld, G., and Klagsbrun, M. (1998). Neuropilin-1 is expressed by endothelial and tumor cells as an isoform-specific receptor for vascular endothelial growth factor. Cell *92*, 735-745.

Sounni, N.E., Dehne, K., van Kempen, L., Egeblad, M., Affara, N.I., Cuevas, I., Wiesen, J., Junankar, S., Korets, L., Lee, J.*, et al.* (2010). Stromal regulation of vessel stability by MMP14 and TGFbeta. Disease models & mechanisms *3*, 317-332.

Spitzer, M.S., Wallenfels-Thilo, B., Sierra, A., Yoeruek, E., Peters, S., Henke-Fahle, S., Bartz-Schmidt, K.U., and Szurman, P. (2006). Antiproliferative and cytotoxic properties of bevacizumab on different ocular cells. The British journal of ophthalmology *90*, 1316-1321.

Springer, M.L., Banfi, A., Ye, J., von Degenfeld, G., Kraft, P.E., Saini, S.A., Kapasi, N.K., and Blau, H.M. (2007). Localization of vascular response to VEGF is not dependent on heparin binding. The FASEB journal : official publication of the Federation of American Societies for Experimental Biology *21*, 2074-2085.

Stalmans, I., Ng, Y.S., Rohan, R., Fruttiger, M., Bouche, A., Yuce, A., Fujisawa, H., Hermans, B., Shani, M., Jansen, S.*, et al.* (2002). Arteriolar and venular patterning in retinas of mice selectively expressing VEGF isoforms. The Journal of clinical investigation *109*, 327-336.

Stamm, S. (2008). Regulation of alternative splicing by reversible protein phosphorylation. The Journal of biological chemistry *283*, 1223-1227.

Starzec, A., Ladam, P., Vassy, R., Badache, S., Bouchemal, N., Navaza, A., du Penhoat, C.H., and Perret, G.Y. (2007). Structure-function analysis of the antiangiogenic ATWLPPR peptide inhibiting VEGF(165) binding to neuropilin-1 and molecular dynamics simulations of the ATWLPPR/neuropilin-1 complex. Peptides *28*, 2397-2402.

Starzec, A., Vassy, R., Martin, A., Lecouvey, M., Di Benedetto, M., Crepin, M., and Perret, G.Y. (2006). Antiangiogenic and antitumor activities of peptide inhibiting the vascular endothelial growth factor binding to neuropilin-1. Life sciences *79*, 2370-2381.

Sternberg, C.N., Davis, I.D., Mardiak, J., Szczylik, C., Lee, E., Wagstaff, J., Barrios, C.H., Salman, P., Gladkov, O.A., Kavina, A.*, et al.* (2010). Pazopanib in locally advanced or metastatic renal cell carcinoma: results of a randomized phase III trial. Journal of clinical oncology : official journal of the American Society of Clinical Oncology *28*, 1061-1068.

Stone, J., Itin, A., Alon, T., Pe'er, J., Gnessin, H., Chan-Ling, T., and Keshet, E. (1995). Development of retinal vasculature is mediated by hypoxia-induced vascular endothelial growth factor (VEGF) expression by neuroglia. The Journal of neuroscience : the official journal of the Society for Neuroscience *15*, 4738-4747.

Strasser, G.A., Kaminker, J.S., and Tessier-Lavigne, M. (2010). Microarray analysis of retinal endothelial tip cells identifies CXCR4 as a mediator of tip cell morphology and branching. Blood *115*, 5102-5110.

Strieter, R.M. (2005). Masters of angiogenesis. Nature medicine *11*, 925-927.

Stuttfeld, E., and Ballmer-Hofer, K. (2009). Structure and function of VEGF receptors. IUBMB life *61*, 915-922.

Suchting, S., Freitas, C., le Noble, F., Benedito, R., Breant, C., Duarte, A., and Eichmann, A. (2007). The Notch ligand Delta-like 4 negatively regulates endothelial tip cell formation and vessel branching. Proceedings of the National Academy of Sciences of the United States of America *104*, 3225-3230.

Sulpice, E., Plouet, J., Berge, M., Allanic, D., Tobelem, G., and Merkulova-Rainon, T. (2008). Neuropilin-1 and neuropilin-2 act as coreceptors, potentiating proangiogenic activity. Blood *111*, 2036-2045.

Sumanasekera, C., Watt, D.S., and Stamm, S. (2008). Substances that can change alternative splice-site selection. Biochemical Society transactions *36*, 483-490.

Suri, C., Jones, P.F., Patan, S., Bartunkova, S., Maisonpierre, P.C., Davis, S., Sato, T.N., and Yancopoulos, G.D. (1996). Requisite role of angiopoietin-1, a ligand for the TIE2 receptor, during embryonic angiogenesis. Cell *87*, 1171-1180.

Takahashi, T., Yamaguchi, S., Chida, K., and Shibuya, M. (2001). A single autophosphorylation site on KDR/Flk-1 is essential for VEGF-A-dependent activation of PLC-gamma and DNA synthesis in vascular endothelial cells. The EMBO journal *20*, 2768-2778.

Takashima, S., Kitakaze, M., Asakura, M., Asanuma, H., Sanada, S., Tashiro, F., Niwa, H., Miyazaki Ji, J., Hirota, S., Kitamura, Y.*, et al.* (2002). Targeting of both mouse neuropilin-1 and neuropilin-2 genes severely impairs developmental yolk sac and embryonic angiogenesis. Proceedings of the National Academy of Sciences of the United States of America *99*, 3657-3662.

Tanaka, K., Yamaguchi, S., Sawano, A., and Shibuya, M. (1997). Characterization of the extracellular domain in vascular endothelial growth factor receptor-1 (Flt-1 tyrosine kinase). Japanese journal of cancer research : Gann *88*, 867-876.

Tanimoto, K., Yoshiga, K., Eguchi, H., Kaneyasu, M., Ukon, K., Kumazaki, T., Oue, N., Yasui, W., Imai, K., Nakachi, K.*, et al.* (2003). Hypoxia-inducible factor-1alpha polymorphisms associated with enhanced transactivation capacity, implying clinical significance. Carcinogenesis *24*, 1779-1783.

Tee, M.K., and Jaffe, R.B. (2001). A precursor form of vascular endothelial growth factor arises by initiation from an upstream in-frame CUG codon. The Biochemical journal *359*, 219-226.

Teng, L.S., Jin, K.T., He, K.F., Zhang, J., Wang, H.H., and Cao, J. (2010). Clinical applications of VEGF-trap (aflibercept) in cancer treatment. Journal of the Chinese Medical Association : JCMA *73*, 449-456.

Terman, B.I., Dougher-Vermazen, M., Carrion, M.E., Dimitrov, D., Armellino, D.C., Gospodarowicz, D., and Bohlen, P. (1992). Identification of the KDR tyrosine kinase as a receptor for vascular endothelial cell growth factor. Biochemical and biophysical research communications *187*, 1579-1586.

Tessler, S., Rockwell, P., Hicklin, D., Cohen, T., Levi, B.Z., Witte, L., Lemischka, I.R., and Neufeld, G. (1994). Heparin modulates the interaction of VEGF165 with soluble and cell associated flk-1 receptors. The Journal of biological chemistry *269*, 12456-12461.

Thomas, M., and Augustin, H.G. (2009). The role of the Angiopoietins in vascular morphogenesis. Angiogenesis *12*, 125-137.

Tirand, L., Frochot, C., Vanderesse, R., Thomas, N., Trinquet, E., Pinel, S., Viriot, M.L., Guillemin, F., and Barberi-Heyob, M. (2006). A peptide competing with VEGF165 binding on neuropilin-1 mediates targeting of a chlorin-type photosensitizer and potentiates its photodynamic activity in human endothelial cells. Journal of controlled release : official journal of the Controlled Release Society *111*, 153-164.

Tischer, E., Mitchell, R., Hartman, T., Silva, M., Gospodarowicz, D., Fiddes, J.C., and Abraham, J.A. (1991). The human gene for vascular endothelial growth factor. Multiple protein forms are encoded through alternative exon splicing. The Journal of biological chemistry *266*, 11947-11954.

Tordjman, R., Lepelletier, Y., Lemarchandel, V., Cambot, M., Gaulard, P., Hermine, O., and Romeo, P.H. (2002). A neuronal receptor, neuropilin-1, is essential for the initiation of the primary immune response. Nature immunology *3*, 477-482.

Tozer, G.M., Akerman, S., Cross, N.A., Barber, P.R., Bjorndahl, M.A., Greco, O., Harris, S., Hill, S.A., Honess, D.J., Ireson, C.R.*, et al.* (2008). Blood vessel maturation and response to vascular-disrupting therapy in single vascular endothelial growth factor-A isoform-producing tumors. Cancer research *68*, 2301-2311.

Uyttendaele, H., Ho, J., Rossant, J., and Kitajewski, J. (2001). Vascular patterning defects associated with expression of activated Notch4 in embryonic endothelium. Proceedings of the National Academy of Sciences of the United States of America *98*, 5643-5648.

Van Cutsem, E., Tabernero, J., Lakomy, R., Prenen, H., Prausova, J., Macarulla, T., Ruff, P., van Hazel, G.A., Moiseyenko, V., Ferry, D.*, et al.* (2012). Addition of Aflibercept to Fluorouracil, Leucovorin, and Irinotecan Improves Survival in a Phase III Randomized Trial in Patients With Metastatic Colorectal Cancer Previously Treated With an Oxaliplatin-Based Regimen. Journal of clinical oncology : official journal of the American Society of Clinical Oncology *30*, 3499-3506.

Van Cutsem, E., Vervenne, W.L., Bennouna, J., Humblet, Y., Gill, S., Van Laethem, J.L., Verslype, C., Scheithauer, W., Shang, A., Cosaert, J.*, et al.* (2009). Phase III trial of bevacizumab in combination with gemcitabine and erlotinib in patients with metastatic pancreatic cancer. Journal of clinical oncology : official journal of the American Society of Clinical Oncology *27*, 2231-2237.

Vander Kooi, C.W., Jusino, M.A., Perman, B., Neau, D.B., Bellamy, H.D., and Leahy, D.J. (2007). Structural basis for ligand and heparin binding to neuropilin B domains. Proceedings of the National Academy of Sciences of the United States of America *104*, 6152-6157.

Varey, A.H., Rennel, E.S., Qiu, Y., Bevan, H.S., Perrin, R.M., Raffy, S., Dixon, A.R., Paraskeva, C., Zaccheo, O., Hassan, A.B.*, et al.* (2008). VEGF 165 b, an antiangiogenic VEGF-A isoform, binds and inhibits bevacizumab treatment in experimental colorectal carcinoma: balance of pro- and antiangiogenic VEGF-A isoforms has implications for therapy. British journal of cancer *98*, 1366-1379.

von Wronski, M.A., Raju, N., Pillai, R., Bogdan, N.J., Marinelli, E.R., Nanjappan, P., Ramalingam, K., Arunachalam, T., Eaton, S., Linder, K.E.*, et al.* (2006). Tuftsin binds neuropilin-1 through a sequence similar to that encoded by exon 8 of vascular endothelial growth factor. The Journal of biological chemistry *281*, 5702-5710.

Wallez, Y., and Huber, P. (2008). Endothelial adherens and tight junctions in vascular homeostasis, inflammation and angiogenesis. Biochimica et biophysica acta *1778*, 794-809.

Waltenberger, J., Claesson-Welsh, L., Siegbahn, A., Shibuya, M., and Heldin, C.H. (1994). Different signal transduction properties of KDR and Flt1, two receptors for vascular endothelial growth factor. The Journal of biological chemistry *269*, 26988-26995.

Wang, E.T., Sandberg, R., Luo, S., Khrebtukova, I., Zhang, L., Mayr, C., Kingsmore, S.F., Schroth, G.P., and Burge, C.B. (2008). Alternative isoform regulation in human tissue transcriptomes. Nature *456*, 470-476.

Wang, L., Mukhopadhyay, D., and Xu, X. (2006). C terminus of RGS-GAIP-interacting protein conveys neuropilin-1-mediated signaling during angiogenesis. The FASEB journal : official publication of the Federation of American Societies for Experimental Biology *20*, 1513-1515.

Wang, L., Zeng, H., Wang, P., Soker, S., and Mukhopadhyay, D. (2003). Neuropilin-1-mediated vascular permeability factor/vascular endothelial growth factor-dependent endothelial cell migration. The Journal of biological chemistry *278*, 48848-48860.

Wang, Z.L., Tang, Z.C., Zhang, Y., Zhang, W., Yao, J., Wei, Z.C., Qi, B., Song, X.M., and Wu, Y.N. (2012). Neuropilin-1 down-regulation impairs cell migration and induces the differentiation of human tongue squamous cell carcinoma cells. Head & neck oncology *4*, 54.

Warner, A.J., Lopez-Dee, J., Knight, E.L., Feramisco, J.R., and Prigent, S.A. (2000). The Shc-related adaptor protein, Sck, forms a complex with the vascular-endothelial-growth-factor receptor KDR in transfected cells. The Biochemical journal *347*, 501-509.

Warren, R.S., Yuan, H., Matli, M.R., Gillett, N.A., and Ferrara, N. (1995). Regulation by vascular endothelial growth factor of human colon cancer tumorigenesis in a mouse model of experimental liver metastasis. The Journal of clinical investigation *95*, 1789-1797.

Wen, Y., Edelman, J.L., Kang, T., Zeng, N., and Sachs, G. (1998). Two functional forms of vascular endothelial growth factor receptor-2/Flk-1 mRNA are expressed in normal rat retina. The Journal of biological chemistry *273*, 2090-2097.

Whitaker, G.B., Limberg, B.J., and Rosenbaum, J.S. (2001). Vascular endothelial growth factor receptor-2 and neuropilin-1 form a receptor complex that is responsible for the differential signaling potency of VEGF(165) and VEGF(121). The Journal of biological chemistry *276*, 25520-25531.

Whittle, C., Gillespie, K., Harrison, R., Mathieson, P.W., and Harper, S.J. (1999). Heterogeneous vascular endothelial growth factor (VEGF) isoform mRNA and receptor mRNA expression in human glomeruli, and the identification of VEGF148 mRNA, a novel truncated splice variant. Clin Sci (Lond) *97*, 303-312.

Whittles, C.E., Pocock, T.M., Wedge, S.R., Kendrew, J., Hennequin, L.F., Harper, S.J., and Bates, D.O. (2002). ZM323881, a novel inhibitor of vascular endothelial growth factor-receptor-2 tyrosine kinase activity. Microcirculation *9*, 513-522.

Woolard, J., Wang, W.Y., Bevan, H.S., Qiu, Y., Morbidelli, L., Pritchard-Jones, R.O., Cui, T.G., Sugiono, M., Waine, E., Perrin, R.*, et al.* (2004). VEGF165b, an inhibitory vascular endothelial

growth factor splice variant: mechanism of action, in vivo effect on angiogenesis and endogenous protein expression. Cancer research *64*, 7822-7835.

Xu, D., Fuster, M.M., Lawrence, R., and Esko, J.D. (2011a). Heparan sulfate regulates VEGF165- and VEGF121-mediated vascular hyperpermeability. The Journal of biological chemistry *286*, 737-745.

Xu, J., Dou, T., Liu, C., Fu, M., Huang, Y., Gu, S., Zhou, Y., and Xie, Y. (2011b). The evolution of alternative splicing exons in vascular endothelial growth factor A. Gene *487*, 143-150.

Xue, Y., Chen, F., Zhang, D., Lim, S., and Cao, Y. (2009). Tumor-derived VEGF modulates hematopoiesis. Journal of angiogenesis research *1*, 9.

Xue, Y., Religa, P., Cao, R., Hansen, A.J., Lucchini, F., Jones, B., Wu, Y., Zhu, Z., Pytowski, B., Liang, Y.*, et al.* (2008). Anti-VEGF agents confer survival advantages to tumor-bearing mice by improving cancer-associated systemic syndrome. Proceedings of the National Academy of Sciences of the United States of America *105*, 18513-18518.

Yager, D.R., Zhang, L.Y., Liang, H.X., Diegelmann, R.F., and Cohen, I.K. (1996). Wound fluids from human pressure ulcers contain elevated matrix metalloproteinase levels and activity compared to surgical wound fluids. The Journal of investigative dermatology *107*, 743-748.

Yamada, Y., Takakura, N., Yasue, H., Ogawa, H., Fujisawa, H., and Suda, T. (2001). Exogenous clustered neuropilin 1 enhances vasculogenesis and angiogenesis. Blood *97*, 1671-1678.

Yamaoka-Tojo, M., Tojo, T., Kim, H.W., Hilenski, L., Patrushev, N.A., Zhang, L., Fukai, T., and Ushio-Fukai, M. (2006). IQGAP1 mediates VE-cadherin-based cell-cell contacts and VEGF signaling at adherence junctions linked to angiogenesis. Arteriosclerosis, thrombosis, and vascular biology *26*, 1991-1997.

Yamazaki, Y., Tokunaga, Y., Takani, K., and Morita, T. (2005). C-terminal heparin-binding peptide of snake venom VEGF specifically blocks VEGF-stimulated endothelial cell proliferation. Pathophysiology of haemostasis and thrombosis *34*, 197-199.

Yaqoob, U., Cao, S., Shergill, U., Jagavelu, K., Geng, Z., Yin, M., de Assuncao, T.M., Cao, Y., Szabolcs, A., Thorgeirsson, S.*, et al.* (2012). Neuropilin-1 stimulates tumor growth by increasing fibronectin fibril assembly in the tumor microenvironment. Cancer research *72*, 4047-4059.

You, W.K., Sennino, B., Williamson, C.W., Falcon, B., Hashizume, H., Yao, L.C., Aftab, D.T., and McDonald, D.M. (2011). VEGF and c-Met blockade amplify angiogenesis inhibition in pancreatic islet cancer. Cancer research *71*, 4758-4768.

Yuan, L., Moyon, D., Pardanaud, L., Breant, C., Karkkainen, M.J., Alitalo, K., and Eichmann, A. (2002). Abnormal lymphatic vessel development in neuropilin 2 mutant mice. Development *129*, 4797-4806.

Zhu, R.Y., Xin, X., Dai, H.Y., Li, Q., Lei, J.Y., Chen, Y., and Jin, J. (2012). Expression and purification of recombinant human serum albumin fusion protein with VEGF165b in Pichia pastoris. Protein expression and purification *85*, 32-37.

Zwang, Y., and Yarden, Y. (2009). Systems biology of growth factor-induced receptor endocytosis. Traffic *10*, 349-363.

Zygalaki, E., Stathopoulou, A., Kroupis, C., Kaklamanis, L., Kyriakides, Z., Kremastinos, D., and Lianidou, E.S. (2005). Real-time reverse transcription-PCR quantification of vascular endothelial growth factor splice variants. Clinical chemistry *51*, 1518-1520.

ANNEX 1

ANNEX 2