

UNIVERSIDADE DE LISBOA
FACULDADE DE CIÊNCIAS
DEPARTAMENTO DE BIOLOGIA VEGETAL



**MOLECULAR MECHANISMS AND RELEVANCE OF
THE NUCLEAR INTERNALIZATION OF VEGF AND
VEGFR2 (KDR) ON ENDOTHELIAL CELLS**

Ana Inês Jones Manteigas Domingues

**DOUTORAMENTO EM BIOLOGIA
(Biologia Celular)**

2011

Cover image: Confocal micrograph of Human Umbilical Vein Endothelial Cell visualizing VEGFR2 (red) and the microtubules (green). Counterstaining of cell nucleus with DAPI (blue). Adapted from: Santos SC, Miguel C, Domingues I, Calado A, Zhu Z, et al. (2007) VEGF and VEGFR-2 (KDR) internalization is required for endothelial recovery during wound healing. *Exp Cell Res* 313: 1561-1574.

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Professora Doutora Susana Constantino

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(Biologia Celular)**

2011

Ao meu Pai.

"Science goes where you imagine it."

Judah Folkman

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ABBREVIATIONS

ANGPT1	Angiopoietin 1
ANGPT2	Angiopoietin 2
AKT	Protein Kinase B
c-Cbl	Casitas B-lineage lymphoma
ChIP	Chromatin Immunoprecipitation
CMR1	Exportin 1
ECs	Endothelial cells
ECM	Extracellular matrix
EGF	Epidermal Growth Factor
EGFR	Epidermal Growth Factor Receptor
EMSA	Electrophoretic Mobility Shift Assays
eNOS	Endothelial Nitric Oxide Synthase
F	Phenylalanine residue
FIGF	c-fos induced growth factor (vascular endothelial growth factor D)
FGF	Fibroblast Growth Factor
FGFR	Fibroblast Growth Factor Receptor
FLT1	fms-related tyrosine kinase 1 (VEGFR1)
FLT4	fms-related tyrosine kinase 4 (VEGFR3)
FRAP	Fluorescence Recovery After Photobleaching
h	hours
HIF	Hypoxia Inducible Factor
HSPGs	Heparan Sulphate Proteoglycans
IDEMSA	Immunodepleting EMSA
Ig	Immunoglobulin
IL	Interleukins
KDR	Kinase insert Domain Receptor (type III RTK), (VEGFR2)
min	minutes
NES	Nuclear Export Signal
NLS	Nuclear Localization Signal
NRP	Neuropilins
PDGF	Platelet-Derived Growth Factor
PDGFR	Platelet-Derived Growth Factor Receptor
PI3K	Phosphatidylinositol-3-kinase

PKC	Protein kinase C
PLCγ	Phospholipase C γ
PIGF	Placenta Growth Factor
RTK	Receptor Tyrosine Kinase
s	seconds
Shb	Src homology 2 domain
SMC	Smooth Muscle Cells
Sp1	Specificity protein 1
svVEGF	snake venom VEGF
TGFβ	Transforming Growth Factor β
TIE	TEK tyrosine kinase, endothelial
TNFα	Tumor Necrosis Factor α
TSAd	T cell specific adapter protein
VE-Cadherin	Vascular Endothelial Cadherin
VEGFA	Vascular Endothelial Growth Factor A
VEGFB	Vascular Endothelial Growth Factor B
VEGFC	Vascular Endothelial Growth Factor C
VEGFR1	Vascular Endothelial Growth factor Receptor 1 (FLT1)
VEGFR2	Vascular Endothelial Growth factor Receptor 2 (KDR)
VEGFR3	Vascular Endothelial Growth factor Receptor 3 (FLT4)
VPF	Vascular Permeability Factor
WB	Western Blot
WT	Wild-type
Y	Tyrosine residue
YY1	Ying and Yang 1 protein

RESUMO

O crescimento de vasos sanguíneos a partir de vasos pré-existentes – Angiogénese – é um processo fundamental durante o desenvolvimento embrionário e que contribui também para a homeostasia dos tecidos adultos. A vascularização assegura o fornecimento de oxigénio e nutrientes, essenciais para o crescimento e reparação dos órgãos. Todos os vasos sanguíneos são revestidos por uma monocamada de células endoteliais vasculares, considerada um componente essencial no processo angiogénico, uma vez que em resposta a diferentes estímulos, as células endoteliais migram e proliferam estabelecendo novos vasos sanguíneos. Em condições fisiológicas a angiogénese é regulada por um balanço crítico entre factores estimuladores (pró-angiogénicos) e inibidores (anti-angiogénicos). No entanto, em condições patológicas, como durante o crescimento tumoral, o excesso de moléculas estimuladoras induz o crescimento descontrolado de vasos sanguíneos facilitando a progressão tumoral e a disseminação de metástases. O factor de crescimento do endotélio vascular (VEGF) é um dos reguladores mais importantes da resposta angiogénica em células endoteliais vasculares. O VEGF é produzido pela maioria das células do organismo em resposta a diferentes estímulos como a hipoxia e actua maioritariamente nas células endoteliais vasculares promovendo a sobrevivência, proliferação e migração destas células.

Os efeitos biológicos do VEGF são mediados por meio de dois diferentes receptores cinase de tirosina, específicos e expressos na superfície destas células: o receptor 1 e 2 designados por VEGFR1 (ou FLT1) e VEGFR2 (ou KDR). Estudos genéticos demonstraram que tanto o VEGF como os seus receptores são necessários para o desenvolvimento normal do sistema vascular. Os embriões de murganhos deficitários em qualquer um destes genes apresentam letalidade entre os dias E8.5-12.5 do desenvolvimento embrionário manifestando graves deficiências na formação inicial dos vasos sanguíneos. Diferentes estudos mostraram que apesar da afinidade do VEGF ser superior para VEGFR1 relativamente a VEGFR2, é a activação do segundo que é responsável pelos efeitos mediados pelo VEGF nas células

endoteliais. O modo de acção do VEGF é classicamente descrito através da sua ligação ao domínio extracelular do VEGFR2, induzindo a sua dimerização e a auto-fosforilação de resíduos de tirosina específicos no domínio intracelular do receptor. A fosforilação destes resíduos gera locais de ligação e activação de mensageiros secundários que induzem a activação de diferentes vias de transdução de sinal e consequentemente a respostas celulares específicas como a proliferação, migração, sobrevivência e permeabilidade. Os mecanismos moleculares de acção do VEGF e VEGFR2 têm sido alvo de numerosos estudos nos últimos 40 anos.

Recentemente, diferentes publicações mostraram que para além da localização na superfície celular, o VEGF e VEGFR2 são também detectados no núcleo nomeadamente em tecidos e células leucémicas, *in vitro* e *in vivo*. Outros trabalhos mostraram a presença de VEGFR2 no núcleo de células endoteliais de rato ou bovino. Estas observações abriram, sem dúvida, a possibilidade de novos mecanismos de acção exercidos por estas moléculas. Assim, no decorrer deste trabalho focámo-nos na investigação dos mecanismos moleculares de internalização nuclear do VEGF e VEGFR2 nas células endoteliais humanas e na relevância funcional deste processo.

Na primeira parte deste trabalho, demonstrámos que após um estímulo de VEGF, tanto o VEGF como o VEGFR2 são internalizados para o núcleo da célula endotelial, num processo dependente de VEGFR1. Verificámos que esta mesma internalização é necessária para a reconstituição de uma monocamada endotelial após a indução de feridas *in vitro*. Estes resultados revelaram uma nova função biológica já anteriormente descrita dos dois receptores de VEGF. Os nossos resultados mostraram também que no processo de internalização é necessária a activação da via de sinalização de PI3K, e que o mesmo envolve a endocitose mediada por caveolina-1, usando os microtúbulos como motores. Para além disto, através da construção de mutantes truncados em diferentes resíduos de tirosina na região intracelular do VEGFR2, demonstrámos que a fosforilação/activação do VEGFR2 é crucial para a translocação nuclear observada. Com o objectivo de identificar quais os resíduos de tirosina envolvidos na translocação do receptor,

construímos várias proteínas de fusão VEGFR2-GFP usando o VEGFR2 nativo ou formas mutadas de VEGFR2; nestas um ou dois resíduos de tirosina foram convertidos em resíduos de fenilalanina e testámos a sua capacidade de serem internalizados para o núcleo de células endoteliais por ensaios de microscopia denominados *Fluorescence Recovery After Photobleaching* (FRAP). Os nossos resultados demonstraram que o resíduo de tirosina Y951 (localizado em um dos domínios intracelulares do receptor), tem uma função importante na translocação nuclear do VEGFR2.

Na segunda parte deste trabalho focámo-nos na investigação da função do receptor VEGFR2 no núcleo das células endoteliais. Num modelo endotelial de sobreexpressão de VEGFR2, os nossos resultados mostraram que a sobreexpressão nuclear de VEGFR2 estava correlacionada com a expressão ou actividade de várias proteínas nucleares. O envolvimento de VEGFR2 na actividade transcricional também foi sugerido em ensaios de incorporação de 5-FU, em que demonstrámos que os níveis de transcrição celulares estavam diminuídos e correlacionados com a diminuição dos níveis nucleares de VEGFR2.

Por análise de espectrometria de massa os nossos resultados mostraram que em células endoteliais, o VEGFR2 nuclear interage com diferentes proteínas nucleares. Confirmámos a interacção de VEGFR2 com o factor de transcrição Sp1, envolvido na regulação de diferentes genes importantes para a resposta angiogénica. Através de ensaios de imunoprecipitação da cromatina (ChIP), mostrámos que o VEGFR2 se liga a uma região do seu próprio promotor e à qual também se liga Sp1. Estes resultados foram confirmados por ensaios de interacção DNA-proteína (EMSA), em que se utilizou a mesma região do promotor de *VEGFR2*. Contudo, os nossos resultados não permitem definir se VEGFR2 se liga ao seu promotor interagindo com Sp1. Verificámos ainda que a ligação de VEGFR2 ao seu promotor está associada à sua activação transcricional. Ensaio repórter usando luciferase, realizados em células 3T3 que permitiu-nos identificar a região entre -300/-116 (relativamente ao local de início da transcrição), como essencial para conferir a actividade transcricional dependente de VEGFR2. Para além destes resultados, e por meio de ensaios

de CHIP, mostrámos que a ligação de VEGFR2 ao DNA é dependente da activação por VEGF, assim como a sua translocação nuclear. Por último e reforçando as observações acima descritas, verificámos também que a ligação de VEGFR2 ao seu próprio promotor é bloqueada pelo tratamento com Bevacizumab ou Sunitinib, dois agentes anti-angiogénicos que inibem a activação de VEGFR2.

O nosso trabalho revela uma nova função do VEGFR2, como um factor de transcrição envolvido na regulação da sua própria transcrição e sugere que este poderá estar implicado na regulação de outros genes importantes para a amplificação da resposta angiogénica. Diferentes estratégias terapêuticas têm vindo a ser desenvolvidas com o objectivo de bloquear a activação de VEGFR2, enquanto proteína membranar. Contudo, estes inibidores angiogénicos não são efectivos em todos os tumores e o benefício é modesto, mesmo para os doentes que inicialmente respondem de forma favorável à terapêutica, indicando que existem mecanismos de resistência à terapia anti-VEGF. A análise da actividade transcricional de VEGFR2 nestes tumores e a identificação de genes regulados por esta proteína irão certamente contribuir para uma maior compreensão da sua função na angiogénese tumoral e na descoberta de novos alvos terapêuticos.

Palavras-chave: Células endoteliais; VEGF; VEGFR2; VEGFR1; internalização nuclear; cicatrização de feridas; regulação génica.

SUMMARY

The angiogenic growth of new blood vessels from pre-existing ones is fundamental during embryonic development and for normal homeostasis of adult tissues. Vascularization ensures the delivery of oxygen and nutrients essential for organ growth and repair. Under physiological conditions, a fine balance between stimulators and inhibitors regulates the complex process of angiogenesis. However, in pathological conditions as tumor progression, the stimulus becomes excessive and induces an angiogenic switch that results in the uncontrolled growth of blood vessels that facilitates tumor growth and metastasis.

The VEGF/VEGFRs system is a potent regulator of the angiogenic response in endothelial cells (ECs). VEGF binds and activates its tyrosine kinase receptors VEGFR1 and VEGFR2 at the surface of ECs. In contrast to VEGFR1, VEGFR2 has a potent tyrosine kinase activity and is considered the major mediator of the signaling responses induced by VEGF. VEGFR2 phosphorylation at the cell membrane induces the activation of intracellular signaling cascades that regulate a wide range of biological responses including survival, proliferation, migration and permeability. The molecular bases of these processes have been the focus of intensive work for the last 40 years. Moreover, the discovery of the presence of VEGF and VEGFR2 not only at the cell surface but also intracellularly, in particular in the cell nucleus, has opened the possibility for new mechanisms of VEGF/VEGFR2 activity.

In this work we focused on the molecular basis and relevance of the nuclear internalization of VEGF and VEGFR2 in ECs. We demonstrated that upon VEGF stimulation, both VEGF and VEGFR2 are internalized to the nucleus in a VEGFR1-mediated process required for EC recovery following *in vitro* wounding. We also showed that the VEGF/VEGFR2 internalization is mediated via caveolae-mediated endocytosis and microtubules and requires the activation of the PI3K pathway. By generating several VEGFR2 deletion mutants in tyrosine residues we demonstrated that VEGFR2 internalization required its phosphorylation. In addition, we showed the importance of the tyrosine residue Y951 for the nuclear internalization of VEGFR2.

Having established the molecular basis for this nuclear internalization process, we focused on the role of VEGFR2 in the nucleus. We showed that VEGFR2 interacts with the Sp1 transcription factor. We also demonstrated that VEGFR2 binds to the Sp1-responsive region of the *VEGFR2* proximal promoter and that the VEGFR2 binding to DNA is linked to the transcriptional activation of the *VEGFR2* promoter. This previously unrecognized function of nuclear VEGFR2 as a putative transcription factor involved in the regulation of its own transcription is a novel mechanism for the amplification of the angiogenic response and can be crucial to develop new therapeutic approaches associated with angiogenesis-dependent diseases.

Keywords: Endothelial cells; VEGF; VEGFR2; VEGFR1; nuclear internalization; wound healing; gene regulation.

SCOPE OF THE THESIS

This thesis is organized in five chapters. Chapter I is a general introduction where I describe the different mechanisms underlying the formation of blood vessels in physiological and pathological conditions and the function and regulation of the VEGF/VEGFR system in these processes.

In Chapters II, III and IV I present the results obtained on the molecular mechanisms and relevance for the nuclear internalization of VEGF/VEGFR in ECs. Each Chapter is organized as: Abstract, Introduction, Results, Discussion, Material and Methods, Acknowledgements, References. In Chapters II and IV additional Supplementary Results and Supplementary References were included.

In Chapter II, I present our results published in the paper: Constantino Rosa Santos S, Miguel C, **Domingues I**, Calado A, Zhu Z, Wu Y, and Dias S. *VEGF and VEGFR2 (KDR) Nuclear Internalization Is Required For Endothelial Recovery During Wound Healing*. *Exp Cell Res*, 2007. 313(8): p. 1561-74.

We described that VEGFR2 translocates to the nucleus of ECs upon VEGF stimulation, in a process mediated by VEGFR1. The translocation process requires the activation of the PI3-kinase pathway and is linked to a caveolae-mediated endocytic pathway, via microtubules. We also focused on the role of VEGFR2 phosphorylation in the process of nuclear internalization. By generating different VEGFR2 tyrosine deletion mutants, we showed that the VEGF-induced VEGFR2 internalization was impaired, suggesting that the activation of the receptor is involved in its nuclear translocation.

In Chapter III, I describe our results concerning the role of the VEGFR2 tyrosine residues in the nuclear translocation process. We generated tyrosine to phenylalanine point mutants and established its expression in an EC model. We analyzed the internalization dynamics of these mutants compared to VEGFR2 wild-type (WT) by FRAP and identified the tyrosine residue Y951 in the kinase insert domain as essential for the nuclear translocation of VEGFR2. Some of these results were included in the revised manuscript version shown in Chapter IV.

In Chapter IV, I present our results obtained on the function of nuclear VEGFR2, submitted to PloS One in the paper: **Domingues I**, Rino, J, Demmers JAA, de Lanerolle P and Constantino Rosa Santos S. *VEGFR2 Translocates to the Nucleus to Regulate its own Transcription*. We showed that nuclear VEGFR2 interacts with several nuclear proteins, identified by mass spectrometry analysis, including the Sp1 transcription factor. By *in vivo* chromatin immunoprecipitation (ChIP) assays, we found that VEGFR2 binds to the Sp1-responsive region of the *VEGFR2* proximal promoter. By reporter assays, we showed that the VEGFR2 DNA binding is directly linked to the transcriptional activation of the *VEGFR2* promoter. Overall, we demonstrate a new mechanism by which VEGFR2 activates its own promoter that could be involved in amplifying the angiogenic response.

Chapter V is a final discussion of the key findings obtained throughout this thesis and the putative relevance of the new mechanisms described for the angiogenesis field.

CHAPTER I

GENERAL INTRODUCTION

1. THE FORMATION OF BLOOD VESSELS

Oxygen and nutrients are essential for cell growth and homeostasis. In primitive and smaller organisms, oxygen simply diffuses to all the cells in the body. Throughout evolution larger and complex organisms developed a network of blood vessels containing carrier molecules to deliver oxygen and nutrients to distant organs and remove the metabolic waste, contributing to body homeostasis [1]. In vertebrates, the cardiovascular system is a closed blood circuit pumped from the heart through arteries that ramify in smaller arterioles and into capillary beds [2]. The blood then returns through venules and veins to the heart [2].

The capillaries are composed of endothelial cells (ECs) surrounded by a basement membrane and a sparse layer of pericytes [3]. The capillary bed is the largest surface of the vascular system and due to its wall structure these vessels form the main site for exchange of gases and nutrients with the surrounding tissues [2,3]. The capillary endothelial layer presents distinctive characteristics depending on the different organs or tissues conferring different levels of permeability. The endothelial layer is continuous in the muscle tissue and discontinuous in the liver sinusoids, while in the kidney is fenestrated. The blood-brain barrier has further specialized ECs that are impermeable to various molecules [3]. Arterioles and venules have an increased coverage of pericytes and smooth muscle cells (SMC) compared to capillaries. The vascular SMCs from the precapillary arterioles are tightly packed with the endothelial layer and form their own basement membrane.

The walls of larger vessels are composed of additional specialized layers that ensure the transport of blood to and from the heart [2]. The inner layer of arteries and veins, *tunica intima* is composed of ECs surrounded by a basement membrane. A middle layer named *tunica media* is composed of SMCs and elastic fibers that control the vessel diameter and the blood flow. The outer layer, *tunica adventitia* is composed of connective tissue, collagen fibers and nerves and has its own blood supply named *vasa vasorum* [3].

Although the walls of arteries and veins are composed of these same layers, both present different characteristics as a result of the pressure and direction of the blood flow. The medial layer in arteries is a robust muscular sheet that ensures the high arterial blood pressure. In contrast blood flows with a lower pressure in veins and as a result, their wall is thinner than arteries with additional semi-lunar valves, which prevent the blood from flowing backwards [2,3].

The molecular mechanisms underlying the formation of blood vessels will be further discussed.

1.1. VASCULOGENESIS AND ANGIOGENESIS

The first step in the formation of blood vessels is the differentiation of vascular ECs, which line the inner surface of all blood vessels. In an early phase of the vasculogenesis, a subset of mesodermal precursors, the hemangioblast, is committed to differentiate into angioblasts and primitive haematopoietic cells [4], (Figure 1).

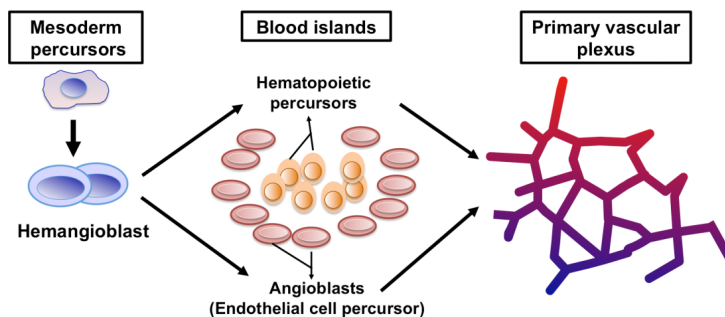


Figure 1. Vasculogenesis.

The mesodermal precursors differentiate in hemangioblasts, the bipotential precursors of angioblasts and hematopoietic cells. The angioblasts aggregate at the surface and the hematopoietic precursors in the center of the blood islands in the yolk sac. The angioblasts then differentiate into ECs and form the primary vascular plexus with the first blood cells in circulation. Adapted from [4].

The angioblasts aggregate in the periphery and the hematopoietic precursors accumulate in the middle of the blood islands in the yolk sac and embryo [4]. The angioblasts then differentiate into ECs to form simple tube-like structures

and organize the primary vascular plexus [4]. The progressive expansion and remodelling of the primary network of vessels occurs by ramification of larger vessels into smaller ones (intussusceptive growth) or by sprouting and proliferation from the existing vessels, a process named angiogenesis [5], (Figure 2).

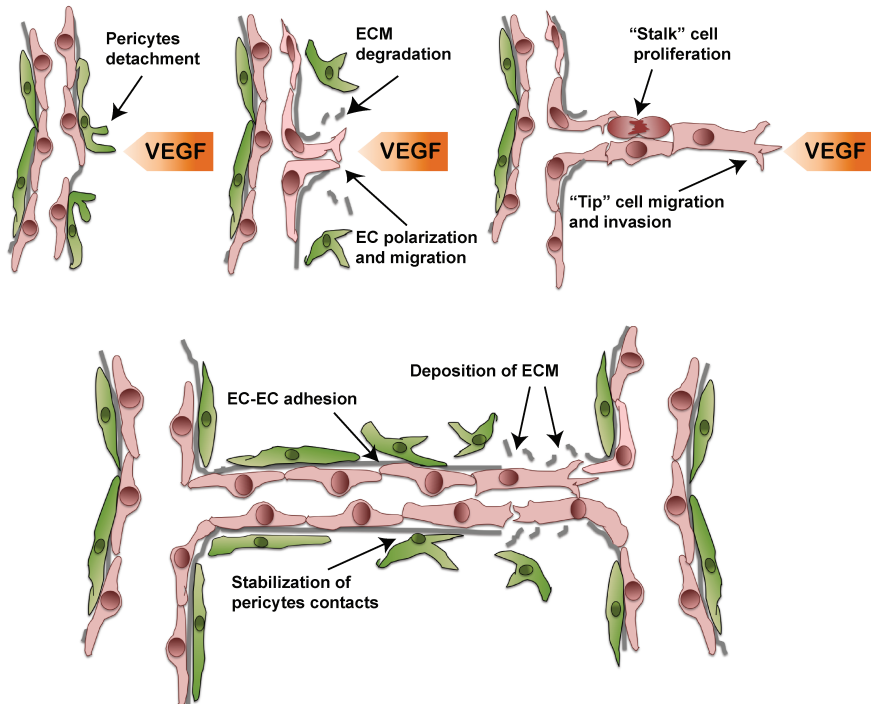


Figure 2. Angiogenesis.

Blood vessels arise from pre-existing capillaries. A VEGF gradient induces the angiogenic program. First, pericytes (in green) detach and blood vessels dilate. The extracellular matrix (ECM, in grey) is degraded. ECs (pink) polarize and start to migrate. The leading “tip” cells extend filopodia towards the angiogenic stimuli while adjacent “stalk” cells proliferate, loosely following each other. Behind the migration columns, ECs adhere to each other and create a lumen, which is accompanied by ECM deposition and pericyte attachment. Finally, blood-vessel sprouts fuse with other sprouts to build new circulatory routes. Adapted from [6,7].

In these early stages of vasculogenesis and angiogenesis the first identified member of the Vascular Endothelial Growth Factor (VEGF) family of glycoproteins, VEGFA, and its receptors VEGFR1 and VEGFR2 are key players. Genetic studies have shown their requirement for the normal development of the vascular system [8]. Embryos deficient for any of these

molecules die *in utero* around embryonic day (E)8.5-12 exhibiting profound defects in early vessel formation [9,10,11,12], (see below). VEGFA is produced by the majority of the cells, in response to different stimuli, such as hypoxia (see below) and acts in a paracrine fashion on ECs, which express the receptors VEGFR1 and VEGFR2 [8].

The angiogenic program is activated when in response to VEGF, specialized endothelial ‘tip’ cells loose the cell – cell contacts, detach from the vascular wall and selectively degrade the ECM [7], (Figure 2). At the front of a sprout, highly polarized and motile endothelial “tip” cells extend filopodia to guide a sprouting vessel towards the angiogenic stimulus [13]. Following behind the tip cells, endothelial “stalk” cells proliferate to elongate the vessel branch [7], (Figure 2). Each of these EC phenotypes (tip and stalk ECs) has a distinct molecular signature. However, these phenotypes are not permanent, but, are plastically regulated by the axis Notch, its ligand Delta-like-4 (DLL4)–and VEGF-VEGFR signaling [13]. The DLL4/Notch model predicts that ECs, exposed to the highest VEGFA levels, gain a competitive advantage to acquire a tip-cell fate and position in the sprout [13]. Recent findings suggested that ECs dynamically compete with each other for the tip cell position [14]. VEGFA induces an upregulation of *DLL4* and consequently, this tip cell produces more *DLL4* than its neighboring cell [13,15,16,17,18,19]. The elevated levels of *DLL4* activate Notch signaling in neighboring cells and consequently, VEGF-signaling is reduced, inducing a decreased *DLL4* expression [13,15,16,17,18,19]. As a result, the tip cell suppresses the same response in stalk cells, which progressively lose competition for the tip [13,15,16,17,18,19]. It was shown that VEGFR levels act upstream of Notch in the selection process by regulating *DLL4* levels [14]. Cells with reduced VEGFR2 levels hardly adopt the tip cell position [14]. Tip cell competition through VEGFR levels establishes a biological function for the dynamic VEGFR regulation during sprouting angiogenesis [14]. A critical step in the angiogenic process is the recruitment of pericytes to the newly formed vessels as these cells are essential for the maturation of endothelial tubes into blood vessels [20].

1.2. MATURATION OF BLOOD VESSELS

The coverage of vessels with pericytes and smooth muscle cells (SMC) contribute to the regulation of vessel perfusion and the maintenance of a mature vasculature, a process named arteriogenesis [1,21]. Pericytes are embedded within the basement membrane of the endothelium and communicate with ECs through gap junctions or paracrine signaling pathways. Pericytes have gained attention in recent years as potential new targets for the anti-angiogenic therapies discussed below [22].

The platelet-derived growth factor β (PDGF β)/ PDGF receptor β (PDGFR β) and angiopoietin/Tie2 signaling systems are important regulators of the blood vessel maturation [3]. PDGF β secreted by ECs in response to VEGF recruits pericytes and SMC to the growing vessels by signaling through PDGFR β [23]. In agreement with this, *Pdgf β ^{-/-}* and *Pdgfr β ^{-/-}* knockout mice are embryonic lethal presenting severe hemorrhage and edema due to the immature vessels that fail to attract pericytes [23].

Also important for vessel growth and maturation is the endothelial specific-TIE2 tyrosine kinase receptor and two ligands of the angiopoietin (ANGPT) family, ANGPT1 and ANGPT2 expressed in SMC and ECs respectively. ANGPT1 is required for the correct organization and maturation of the newly formed vessels presumably by facilitating the EC-SMC interconnection through the activation of TIE2 [3]. Consistent with this, *Angpt1^{-/-}* and *Tie2^{-/-}* deficient mice are also embryonic lethal, having similar phenotypes characterized by severe defects in vascular remodelling and maturation [24,25]. ANGPT2 plays a double role depending on the presence or absence of VEGF [26]. In the absence of VEGF, ANGPT2 acts as an antagonist of ANGPT1 leading to the regression of blood vessels. In the presence of VEGF, ANGPT2 facilitates vascular remodelling, inducing vessel destabilization [26,27]. Additionally, other signaling molecules, such as the transforming growth factor β (TGF β) and the sphingosine-1-phosphate receptor (S1PR1), are also involved in the vessel maturation processes [3,21].

The concerted action of these molecules results in the establishment of a large and highly organized vascular network, with larger vessels that progressively ramify into smaller vessels, terminating in capillaries and contributing to organ growth [1].

1.3. ENDOTHELIAL CELL SPECIFICATION

Although the molecular processes underlying the arterial-venous EC specification are not fully understood, increasing evidence suggest that this process is regulated by the concerted action of different molecules [28]. Two members of the Eph-Ephrin subclass of receptor tyrosine kinases, the ligand ephrinB2 and its receptor EphB4, are specific markers for arteries or veins respectively [29]. These markers are detected in blood vessels of the embryo before a proper circulation is observed, indicating that the arterial-venous commitment occurs early in development [29]. Consistent with this, genetic studies demonstrated that *EphrinB2*^{-/-} [29] and *EphB4*^{-/-} [30] knockout mice exhibited defects in remodeling of the primary capillary plexus into arteries and veins. However, the action of other upstream factors seems to be required for the determination of arterial or venous EC fate [31].

Among these factors, the Notch pathway has been shown to have a crucial role in arterial–venous specification *in vivo*, promoting arterial EC commitment and repressing venous differentiation [28]. The receptor Notch4 and the ligand DLL4 are both specifically expressed in arteries [32,33]. Consistently, *Notch1/4*^{-/-} and *Dll4*^{-/-} mice display defects in vascular development, including arterial specification [34,35]. In *Dll4*^{-/-} mice disrupted arterial EC differentiation is accompanied by decreased *EphrinB2* expression and simultaneously increased *EphB4* expression [34]. Studies in zebrafish have shown that Sonic Hedgehog and VEGF act upstream of Notch in the regulation of the arterial fate [36].

Conversely, the orphan receptor COUP-TFII is specifically expressed in venous, but not in arterial ECs [37]. The conditional inactivation of COUP-TFII in ECs results in the acquisition of arterial characteristics in veins and

increased expression of arterial markers such as *Notch4* and *EphrinB2* [37]. These results suggested that COUP-TFII promotes the venous cell fate by suppressing the Notch pathway [28].

Another important form of endothelial differentiation is the formation of the lymphatic ECs. Around E10 of mouse development a subpopulation of venous ECs expressing the prospero homeobox transcription factor *Prox1* trans-differentiates into lymphatic ECs [38]. The molecular mechanisms for growth, migration and survival of the lymphatic ECs are also intimately related to members of the VEGF family of ligands and receptors, most particularly VEGFC and FIGF (previously named VEGFD) and the VEGF receptor 3 (VEGFR3), [39,40].

2. PHYSIOLOGICAL AND PATHOLOGICAL ANGIOGENESIS

In adulthood, the vasculature remains quiescent and angiogenic processes occur only in specific situations, such as wound healing, the regeneration of the endometrium during the menstrual cycle or in the placenta during pregnancy [1]. Quiescent ECs retain the ability to rapidly migrate and proliferate in response to different stimuli [1]. Therefore, in physiological situations, the angiogenic events that occurred during normal vascular development in the embryo are reactivated [8]. Conversely, a de-regulation of the angiogenic process contributes to many malignant, inflammatory, ischemic, infectious and immune disorders [41]. Several diseases are characterized by excessive angiogenesis, such as cancer, age-related macular degeneration (AMD) and rheumatoid arthritis among others [21]. However, insufficient angiogenesis also characterizes several diseases. Heart ischemia, hypertension, atherosclerosis and diabetes are some examples of processes where insufficient angiogenesis can be observed [21]. The great impact that angiogenesis has in health and disease has been pushing the research towards the study of the molecular mechanisms that regulate this process.

2.1. ANGIOGENIC BALANCE

Angiogenesis is a complex process regulated by a fine-tuned balance of pro- and anti-angiogenic molecules. In adults, the balance can be disrupted to favor increased blood vessel formation in physiologic repair settings (such as wound healing) but also in many pathological conditions (such as cancer). A classical view of this model proposes that in a quiescent state, the balance between angiogenic stimulators and inhibitors favors inhibition and consequently capillary growth is restrained [42]. Additionally, it was recently proposed that a dynamic equilibrium exists between of pro- and anti-angiogenic factors. The physiologic status of this balance could determine the predisposition of an individual to turn the switch “ON” during pathologic events [43].

Several positive regulators of angiogenesis have been described, including growth factors and cytokines, such as VEGF, placental growth factor (PlGF), fibroblast growth factors (FGFs), PDGF β , transforming growth factor α and β (TGF α/β), tumor necrosis factor-alpha (TNF α) and interleukin 3 and 8 (IL3, IL8) among others [44]. The action of negative angiogenic regulators, such as thrombospondin 1 (THBS1), angiostatin (fragment of plasminogen) and endostatin (fragment of type VIII collagen that binds integrins), balances the effect of the pro-angiogenic molecules [44]. In a physiological situation like wound healing the release of pro-angiogenic molecules into the wound bed after injury shifts the local equilibrium between stimulators and inhibitors towards vascular growth, driving the quiescent ECs to begin the angiogenic program. The newly formed vessels rapidly mature and become stable [6]. However, under certain pathological conditions, such as tumors, local inhibitory controls are unable to contain the augmented activity of angiogenic inducers promoting the “angiogenic switch” [6]. In these conditions a disruption in the angiogenic balance is under the control of both the genetic composition of any individual cancer cell and its microenvironment within the tumor. [6]. Tumors described as “wounds that never heal” have a constant growth of new

blood vessels that fail to become quiescent [6,45]. VEGF signaling mediated by VEGF receptors is considered one of the most important factors for the activation of the angiogenic events both in physiological and pathological settings [1]. The VEGF-mediated effects in wound healing and tumor angiogenesis will be further analyzed.

2.2. WOUND HEALING

The healing process after injury follows a series of overlapping phases including angiogenesis, inflammation, re-epithelialization and tissue remodeling [46]. After injury, a fibrin/fibronectin-rich clot is formed at the wound site and the granulation tissue fills the wound bed [46]. A good vascularization ensures the delivery of oxygen, nutrients, and immune/stem cells to facilitate repair [46]. The release of several growth factors and cytokines such as VEGF induces the migration and proliferation of ECs to the wound bed [46,47]. The inflammatory response is initiated with the recruitment of circulating monocytes and macrophages through the activation of VEGFR1 by VEGF [46]. The endothelial plexus is further stabilized by the recruitment of pericytes and establishment of cell-cell and cell-ECM interactions through the activation of integrins and cadherins, contributing to the physiological tissue repair [46]. Failure or insufficient blood vessel formation impedes adequate cellular responses to heal and thus the tissue repair is delayed or blocked, leading to chronic ischemic wounds [48].

2.3. TUMOR ANGIOGENESIS

Early tumor formation is based on a succession of genetic and epigenetic alterations leading to an increased proliferation rate and/or decreased cell apoptosis that progressively guide the conversion of normal cells into cancer cells [49]. Like normal tissues, tumors require the constant supply of oxygen and nutrients allowing them to grow beyond a certain size. In 1971, Dr. Judah Folkman proposed that tumor growth and metastasis are angiogenesis-dependent and therefore the inhibition of angiogenesis (anti-angiogenesis)

would be a successful strategy to manage tumor growth [50]. Folkman, often considered as “the father of the tumor angiogenesis field”, also hypothesized that a factor produced by tumor cells (tumor angiogenesis factor – TAF) and mitogenic to ECs would stimulate the formation of new vessels. VEGF was later identified [51,52] as being the angiogenic factor proposed and partially isolated by Folkman [53]. Since then, researchers worldwide have identified several of the pro- and anti-angiogenic molecules described above.

Solid tumors are highly complex structures composed of neoplastic cells and stroma. Tumor cells actively recruit stromal cells such as tumor-associated fibroblasts, ECs, pericytes and SMCs that contribute for the tumor microenvironment and play an important role in the initiation and progression of tumors [54].

Classically, tumor-growth is composed of two phases: an initial avascular phase that corresponds to a small mass of neoplastic cells that grows until 1-2 mm [6]. These so-called *in situ* or dormant tumors need to recruit their own blood supply to ensure the delivery of oxygen and nutrients allowing tumor growth [6,55]. Interestingly, these dormant lesions were found during autopsies of individuals who died of causes other than cancer, suggesting that only a subset of these tumors enter the second vascular phase [6,55]. Angiogenesis is therefore considered a rate-limiting step in tumor growth and if the “angiogenic switch” is not turned “ON”, tumors can stay dormant by reaching a balance between cell proliferation and cell death [6,54,55]. Both tumor and stromal cells that enter the vascular phase secrete an excess of pro-angiogenic factors stimulating a switch in the angiogenic phenotype and leading to the activation of the “angiogenic switch” [54]. This results in the expansion of the tumor mass that becomes more likely to metastasize and is potentially lethal [54].

VEGFA is expressed in most tumors and its expression correlates with tumor vessel density and growth [6]. VEGFA-stimulated ECs mitogenesis and permeability are primarily mediated by VEGFR2 [56]. Tumor vessels are architecturally different from normal blood vessels, exhibiting an irregular,

dilated and tortuous shape. Consequently, they are characterized by a variable diameter and irregular blood flow. The increased and constant production of VEGFA is partly responsible for the leaky and tortuous phenotype of the tumor vessel network [7]. These vessels also do not mature properly and show a poor coverage with pericytes and SMCs, which contributes for a decreased blood flow. As a consequence nutrients and oxygen are not correctly delivered and the tumor remains hypoxic, resulting in the constant overproduction of VEGFA and abrogation of a normal vessel phenotype [54]. Other members of the VEGF family, such as PlGF, are responsible for an enhanced activation of the VEGFA-VEGFR2 mediated responses, contributing to constant vessel growth [57] (see below). The observation that tumor cells (solid and hematologic tumors) express not only VEGF but also its receptors, suggested that autocrine loops might also mediate tumor growth [58,59]. Interestingly, it has been reported that VEGF and its receptors may localize not only to the cell surface but also internally [60,61], promoting cell survival via intracrine loops [60].

The importance of the VEGF/VEGFR contribution for tumor angiogenesis led to the development of anti-angiogenic therapies targeting this system [62]. Different monoclonal antibodies as well as several small molecule inhibitors of the VEGFRs have also been developed [63], (Table 1).

In February 2004, Bevacizumab (Avastin®, Genentech, Roche) became the first anti-angiogenic drug to receive approval from the Food and Drug Administration (FDA, USA) for treatment of metastatic colorectal cancer in combination with 5-fluorouracil-based chemotherapy [64]. Bevacizumab is a humanized monoclonal antibody that binds to and inhibits the biological activity of all VEGFA isoforms [64]. The small molecule inhibitors target the ATP-binding site of the receptors blocking the downstream intracellular signaling pathways [62,63].

Sunitinib (Sutent®, Pfizer) is a multi-targeted receptor tyrosine kinase (RTK) inhibitor of VEGFRs, PDGFRs and RET, which exhibits anti-tumor activity [65]

and has been approved by the FDA for the treatment of advanced renal cell carcinoma [66].

These and other agents have prolonged the life of numerous cancer patients and have revolutionized the face of clinical oncology. However, clinical experience also showed that VEGF-targeted therapy often prolongs overall survival of patients by only months, without offering an enduring cure [67]. Recent studies in mice have shown that although these VEGF-targeted drugs inhibit primary tumor growth, they may also promote tumor invasiveness and metastasis, decreasing the survival rates [68,69].

Table 1. FDA-approved anti-angiogenic therapies. Monoclonal antibodies and small molecule tyrosine kinase inhibitors targeting angiogenesis.

Monoclonal Antibody Therapies		
Agent	Target	Phase
Bevacizumab (Avastin®) Genentech, Roche	Humanized monoclonal anti-VEGF antibody	Approved - metastatic colorectal cancer (mCRC), non-small cell lung cancer (NSCLC), advanced breast cancer
Cetuximab (Erbix®) Bristol-Myers Squibb, ImClone	Chimeric IgG1 monoclonal antibody that binds the extracellular domain of epidermal growth factor receptor (EGFR)	Approved - mCRC, head and neck cancer
Panitumumab (Vectibix™) Amgen	A fully humanized IgG2 anti-EGFR monoclonal antibody	Approved - mCRC
Trastuzumab (Herceptin®) Genentech, Roche	A humanized IgG1 monoclonal antibody that binds the extracellular domain of HER-2	Approved - Breast cancer
Small Molecule Tyrosine Kinase Inhibitors (TKIs)		
Agent	Target	Phase
Sunitinib (Sutent®) Pfizer	Small molecule TK inhibitor of VEGFR1, VEGFR2, VEGFR3, PDGFR β and RET	Approved - Advanced renal cell carcinoma, gastrointestinal stromal tumor (GIST)
Sorafenib (Nexavar®) Bayer, Onyx	Small molecule TK inhibitor of VEGFR-1, VEGFR2, VEGFR3, PDGFR β and Raf-1	Approved - Advanced renal cell carcinoma, advanced hepatocellular carcinoma
Erlotinib (Tarceva®) Genentech, Roche	Small molecule TK inhibitor of EGFR	Approved - NSCLC, pancreatic cancer

These findings contribute to understand the intrinsic and evasive resistance to these drugs [67]. Therefore, it is crucial to develop new strategies that will allow us to optimally exploit the potential of VEGF therapy to block primary tumor growth, while at the same time suppressing pro-metastatic effects.

3. VEGFs AND VEGF RECEPTORS – THE REGULATORS OF ANGIOGENESIS

VEGFs are secreted, dimeric glycoproteins that belong to the cysteine-knot growth factor superfamily [70]. VEGFs contain an approximately 100 amino acid VEGF homology domain characterized by eight spaced cysteine residues, structurally related with PDGF [70]. The VEGF family consists of seven members [71]: five of them, namely VEGFA (the original form of VEGF), PIGF, VEGFB, VEGFC and c-fos induced growth factor (FIGF, previously named vascular endothelial growth factor D) are present in mammals. The other two members are the parapoxvirus VEGFE and the snake venom VEGF (svVEGF) [72]. Although VEGFs usually form homodimers, the formation of heterodimers between VEGFA and PIGF [57,73] or VEGFA and VEGFB [74] has been reported. The different members of the VEGF family interact with a set of cell-surface receptors that initiate signal transduction cascades in response to these factors [71], (Figure 3).

These three closely related receptors are members of the RTK superfamily: VEGFR1, VEGFR2 and VEGFR3 (described in detail further below), [71,75]. Additionally, VEGF interacts with a class of co-receptors, neuropilin 1 (NRP1) and NRP2, initially described as semaphorin receptors involved in axon guidance in the nervous system, that have been shown to modulate VEGF binding to the main receptors [71,75].

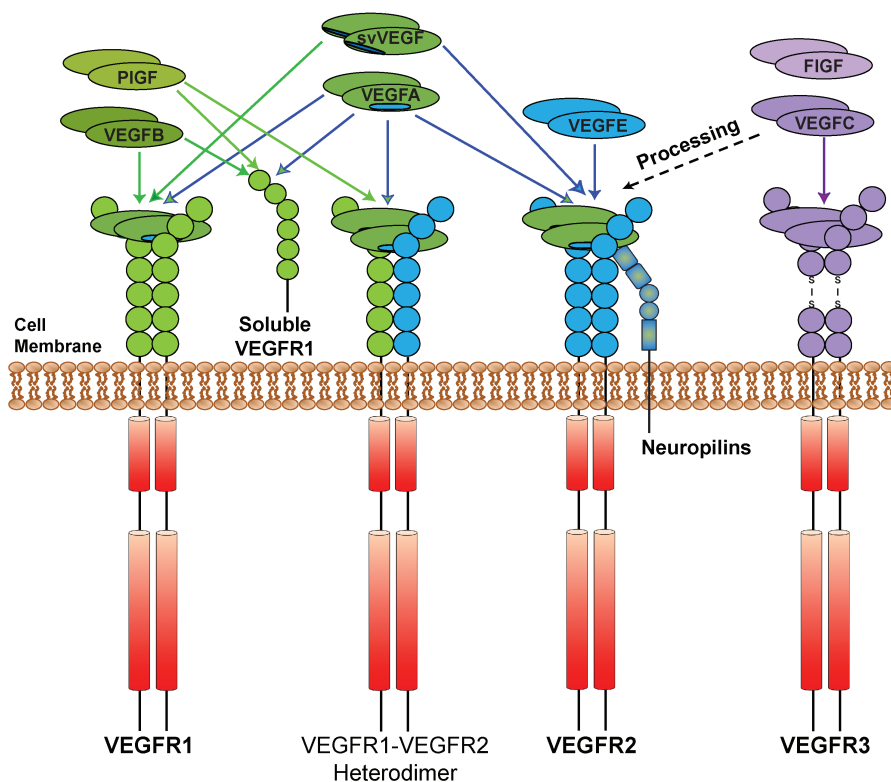


Figure 3. The VEGF ligand family and their receptors.

The VEGF family includes VEGFA, B, C, E, FIGF, snake venom (sv) and placenta growth factor (PIGF), which have different binding properties to three different receptor tyrosine kinases, VEGFR1 (green), VEGFR2 (blue) and VEGFR3 (purple). Additionally, VEGFs and VEGFRs bind to co-receptors such as neuropilins. The extracellular domain of VEGFR1 is also expressed as a soluble protein (sVEGFR1). Both PLGF and VEGFB are selective ligands for VEGFR1. VEGFE is a selective ligand for VEGFR2. Snake venom VEGF (svVEGF) isoforms bind to VEGFR1 and/or VEGFR2. Unprocessed VEGFC and FIGF are selective ligands for VEGFR3. VEGFA isoforms bind sVEGFR1 and VEGFR2. Proteolytic processing of VEGFC and FIGF allows for binding to VEGFR2. Adapted from [71,72,75].

3.1. VEGFA

VEGFA was the first identified member of the VEGF family. It was first partially purified in Harold Dvorak's laboratory in 1983 from the conditioned media of a guinea-pig tumor cell line and initially described as vascular permeability factor (VPF) since it was able to induce vascular leakage in the skin [52]. In 1989, Ferrara and Henzel described the EC specific-mitogenic activity of a molecule designated VEGF [51] and the molecular cloning of VEGF [76] and VPF [77] later that year demonstrated that these were the same molecule. These

findings showed that VEGF was an endothelial-specific mitogen and permeability inducer suggesting that it could be a critical regulator of the physiological and pathological growth of blood vessels [78].

Genetic studies further demonstrated the importance of VEGFA for normal vascular development since the loss of a single VEGFA allele in mice resulted in embryonic lethality between E11-12 [9,10]. Both *Vegfa*^{-/-} null and *Vegfa*^{+/-} heterozygous embryos show abnormal vascular development with several defects in the cranial region and heart [10], presenting decreased EC density and proliferation, with smaller calibre vessels and a disorganized vascular plexus [9]. The severe phenotypes observed indicated that decreased VEGFA levels below a certain threshold during embryonic development results in disruption of normal organogenesis [10]. The critical VEGFA gene-dosage dependence during development was also observed in mice overexpressing VEGFA. In particular, disruption of the regulatory 3' untranslated region (UTR) of the *Vegfa* gene led to a 2-3 fold expression increment resulting in embryonic death at E12.5 due to cardiac failure [79].

An additional level of complexity in the regulation of VEGFA results from alternative splicing of the eight exons that compose the *VEGFA* gene [63]. Several isoforms are generated containing 121 to 206 amino acids after signal sequence cleavage in humans and one amino acid less in mice: VEGFA₁₂₁, VEGFA₁₄₅, VEGFA₁₆₂, VEGFA₁₆₅, VEGFA_{165b}, VEGFA₁₈₃, VEGFA₁₈₉ and VEGFA₂₀₆ [80,81], (Figure 4).

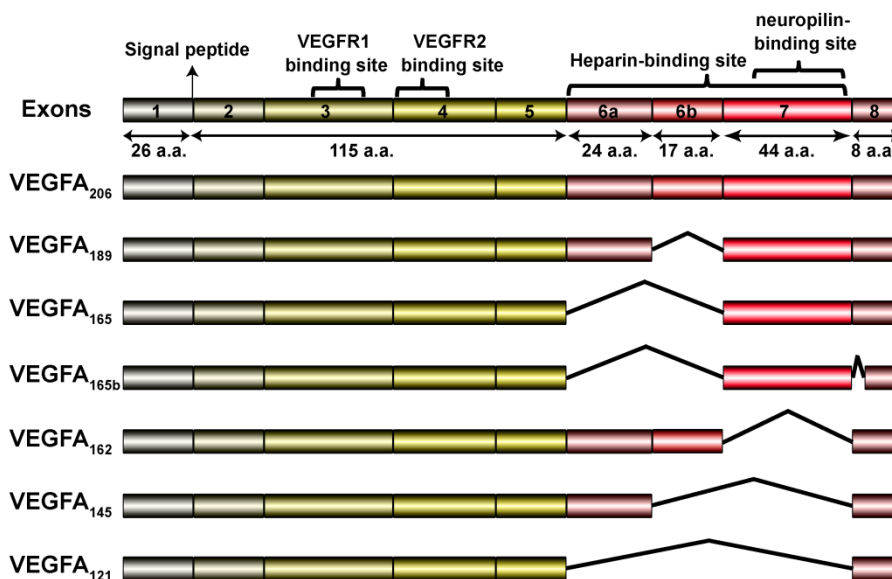


Figure 4. The splicing variants of the human *VEGFA* gene.

The gene encoding *VEGFA* consists of eight exons encoding different structural motifs. Alternative splicing of a single pre-mRNA species produces at least eight different *VEGFA* isoforms that vary in total amino acid number: *VEGFA*₁₂₁, *VEGFA*₁₄₅, *VEGFA*₁₆₂, *VEGFA*₁₆₅, *VEGFA*_{165b}, *VEGFA*₁₈₉ and *VEGFA*₂₀₆. *VEGFA*₁₆₅ is the predominant isoform. Isoforms *VEGFA*₁₄₅, *VEGFA*₁₆₂ and *VEGFA*₁₈₃ are less frequent. Adapted from [70,72,75].

*VEGFA*₁₂₁, *VEGFA*₁₆₅ and *VEGFA*₁₈₉ are preferentially expressed isoforms, presenting a wide tissue distribution [70]. These isoforms display different abilities to bind heparin, heparan sulphate proteoglycans (HSPGs) and ECM, due to the presence or absence of the sequences encoded by exons 6 and 7 [80,81], (Figure 4).

The increased affinity to heparin regulates the functional activity of *VEGFA*₁₈₉ and *VEGFA*₂₀₆ isoforms, as they are kept sequestered in the ECM and cell surface, only becoming available once released by heparinases or from proteolysis following plasminogen activation [81,82,83]. By contrast, *VEGFA*₁₂₁ that lacks exons 6 and 7 does not bind heparin and is a freely diffusible protein. *VEGFA*₁₆₅, the predominant *VEGFA* isoform, contains only the heparin-binding domain encoded by exon 7 and is therefore moderately diffusible and is the major inducer of the VEGF activity [70,82]. *VEGFA*_{165b} is another *VEGFA*₁₆₅ isoform, which results from the use of an alternative distal

splice acceptor site in exon 8 and has been proposed to be a natural antagonist of VEGFA₁₆₅ [84,85].

We are still far from understanding the relative contributions of the different isoforms to the VEGFA physiological activities. Genetic studies have shown the requirement of the heparin-binding VEGFA isoforms. In particular mice expressing exclusively the VEGFA₁₂₀ isoform (*Vegfa*^{120/120}) die shortly after birth due to cardiac failure and present deficiencies in vascular outgrowth, impaired myocardial angiogenesis [86] and retarded bone development [87]. Therefore, the absence of VEGFA₁₆₄ and VEGFA₁₈₈ in mice leads to ischemic cardiomyopathy [86], demonstrating the functional importance of the two heparin-binding domain encoded in exons 6 and 7 of the VEGFA-expressing isoforms. Mice expressing exclusively the VEGFA₁₈₈ isoform (*Vegfa*^{188/188}) displayed impaired retinal arterial development although normal venular growth was reported [88]. *Vegfa*^{188/188} mice also showed impaired vascularization surrounding the epiphysis, resulting in the defective development of secondary ossification centers among other bone deficiencies [87]. This indicates that this isoform alone is able to fulfil many, but not all the functions of VEGFA. Interestingly, mice expressing exclusively the VEGFA₁₆₄ isoform (*Vegfa*^{164/164}) are viable and healthy indicating that this isoform is sufficient for the normal outgrowth and remodeling of blood vessels [89].

VEGFA gene expression is controlled at different levels including transcription, mRNA stability, alternative splicing and translation [90]. Hypoxia is one of the best studied stimulus regulating VEGFA gene expression and is considered essential for the development of new blood vessels during embryonic development and for tumor vascularization [90]. VEGFA gradients attract and guide the sprouting ECs to the most oxygen-depleted areas in the tissue reflecting the nutrient needs of the cells. Hypoxia-inducible factor 1 (HIF1) is an oxygen-regulated transcriptional activator that functions as a master regulator of oxygen homeostasis [91]. HIF1 is a heterodimer composed of HIF1 α and HIF1 β subunits. Whereas HIF1 β is constitutively expressed, HIF1 α expression is induced in hypoxic cells with an exponential increase in

expression as cells are exposed to low O₂ concentrations [91]. In non-hypoxic conditions, HIF1 α is ubiquitinated and subjected to proteasomal degradation [91]. Under hypoxic conditions, the fraction of HIF1 α that is ubiquitinated decreases dramatically, resulting in an accumulation of the protein [91]. A functional hypoxia response element (HRE) in the 5' region of the *VEGFA* human promoter was found to bind the heterodimer HIF1 β /HIF1 α [92].

In addition to this major regulator, other transcription factors such as Sp1 and AP-2 were found to regulate the *VEGFA* gene expression by hypoxia-independent mechanisms, triggered by distinct stimuli [90].

3.2. VEGF-RELATED PROTEINS

3.2.1. PLGF

PlGF was first isolated from a placenta cDNA library and identified as a *VEGFA* homolog [93]. PlGF is also expressed in the heart, lung, thyroid and skeletal muscle, although at low levels [94]. PlGF is a \approx 46 kDa dimeric secreted glycoprotein that shares 42% amino acid sequence homology with *VEGFA* [95]. The human gene for *PlGF* is encoded by 7 exons that undergo alternative splicing generating 3 isoforms: PlGF-1, PlGF-2 and PlGF-3 [96,97]. Another PlGF isoform termed PlGF-4 was isolated from human trophoblasts [98]. The predominant isoforms PlGF-1 (134 amino acids) and PlGF-2 (152 amino acids) differ in size by the insertion of a basic 21-amino acid stretch encoded by exon 6 that enables PlGF-2 to bind heparin [97] and NRP1 [99].

The biological effects of PlGF are still under debate due to the apparent contradictory results obtained so far. For instance, PlGF alone has demonstrated little mitogenic and permeability-enhancing activity *in vitro* or *in vivo*, but it was able to potentiate the effects of low concentrations of VEGF [100]. Consistent with this, the *Plgf*^{-/-} knockout mice presented normal vascular development [101]. However, PlGF deficiency impaired angiogenesis and permeability during ischemia and wound healing and also in pathological conditions as inflammation and cancer [101]. Different hypotheses have been raised to explain the differential role of PlGF in developmental and

pathological angiogenesis. Since PlGF is a selective and high affinity ligand for VEGFR1 it was suggested that it could displace VEGF bound to VEGFR1 and therefore increased its availability to bind VEGFR2 [100]. Another important feature of PlGF is its ability to naturally form heterodimers with VEGFA [73], which are potentiated under pathological conditions such as cancer [102]. The VEGFA/PlGF heterodimers, but not PlGF homodimers were able to induce mitogenic effects as potent as VEGFA homodimers *in vitro* [73]. Furthermore, VEGFA/PlGF heterodimers are able to bind not only VEGFR1, but also VEGFR2 and enhance angiogenesis by activation of intermolecular VEGF receptor crosstalk through the formation of VEGFR1/VEGFR2 heterodimers (further discussed below), [57].

3.2.2. VEGFB

VEGFB was the third VEGF ligand identified as selectively binding VEGFR1 [74,103]. VEGFB shares 45% homology with VEGFA [104]. The human gene for *VEGFB* is organized in 7 exons [105] and alternative splicing of the exon 6 generates two isoforms: VEGFB₁₆₇ [106] and VEGFB₁₈₆ [104]. VEGFB₁₆₇ contains a highly basic C-terminus domain encoded by exon 6b highly homologous to sequence encoded by exon 7 in VEGFA₁₆₅ [104]. Like VEGFA₁₆₅, VEGFB₁₆₇ binds heparin and the ECM, while VEGFB₁₈₆ is freely secreted from cells [104]. VEGFB₁₆₇ is the predominant isoform *in vivo* being highly expressed in the heart, skeletal muscle and pancreas [104]. VEGFB is also able to heterodimerize with VEGFA₁₆₅ *in vitro* [104,106]. However, the biological significance of the heterodimers *in vivo* remains to be determined. Additionally, *Vegfb*^{-/-} knockout mice are healthy and fertile, although these animals showed a reduced heart size and displayed a deficient recovery from experimentally induced ischemia [107]. Although the precise function of VEGFB is still unclear, these findings suggest a role in the establishment of a functional coronary vasculature [107].

3.2.3. VEGFC AND FIGF

VEGFC and its homologue FIGF (c-fos induced growth factor previously named vascular endothelial growth factor D) constitute a subfamily of VEGF ligands characterized by unique N- and C-terminal extensions of the VEGF-homology domain [74,108,109]. VEGFC and FIGF are secreted as homodimeric precursor proteins that undergo proteolytic processing. The C-terminal domain is cleaved upon secretion, remaining bound to the N-terminal portion of the protein by disulphide bridges. Upon further proteolytic processing of the N-terminal, the mature protein is released and comprises only the VEGF-homology domain [40,110]. The proteolytic processing of both VEGFC and FIGF modulates receptor-binding affinities, since the mature forms are able to bind both VEGFR2 and VEGFR3, while the unprocessed forms bind exclusively VEGFR3 [40,110]. *Vegfc*^{-/-} knockout mice die *in utero* at E15.5 due to large tissue edema as a result of the deficient lymphatic system [111]. *Vegfc*^{-/-} lymphatic endothelial cells fail to sprout from embryonic veins to form the lymph vessels. However, the commitment to the lymphatic lineage is not abrogated, indicating that VEGFC is crucial for the sprouting and growth of the lymphatic vasculature but not for its induction [111]. Interestingly, *Vegfc*^{+/-} mice are viable but develop lymphoedema, suggesting that both *Vegfc* alleles are needed to provide the dose required for the correct establishment and function of the lymphatic vasculature [111].

Contrary to *Vegfc*^{-/-} mutants, *Figf*^{-/-} deficient mice are healthy and fertile, without any severe lymphatic deficiency, presenting only a small reduction in the number of lymphatic capillaries surrounding the bronchioles in the lungs, where FIGF expression is usually elevated [112]. These results suggested a minor role for FIGF in lymphatic development or that the lack of FIGF is compensated by VEGFC [112].

3.2.4. VEGFE AND svVEGF

VEGFE is the generic name of a subfamily of VEGF-related proteins encoded in the genome of the strains NZ-7, NZ-2 [113], D1701 [114] and NZ-10 [115] of

Orf virus. VEGFE seems to contribute to the enormous capillary proliferation and dilation in skin lesions observed upon viral infection [113,116]. Another VEGF-related protein (VEGFE_{VR634}) was isolated from the *Pseudocowpox virus*, a distant species of the *Parapoxvirus* genus [115]. Interestingly, VEGFE family members share only 20 to 25% amino acid identity with VEGFA and do not contain a heparin-binding domain [113,117]. However, VEGFE induces a potent mitogenic activity on ECs *in vitro* and mice engineered to express *Vegfe*_{NZ7} under the control of the skin K-14 promoter showed enhanced angiogenesis in subcutaneous tissue without side effects [118]. The VEGFE family members are selective ligands for VEGFR2 [114,115,116,118,119]. VEGFE_{NZ2} [119] and VEGFE_{NZ10} [115] were reported to bind also NRP1. VEGF-related proteins have also been identified in the snake venom of different species and generically designated as svVEGFs [120,121,122]. The cysteine knot motif is conserved in these proteins but the biological affinities for the VEGFRs differ, despite sharing a 50% homology with VEGFA₁₆₅ [72].

3.3. VEGF RECEPTORS

The biological effects of the VEGFs are mediated through the binding and activation of two related members of the RTK superfamily: VEGFR1 (also named as fms-like tyrosine kinase 1, FLT1) and VEGFR2 (also referred to as kinase insert domain, KDR). A third member of this receptor family, VEGFR3 (also named as fms-like tyrosine kinase 4, FLT4), binds VEGFC and FIGF and is primarily involved in the lymphatic vessel development and lymphangiogenesis [71]. Genetic ablation of *Vegfr3* in mice (*Vegfr3*^{-/-}) leads to embryonic death between E9.5-14 due to fluid accumulation in the pericardial cavity and cardiovascular failure [123]. Both vasculogenesis and angiogenesis occur in these embryos. However, defects in vascular remodelling were observed, suggesting that VEGFR3 is not involved in initial steps of the vascular assembly, but rather in the remodelling of the vascular plexus [123]. These effects could also be due to increased availability of VEGFC and FIGF for activation of VEGFR2 [124]. In the adult VEGFR3 expression is almost

restricted to lymphatic vessels and it is in the lymphatic vasculature development and lymphangiogenesis that VEGFR3 has a central role [38].

A different class of VEGF receptors are the neuropilins, NRP1 and NRP2, initially described as mediators of neuronal guidance, which are also involved in angiogenesis [125]. Neuropilins are single-pass transmembrane non-tyrosine kinase proteins with a large extracellular domain and a short cytoplasmic domain [125]. Neuropilins are co-receptors for both the semaphorin family of axonal guidance molecules and the VEGF family [125]. The co-expression of NRP1 and VEGFR2 in porcine aortic ECs enhanced the binding and bioactivity of VEGFA₁₆₅, suggesting that NRP1 acts as a co-receptor for VEGFR2 [126]. Genetic studies have shown that *Nrp1*^{-/-} knockout mice die at E13 from cardiovascular defects and deficient neural vascularization [127]. Also, mice overexpressing NRP1 die *in utero* at E17.5 due to cardiac defects and excessive and hemorrhagic blood vessels [128]. *Nrp2*^{-/-} knockout mice are viable and show a normal vascular phenotype [129]. However, double knockout mice (*Nrp1*^{-/-}*Nrp2*^{-/-}) die *in utero* at E8.5 and show a severe vascular phenotype with greatly diminished yolk sac vasculature and disorganized blood vessels, resembling the *Vegfa*^{-/-} and *Vegfr2*^{-/-} knockout mice [130]. These genetic studies thus demonstrated a partial genetic redundancy between NRP1 and NRP2, and support an essential role for neuropilins in VEGF signaling [125].

Structurally, the VEGF receptor-protein tyrosine kinases consist of an extracellular region containing seven immunoglobulin (Ig)-like domains (in VEGFR3 the fifth Ig-like domain is substituted by a disulfide bridge); a single transmembrane region; a juxtamembrane domain; a split tyrosine kinase domain, that is interrupted by a 70-amino acid kinase insert, and a C-terminal tail [75], (Figure 5).

Classically, as for other RTKs, binding of VEGF to the extracellular domain of the transmembrane VEGFR leads to receptor dimerization, trans autophosphorylation and protein kinase activation [75].

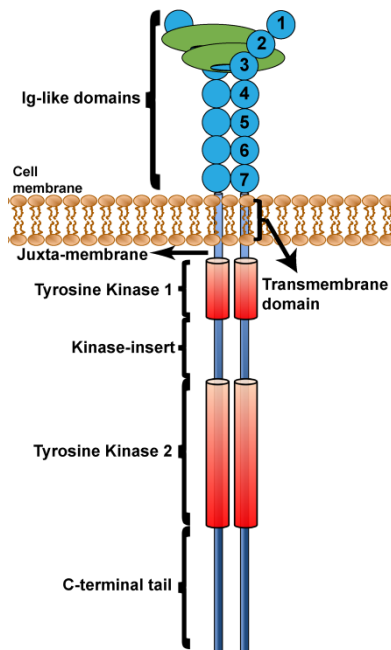


Figure 5. Schematic structure of the VEGF receptors.

The structure of VEGFRs is composed of an extracellular region with Ig-like domains; a single transmembrane domain and an intracellular domain comprising a kinase domain split by a kinase insert and a C-terminal tail. Adapted from [71,75].

The intracellular recruitment of adaptors and second messengers activates complex signaling cascades leading to the modulation of a variety of cellular functions, such as vascular permeability, migration, proliferation and survival [75]. Upon VEGFR activation by VEGF, ECs activate the angiogenic program [75].

3.3.1. VEGFR1 – A DUAL FUNCTION RECEPTOR

VEGFR1 was cloned and characterized from a human placenta cDNA library [131] and was the first high affinity VEGF receptor to be identified [132]. Human *VEGFR1* gene encodes a 1338 amino acids protein of 180 kDa [131]. Structural and mutational experiments have identified the second Ig-like domain as the VEGFA, PlGF and VEGFB binding site and the first Ig-like domain as a receptor dimerization motif [133].

The binding affinity of VEGFR1 for VEGFA is at least 10 fold higher than that of VEGFR2 (dissociation constant $K_d=10$ pM), [62]. However, despite binding

VEGF with high affinity, VEGFR1 presents weak tyrosine kinase phosphorylation activity following VEGF stimulation [62]. Gene-targeting studies demonstrated the essential role of VEGFR1 in vascular development. *Vegfr1*^{-/-} mice die *in utero* between E8.5-9.0 exhibiting a severe disorganization of the vasculature and an increased number of ECs [11]. The phenotype observed was due to an increased mesenchymal to hemangioblast commitment resulting in an excess of the EC population that leads to the development of a disorganized vascular plexus [134]. Based on the biochemical and genetic data it was proposed that VEGFR1 could be a negative regulator of the VEGF activity, acting as a “decoy” receptor to sequester VEGF, thus rendering it less available for interacting with VEGFR2 [72]. Further genetic studies supported this idea since VEGFR1 tyrosine kinase (TK)-deficient mice (*Vegfr1*^{TK-/-}) are viable and fertile and do not exhibit deficiencies in vascular development, indicating that the VEGFR1 TK activity is dispensable for embryonic angiogenesis and that the extracellular domain of VEGFR1 is sufficient to rescue lethality in *Vegfr1* null mutant mice [135].

Alternative splicing generates a soluble truncated form of the VEGFR1 that encodes only the first six Ig-like domains of the extracellular region of the receptor [72]. sVEGFR1 binds VEGF strongly and efficiently blocks the VEGF-activity when present in a 10 fold excess over VEGF [136]. High sVEGFR1 levels are expressed in the placenta, where it might control VEGF activity at particular stages of pregnancy [136]. Interestingly, 50% of the mice expressing exclusively the extracellular region of VEGFR1 (*Vegfr1*^{(TM-TK)-/-}) die *in utero* at E8.5 due to poor blood vessels development, suggesting that the VEGFR1 transmembrane domain (TM) is important for maintaining the ligand-binding domain attached to the cell membrane and directly regulate the levels of ligands near the cell surface [137].

3.3.1.1. SIGNAL TRANSDUCTION

Despite the proposed negative regulation of the VEGF activity, other studies suggest a positive role for VEGFR1 signaling. VEGFR1 overexpression in

porcine EC induces a moderate increase in the VEGFR1 TK activity [138]. In these conditions, biochemical experiments identified six potential tyrosine phosphorylation sites in the C-terminal tail of VEGFR1 (Figure 6): Y1169; Y1213; Y1242; Y309; Y1327 and Y1333.

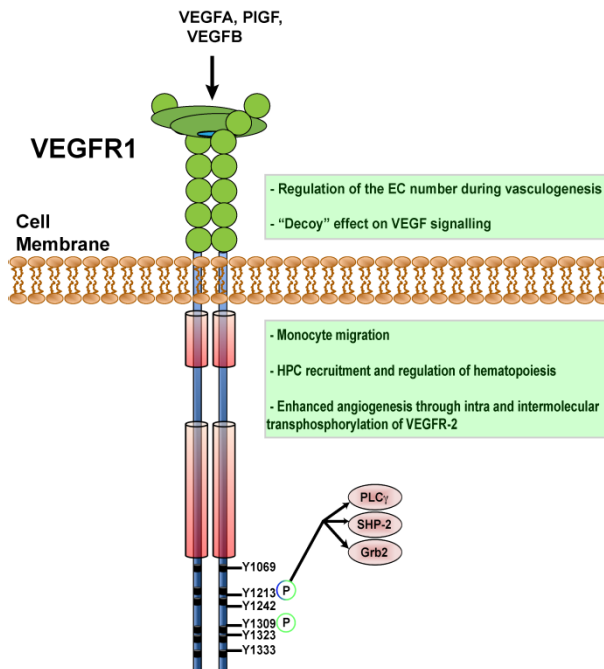


Figure 6. Schematic structure of VEGFR1

The extracellular domain, with the seven Ig-like structures, binds VEGF and PlGF and is involved in the regulation of the EC number during development by acting as a “decoy receptor” as explained in the text. Residues Y1069, Y1213, Y1242, Y1309, Y1323 and Y1333 were identified in the C-terminal tail. VEGF induces phosphorylation (P) of Y1213 and PlGF induces phosphorylation (P) of Y1309. Three SH2-domain containing molecules bind to PY1213: SHP-2, PLC γ and Grb2. The intracellular signaling of VEGFR1 is primarily involved in the monocyte migration and regulation of haematopoiesis and enhances angiogenesis through intra- and intermolecular phosphorylation of VEGFR2 as explained in the main text. Adapted from [71, 139].

Tyrosine residue Y1213, was identified as docking site for three Src homology 2 domain (SH2)-binding proteins namely SHP-2, phospholipase C γ (PLC γ) and growth factor receptor-bound 2 (Grb2) [136,138]. Consistently, increased tyrosine phosphorylation of PLC γ was observed in VEGFA-stimulated porcine aortic EC overexpressing VEGFR1 [136]. A specific

biological response that requires the VEGFR1 TK activation is the migration of monocytes in response to VEGF and PlGF as *Vegfr1*^{TK-/-} mice shows defective mobilization of monocytes [57]. Consistent with this, the monocyte/macrophage lineage expresses high levels of VEGFR1, but not VEGFR2, suggesting the involvement of VEGFR1 in the VEGF-dependent signaling [62].

In monocytes, it was shown that activation of VEGFR1 through PlGF results in the activation of the PI3-kinase (PI3K)/AKT and Erk-1/2 pathways leading to chemotaxis and to the production of several inflammatory cytokines [140]. Also, activation of VEGFR1 by PlGF is involved in the recruitment of VEGFR1-positive hematopoietic progenitor cells (HPCs) from the bone marrow [141]. These VEGFR1⁺HPCs cells and other accessory cells released from the bone marrow can create a favorable microenvironment for cancer cell spreading [142,143]. The migration and influx of these activated VEGFR1⁺HPCs to distant tissues induces early changes in the local microenvironment, termed the “pre-metastatic niche”, priming the tissues for tumor cell implantation and proliferation [142,143]. These VEGFR1⁺HPCs clusters preserve the expression of primitive cell surface markers, rather than undergoing lineage-committed maturation. The recruitment of VEGFR1⁺HPCs might establish the metastatic signature, determining the tumor-pattern of metastatic spread and contributing for cancer cell proliferation [142,143].

Although VEGF and PlGF bind equally to VEGFR1 each activates VEGFR1 in a distinct manner. For instance, VEGFA stimulates VEGFR1 Y1213 phosphorylation whereas PlGF stimulates Y1309 phosphorylation [57]. PlGF binding, but not VEGF is able to alter the gene expression profile in mouse capillary ECs. It was shown *in vivo* that PlGF can enhance VEGFA-mediated angiogenesis in ischemic conditions [101], through a molecular process of crosstalk between the two VEGF receptors [57].

In the absence of PlGF, VEGFR1 acts as a “reservoir” for VEGF regulating the availability of VEGF for binding VEGFR2 [57]. When PlGF binds and activates

VEGFR1, potentiates angiogenesis through different mechanisms: 1) VEGF is displaced from VEGFR1 and becomes more available to bind and activate VEGFR2; 2) PlGF-mediated VEGFR1 activation leads to intermolecular trans-phosphorylation of VEGFR2, increasing VEGFR2 phosphorylation; this mechanism requires VEGFR1 TK signaling, since the increased phosphorylation levels of VEGFR2 were not observed in *Vegfr1^{TK-/-}* cells; 3) Another crosstalk mechanism is observed in VEGFR1/VEGFR2 heterodimers, where the trans-phosphorylation occurs intermolecularly induced either by PlGF/VEGF heterodimers or VEGF homodimers [57]. Together these data suggest an important regulatory role for VEGFR1 in VEGF and PlGF mediated angiogenesis, specially potentiated in pathological conditions such as cancer [57].

3.3.2. VEGFR2 – A PRO-ANGIOGENIC RECEPTOR

The human *VEGFR2* gene encodes a 200-230 kDa protein with 1356 amino acids. Mutation analyses of the VEGFR2 extracellular domain have shown that the second and third Ig-like domains were high affinity VEGF binding sites and that the first and fourth Ig-like domains were important for the receptor dimerization [144]. The fifth and sixth Ig-like domains of VEGFR2 seem to be important to preserve ligand binding [145]. Both VEGFR1 and VEGFR2 undergo post-translational modifications. In the case of VEGFR2, but not VEGFR1, only the glycosylated or mature form of the receptor is efficiently autophosphorylated upon ligand binding [70].

VEGFR2 binds to VEGF with a lower affinity than VEGFR1 ($K_d=75$ pM), but in contrast to VEGFR1 it has high tyrosine kinase activity [62].

Knockout *Vegfr2^{-/-}* embryos die *in utero* between E8.5-9.0 as a result of profound defects in vasculogenesis and angiogenesis. *Vegfr2^{-/-}* embryos fail to develop yolk-sac blood islands and organized blood vessels and show a reduced number of hematopoietic and EC precursors [12]. These results suggested a pivotal role for VEGFR2 in vascular development and therefore it is considered the major mediator of the VEGF signaling during vasculogenesis

and angiogenesis [62]. The critical role of VEGFR2 in these processes led to the intense scrutiny of the signaling cascades mediated by its activation and that will be further discussed in the next section.

3.3.2.1. SIGNAL TRANSDUCTION

Several tyrosine residues in the intracellular region of VEGFR2 were identified, using different systems and shown to be autophosphorylated upon ligand binding: Y951 and Y996 in the insert kinase domain; Y1054 and Y1059 in the kinase domain 2 [146]; Y1075 and Y1214 in the C-terminal tail [147,148]. Phosphorylated Y1305, Y1309 and Y1319 in the C-terminal tail have also been reported [149]. Phosphorylated VEGFR2-tyrosine residues serve as docking sites for molecules that initiate different signaling cascades leading to cellular responses such as proliferation, migration, survival and permeability [75]. The relative contribution of the different phosphorylated tyrosine residues for the VEGFR2 function *in vivo* is still largely unknown. Genetic studies have shown that mice carrying a *Vegfr2* tyrosine (Y) to phenylalanine (F) Y1173F mutation (Y1175, in human), (*Vegfr2*^{Y1173F}) die around E8.5-9.0 with a similar phenotype to that observed in *Vegfr2*^{-/-} embryos. Additionally, mice exclusively expressing *Vegfr2*^{Y1212F} mutation (equivalent to human Y1214) are viable and fertile. These studies suggested that the activation of Y1175, but not Y1214 is critical for the VEGF-mediated actions *in vivo* [150]. Several *in vitro* studies have described some of the molecular cascades that can be initiated upon VEGFR2 activation (Figure 7).

The phosphorylation of Y1054 and Y1059 is required for maximal kinase activity [151]. VEGFR2 phosphorylated on Y1175 binds to and activates PLC γ , which mediates the activation of the extracellular regulated kinase (Erk) pathway leading to cell proliferation [71], (Figure 7).

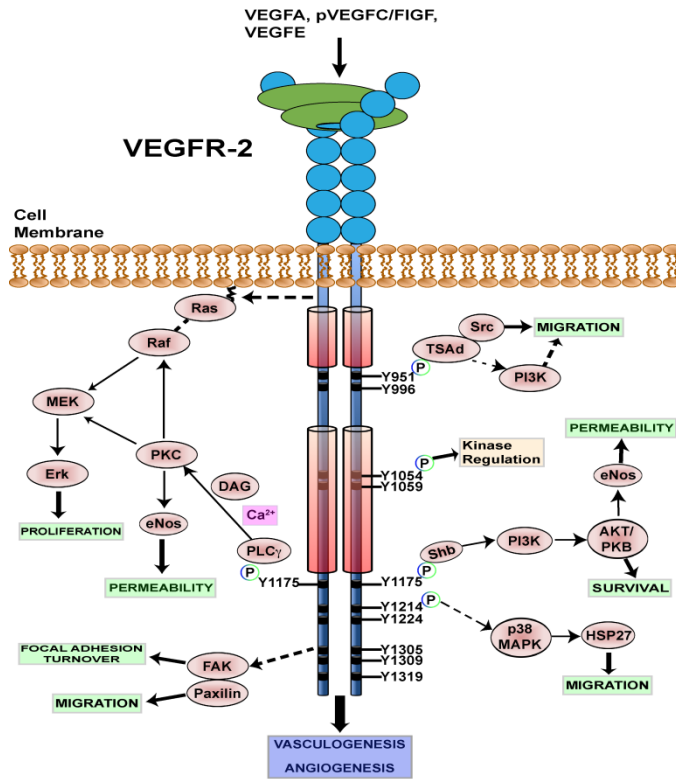


Figure 7. VEGFR2 schematic structure and signal transduction.

The schematic structure of the VEGFR2 is identical as shown before for VEGFR1. The extracellular domain, binds VEGFA, processed (p) VEGFC, FIGF, VEGFE and svVEGF. Several intracellular proteins bind to specific phosphorylated (P) tyrosine residues in the intracellular region of VEGFR2, via their SH2 domains, leading to the phosphorylation and activation of these proteins. PY1175 binds PLC γ , which by hydrolysis generates second messenger DAG and leads to the release of intracellular calcium (Ca²⁺). DAG is a physiological activator of PKC that in turn activates the Raf-MEK-Erk pathway leading to cell proliferation. The activation of Ras via VEGFR2 is unclear. PKC also activates eNOS that induces the NO production and therefore increases permeability. TSA δ binds to PY951 and Src inducing cell migration. TSA δ can also activate PI3K-dependent migration, however is not clear if this pathway is activated in EC. Binding of Shb to PY1175 regulates activation PI3K and the AKT/PKB survival pathway. AKT can also induce permeability by activation of eNOS. PY1214 results in activation p38 MAPK and HSP27 leading to actin remodelling and cell migration. The activation of FAK and paxilin is also mediated by VEGFR2 activation and regulates the focal adhesion turnover and cell migration, however is not clear the specific tyrosine involved. Adapted from [71].

VEGF activation of VEGFR2 also induces cell survival. This effect seems to be mediated by binding of the adapter Src homology 2 domain (Shb) to Y1175,

which then activates PI3K, leading to the downstream activation of protein kinase B PKB/Akt that mediates cell survival [71].

Another important EC response to VEGF is the activation of signaling pathways leading to cell migration, mediated by VEGFR2. This effect seems to be mediated by the binding of the T-cell-specific adapter (TSA), a VEGF receptor-associated protein, to phosphorylated Y951 and its activation, which promotes its association with Src leading to actin reorganization and cell migration [149], (Figure 7). Additionally, TSA binds PI3K that can also be involved in cell migration [152]. The activation of PI3K-AKT also leads to the activation of endothelial nitric oxide synthase (eNOS), inducing the production of nitric oxide (NO) and thereby increasing the vascular permeability [71]. Interestingly, the phosphorylation levels of Y951 were found to be increased in embryonic bodies (EB) sprouting vessels but not in EB resting vessels, further suggesting a role for Y951 in VEGFA dependent migration [149]. Phosphorylation of Y1214 was shown to activate p38 MAPK resulting in the phosphorylation of the heat-shock protein-27 (HSP27) a chaperone that positively regulates VEGF-induced actin remodeling and motility [71], (Figure 7). The VEGFR2-mediated activation of Focal adhesion kinase (FAK) and paxilin, involved in the regulation of the focal adhesion turnover have also been implicated in VEGF/VEGFR2-induced migration. Although it is not clear which VEGFR2 tyrosine residue is involved in this activation [71].

3.3.2.2. REGULATION OF THE VEGFR2 GENE EXPRESSION

VEGFR2 is the earliest marker of mesodermal vascular precursors in mice [8]. VEGFR2 is strongly and specifically expressed in vascular ECs, although it has been observed in other cell types, such as retinal progenitor cells, neurons, osteoblasts, pancreatic duct cells and megakaryocytes [62]. The human *VEGFR2* gene promoter shows a class II promoter structure: it does not have a TATA box region but is highly complex containing multiple regulatory elements [153], (Figure 8).

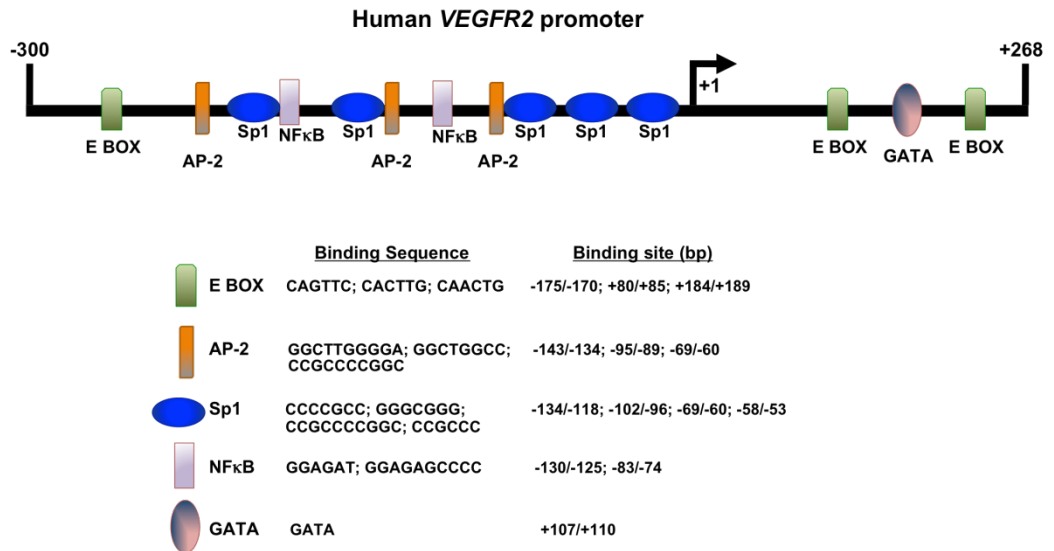


Figure 8. Schematic structure of the human *VEGFR2* promoter.

Several consensus-binding sequences for the indicated transcription factors were predicted from the human *VEGFR2* promoter sequence between -300/+268 bp relative to the transcription start site (indicated with black arrow). Adapted from [153].

The cloning and functional analysis of the human *VEGFR2* gene promoter identified a fragment from -225 to +268 base pairs (bp) relative to the transcription start site as required for maximal promoter activity [153,154]. This segment, defined as the core promoter for human *VEGFR2* gene, contains putative binding sites for the transcription factors AP-2, NFκB, and Sp1. Also, GATA elements and E-Box sequences have been identified in this region [153,154], (Figure 8).

Studies using electrophoretic mobility shift assays (EMSA) and *in vitro* DNase I footprinting have shown that the transcription factor specificity protein 1 (Sp1) binds to five CG-rich regions of the human *VEGFR2* promoter in ECs but not in other cell types, suggesting that this element is essential for the specific *VEGFR2* expression in ECs [154]. Different studies also showed that binding of Sp1 to the GC-region (-79 to -68) enhanced *VEGFR2* expression while binding of Sp3 to the same region attenuated this response [155].

Sp1 is a ubiquitous transcription factor that binds to GC-rich regions in gene promoters and was initially implicated in the regulation of housekeeping genes

[156]. Interestingly, the Sp1 gene is auto-regulated and is highly expressed in the heart of mouse embryos [156], suggesting an important role for this transcription factor in the regulation of the vasculature. Sp1 has also been implicated in the regulation of the *VEGF* gene [90]. Additionally, hormone-induced activation of the *VEGFR2* promoter has also been reported to be dependent of Sp proteins [157].

Other mechanisms such as epigenetic regulation might also contribute to the *VEGFR2* expression [158]. Interestingly, a hypoxia responsive element (HRE) was not identified in the human *VEGFR2* gene promoter, indicating that unlike *VEGF* and *VEGFR1*, *VEGFR2* transcription is not directly regulated by hypoxia [159].

3.3.2.3. INTRACELLULAR TRAFFICKING

VEGFR2 intracellular trafficking is potentially an important mechanism through which angiogenesis and other endothelial functions can be regulated, in addition to the previously described signaling from cell surface-located receptors. Although the signal transduction activated by VEGFR2 upon VEGF stimulation has been the object of intense study, the mechanisms mediating the endocytosis and membrane trafficking of VEGFR2 are still poorly understood.

The majority of the data available on RTK trafficking is based on the epidermal growth factor receptor (EGFR) and its ligand epidermal growth factor (EGF) [160]. Until recently RTK internalization was thought to be exclusively for downregulation of receptor activity through degradation. In the absence of ligand stimulation, EGFR resides largely at the cell surface. Upon EGF binding, soluble clathrin is recruited to the cell membrane to form clathrin-coated membrane invaginations surrounding the activated receptor and trigger an endocytosis process to sort EGFR into early endosomes. From early-endosomes EGFR can either be recycled from this compartment back to the plasma membrane or under ligand saturation conditions the receptor undergoes degradation through late endosomal sorting to the lysosome [160].

Therefore, initially RTK internalization was considered an important mechanism to control the intensity and duration of the signal transduction [160].

Two studies on VEGFR2 trafficking suggested that VEGFR2 is ubiquitinated by the E3 ubiquitin ligase, c-Cbl [161,162]. However, a third study suggested that activated PKC, but not c-Cbl, marks the receptor for internalization and degradation [163]. Despite the leading idea of the receptor internalization for degradation to stop the signaling process, increasing evidence suggests that receptor activity may be sustained on endosomes [164,165]. It has been shown that cell confluency inhibits EC growth and proliferation, in a process mediated by increased cell-cell junctions [164]. These adherens junctions are modulated by a class of adhesive transmembrane proteins called cadherins localized at the intercellular junctions forming zipper-like structures once cells get in contact. ECs express a cell-specific cadherin called vascular endothelial cadherin (VE-cadherin or VEC) that forms a complex with VEGFR2 and inhibits its phosphorylation and mitogen activity. It was described that in the absence of VE-cadherin, VEGFR2 is rapidly internalized to endosomes via a clathrin-dependent pathway, maintaining its signaling activity rather than being degraded [164]. The internalization of VEGFR2 and sustained receptor signaling was recently shown to be required for VEGF-induced tip cell filopodial extension [165]. EphrinB2 at the tip cell filopodia was shown to regulate VEGFR2 endocytosis and intracellular signal to direct filopodial extension *in vivo* [165].

Other studies have shown that activated VEGFR2 is internalized preferentially via a caveolar pathway and transported to perinuclear caveosomes, colocalizing with caveolin-1 [166]. Caveolae are lipid raft-enriched, flask-shaped invaginations, present in the plasma membrane of different cell types especially in ECs and adipocytes [167,168]. Caveosomes are intracellular caveolin-1-containing membrane-bounded structures of neutral pH, distinct from classical low pH endosomal compartments [167,169]. The VEGFR2, caveolin-1 interaction has been reported at the plasma membrane level and

caveolin-1 was suggested to act as a negative regulator of VEGFR2 activity in resting conditions [170]. However, in the presence of VEGF, the localization within caveolae domains is important for the VEGF-induced phosphorylation of VEGFR2 [170]. Importantly, removal of VEGFR2 from caveolae domains at cell surface results in inhibition of EC migration [170]. A similar effect was observed in caveolin-1 knockout mice (*Cav^{-/-}*), [171,172]. A defective post-ischemic neo-vessel formation due to a failure in VEGFR2 compartmentalization in caveolae domains was reported [171].

Other proteins were also shown to be involved in the correct positioning of VEGF-VEGFR2 in caveolae vesicles, such as dynamin-2 [173]. The reported perinuclear colocalization of caveolin-1, dynamin-2, and VEGFR2 suggests that these three components are in a complex probably located in the trans-Golgi region [173]. Dynamin-2 mutants induced an enhanced VEGFR2 degradation by lysosomal and proteasomal proteases since VEGFR2 failed to localize in caveosomes [173,174]. Also, it was reported that dynamin-2 inhibitors abolished the VEGF-induced internalization of VEGFR2, resulting in decreased tip cell filopodial extensions [165]. From caveosomes the internalized cargo can be delivered to the endoplasmic reticulum through different pathways or possibly undergo a retrograde transport pathway to the nucleus [160]. This last idea is particularly interesting considering recent publications reporting the presence of VEGF or phosphorylated VEGFR2 in the nucleus of a large number of cell types in a variety of physiological and pathological conditions. For instance, nuclear VEGFR2 was observed in normal and neoplastic renal tissues [175,176], in the nucleus of MCF-7, HeLa and HL60 cells [176] and in bovine and rat ECs [166,177]. A similar finding was reported in another study showing that VEGFR2 is constitutively phosphorylated and located in the nucleus of VEGF-producing leukemias [60]. The mechanisms regulating VEGFR2 intracellular trafficking towards the cell nucleus are not known and will be one of the focuses of this thesis. Furthermore, the nuclear localization of VEGFR2 opens the possibility of its direct involvement in transcriptional regulatory activities.

4. AIM OF THIS THESIS

The aim of this work is to study the molecular mechanisms and relevance of the nuclear internalization of VEGF and VEGFR2 in ECs. Until recently, VEGFR2 was described as a tyrosine kinase membrane receptor. In this work, I focused on the molecular mechanisms underlying the VEGFR2 nuclear translocation in ECs. Furthermore, I investigated the role of VEGFR2 as a nuclear protein and defined the basis for a novel function for VEGFR2 in the transcriptional regulation of its own expression.

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CHAPTER II

VEGF AND VEGFR2 (KDR) NUCLEAR INTERNALIZATION IS

REQUIRED FOR ENDOTHELIAL RECOVERY DURING

WOUND HEALING

2.1. OVERVIEW OF THE ARTICLE:

VEGF and VEGFR2 (KDR) Internalization is Required for Endothelial Recovery during Wound Healing

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The angiogenic growth of new blood vessels is crucial during embryonic development and in adult tissues in different physiological and pathological situations such as wound healing or tumor growth, among others. Endothelial cells (ECs) lining the inside of all blood vessels are key components in the angiogenic process. Following injury, quiescent vascular ECs are reactivated in response to different stimuli. ECs migrate and proliferate towards the wound bed inducing tissue vascularization and contributing to the healing process.

Vascular Endothelial Growth Factor (VEGF) is one of the most important pro-angiogenic factors. VEGF is produced in tissues in response to hypoxia and binds its receptors VEGFR1 and VEGFR2 at the EC surface. The molecular processes triggered upon VEGF activation of its receptors are extremely important and have been the focus of intense work. Although VEGFR2 has a lower affinity for VEGF than VEGFR1, it is the major mediator of the mitogenic activity induced by VEGF in ECs. VEGFR1, despite its higher affinity for VEGF, has a weaker kinase activity and until recently, it was thought to act mainly as a “decoy” receptor for VEGF in ECs. However, it was demonstrated that VEGFR1 activation leads to intermolecular trans-phosphorylation of VEGFR2, increasing VEGFR2 phosphorylation and activation.

In this paper, we demonstrated that VEGF and VEGFR2 are translocated to the EC nucleus in a process mediated by VEGFR1 and that this internalization is required for EC recovery following *in vitro* wounding.

- We showed that, upon VEGF stimulation, VEGF and VEGFR2 translocate to the nucleus of ECs. A neutralizing antibody against VEGFR1 (6.12 Ab) blocked the VEGF-VEGFR2 nuclear accumulation, showing a functional relevance for the receptors' crosstalk.
- We also demonstrated that VEGFR2 internalization is blocked by drugs inhibiting its phosphorylation (KDRi). Additionally, VEGFR2 tyrosine deletion mutants showed impaired nuclear accumulation further supporting that the phosphorylation of VEGFR2 is important for its nuclear translocation.
- We explored the VEGF-VEGFR2 internalization process and showed that it requires the activation of the PI3K pathway and is linked to a caveolae-mediated endocytic pathway, via microtubules.
- The functional relevance of the VEGF and VEGFR2 nuclear translocation was supported by wound healing assays *in vitro*. The blockage of the VEGFR1-mediated VEGF-VEGFR2 nuclear translocation led to a delayed EC recovery following injury.

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Research Article

VEGF and VEGFR-2 (KDR) internalization is required for endothelial recovery during wound healing

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ABSTRACT

Vascular endothelial growth factor (VEGF) receptor activation regulates endothelial cell (EC) survival, migration and proliferation. Recently, it was suggested the cross-talk between the VEGF receptors-1 (FLT-1) and -2 (KDR) modulated several of these functions, but the detailed molecular basis for such interactions remained unexplained. Here we demonstrate for the first time that VEGF stimulation of EC monolayers induced a rapid FLT-1-mediated internalization of KDR to the nucleus, via microtubules and the endocytic pathway, internalization which required the activation of PI 3-kinase/AKT. KDR deletion mutants were generated in several tyrosine residues; in these, VEGF-induced KDR internalization was impaired, demonstrating this process required activation (phosphorylation) of the receptor. Furthermore, we demonstrate that in vitro wounding of EC monolayers leads to a rapid and transient internalization of VEGF+KDR to the nucleus, which is essential for monolayer recovery. Notably, FLT-1 blockade impedes VEGF and KDR activation and internalization, blocking endothelial monolayer recovery. Our data reveal a previously unrecognized mechanism induced by VEGF on EC, which regulates EC recovery following wounding, and as such indicate novel targets for therapeutic intervention.

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Introduction

Vessel integrity is important for tissue and organ homeostasis and plays a crucial role in physiological and pathological situations including wound healing and tumour growth, among many others [1–3]. In such situations, endothelial cells (ECs) of vessels respond to local and

systemic stimuli by resisting apoptosis, proliferating and migrating in response to specific cues, which have been under intense scrutiny. One of the crucial molecular pathways for EC responses during situations of vascular trauma involves vascular endothelial growth factor (VEGF) and its receptors (VEGFR-1, FLT-1 and VEGFR-2, KDR). VEGF is produced by the majority of cells in the body but acts mainly

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on EC, promoting their survival, proliferation or migration in situations such as the ones described above. In particular, the role of VEGF in tumour angiogenesis (formation of new tumour blood vessels, essential for tumour growth), has been studied extensively [1,2]. VEGF binding to KDR induces autophosphorylation of KDR intracellular tyrosine residues and consequently several signalling pathways are activated. In contrast to KDR, FLT-1 has weaker kinase activity and its role in VEGF-stimulated cellular responses remains to be determined. Furthermore, the molecular basis explaining the interactions between the 2 VEGF receptors and the relevance of such mechanism for EC functions remains undisclosed. For instance, the cross-talk between FLT-1 and KDR was only recently demonstrated [4].

In the present report, we observed a rapid and transient internalization (to the nucleus) of KDR after VEGF stimulation of EC. We exploited the molecular basis for this transport and reveal novel molecular interactions including the microtubular cytoskeleton, the endocytic pathway and activation of specific signalling pathways. In addition, we demonstrate that VEGF is also internalized and the importance of KDR/VEGF internalization using an *in vitro* wound healing model; here, FLT-1 blockade impeded KDR/VEGF internalization and delayed EC monolayer recovery following wounding.

Our results demonstrate the importance of VEGF receptor cross-talk and activation, namely in mediating VEGF/KDR internalization, for EC monolayer recovery following injury.

Materials and methods

Reagents

Antibodies against KDR (clones IMC-1C11, used at 1 µg/ml for neutralization experiments or 6.64) or FLT-1 (clone 6.12, used at 1 µg/ml for neutralization experiments or FB5) were kindly provided by ImClone Systems (NY, USA). Ly294002 (used at 25 µM), Nocodazole (used at 1 µg/ml) and Cytochalasin D (used at 1 µg/ml), VEGF (used throughout at 20 ng/ml) and PlGF (used at 10 ng/ml) were purchased from Sigma. U0126 (used at 10 µM) was purchased from New England Biolabs. Bay 11-7082 (used at 5 µM) and the KDR-specific inhibitor (KDRi, used at 70 nM) were obtained from Calbiochem.

Cell culture

Primary human umbilical vein endothelial cells (HUVEC) were kindly provided by Dr. Shahin Rafii (Cornell University Medical College, NY, USA). Primary human cardiac microvascular endothelial cells (HMEC-C) were purchased from Cambrex. HUVEC and HMEC-C were cultured in growing endothelial medium (basal medium with supplements including VEGF, hEGF, hFGF-B, R3-IGF-1, ascorbic acid, BBE, hydrocortisone, heparin and 5% of serum), as provided by the manufacturers (Cambrex, USA). Upon reaching confluence, the cells were passed onto other gelatin-coated flasks or coverslips (see below) and used up to passage 6, in the experiments described in the present report. NIH 3T3 cells were cultured in Dulbecco's modified Eagle's minimal essential medium supplemented with 10% fetal calf serum.

KDR mutants and 3T3 transfection

The vector KDR-pEGFP(N1) was kindly provided by Andrew Reynolds (Tumor Biology Laboratory, Cancer Research UK Clinical Center, UK). The KDR deletion mutants (KDRΔ1174-GFP, KDRΔ1053-GFP and KDRΔ950-GFP) were generated by RT-PCR cloning using the KDR-pEGFP(N1) vector. The forward primer 5' CGTCATGGATCCAGATGAACCTCC 3' which contains the *Bam*HI site is used in all of the RT-PCR reactions. The reverse primers for each of the mutants are indicated below. All of them contain an *Age*I site at 5' end.

KDRΔ1174: 5'-GCGCACCGGTGTCTTTGCCATCCTGCTGAGC-3'
 KDRΔ1053: 5'-GCGCACCGGTAATATCCCGGCCAAGCCAAAAG-3'
 KDRΔ950: 5'-GCGCACCGGTGTCTTTCCCTTGACGGAATCG-3'

The PCR products were double digested with *Bam*HI and *Age*I, gel-purified and subcloned into pEGFP(N1)-KDR by using the same restriction enzymes. NIH 3T3 were transfected using Fugene6 transfection reagent with either empty vector (pEGFP(N1)) or the full-length or the deletion mutants of KDR, according to the manufacturer's protocol. After 2 days, G418 (1 mg/ml) was added to the cultured medium and stably transfected cells displaying neomycin resistance were obtained.

Ten independent pools of the full-length or deletion mutants of KDR and 5 pools of 3T3 transfected with pEGFP(N1) vector were obtained.

RT-PCR assay

Total RNA was obtained using TRIzol® (Life Technologies) and treated with DNA-free TM (Ambion, Inc.) according to the manufacturer's protocol. One microgram of total RNA was converted to cDNA by incubation with 200 U SuperScript II reverse transcriptase (Life Technologies) and 3 µg/µl random hexamers in 50 mM Tris pH 8.3, 75 mM KCl, 3 mM MgCl₂, 100 mM dithiothreitol (DTT) and 0.5 mM dNTPs at 37 °C, for 90 min.

PCR for GFP was performed using 3 µl of cDNA as a template and the primers 5' CATGGTGAGCAAGGGCGAGG 3' and 5' TAGGTCAGGGTGGTCACGAG 3'. PCR (45 s at 95 °C, 45 s at 60 °C, 1 min at 72 °C) was performed during 35 cycles. PCR for KDR was provided at 35 cycles (45 s at 95 °C, 45 s at 56 °C, 1 min at 72 °C) using 3 µl of cDNA and the primers 5' CACGATCACAGAAACCTGGAG 3' and 5' CCAAGTTGCTCTTTCCCTGGGC 3'. The PCR products were 201 and 291 bp in GFP and KDR reactions, respectively.

Whole cell extracts: subcellular protein extraction (cytoplasmic, nuclear and membrane fractions)

Whole cell extracts were prepared by resuspending the cell pellets in the following buffer: 1% NP40, 50 mM Tris pH 7.5, 10% (v/v) Glycerol, 150 mM NaCl, 1 mM EDTA supplemented with protease and phosphatase inhibitors. After incubation 30 min on ice, the cells were centrifuged, for 15 min, at 4 °C and 9800×g and the supernatants were recovered. In order to obtain membrane extracts, 1×10⁷ cells were resuspended in 1 mM Tris pH 7.4, 1 mM EDTA and incubated, for 60 min, at 4 °C. The cell lysate was centrifuged at 9800×g, for 30 min, at 4 °C. Then, the pellet of membranes was resuspended in

20 mM Tris pH 7.4, 0.1 mM EGTA pH 7.4, incubated for 30 min at 4 °C and ultracentrifuged at 100000×g, for 30 min, at 4 °C. Membranes were then solubilized with 1% (v/v) Triton X-100 in 100 mM Tris pH 8.0, for 60 min, at 4 °C, ultracentrifuged at 100000×g, for 60 min, at 4 °C and supplemented with protease and phosphatase inhibitors.

Cytoplasmic extracts were prepared by resuspending cell pellets in a buffer containing 20 mM HEPES pH 7.9, 10 mM KCl, 1 mM EDTA, 0.2% (v/v) NP40, 10% (v/v) Glycerol, 1 mM DTT, supplemented with protease and phosphatase inhibitors, and then centrifuged, for 2 min, at 4 °C and 9800×g. To obtain nuclear extracts, the pellets resulting from the cytoplasmic extraction were treated with a buffer containing 20 mM HEPES, pH 7.9, 400 mM NaCl, 10 mM KCl, 1 mM EDTA, 20% (v/v) Glycerol, 1 mM DTT and supplemented with protease and phosphatase inhibitors. After 30 min on ice, lysates were centrifuged, for 5 min, at 4 °C and 9800×g.

Nuclear–cytoplasm fractionation by sucrose gradient was also prepared. HUVECs were resuspended in isotonic buffer (10 mM Tris–HCl pH 7.5, 140 mM NaCl, 5 mM MgCl₂) containing 0.5% of NP40, incubated, for 15 min, at 4 °C, and centrifuged at 900×g, for 5 min, at 4 °C. The supernatant is kept as the cytoplasmic fraction. The pellet is briefly washed with the isotonic buffer containing 0.1% of NP40 and again centrifuged in the same conditions. The supernatant liquid was pooled in the cytoplasmic fraction and the pellet containing the nucleus was resuspended in the purification buffer (10 mM Tris–HCl pH 7.5, 140 mM NaCl, 5 mM MgCl₂ and 11% sucrose) and centrifuged, at 2500×g, for 10 min, at 4 °C, over a layer of isotonic buffer with 11% sucrose, establishing a density gradient. The pellet was then resuspended in the SDS loading buffer, the sample was boiled, for 5 min, and analysed by Western blot.

Separation of whole cell protein into Triton X-100 soluble and insoluble (cytoskeleton) fractions

Cells were resuspended in 100 µl of PHEM buffer (60 mM Pipes, 2 mM HEPES, 10 mM EGTA, 2 mM MgCl₂ and protease/phosphatase inhibitors) containing 0.1% (v/v) Triton X-100. After 2 min on ice, the Triton X-100 soluble and insoluble fractions were centrifuged, for 30 min, at 4 °C and 9800×g. Supernatant (Triton X-100 soluble fraction) was removed to a new tube and diluted 1:1 with 2× reduced sample buffer. The pellet (Triton X-100 insoluble cytoskeleton fraction) was washed with PHEM buffer containing 0.1% Triton X-100 to eliminate the residual soluble element. The cytoskeleton fraction was then resuspended in 100 µl of PHEM buffer and diluted 1:1 with 2× reduced sample buffer. Insoluble fraction was boiled, for 5 min, and then centrifuged, for 2 min, at 4 °C and 9800×g.

Immunoprecipitation studies and Western blotting

Whole, cytoplasmic and nuclear extracts, soluble and insoluble fractions were used to immunoprecipitate. Lysates were precleared with 50 µl of protein G-Sepharose beads. Supernatants were incubated with the specific antibody (rabbit anti-KDR or mouse anti-GFP), for 12 h, at 4 °C, and incubated with protein G-Sepharose beads for an additional

hour, at 4 °C. Beads were washed in a lysis buffer and resuspended in SDS loading buffer before electrophoresis. For Western blotting, equal protein amounts were separated by SDS–PAGE and transferred to nitrocellulose membranes. Blots were incubated with antibodies raised against the intracellular (I) or extracellular (E) region of KDR, FLT-1, VEGF, caveolin-1, cyclin-E, P-IkB or P-tyrosine (Santa Cruz, CA, USA). The anti-GFP antibody was obtained from Roche (Germany, EU).

Immunofluorescence, confocal microscopy

For these experiments, HUVEC were cultured on ethanol-treated, gelatin-coated, glass coverslips, which were placed in 24-well plates. The cells were fixed in 1% (v/v) formaldehyde/PBS, for 10 min, at 4 °C and washed in PBS. After permeabilization with 0.1% (v/v) Triton X-100 plus 5% (v/v) normal serum, the cells were incubated with the primary antibodies, for 12 h, at 4 °C (mouse anti-human KDR, clone 6.64 from ImClone; mouse anti-human FLT-1, clone FB5 from ImClone; rabbit anti-human KDR from Santa Cruz; goat anti-human KDR from Santa Cruz; mouse anti-human VEGF from Sigma; mouse anti-human β-tubulin from Sigma; mouse anti-human β-actin from Sigma). After primary antibody incubation, the cells were washed and incubated with secondary antibodies (Alexa fluor 594 and Alexa fluor 488, Molecular Probes, US), for 1 h, at room temperature. The samples were mounted in Vectashield and analysed by fluorescence microscopy (Axio-plan Microscope, Zeiss, Germany) and by confocal microscopy (in the case of KDR, VEGF and GFP). Regarding the latter, sets of optical sections with 0.5-µm intervals along the z-axis were obtained from the bottom to the top of cells using a laser scanning confocal microscope (True Confocal Scanner Leica TCS SP2; Leica Microsystems); objectives HCX PL APOCS 63× 1.4 oil. The laser lines relative powers were set in order to have the identical light intensity for the same sample. This was performed using the fieldmaster (Coherent) with the head LM2. Acquisition and image treatment were performed with the LSC software (Leica). As mentioned above, the experiments involving immunofluorescence or confocal microscopy were done using cells from different passage numbers and repeated at least 3 times.

Quantification of VEGF levels in EC culture supernatants

An ELISA kit (Oncogene Research Products) specific for human VEGF was used to determine VEGF concentrations in the supernatants of EC cultures after and before wounding. Test samples were assayed in duplicate and were developed according to the manufacturer's instructions.

Results

VEGF receptor cross-talk and activation regulates KDR nuclear translocation on ECs

HUVEC and HMEC-C were cultured in growing medium and KDR localization was investigated by Western blotting using subcellular protein fractions (Figs. 1A, B). As shown by the use

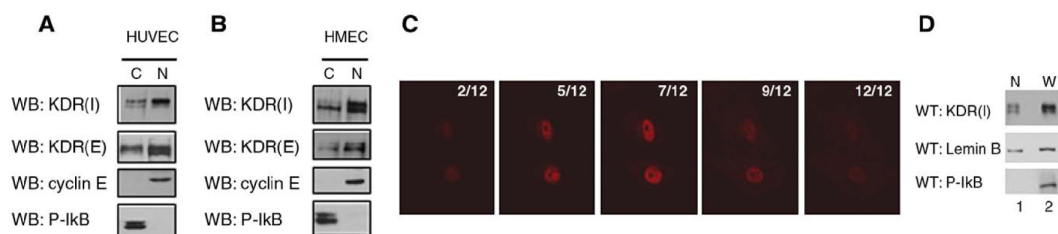


Fig. 1 – HUVEC (A) and HMEC-C (B) were cultured in growing medium. Cytoplasmic (C) and Nuclear (N) extracts were analysed by Western blot (WB) with an antibody against the internal (KDR(I)) and external (KDR(E)) portion of KDR or with anti-cyclin E and anti-P-IkB antibodies. (C) Confocal microscopy of HUVEC cultured on gelatin-coated slides was performed using the rabbit anti-human KDR(I) antibody. Internal section (5/12) demonstrates that KDR locates into the nucleus of VEGF-stimulated HUVEC. Results shown are representative of at least 3 experiments. (D) Nuclear (N) and whole (W) extracts were analysed by WB with the anti-KDR(I) or anti-lamin B and anti-P-IkB antibodies.

of antibodies against the internal (KDR(I)) and external (KDR(E)) portions of KDR, there is a clear expression of KDR not only in the cytosol but also in the nucleus, both in HUVEC (Fig. 1A) and in HMEC-C (Fig. 1B). Both membranes were incubated with anti-cyclin E (nuclear protein) and anti-P-IkB (cytoplasmic protein) antibodies, demonstrating the lack of contamination in the cytoplasmic and nuclear fractions respectively. The nuclear KDR localization in HUVEC was confirmed by confocal microscopy (Fig. 1C). We also performed nuclear–cytoplasm fractionation by sucrose gradient (Fig. 1D). The nuclear fraction (lane 1) and a whole extract (as a control) (lane 2) were analysed by WB using the anti-KDR(I). Immunoblotting for anti-lamin B and anti-P-IkB were performed as nuclear and cytoplasmic markers, respectively. The expression of KDR in the nuclear fraction was detected.

Moreover, we demonstrate that the KDR cellular localization may be changed upon VEGF stimulation. As shown by Western blotting, when the HUVEC were cultured in basal medium (without supplements or serum), for 48 h, a decrease in nuclear KDR expression was observed, which is restored after VEGF stimulation, shifting from the cytosol to the nucleus (Fig. 2A). These results were confirmed by confocal microscopy (Fig. 2B). Using the anti-KDR(E) antibody we show that after 30 min of VEGF stimulation (20 ng/ml), KDR is rapidly internalized and localized, not only at the cell surface, but also into the nucleus. The KDR localization was followed for 7 h after VEGF stimulus and we can see that the nuclear localization of KDR is maintained; however, the nuclear and surface KDR levels undergo variations during this time.

Next, we asked how VEGF–receptor interactions led to KDR internalization. Co-treatment of HUVEC with VEGF and an intracellular inhibitor of KDR phosphorylation (KDRi) blocked the VEGF-induced KDR nuclear translocation (Figs. 2A and C) suggesting that VEGF induces KDR phosphorylation, thereby allowing internalization to occur. However, VEGF treatment in the presence of a neutralizing antibody against KDR (IMC-1C11Ab) failed to completely block KDR internalization and nuclear accumulation, while a neutralizing monoclonal antibody against FLT-1 (6.12Ab) impeded this process (Fig. 2D). The expression of KDR was also determined by Western blotting, and the results clearly demonstrate a decrease of nuclear KDR, accompanied by an accumulation of membrane KDR after

6.12Ab treatment (Fig. 2E). As above, the same extracts were analysed with anti-cyclin E (nuclear protein) and anti-FLT-1 (membrane protein) antibodies, as a control for the extracts specificity and equal amount of protein loading.

Importantly, FLT-1 expression is predominantly located at the EC surface, even in the presence of VEGF (Fig. 2F).

These results suggest that VEGF binds FLT-1, and the molecular interaction between the two VEGF receptors results in KDR activation and transport into the nucleus.

In order to study the cross-talk between the 2 VEGF receptors, HUVEC were cultured in basal medium, for 36 h, and then treated or not with KDRi, or 6.12Ab, for 12 h. Finally, the cells were left unstimulated or stimulated with Placental growth factor (PIGF) (10 ng/ml) for 30 min. Whole protein extracts were analysed by Western blot. As shown (Fig. 2G), activation of FLT-1 alone, through the use of PIGF, results in KDR phosphorylation by a mechanism inhibited by the KDRi or 6.12Ab. The same membrane was incubated with the anti-KDR(I) antibody confirming that the neutralizing 6.12Ab acts only in the activation and not in the expression of KDR.

These data are in agreement with recent reports, demonstrating the activation of FLT-1 alone, through the use of PIGF, results in KDR phosphorylation [4].

The effect of KDR Deletion Mutants in KDR intracellular localization

The results described above suggest that KDR phosphorylation is crucial for KDR internalization. Several auto-phosphorylation sites have been identified in the intracellular region of KDR: Tyr951 and Tyr996 in the kinase insert; Tyr1054 and Tyr1059 in the kinase domain and Tyr1175, Tyr1212, Tyr1214 and Tyr1305 in the C-terminal tail. In order to reveal the role of these different tyrosines residues in the process of KDR internalization, we established 3 KDR deletion mutants for the intracellular region by RT-PCR using the KDR-pEGFP(N1) vector.

The definitions of KDR deletion mutants are as follows (see also Fig. 3A): KDRΔ1174 (deletion of aa1175–1355); KDRΔ1053 (deletion of aa1054–1355); and KDRΔ950 (deletion of aa951–1355). These were generated in 3T3 cells (details are described in Materials and methods). mRNA from 4 pools of each KDR transfection (full-length and deletion mutants) were obtained

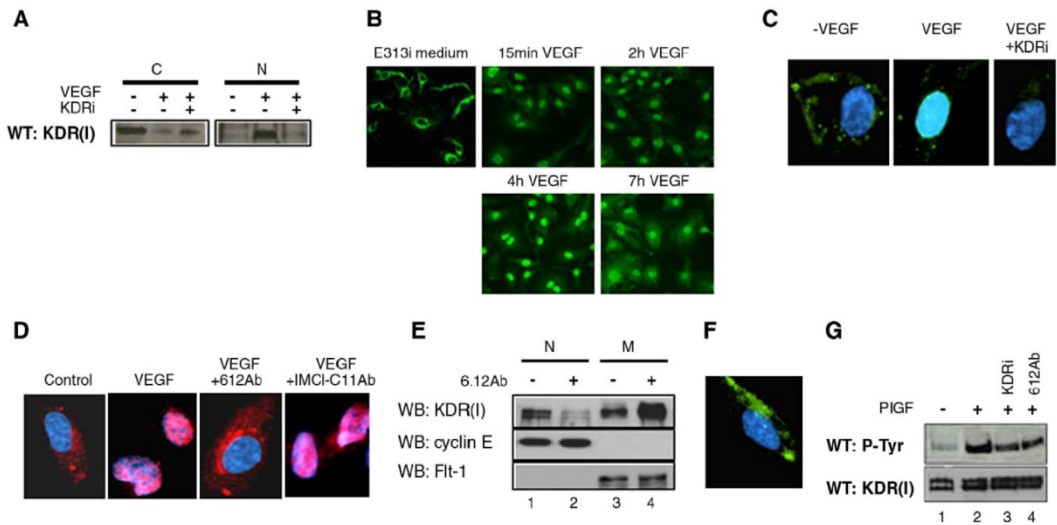


Fig. 2 – (A and C) HUVEC were cultured in basal medium, for 48 h, and then left untreated or stimulated with VEGF (20 ng/ml), for 30 min, in the presence or absence of the KDR-specific inhibitor (KDRi, used at 70 nM), pre-incubated for 12 h. (A) Cytoplasmic (C) and nuclear (N) protein extracts were analysed by Western blot (WB) with the KDR(I) antibody. (C) Immunofluorescence analysis was performed. These cells were stained with a mouse anti-human KDR antibody (green fluorescence) and DAPI (blue fluorescence). Note the nuclear expression of KDR in response to VEGF stimulation. Magnification: 630 \times . Results shown are representative of at least 3 experiments. (B) HUVEC were cultured in basal medium, for 48 h (left panel) and then stimulated with VEGF (20 ng/ml) for the indicated times. The cells were stained with the goat anti-human KDR(E) antibody. (D) HUVEC were cultured in basal medium, for 36 h, then treated or not with a neutralizing monoclonal antibody (moAb) against FLT-1 (clone 6.12, used at 1 μ g/ml) or a moAb against KDR (clone IMC-1C11, used at 1 μ g/ml), for 12 h, and finally stimulated or not with VEGF (20 ng/ml), for 30 min. Cells were stained with the rabbit anti-human KDR(I) antibody (red fluorescence) and DAPI (blue fluorescence). Note the nuclear accumulation of KDR in cells treated with VEGF alone and the dramatic effect of the neutralizing 6.12Ab at blocking this effect. These images represent approximately 95% of cells in a given condition and were obtained from experiments repeated at least 3 times (at different cell passages). Magnification: 630 \times . (E) HUVEC were cultured in growing medium and treated (lanes 2 and 4) or not (lanes 1 and 3) with the 6.12Ab (1 μ g/ml), for 12 h. Nuclear (N) and membrane (M) extracts were analysed by Western blot (WB) with the anti-KDR(I) or with anti-cyclin E and anti-FLT1 antibodies. (F) HUVEC were cultured in basal medium, for 48 h, and then stimulated with VEGF (20 ng/ml), for 30 min, and stained with a mouse anti-human FLT-1 antibody (clone FB5) (green fluorescence) and DAPI (blue fluorescence). Note the surface (membrane) and cytosolic FLT-1 expression. Magnification: 630 \times . Results shown are representative of at least 3 experiments. (G) HUVEC were cultured in basal medium, for 36 h, and then treated with KDRi (used at 70 nM) (lane 3), or with the 6.12Ab (1 μ g/ml), for 12 h (lane 4). Finally, the EC were not stimulated (lane 1) or stimulated with PIGF (10 ng/ml) (lanes 2–4) for 30 min. Whole protein extracts were analysed by Western blot (WB) using anti-P-tyrosine and the anti-KDR(I) antibodies.

and analysed by RT-PCR using GFP and KDR-specific primers (Fig. 3B). The same was performed with mRNA from 3T3 transfected with pEGFP(N1) or from HUVEC. Simultaneously, we used the vector pEGFP(N1)-KDR as a positive control in GFP or KDR RT-PCR reactions or nuclease-free water as a negative control. The results confirm the expression of KDR and GFP transcript in all the 3T3 pools transfected with KDR full-length or deletion mutants. As expected, a control transfection (3T3 transfected with pEGFP(N1)) and HUVEC do not express KDR and GFP, respectively.

Next, we obtained cytoplasmic and nuclear protein extracts from 3T3 transfected with the full-length and deletion mutants. KDR immunoprecipitation was performed, followed by immunoblotting with an anti-KDR antibody (Fig. 3C, left

panel). As a control, the same experiment was performed with 3T3 transfected with the pEGFP(N1) vector (Fig. 3C, right panel). The results show the presence of the fusion protein in cytoplasmic and nuclear extracts of 3T3 transfected with KDR-GFP, KDR Δ 1174-GFP and KDR Δ 1053-GFP. Note the high level of the fusion protein in nuclear extracts compared with cytoplasmic extracts in 3T3 KDR-GFP and 3T3 KDR Δ 1174-GFP. Interestingly, the fusion protein is only detected in cytoplasmic extracts in 3T3 transfected with KDR Δ 950-GFP.

The localization of the fusion protein was also investigated by confocal microscopy, and the results suggest a nuclear expression of KDR in 3T3 transfected with the full-length KDR (Fig. 4A), as well as in the deletion mutants KDR Δ 1174-GFP (Fig. 4B) and KDR Δ 1053-GFP (Fig. 4C). However, in contrast with the

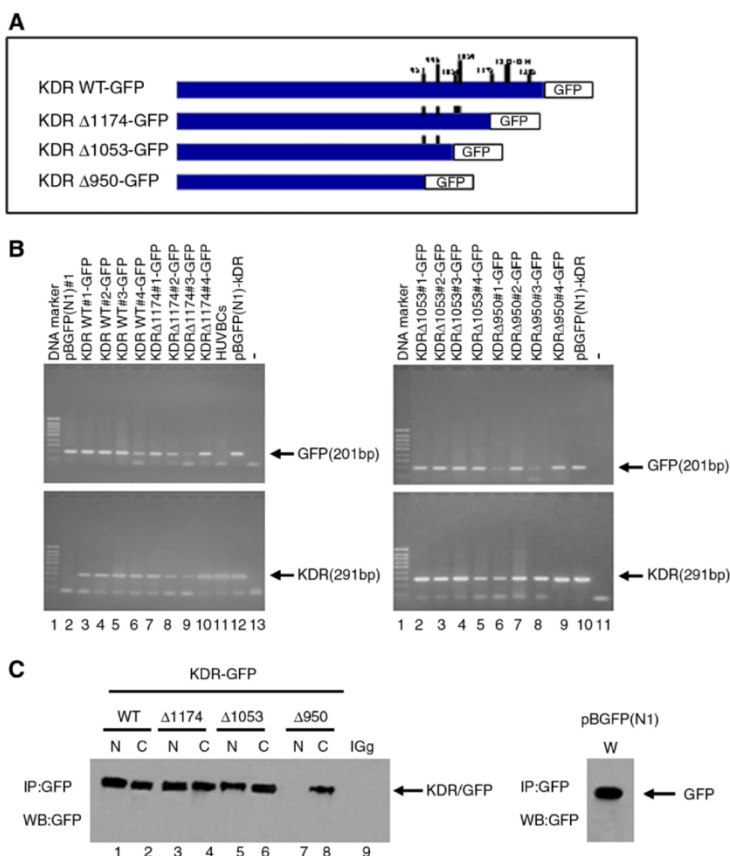
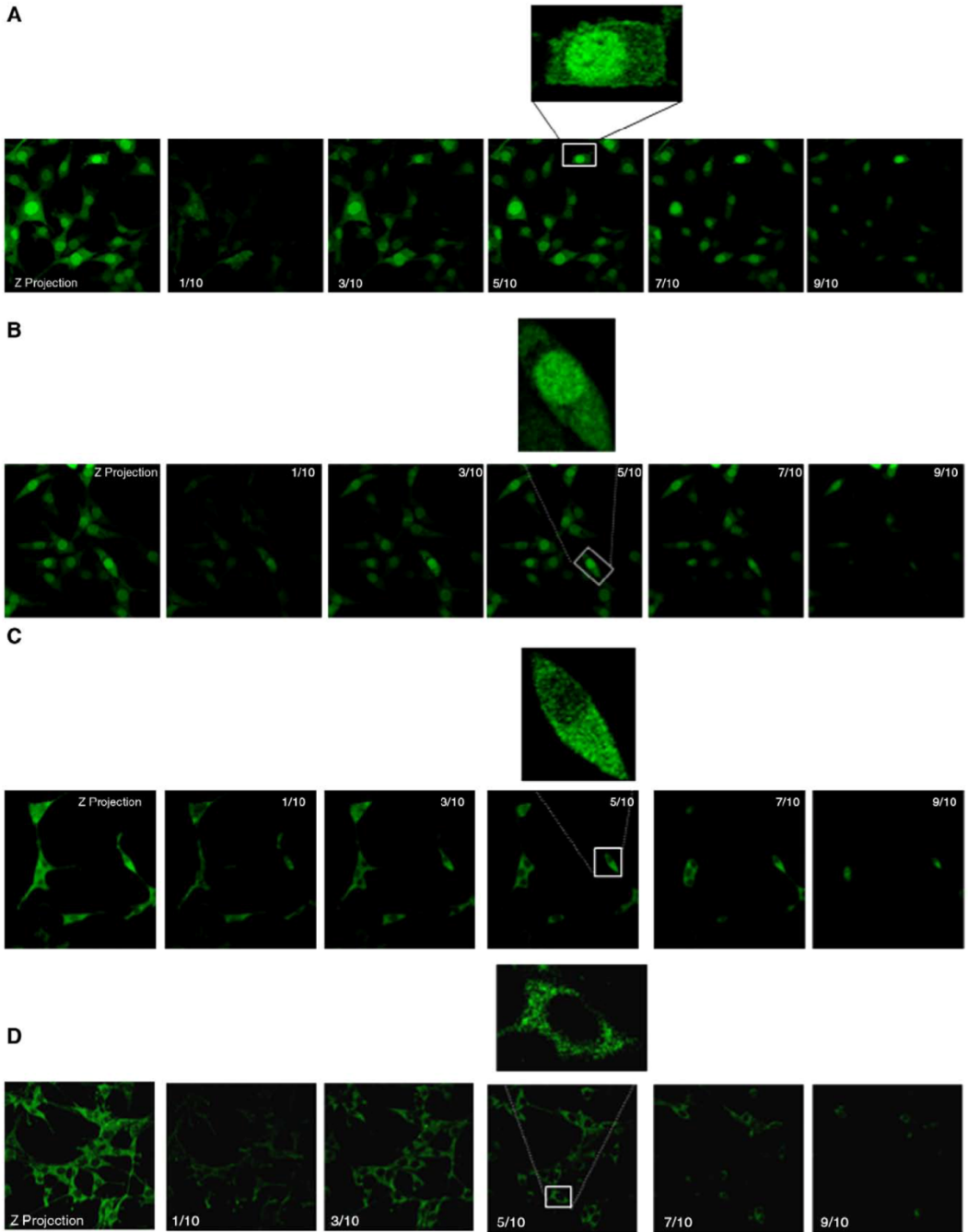


Fig. 3 – (A) Schematic representation of wild-type (WT) and mutants of KDR fused to GFP. KDR Δ 1174-GFP, KDR Δ 1053-GFP and KDR Δ 950-GFP lack 181, 302 and 405 amino acids from the C-terminal region respectively. The tyrosines (Tyr) are identified in bold. (B) GFP (201 bp-upper panels) and KDR (291 bp-lower panels) transcripts in 3T3 transfected with KDR WT-GFP (lanes 3–6, left panel), KDR Δ 1174-GFP (lanes 7–10, left panel), KDR Δ 1053-GFP (lanes 2–5, right panel) and KDR Δ 950-GFP (lanes 6–9, right panel). 3T3 transfected with pEGFP(N1) vector (lane 2, left panel) or HUVEC (lane 11, left panel) were used as positives and negatives controls for GFP and KDR reactions. The plasmids DNA containing the KDR-GFP gene (lane 12, left panel and lane 10, right panel) and nuclease-free water (lane 13, left panel and lane 11, right panel) were used as positive and negative controls, respectively, in all RT-PCRs reactions. (C) Nuclear (N), cytoplasmic (C) (left panel) and whole lysates (T) (right panel) from 3T3 transfected with the KDR WT-GFP (lanes 1 and 2, left panel), KDR Δ 1174-GFP (lanes 3 and 4, left panel), KDR Δ 1053-GFP (lanes 5 and 6, left panel), KDR Δ 950-GFP (lanes 7 and 8, left panel) and pEGFP(N1) vector (right panel) were obtained. Immunoprecipitation (IP) and Western blot (WB) were conducted with an anti-GFP antibody. Positions of the fusion protein KDR/GFP or GFP were indicated. Note the absence of the nuclear fusion protein in 3T3 KDR Δ 950-GFP (lane 7, left panel).

full-length and KDR Δ 1174-GFP, the cells that express KDR Δ 1053-GFP show a decrease in KDR nuclear accumulation and the levels of the fusion protein appear increased in the cell

cytoplasm. Notably, cells transfected with the mutant KDR Δ 950-GFP (Fig. 4D) show a clear absence of nuclear KDR expression.

Fig. 4 – 3T3 cells transfected with the KDR WT-GFP (A), KDR Δ 1174-GFP (B), KDR Δ 1053-GFP (C) and KDR Δ 950-GFP (D) were analysed by confocal microscopy for GFP fusion proteins. The numbers of the different sections were indicated. A detail of the internal section (5/10) for each fusion protein was performed. Note the nuclear accumulation of the fusion protein (green fluorescence) in 3T3 KDR WT-GFP (A) and KDR Δ 1174-GFP (B). A decrease in KDR nuclear accumulation is observed in cells expressing KDR Δ 1053-GFP (C) and 3T3 cells transfected with the mutant KDR Δ 950-GFP show a clear absence of nuclear protein fusion expression (D).



A VEGF/KDR complex is transported to the nucleus of EC

Since it is known for other ligand/receptors to be transported in a complex, to the cell nucleus, here we investigated if VEGF and KDR, are also internalized as a complex. First, as shown in Fig. 5A, we demonstrate that VEGF itself is also rapidly internalized after VEGF stimulation of HUVEC.

Confocal microscopy studies (Fig. 5B) demonstrated that, the cytoplasmic KDR co-localizes with VEGF, while immunoprecipitation of whole cell extracts with a KDR antibody results in the pull-down of VEGF 165/165 homodimers, as shown by Western blotting with anti-VEGF antibody (Fig. 5C).

These results suggest that a direct VEGF/KDR interaction is involved in their internalization, intracellular trafficking and nuclear localization.

Finally, we also demonstrated that the internalization of VEGF and KDR ultimately results in the transport of this complex into the nucleus, via the nuclear pore complex (Supplementary Fig. 1).

Microtubules and caveolae are involved in KDR/VEGF internalization

Given the involvement of elements of the cell structure in mediating the internalization and intracellular transport of

other receptors and signaling intermediates such as the EGFR, we asked whether VEGF-induced KDR/VEGF nuclear accumulation required the interaction with cell structural proteins, microtubules and/or actin filaments.

To answer this, EC were cultured in basal medium, for 48 h, pre-incubated with a microtubule de-polymerizing drug (nocodazole), or one that affects actin assembly (cytochalasin D), and then stimulated with VEGF (20 ng/ml), for 30 min. KDR expression/localization was determined by immunofluorescence (Figs. 6A, B).

As seen by fluorescence microscopy, VEGF treatment of EC had little effect on the microtubules or actin filaments; however, it induced KDR nuclear translocation, as described above. As shown in Fig. 6A (middle panel), nocodazole treatment (15 min) of EC induced a pronounced de-polymerization of microtubules and reduced KDR expression significantly, which now localized in the cell membrane. Notably, once nocodazole is removed from the cultures and ECs are stimulated with VEGF, a clear recovery in microtubule assembly is seen, while KDR expression is restored and once again relocates to the nucleus, demonstrating that this effect is reversible if the cells are exposed to the drug for a short period of time (Fig. 6A, right panel). With regards to actin, although cytochalasin D had a clear effect on the cell structure, reducing actin expression to a minimum before

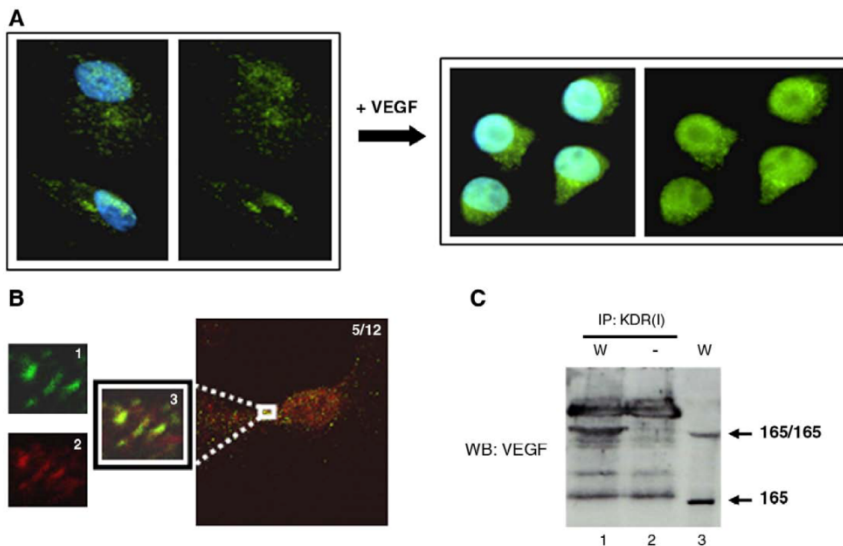


Fig. 5 – (A) HUVEC were cultured in basal medium, for 48 h, and then stimulated with VEGF (20 ng/ml) for 30 min. These cells were stained with a mouse anti-human VEGF antibody (green fluorescence) and with DAPI (blue fluorescence). Note the nuclear localization of VEGF in response to VEGF stimulation. Magnification: 630 \times . Results shown are representative of at least 3 experiments. (B) Confocal microscopy for co-localization of KDR with VEGF was performed. HUVEC were sequentially labeled on the same slide with the rabbit anti-human KDR(I) and a mouse anti-human VEGF antibodies. Superimposition of both pairs of images showed co-localization of KDR and VEGF from the yellow color and represents an internal section (optical slide 5/12). A detail of previous image shows the localization of VEGF (panel 1-green fluorescence); KDR (panel 2, red fluorescence) and VEGF and KDR (panel 3, yellow fluorescence). (C) Immunoprecipitation (IP) of cell lysates was conducted with the anti-KDR(I) antibody followed by Western blot (WB) with an anti-VEGF antibody (lane 1). Positions of the VEGF homo-dimer (165/165) and 165 VEGF isoform were indicated (black arrows). Immunoprecipitation-negative control (lane 2) and a control cellular lysate (lane 3) were also included in the experiment.

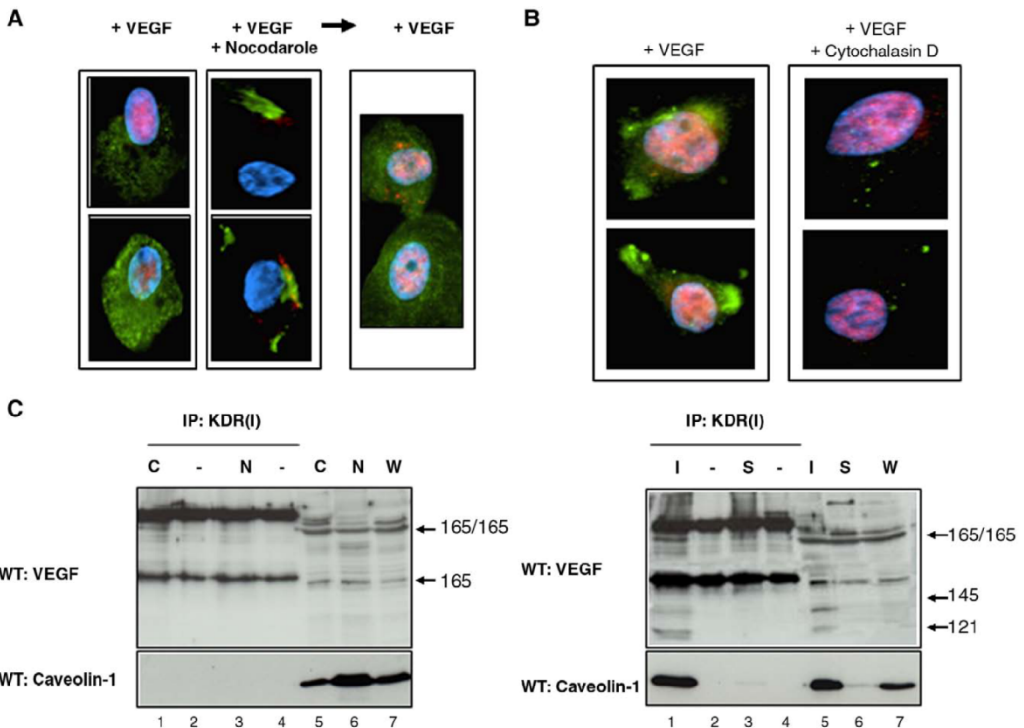


Fig. 6 – (A) HUVEC were cultured, on gelatin-coated slides, in basal medium, for 48 h, and then stimulated with VEGF (20 ng/ml), for 30 min, in the presence (middle panel) or absence (left panel) of nocodazole (1 µg/ml), pre-incubated for 30 min. In the right panel and after nocodazole treatment, the cells were washed and free of nocodazole and the EC were stimulated with VEGF (20 ng/ml) for 30 min (right panel). In all the following conditions, EC were stained with the rabbit anti-human KDR(I) antibody (red fluorescence), mouse anti-human β-tubulin antibody (green fluorescence) and DAPI (blue fluorescence). Note the apparent re-distribution of KDR under these conditions. Magnification: 630×. **(B)** HUVEC were cultured in basal medium for 48 h and then stimulated with VEGF (20 ng/ml) for 30 min, in the presence (right panel) or absence (left panel) of cytochalasin D (1 µg/ml), pre-incubated for 30 min. The cells were stained with the rabbit anti-human KDR(I) antibody (red fluorescence), mouse anti-human β-actin (green fluorescence) and DAPI (blue fluorescence). Note the absence of a defined actin structure and the sustained KDR nuclear expression after cytochalasin D treatment. The results shown are representative of at least 3 independent experiments. Magnification: 630×. **(C)** Immunoprecipitation (IP) of cytoplasmic (C) and nuclear (N) extracts (left panel) or insoluble (I) and soluble (S) fractions (right panel) was conducted with the anti-KDR(I) antibody followed by Western blot (WB) with an anti-VEGF antibody. Immunoprecipitation-negative controls (lanes 2 and 4, left and right panel) and the different cellular (C, N, I, S and whole (W)) extracts (lanes 5–7, left and right panel) were also included in the experiment. The membranes were re-probed with an anti-caveolin-1 antibody. Positions of VEGF isoforms were indicated (black arrows).

compromising cell integrity, interestingly it failed to impede VEGF-induced KDR nuclear localization (Fig. 6B).

Moreover, our data (confocal and immunoprecipitation studies) suggest that VEGF interacts with KDR in the cytoplasm, and given the involvement of the microtubules in the VEGF and KDR internalization, we were interested to know in which cell fraction VEGF interacts with KDR. For this purpose, cytoplasmic and nuclear extracts (Fig. 6D, left panel) and soluble and insoluble cytoskeleton fractions (Fig. 6C, right panel) were prepared from EC and KDR immunoprecipitation was performed followed by immunoblotting with an anti-VEGF antibody. Interestingly, the results suggest that 121 and

145 isoforms of VEGF, and 165/165 VEGF homodimers only interact with KDR in insoluble (cytoskeleton) fraction.

As previously described, different proteins are internalized and traffic to their destination through vesicles that travel along cytoskeletal filaments with the aid of molecular motors. In order to investigate the role of caveolae vesicles in the intracellular movement of KDR and VEGF, the same membranes (Fig. 6C, left and right panel) were probed with an anti-caveolin-1 antibody. As shown, the interactions between KDR and caveolin-1 were not detected in cytoplasmic or nuclear extracts (Fig. 6C, left panel). In contrast, an important interaction between caveolin-1 and KDR is detected in

insoluble extracts while it is absent in soluble extracts (Fig. 6C, right panel). These results indicate that KDR interacts with VEGF in cytoskeleton fraction, with caveolin-1. Thus, KDR and VEGF may be internalized via caveolae-mediated endocytosis. The relative contribution of either mechanism for the process of KDR and VEGF internalization remains to be defined, although recent reports have suggested KDR interacts predominantly with caveolin-1 [5] and consequently activate downstream signaling pathways.

Activation of PI-3 kinase is required for KDR/VEGF internalization

Having defined the structural basis for the internalization and transport of KDR and VEGF on EC, next we investigated the involvement of specific signaling pathways in this process.

For this purpose, HUVEC were cultured in basal medium, for 48 h, and pre-treated or not with inhibitors of the MAP-kinase, PI 3-kinase and NF- κ B pathways, for 30 min, and then stimulated with VEGF (20 ng/ml), for 30 min. As shown (Fig. 7), the inhibitors had different effects on VEGF-induced KDR transport and nuclear localization. Compared to EC treated with VEGF alone, incubation with LY294002 (PI 3-kinase) impeded KDR nuclear localization (Fig. 7B), while U0126 (MAP-kinase) and Bay 11-7082 (NF- κ B) had a marginal effect at blocking this intracellular mechanism (Figs. 7C, D). These results suggest that the PI 3-kinase/AKT pathway is involved in KDR intracellular transport and its subsequent nuclear accumulation. Similar results were seen with regards to VEGF transport (data not shown). These results, together with previous reports showing PlGF stimulation of FLT-1 results in AKT activation [4], highlight the importance of this signaling pathway in regulating the cross-talk between the 2 VEGF receptors, and, as shown here, in controlling VEGF/KDR intracellular transport.

Endothelial monolayer recovery following injury is accompanied by VEGF/KDR internalization

Next, we tested our mechanistic findings described above in an *in vitro* wound healing model, where confluent EC monolayers, cultured in growing medium, suffer mechanical wounding. We observed by immunofluorescence staining (Fig. 8) that KDR and VEGF accumulate intracellularly in EC facing (closer) to the wounded area (Fig. 8A), in contrast to those further away from the wound (Fig. 8B), where expression of either is quite low. This KDR/VEGF accumulation preceded monolayer recovery (the first evidence for this accumulation is seen 15 min after wounding), suggesting this mechanism might be important for EC recovery following mechanical stress. Moreover, the sudden increase in intracellular VEGF and KDR could not be explained by protein synthesis and therefore had to derive from a rapid internalization process. We measured the VEGF levels in growing medium before wounding and 15 min after wounding, by ELISA, and confirmed the latter hypothesis. As shown in Table 1, VEGF levels 15 min after wounding decrease almost 5-fold in the culture supernatant, coherent with the accumulation of intracellular VEGF seen by immunofluorescence. Importantly, the levels of VEGF in the culture medium go back to baseline (before wounding) levels 7 h after wounding (Table 1), which coincides with EC monolayer recovery. Simultaneously, we verified by immunofluorescence that, after EC monolayer recovery, the majority of EC shows basal levels of intracellular KDR and VEGF (Fig. 8C).

FLT-1 blockade delays recovery of EC monolayer following injury

Having defined the crucial molecular events involved in the transport of a VEGF/KDR complex inside EC, leading to its

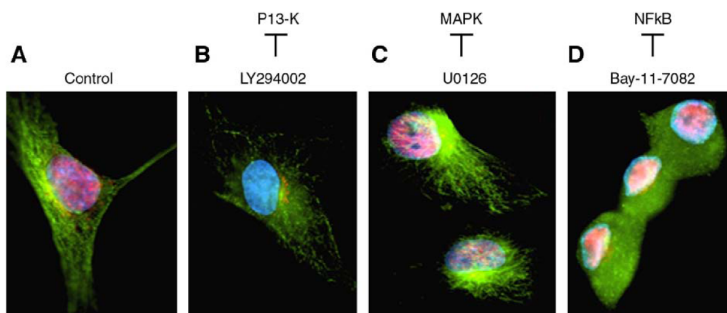


Fig. 7 – In all the following conditions, HUVEC were stained with DAPI (blue fluorescence) and the following antibodies: rabbit anti-human KDR(I) (red fluorescence) and mouse anti-human β -tubulin (green fluorescence). HUVEC were cultured in basal medium, for 48 h, and then (A) stimulated with VEGF (20 ng/ml), for 30 min. Note the nuclear accumulation of KDR. (B) treated with LY294002 (25 μ M) for 30 min and then stimulated with VEGF (20 ng/ml) for 30 min. Note the absence of nuclear KDR in these cells. (C) Treated with U0126 (10 μ M), for 30 min, and then stimulated with VEGF (20 ng/ml), for 30 min. In contrast to LY294002, U0126 failed to block the VEGF-induced KDR nuclear localization. (D) Treated with Bay 11-7082 (5 μ M) for 30 min and then stimulated with VEGF (20 ng/ml) for 30 min. As shown, Bay 11-7082 failed to block KDR internalization and nuclear localization. The results shown are representative of 3 independent experiments for all conditions, and the effects were seen in 95% of the treated cells. Magnification: 630 \times .

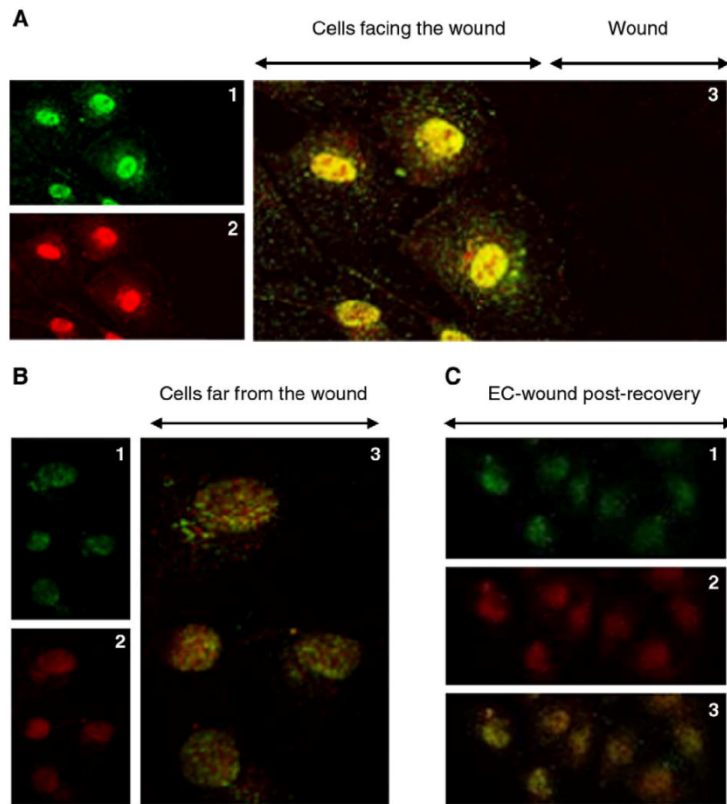


Fig. 8 – HUVEC were cultured in growing medium and a wound was performed in a confluent EC monolayer. After 15 min of post-wounding (A, B) or 7 h of post-wounding (C), the cells were fixed in 1% formaldehyde/PBS. The EC were sequentially labeled on the same slide with the rabbit anti-human KDR(I) antibody (red fluorescence) and a mouse anti-human VEGF antibody (green fluorescence) and the Z-projections of confocal images were obtained. (A) concerns to EC far from the wound and (B) concerns to EC facing the wound. The images 1 and 2 of panels A–C represent VEGF and KDR localizations, respectively. Superimposition of both pairs of images showed co-localization of KDR and VEGF, in yellow (image 3, A–C). Magnification: 630 \times . Results shown are representative of at least 3 experiments.

nuclear accumulation, we tested some of these mechanistic observations in our wound healing model mentioned above. HUVEC were cultured in growing medium and incubated or not with the neutralizing FLT-1 antibody (6.12Ab). As shown in Figs. 9A and B, pre-treatment of the EC monolayer prior to

wounding with anti-FLT-1 antibody (Fig. 9B) delayed monolayer recovery significantly, compared with a control EC monolayer (Fig. 9A) (that is, EC treated with the 6.12Ab took significantly longer to re-populate the wounded area). Accordingly, as determined by immunofluorescent staining, no measurable uptake and no VEGF and KDR internalization was detected either in cells facing (Fig. 9C, right panel) or away from the wound (Fig. 9C).

Table 1 – The extracellular medium before (–), after 15 min of wounding (+) and post-recovery was removed and the level of VEGF measured by VEGF ELISA Kit as described in Materials and methods

Wound	VEGF ($\mu\text{g/ml}$)
–	225.2
+	41.2
Post-recovery	258.3

Discussion

The involvement of VEGF and its receptors (FLT-1 and KDR) in modulating the vascular responses during wound healing and tumour growth has been amply demonstrated [2,3], culminating with the recent success of anti-VEGF antibodies in treating

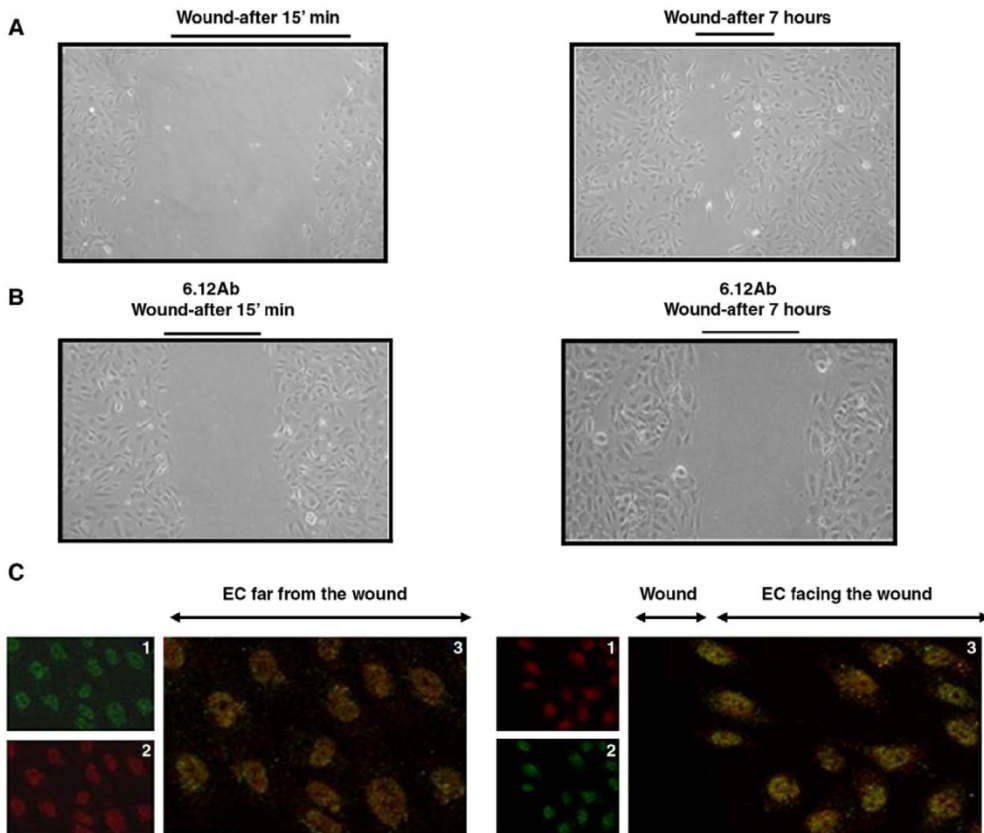


Fig. 9 – HUVEC were cultured in growing medium and incubated (B and C) or not (A) with the neutralizing monoclonal FLT-1 antibody, for 12 h, before wounding. (A and B) While the left panels concern to EC monolayer after 15 min post-wounding, the right panels concern to EC monolayer after 7 h post-wounding. (C) Fifteen minutes after wounding, the cells were sequentially labeled on the same slide with the rabbit anti-human KDR(I) antibody (red fluorescence) and a mouse anti-human VEGF antibody (green fluorescence) and the Z-projections of confocal images were obtained. The left panel concerns to EC far from the wound and the right panel the EC facing the wound. The images 1 and 2 (left and right panel) represent VEGF and KDR localizations respectively. Superimposition of both pairs of images showed co-localization of KDR and VEGF, in yellow (left and right panel-image 3). In contrast to control EC, FLT-1 blockade reduces intracellular VEGF/KDR accumulation. Magnification: 630 \times .

different cancers [2]. The properties of VEGF as a key molecule in regulating vessel integrity and growth have also attracted the attention of researchers in the field of tissue engineering [6], particularly in the cases of severe trauma.

In the present report, we observed a rapid internalization of VEGF and KDR, but not FLT-1, following VEGF stimulation of confluent EC monolayers. In addition, we define a crucial role for FLT-1 in mediating the internalization process and revealed the molecular pathways followed by the VEGF/KDR complex inside EC. As mentioned above, a role for FLT-1 in mediating KDR activation was recently shown [4]. We add on to those findings, by showing that one of the effects of FLT-1 is to activate KDR and mediate the internalization of VEGF and KDR to the nucleus of EC. We also described KDR deletion

mutants in tyrosine residues that failed to internalize KDR, suggesting that specific tyrosine residues may be essential for KDR activation and KDR/VEGF internalization. In the future, we will determine which tyrosine kinase(s) is specifically involved in these processes.

Having shown that the cross-talk between the 2 VEGF receptors results in the internalization of VEGF and KDR on EC, we investigated the mechanisms involved in the internalization process. We describe the intracellular interaction of VEGF and KDR, transported as a complex, with caveolin-1. The presence and importance of caveolin [7] for the internalization of extracellular molecules on EC has already been shown. Among receptor tyrosine kinases, it was described that KDR may be uniquely internalized via a caveolar pathway and

transported to perinuclear caveosomes [8–11]. Caveolae are static structures in an unstimulated state; following receptor activation, caveolar budding occurs and leads to the formation of cavicles which move their cargo along microtubules to and from caveosomes. From caveosomes, internalized cargo may be delivered to the Golgi or the endoplasmic reticulum, possibly providing a transport pathway to the nucleus. Caveosomes may also fuse with the classic endocytic pathway for proteosomal degradation. The relative contribution of either process for the internalization of VEGF or KDR, under stress conditions, remains to be demonstrated. However, recent publications suggest caveolin-1 is crucial for VEGF-induced KDR activation and re-vascularization of ischemic limbs [12], while its deficiency also results in abrogated angiogenesis [13], highlighting its importance in VEGF-mediated effects.

We also demonstrate that the cell cytoskeleton, specifically microtubules, is actively involved in the VEGF/KDR internalization process, revealing a mechanism whereby drugs that affect microtubule integrity, such as Taxol, may exert anti-angiogenic effects [5].

Other mechanisms shown here to be required for VEGF/KDR internalization involve activation of the PI-3 kinase and AKT pathways. This is perhaps not surprising, given the involvement of these pathways in cell survival, and the FLT-1-mediated activation of PI-3 kinase, via KDR activation, as previously reported [4].

Previous studies describing KDR internalization have focused on factors regulating receptor ubiquitylation and degradation, demonstrating that the endocytosis of the receptor may be involved in the regulation of the degree and duration of different signals mediated by KDR, at the cell surface [14–16]. However, the nuclear KDR localization following VEGF stimulation (our data) strongly suggests that KDR internalization is not involved only in the down regulation of the receptor.

In our report, the relevance of the VEGF/KDR internalization process was shown, in assays where we blocked *in vitro* wound healing; in fact, we demonstrate that FLT-1 neutralization impedes endothelial monolayer recovery following wounding, and this is accompanied by a lack of internalized KDR and VEGF, thus highlighting the importance of FLT-1 in this process. This observation may have clinical relevance since it was previously suggested that local cancer recurrence may be preceded by a wound-related angiogenic surge [17].

Notably, VEGF levels in culture supernatants decrease after EC wounding assay. Here we demonstrate that the exogenous VEGF gets rapidly internalized and locates to the nucleus of EC, suggesting this is a mechanism to resist pro-death stimuli and restore EC monolayer integrity following mechanical stress. Importantly, VEGF levels are restored to baseline values when monolayer integrity is restored. There are numerous observations suggesting that VEGF levels may predict cancer relapse following surgery [18–23]. Furthermore, Hornbrey et al. [20] demonstrated that VEGF levels following surgery decrease at day 1, followed by an early peak at day 2. In agreement, here we show there is a quick release of internalized VEGF following EC monolayer recovery. The significance of this VEGF release for vessel recovery, angiogenesis surges and eventually tumour relapse remains undisclosed.

Taken together, the present report contributes to our further understanding of the intricate molecular pathways regulated by VEGF receptors and identifies several previously unrecognized molecular interactions that may be targeted in situations of wound healing failure or tumour angiogenesis.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.yexcr.2007.02.020.

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2.3. SUPPLEMENTARY RESULTS

VEGF AND VEGFR2 NUCLEAR TRANSLOCATION IS MEDIATED BY THE NUCLEAR PORE COMPLEX

We further investigated the nuclear translocation process of VEGF/VEGFR2 in ECs. We treated ECs with a lectin, wheat germ agglutinin (WGA), which inhibits the nuclear protein transport and interacts directly with the nuclear pore complex. We observed by confocal microscopy the accumulation of VEGF and VEGFR2 in the cytoplasmic face of the nuclear pore envelope in cells treated with WGA (Figure S1) suggesting, that the internalization of this complex ultimately results in the transport to the cell nucleus, via the nuclear pore complex.

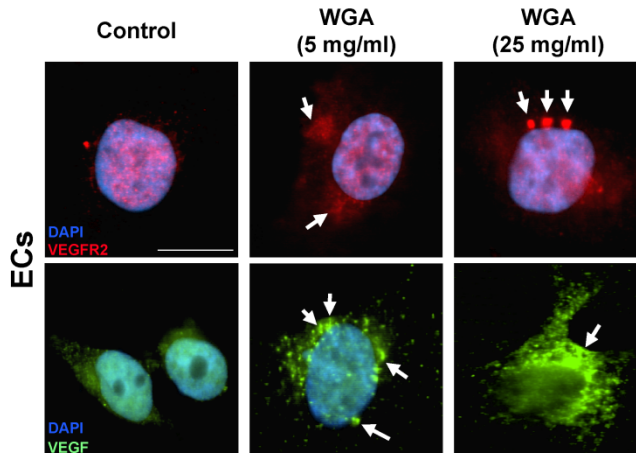


Figure S1. VEGF and VEGFR2 are transported to the nucleus via the nuclear pore complex.

ECs were cultured in growing medium and left untreated or treated with WGA at the indicated concentrations. These cells were stained with a rabbit anti-human VEGFR2 (I) antibody (Alexa 594) and DAPI (blue). Cytoplasmic accumulations of VEGFR2 are indicated with white arrows (upper panel). A similar experiment was performed for VEGF and ECs were stained with a mouse anti-human VEGF antibody (Alexa 488) and DAPI (blue). Cytoplasmic accumulations of VEGF are indicated with white arrows (lower panel) Scale bar 10 μm .

The presence of nuclear localization signals (NLS) in the VEGFR2 sequence were neither described in the literature nor identified in bioinformatics analysis performed by us (data not shown). Interestingly, five potential NLS sequences were identified in the C-terminal region of VEGF [1]. We can then speculate

that the recognition of the VEGF NLS signal is implicated in the nuclear trafficking of the VEGF/VEGFR2, via the nuclear pore complex.

Taken together, the results presented here indicate that VEGF and VEGFR2 are actively transported to the cell nucleus, suggesting a biological function for the internalization of VEGF/VEGFR2.

Our results also showed that the VEGF-VEGFR2 nuclear internalization is a dynamic process suggesting that these proteins are also exported back to the cytoplasm/cell surface. Therefore, we analyzed the nucleo-cytoplasmic translocation of VEGF and VEGFR2 by treating ECs with Leptomycin B, a nuclear export inhibitor (Figure S2).

By immunofluorescence, we observed that VEGF, but not VEGFR2, accumulated in the nucleus of ECs treated with increasing concentrations of Leptomycin B for 1 hour (Figure S2).

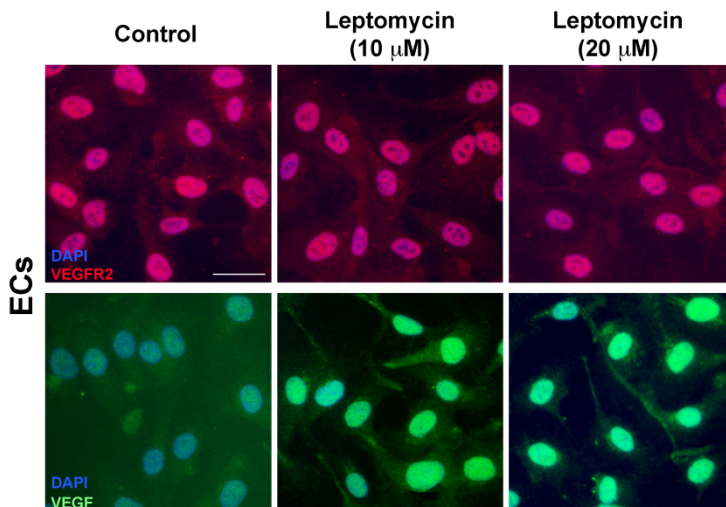


Figure S2. VEGF accumulates in the nucleus after treatment with Leptomycin B.

ECs were cultured in growing medium and left untreated or treated with leptomycin B at the indicated concentrations, for 1 h. Immunofluorescence analysis with a rabbit anti-human VEGFR2 (l) antibody (Alexa 594) and DAPI (blue), (upper panel). A similar experiment was performed for VEGF and ECs were stained with a mouse anti-human VEGF antibody (Alexa 488) and DAPI (blue). Note the VEGF nuclear accumulation in ECs treated with 10 and 20 μM Leptomycin B, (lower panel). Scale bar 20 μm.

These results suggest that VEGF, but not VEGFR2, is exported from the nucleus by a process inhibited by leptomycin B, suggesting the involvement of a nuclear export signal (NES)-dependent mechanism.

VEGFR2 INTERNALIZATION DOES NOT INVOLVE RECEPTOR DEGRADATION

We could not exclude the possibility that the endocytosis of VEGF/VEGFR2 might be involved in the downregulation of the receptor at the cell surface resulting in the attenuation of the signal mediated by VEGF-VEGFR2. It was described that c-Cbl is involved in the downregulation of several RTKs, upon recruitment to specific autophosphorylated forms of these receptors enhancing their degradation through ubiquitylation [2]. Regarding VEGFR2 there is no consensus for the involvement of c-Cbl in the process of endocytosis and degradation of the receptor [3,4]. In order to investigate the involvement of c-Cbl in the degradation of VEGFR2 in ECs, we analyzed the interaction of these proteins in VEGFR2 whole cell immunoprecipitates by western-blot (Figure S3A).

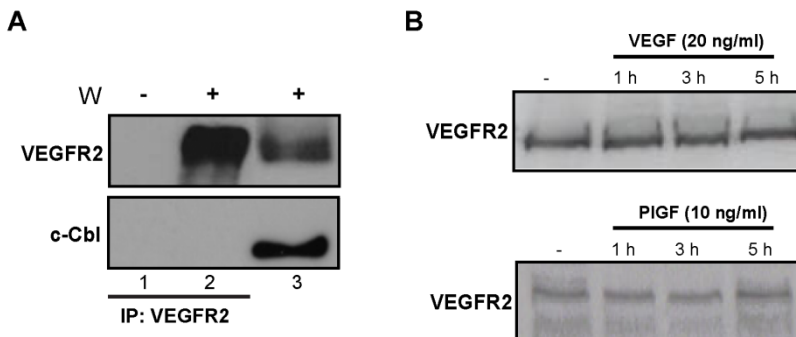


Figure S3. VEGFR2 does not interact with c-Cbl.

(A) ECs were cultured in growing medium and whole protein extracts were prepared. Immunoprecipitation (IP) of whole cell lysates (W) was conducted with the anti-VEGFR2 (I), (lane 2) followed by western blotting using the anti- VEGFR2 (I) and anti-c-Cbl antibodies. VEGFR2 antibody plus beads (without W) was used as negative control for immunoprecipitation (lane 1). A control lysate was also included in the experiment (lane 3). (B) ECs were left unstimulated or stimulated with 20 ng/ml VEGF (upper panel) or 10 ng/ml PIGF (lower panel). VEGFR2 protein levels were analyzed by western blot in whole protein extracts obtained 1, 3 and 5 h upon stimulation.

Under these experimental conditions, we failed to detect any interaction between VEGFR2 and c-Cbl (Figure S3A). Moreover, VEGFR2 protein levels

remained unchanged after 5 h of stimulation with VEGF (Figure S3B, upper panel) or PIGF (Figure S3B, lower panel). Together, the data presented here suggests that VEGFR2 is not degraded upon VEGF stimulation, but rather its nuclear internalization might be involved in new biological functions, such as gene transcription.

2.4. SUPPLEMENTARY REFERENCES

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CHAPTER III

ESSENTIAL ROLE FOR VEGFR2 TYROSINE RESIDUE Y951 IN THE NUCLEAR INTERNALIZATION OF THE RECEPTOR

Essential role for VEGFR2 Tyrosine Residue Y951 in the Nuclear Internalization of the Receptor

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Running head

Y951 is involved in the VEGFR2 nuclear translocation.

Abbreviations

VEGFR2, Vascular Endothelial Growth Factor Receptor 2; VEGF, Vascular Endothelial Growth Factor; ECs, endothelial cells; FRAP, Fluorescence Recovery After Photobleaching; Y, amino acid tyrosine; F, amino acid phenylalanine.

3.1. ABSTRACT

The vascular endothelial growth factor (VEGF) is one of the most important pro-angiogenic factors. VEGF binds to the membrane receptor VEGFR2 that activates signaling cascades leading to cell proliferation, survival and migration. Previously, we showed that VEGF and VEGFR2 translocate to the endothelial cell (EC) nucleus and that this nuclear internalization is required for EC recovery during wound healing. We also showed that the VEGF-induced VEGFR2 internalization was impaired in VEGFR2 tyrosine deletion mutants suggesting that the activation/phosphorylation of the receptor is crucial for its nuclear translocation. In order to identify the tyrosine residues involved in the translocation of the receptor, we constructed several VEGFR2-GFP fusion proteins using the wild type (WT) or mutant forms of VEGFR2 in which one or more tyrosine residues were converted into phenylalanine, and tested their ability to be translocated to the nucleus in endothelial cells (ECs). Our preliminary results suggest that the tyrosine residue Y951 located in the

receptor kinase-insert domain is essential for the VEGFR2 nuclear internalization in ECs.

3.2. INTRODUCTION

Vascular Endothelial Growth Factor (VEGF) signaling through VEGF receptors (VEGFRs) is a crucial regulatory mechanism for vascular development and neovascularization in physiological and pathological processes [1]. VEGFA binds predominantly to two VEGF tyrosine kinase receptors VEGFR1 and VEGFR2 at the surface of vascular ECs [1]. Disruption of the *Vegfr1* or *Vegfr2* genes in mice results in embryonic lethality due to obstruction of the vessels by an overgrowth of endothelial progenitors [2] or by a deficient development of endothelial and hematopoietic precursors [3], respectively. The VEGF receptors are members of the receptor tyrosine kinase (RTK) superfamily and consist of an extracellular domain comprising 7 Ig-like domains, a transmembrane domain, followed by an intracellular split tyrosine-kinase domain and a C-terminal tail [1]. VEGF has a higher affinity for VEGFR1 than for VEGFR2. However, the former receptor has a low kinase activity and a variety of studies suggest that it acts primarily as a “decoy” receptor for VEGF, regulating the availability of VEGF to VEGFR2 in developing blood vessels [4]. Therefore, the VEGF mitogenic effects are mediated through VEGFR2 in ECs. Dimerization of VEGFR2 upon VEGF-binding to its extracellular domain results in the autophosphorylation of tyrosine residues in its intracellular domain resulting in the activation of signaling pathways leading to proliferation, survival, permeability and migration [1]. Several autophosphorylation sites have been identified: Tyrosines Y951 and Y996 located in the kinase insert domain; tyrosines Y1054 and Y1059 in the kinase catalytic domain; tyrosines Y1175 and Y1214 in the C-terminal tail [1]. Tyrosine Y951 has been identified as a binding site for T-cell-specific adapter (TSA_d), responsible for the cytoskeleton regulation and migratory responses of ECs to VEGFA [5,6]. Tyrosines Y1054 and Y1059 are involved in the positive regulation of the receptor kinase activity [7,8]. Phosphorylated tyrosine Y1175 (P-Y1175) binds

and activates phospholipase C γ (PLC γ) leading to the activation of the Erk pathway resulting in cell proliferation [9]. P-Y1175 also binds the adapter molecule Shb, activating the PI3K pathway and regulating cell survival [10].

We have previously described that besides the classical signaling through the membrane receptor, there is a rapid nuclear internalization of VEGF and VEGFR2 in ECs, upon VEGF stimulation [11]. Furthermore, we have generated several VEGFR2 deletion mutants comprising different tyrosine residues and showed that VEGFR2 nuclear translocation requires the phosphorylation/activation of the receptor.

Here, ECs were transduced with lentivirus expressing VEGFR2 WT or VEGFR2 tyrosine to phenylalanine double mutants. We analyzed the nuclear translocation dynamics of these mutants and our results showed that a sub-population of the VEGFR2(Y951F/Y996F) mutant protein is immobilized in the cytoplasm and not translocated to the cell nucleus. The analysis of the translocation dynamics of the VEGFR2 tyrosine to phenylalanine single point mutants identified the tyrosine residue Y951 as the one required for the nuclear internalization of VEGFR2.

3.3. RESULTS

ESTABLISHMENT OF AN EC MODEL EXPRESSING VEGFR2 TYROSINE TO PHENYLALANINE POINT MUTANTS

Our previously reported results suggested that activation/phosphorylation of VEGFR2 was important for its nuclear translocation (Chapter II, [11]). To further identify the tyrosine residues involved in the nuclear internalization of the receptor, we established an EC model of VEGFR2 overexpression fused to GFP (EC VEGFR2-GFP) using a lentiviral infection approach (Figure 1A). We generated two (tyrosine to phenylalanine) double mutants Y951F/Y996F (tyrosine residues in the kinase insert domain) and Y1054F/Y1059F (tyrosine residues in the kinase catalytic domain) fused to GFP (Figure 1A).

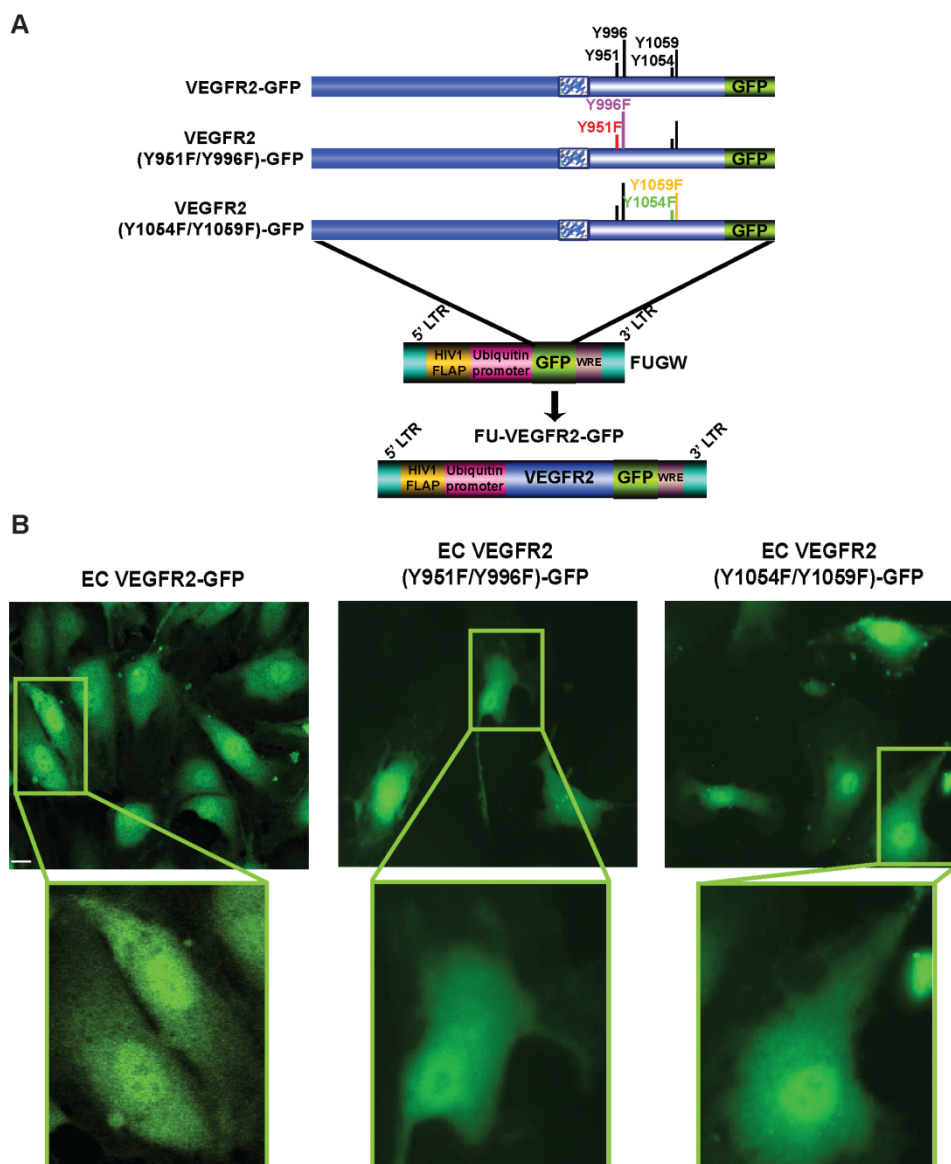


Figure 1. Establishment of an EC model expressing VEGFR2 tyrosine to phenylalanine point mutants.

(A) Schematic representation of the cloning strategy in the lentiviral plasmid FUGW of VEGFR2 WT or tyrosine to phenylalanine mutants fused to GFP. (B) Live cell confocal imaging of EC VEGFR2-GFP (left panel), EC VEGFR2(Y951F/Y996F)-GFP (middle panel) and EC VEGFR2(Y1054F/Y1059F)-GFP (right panel). Lower panels show image detail of the cytoplasmic and nuclear distribution of the respective fusion protein VEGFR2-GFP. Scale bar 10 μ m.

To establish this model, the transduced cells were sorted to obtain a 100 % GFP positive population.

Since VEGFR2 activation is involved in the EC migration, we evaluated the migratory response of EC VEGFR2-GFP compared to control EC GFP (expressing only GFP) using an *in vitro* wound healing assay (Figure 2).

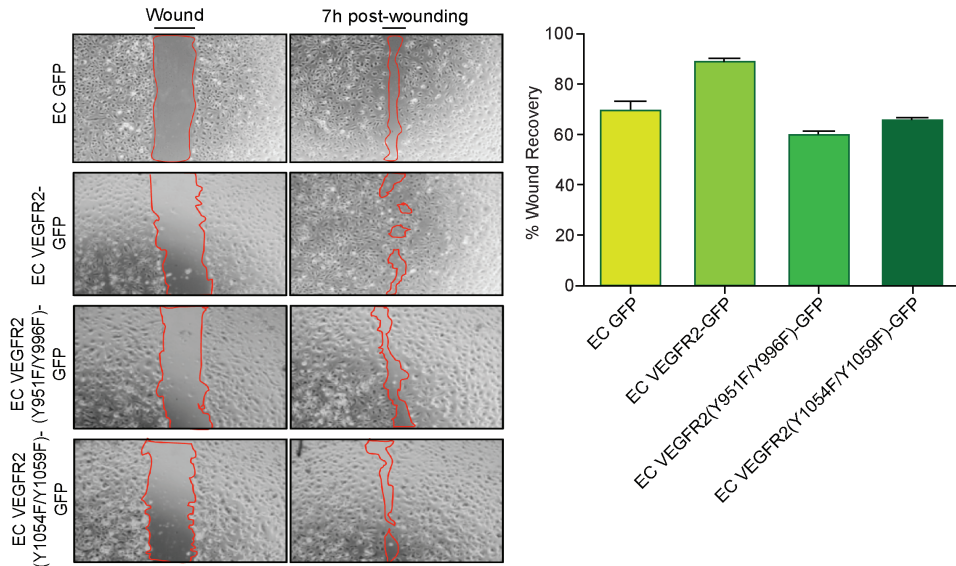


Figure 2. VEGFR2 tyrosine to phenylalanine point mutants have a decreased migratory capacity. *In vitro* wound healing analysis of the migratory response of EC VEGFR2(Y951F/Y996F)-GFP and EC VEGFR2(Y1054F/Y1059F)-GFP compared to EC VEGFR2-GFP and control EC GFP (left panel) and % of wound recovery 7 h post-wounding (right panel).

As expected, we observed that the wound recovery rate was higher in EC VEGFR2-GFP in comparison to control EC GFP (Figure 2), indicating that the VEGFR2 overexpression is correlated with a higher migratory phenotype. Furthermore, we also found that both EC VEGFR2(Y951F/Y996F)-GFP and EC VEGFR2(Y1054F/Y1059F)-GFP migrated less when compared to control EC VEGFR2-GFP (Figure 2).

Next, the VEGFR2-GFP overexpression was analyzed by confocal microscopy (Figure 1B). As expected, VEGFR2-GFP is localized to the cell membrane and present both in the cytoplasm and in the nucleus of EC VEGFR2-GFP (Figure 1B, left panel). Surprisingly, positive transduced EC VEGFR2(Y951F/Y996F)-GFP and EC VEGFR2(Y0154F/Y1059F)-GFP showed a similar VEGFR2

expression pattern as EC VEGFR2-GFP, both in the cytoplasm and nucleus (Figure 1B, middle and right panel).

Together, these results showed that EC VEGFR2(Y951F/Y996F)-GFP and EC VEGFR2(Y1054F/Y1059F)-GFP have a decreased migratory capacity although the VEGFR2 nuclear localization looked similar to EC VEGFR2-GFP.

VEGFR2 (Y951F/Y996F) MUTANT HAS A SLOW NUCLEAR TURNOVER

In order to study the nuclear translocation dynamics of the VEGFR2 WT and VEGFR2 tyrosine double point mutants, Fluorescence Recovery After Photobleaching (FRAP) analysis were performed. The GFP fluorescence was irreversibly bleached by a high intensity short laser pulse in the whole nuclear area (Figure 3A). The recovery of the fluorescence signal from the bleached area was measured every 5 s for 280 s and normalized to the initial intensity, after correcting for total bleaching loss and bleaching due to imaging. We compared the nuclear fluorescence recovery of EC VEGFR2(Y951F/Y996F)-GFP and EC VEGFR2(Y1054F/Y1059F)-GFP to control EC VEGFR2-GFP (Figure 3B). In EC VEGFR2-GFP, the signal was fully recovered within 100 s (Figure 3A and 3B) suggesting a rapid turnover of VEGFR2-GFP between the cytoplasm and the nucleus. EC VEGFR2(Y1054F/Y1059F)-GFP showed a similar recovery kinetics as control EC VEGFR2-GFP (Figure 3B).

However, EC VEGFR2(Y951F/Y996F)-GFP showed a much slower turnover rate, compared to EC VEGFR2-GFP and the normalized fluorescence intensity did not fully recover to the initial levels within 280 s (Figure 3B).

These results indicate that at least a fraction of the mutant VEGFR2(Y951F/Y996F) population is immobilized in the cytoplasmic region and is not translocated to the nucleus. Interestingly, the steady state (i.e., before bleaching) nucleus/cytoplasm fluorescence ratio of the VEGFR2 (WT)-GFP and the two mutants is similar (Figure 3C) but the absolute values of intensity are not (Figure 3D) indicating that some mutations also have an effect on the expression levels of the mutant proteins.

Taken together our results suggest that VEGFR2 tyrosine residues Y951 and/or Y996 are involved in the VEGFR2 nuclear translocation.

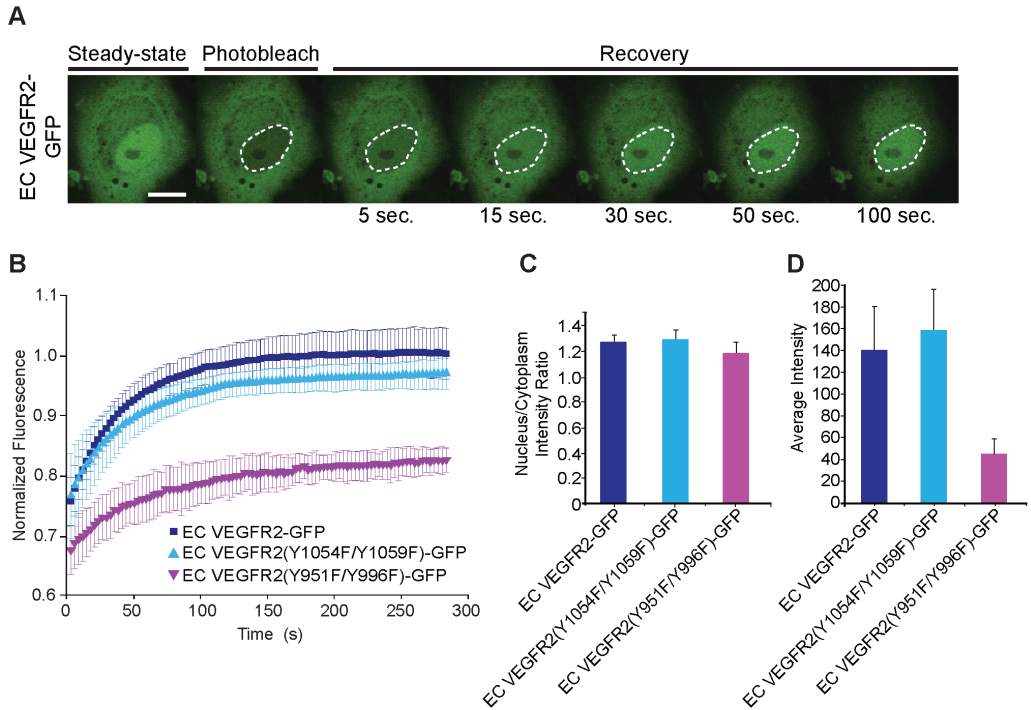


Figure 3. VEGFR2(Y951F/Y996F)-GFP has a slow nuclear turnover.

FRAP analysis of VEGFR2-GFP in EC VEGFR2-GFP and mutants EC VEGFR2(Y951F/Y996F)-GFP and EC VEGFR2(Y1054F/Y1059F)-GFP. (A, B) Live cells were imaged before and at defined time intervals after bleaching the entire nucleus. (A) The image sequence shows representative time points before, immediately after bleaching and 5, 15, 30, 50 and 100 s of fluorescence recovery of EC VEGFR2-GFP. White circles indicate the bleached region. (B) Fluorescence intensity in the bleached region was measured every 5 s for 280 s and normalized for the initial intensity. Results represent 3 independent experiments, with at least 10 different cells analyzed in each case. (C) Steady state (pre-bleach) nucleus/cytoplasm fluorescence intensity ratio measured for each cell pool. (D) Steady-state (pre-bleach) average intensity values of the indicated pools. Error bars represent standard deviation (SD).

VEGFR2 TYROSINE RESIDUE Y951 IS REQUIRED FOR THE NUCLEAR TRANSLOCATION PROCESS

To further evaluate the relative contribution of the tyrosine residues Y951 and Y996 to the VEGFR2 nuclear translocation, we performed a similar experiment with ECs expressing single mutants VEGFR2 Y951F or Y996F (Figure 4A). Interestingly, EC VEGFR2(Y951F)-GFP, but not EC VEGFR2(Y996F)-GFP showed a slower recovery rate, compared to EC VEGFR2-GFP (Figure 4C).

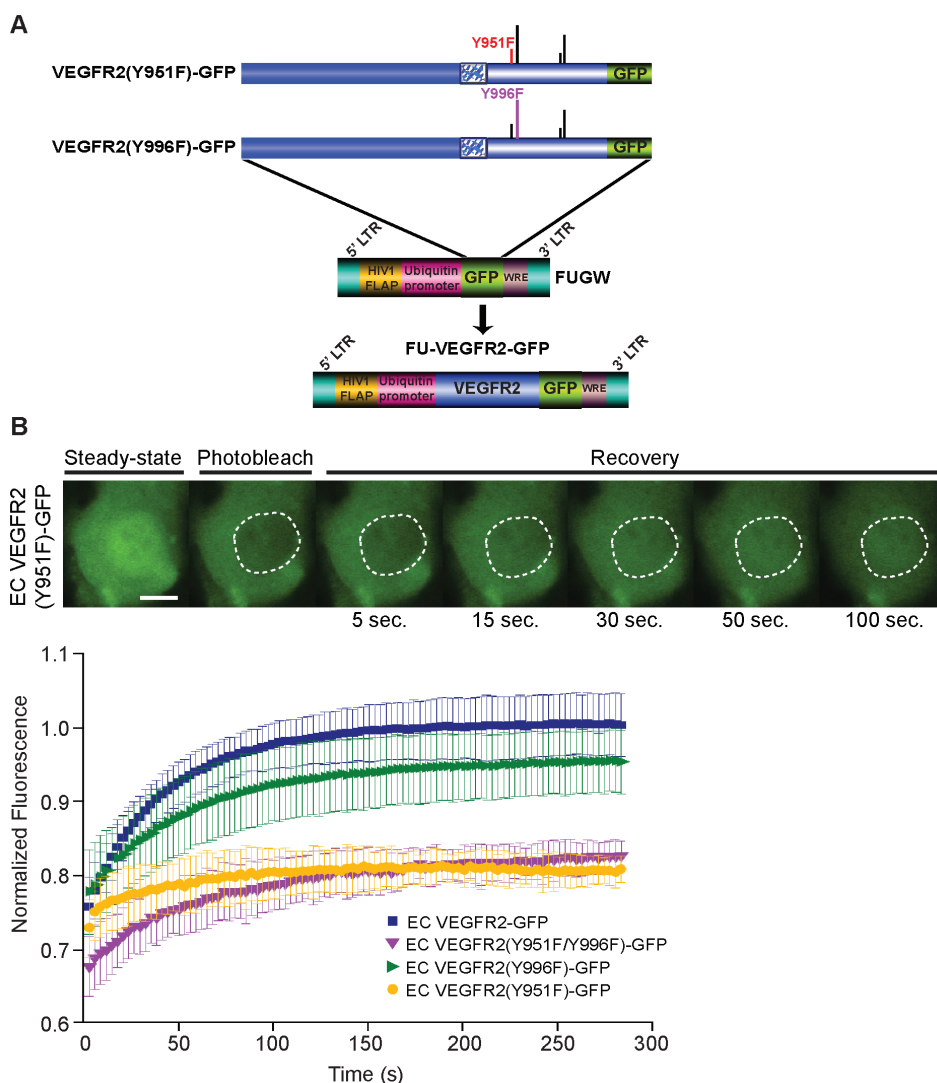


Figure 4. VEGFR2(Y951F)-GFP but not VEGFR2(Y996F)-GFP also has a slow nuclear turnover.

(A) ECs expressing single mutants VEGFR2(Y951F)-GFP and VEGFR2(Y996F)-GFP were generated.

(B) The image sequence shows representative time points before, immediately after bleaching and 5, 15, 30, 50 and 100 s of fluorescence recovery of EC VEGFR2(Y951)-GFP. White circles indicate the bleached region.

(C) FRAP analysis of EC VEGFR2-GFP, double mutant EC VEGFR2(Y951F/Y996F)-GFP and single mutants EC VEGFR2(Y996F)-GFP and EC VEGFR2(Y951F)-GFP. Fluorescence intensity in the bleached nuclear region was measured every 5 s for 280 s and normalized for the initial intensity. Results represent 3 independent experiments with at least 10 different cells analyzed for each case. Error bars represent standard deviation (SD).

As observed in EC VEGFR2(Y951F/Y996F)-GFP, the VEGFR2-GFP signal was not fully recovered within 280 s in EC VEGFR2(Y951F)-GFP (Figure 4B

and 4C). These results strongly suggest that the phosphorylation of the VEGFR2 tyrosine residue Y951 is required, if not sufficient, for the nuclear translocation of this receptor.

3.4. DISCUSSION

VEGF binding to the extracellular domain of VEGFR2 induces receptor dimerization and autophosphorylation of tyrosine residues located in its intracellular domain [12]. We have shown that the ligand-dependent activation is essential for the nuclear internalization of VEGF/VEGFR2 [11]. Additionally, we observed that VEGFR2 deletion mutants had a deficient nuclear translocation in a non-EC system [11] further supporting the model that phosphorylation is required for receptor internalization. In order to identify the tyrosine residues involved in this process, tyrosine to phenylalanine VEGFR2 point mutants were constructed. EC transduced with lentivirus expressing VEGFR2 WT or mutants fused to GFP were generated. The four tyrosine residues Y951, Y996, Y1054 and Y1059 were primarily identified as autophosphorylation sites in the receptor intracellular domain after analysis of bacterially expressed VEGFR2 [13]. By performing an *in vitro* wound healing assay, we observed that EC VEGFR2(Y951F/Y996F)-GFP and EC VEGFR2(Y1054F/Y1059F)-GFP had a lower migratory capacity compared to EC VEGFR2-GFP. Studies using mutant chimeras EGFR-VEGFR2 have shown that Y1059 is required for VEGF-induced proliferation, through the activation of MAPK pathway, but not migration [14]. Additionally, the autophosphorylation of the tyrosine residues Y1054 and Y1059, in the kinase catalytic domain, was described as critical for maximal receptor catalytic activity [7,8]. These data indicate that the cell effects mediated by tyrosine residues Y1054 and Y1059 are primarily associated with cell proliferation. However, our data with EC VEGFR2(Y1054F/Y1059F)-GFP suggest that these residues might also play a role in EC migration, since we observed a diminished migratory capacity compared to EC VEGFR2-GFP.

An indirect effect, mediated by the lack of kinase activity might explain these apparently contradictory results, however further experiments are needed to

support this. A similar decrease in the EC migratory phenotype was observed in EC VEGFR2(Y951F/Y996F)-GFP, consistent with other studies that implicated Y951 in cell migration [5,14]. We took advantage of the VEGFR2-GFP fusion strategy to directly investigate the effect of these mutations in the nuclear internalization mechanism, by confocal microscopy. Previously, we observed that NIH 3T3 cells expressing VEGFR2 Δ 1053-GFP deletion mutant showed a decreased VEGFR2 nuclear accumulation level and NIH 3T3 cells expressing VEGFR2 Δ 950-GFP deletion mutant showed a clear absence of nuclear VEGFR2 [11]. In contrast, the tyrosine to phenylalanine mutants analyzed here showed the presence of VEGFR2-GFP both at cell surface and nucleus of ECs.

As native VEGFR2 is present in ECs transduced with lentivirus expressing the different VEGFR2 mutant constructs a possible explanation for the common distribution pattern is that upon VEGF binding, “heterodimerization” between native and mutant VEGFR2 occurs. This could result in the nuclear translocation of the receptor observed even in cells expressing the VEGFR2 tyrosine to phenylalanine mutants. This hypothesis is consistent with reports indicating that the binding properties of the VEGF ligands lead to the formation of both VEGFRs homodimers and heterodimers [15,16]. Testing this hypothesis would involve the analysis of the nuclear internalization dynamics of these mutants in an EC system with reduced or absent endogenous levels of native VEGFR2, such as in Porcine Aortic Endothelial Cells (PAEC). However, in these ECs, we would have to confirm the nuclear internalization of VEGFR2 and if it occurs, we would need to validate the molecular mechanisms previously found in our EC system.

Since we have shown that VEGFR2 nuclear translocation is a dynamic process [11], we sought to analyze differences in the translocation kinetics of these VEGFR2 tyrosine mutants. We compared the fluorescence recovery rates after photobleaching of EC VEGFR2(Y951F/Y996F)-GFP, EC VEGFR2(Y1054F/Y1059F)-GFP and EC VEGFR2-GFP. Our results showed similar kinetics and recovery rates between EC VEGFR2(Y1054F/Y1059F)-

GFP compared to EC VEGFR2-GFP. However, EC VEGFR2(Y951F/Y996F)-GFP showed slower recovery kinetics and the fluorescent signal was not fully recovery in the time frame of the FRAP experiment (280 s). These results indicated that at least a fraction of the mutant VEGFR2(Y951F/Y996F)-GFP population was not internalized to the cell nucleus, suggesting that tyrosine residues Y951 and/or Y996 were involved in the nuclear translocation process. The FRAP analysis of EC expressing the single point mutants VEGFR2(Y951F)-GFP or VEGFR2(Y996F)-GFP revealed that EC VEGFR2(Y951F)-GFP had a comparable slower recovery rate as observed for EC VEGFR2(Y951F/Y996F)-GFP, suggesting that the residue Y951, but not Y996, is essential for the nuclear translocation of the receptor. Interestingly, it was reported that the phosphorylation of Y951 is markedly observed in sprouting vessels, suggesting that the signal transduction dependent of the Y951 phosphorylation is required during angiogenesis [5]. These results are consistent with our previous observations that VEGF and VEGFR2 are rapidly internalized to the nucleus following injury in ECs facing the wounded area [11]. Another interesting aspect concerning signaling through phosphorylated Y951 is the binding and activation of the T-cell-specific adapter (TSA) a VEGF receptor-associated protein, which associates with Src and PI3K [5,6]. Blocking of PI3K pathway also results in an impaired nuclear translocation of VEGFR2 [11] further suggesting that Y951 is implicated in the nuclear translocation process of VEGFR2 in ECs. Taken together, the data presented here suggest that the phosphorylation of VEGFR2 tyrosine Y951 is essential for the nuclear translocation process of the receptor in ECs. Further studies are required to elucidate the biological relevance of this receptor in the cell nucleus.

3.5. MATERIAL AND METHODS

CELL CULTURE

Primary Human Umbilical Vein Endothelial Cells (HUVEC referred as ECs) were kindly provided by Dr Shahin Rafii (Cornell University Medical College,

New York, USA). ECs, passage 4-8, were cultured in 0.02 % gelatin-coated dishes in growing endothelial medium (basal EBM-2 medium supplemented with EGM-2 singlequots, BBE and 5 % of Fetal Bovine Serum (FBS)) as provided by the manufacturers (Lonza, USA). HEK-293 T cells were cultured in DMEM (Invitrogen Corporation, USA) supplemented with 10 % FBS.

CONSTRUCTION OF THE VEGFR2 POINT MUTANTS BY SITE DIRECTED-MUTAGENESIS

Tyrosine to phenylalanine VEGFR2 point mutants were generated by site-directed mutagenesis using the overlap extension method [17]. Two separate amplification reactions were first performed using the pEGFP-VEGFR2 as template; one using the primer A: 5' C GTC ATG GAT CCA GAT GAA CTC C 3' (sense) and the mutated antisense primer (listed below), the other using the mutated sense primer (listed below) and the primer B: 5' TA G GT CAG GGT GGT CAC GAG 3' (antisense).

The mutated primers designed to replace tyrosine (Y) to phenylalanine (F-bold) residues in Y951 were: 5' GGG AAA GAC TTC GTT GGA GCA 3' (sense) and 5' TGC TCC AAC GAA GTC TTT CCC 3' (antisense); in Y996 were 5' T CCT GAA GAT CTG **TTT** AAG GAC TTC CTG 3' (sense) and 5' G GAA GTC CTT **AAA** CAG ATC TTC AG 3' (antisense); in Y1054 were 5' GCC CGG GAT ATT **TTT** AAA GAT CCA G 3'(sense) and 5' TGG ATC TTT **AAA** AAT ATC CCG GGC C 3' (antisense); in Y1059 were 5' GAT CCA GAT **TTT** GTC AGA AAA GGA G 3' (sense) and 5' C TCC TTT TCT GAC **AAA** ATC TGG ATC T 3' (antisense).

The thermal amplification conditions were 95 °C/5 min, 35 cycles (95 °C/1 min, 61 °C/1 min, 72 °C/1 min), 72 °C/10 min. An overlapping reaction was performed using the mutated products from the first PCR (2–5%) and the sense A and antisense B primers. The thermal amplification conditions were 95 °C/5 min, 35 cycles (95 °C/1 min, 58 °C/1 min, 72 °C/1 min), 72 °C/10 min. PCR products were inserted into the *Bam*HI/ *Ap*al sites of pEGFP-VEGFR2. All constructs were confirmed by DNA sequencing.

GENERATION OF LENTIVIRAL VECTORS EXPRESSING VEGFR2

The VEGFR2 WT and tyrosine to phenylalanine mutants fused to GFP were released from pEGFP-VEGFR2 using the *Sall*/*HpaI* restriction sites and were cloned in the lentiviral plasmid FUGW (kindly given by Dr. Pedro Simas, Instituto de Medicina Molecular, Lisbon, Portugal) in the *BamHI*/*EcoRI* restriction sites, using blunt-end cloning, generating FU-VEGFR2-GFP. All constructs were confirmed by DNA sequencing

LENTIVIRAL PRODUCTION

Lentiviral particles were obtained with the transfection of HEK-293T cells using a standard calcium phosphate precipitation protocol. HEK-293T cells (\approx 50% confluent) were transfected with the lentiviral vector plasmid FUGW or FU-VEGFR2-GFP together with the HIV-1 packaging vector Delta 8.9 and the VSV-g envelope glycoprotein. The viral supernatants were collected 60 h post-transfection and filtered through a 0.45 μ m pore size filter. ECs were seeded at 7.5×10^4 cells (12 well plate) 24 h before transduction and then exposed to 500 μ l of virus supernatant (supplemented with polybrene to a final concentration of 4 μ g/ml). 72 h post-infection the GFP positive cells were sorted. ECs expressing FUGW were named EC GFP; ECs expressing FU-VEGFR2(WT)-GFP were named EC VEGFR2-GFP; ECs expressing FU-VEGFR2(Y951F/Y996F)-GFP were named EC VEGFR2(Y951F/Y996F)-GFP; ECs expressing FU-VEGFR2(Y1054F/Y1059F)-GFP were named EC VEGFR2(Y1054F/Y1059F)-GFP; ECs expressing FU-VEGFR2(Y996F)-GFP were named EC VEGFR2(Y996F)-GFP; ECs expressing FU-VEGFR2(Y951F)-GFP were named EC VEGFR2(Y951F)-GFP.

CONFOCAL MICROSCOPY

Live cell imaging was performed at 37 °C and 5 % CO₂ on a Zeiss LSM 510 META (Carl Zeiss, Germany) inverted laser scanning confocal microscope equipped with a large incubator for temperature control and a stage incubator for CO₂ supply (PeCon, Germany). Images were acquired using a Plan-

Apochromat 63x/1.4 oil immersion objective. GFP fluorescence was detected using the 488 nm line of an Ar laser (45 mW nominal output) and a BP 505-550 nm filter.

FLUORESCENCE RECOVERY AFTER PHOTBLEACHING (FRAP)

Each FRAP analysis started with a single image scan followed by a bleach pulse at 100% laser power in a region of interest (ROI) that coincided with the cell nucleus (~ 350 μm^2 area). A series of 56 single-section images were then acquired at 5 s intervals for 280 s, with the first image being acquired 2 ms after the end of the photobleaching. Image acquisition was performed with laser power attenuated to 1% of the bleaching intensity.

Fluorescence intensity quantification was performed for each FRAP time series using ImageJ software (<http://rsbweb.nih.gov/ij>). The average fluorescence in the nucleus of bleached cells $I(t)$ and the total cell fluorescence $T(t)$ were calculated for each background-subtracted image at time t . FRAP curves for bleached cells were then normalized and corrected for loss of fluorescence due to imaging,

$$I_N(t) = I(t)/I_0 \cdot T_0/T(t)$$

where I_0 and T_0 are the nuclear and total fluorescence intensities before bleaching started [18]. The ratio between steady-state fluorescence in nucleus and cytoplasm was calculated by simply dividing pre-bleach nuclear and cytoplasmic background-subtracted intensities.

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CHAPTER IV

VEGFR2 TRANSLOCATES TO THE NUCLEUS TO REGULATE ITS OWN TRANSCRIPTION

VEGFR2 Translocates to the Nucleus to Regulate its own Transcription

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Running head

VEGFR2 Regulates its own Transcription.

Abbreviations

VEGFR2, Vascular Endothelial Growth Factor Receptor 2; VEGF, Vascular Endothelial Growth Factor; ECs, endothelial cells; ChIP, Chromatin Immunoprecipitation; TFs, transcription factors.

4.1. ABSTRACT

Vascular Endothelial Growth Factor Receptor 2 (VEGFR2) is the major mediator of the angiogenic effects of VEGF. In addition to its well-known role as a membrane receptor that activates multiple signaling pathways, VEGFR2 also has a nuclear localization. However, what VEGFR2 does in the nucleus is still unknown.

In the present report we show that, in endothelial cells (ECs), nuclear VEGFR2 interacts with several nuclear proteins, including Sp1, a transcription factor that has been implicated in the regulation of genes needed for angiogenesis.

By *in vivo* chromatin immunoprecipitation (ChIP) assays, we found that VEGFR2 binds to the Sp1-responsive region of the human *VEGFR2* proximal promoter. These results were confirmed by EMSA assays, using the same region of the *VEGFR2* promoter. Importantly, we show that the VEGFR2 DNA binding is directly linked to the transcriptional activation of the *VEGFR2* promoter. By reporter assays, we found that the region between -300/-116 relative to the transcription start site is essential to confer VEGFR2-dependent transcriptional activity. It was previously described that nuclear translocation of the VEGFR2 is dependent of its activation by VEGF. Consistently, we observed that the binding of VEGFR2 to DNA requires VEGF activation, being blocked by Bevacizumab and Sunitinib, two anti-angiogenic agents that inhibit VEGFR2 activation.

Our findings demonstrate a new mechanism by which VEGFR2 activates its own promoter that could be involved in amplifying the angiogenic response.

4.2. INTRODUCTION

Angiogenesis is the formation of new blood vessels from a pre-existing vascular net. This process is essential during embryonic development and for normal homeostasis of adult tissues. In addition, angiogenesis was recognized to be fundamental in the progression of many pathological diseases such as cancer because it is an essential event in tumor growth and metastatic dissemination [1]. Angiogenesis is a complex dynamic process regulated by a balance between pro-angiogenic and anti-angiogenic factors. Vascular Endothelial Growth Factor (VEGF) is one of the most important pro-angiogenic factors. VEGF stimulates angiogenesis by binding to the VEGF receptor (VEGFR) 1 and VEGFR2 receptor tyrosine kinases (RTKs) on the cell surface of ECs, [2]. Both VEGFR1 and VEGFR2 have seven Ig-like-domains in the extracellular domain, a single transmembrane region and a split tyrosine kinase intracellular domain [2]. VEGFR2 is considered to be the major mediator of several physiological and pathological effects of VEGF on ECs. These include proliferation, survival, migration and permeability [2].

VEGF binds to the extracellular domain of VEGFR2 inducing receptor dimerization and autophosphorylation of specific intracellular tyrosine residues leading to the activation of different signaling pathways [2].

Recognition of the VEGF pathway as a key regulator of angiogenesis has led to the development of several VEGF-targeted agents demonstrating therapeutic efficacy in several human cancers [3]. Therefore, several approaches have been developed to inhibit VEGF signaling, including neutralization of the ligand or receptor by antibodies, and blocking VEGF receptor activation and signaling with tyrosine kinase inhibitors [4]. The pioneers of the clinical proof-of-concept for angiogenesis inhibitors are Bevacizumab (Avastin®, Genentech/Roche), a ligand-trapping monoclonal antibody [5], and Sunitinib (Sutent®, Pfizer), which target receptor tyrosine kinases [6], principally VEGFR2. Their goal is to block the VEGF signaling mediated by the plasma membrane receptor VEGFR2.

Besides the membrane localization of VEGFR2, it was demonstrated that it could also be found in the cell nucleus. In particular, phosphorylated VEGFR2 has been observed in the nucleus of proliferative tumor and leukemia cells [7,8,9,10,11], suggesting that molecular mechanisms that contribute to tumor angiogenesis might require a specific activity of this protein in the nucleus. Moreover, we also demonstrated the role of the nuclear internalization of VEGFR2 in EC recovery following injury [12]. Taken together, these studies document the presence of VEGFR2 in the nucleus and suggest that nuclear VEGFR2 might amplify the angiogenic response. However, the precise activity of VEGFR2 in the nucleus is unknown.

Here, we demonstrate a previously unrecognized function for nuclear VEGFR2 as a putative transcription factor that is involved in the regulation of its own transcription. We show that VEGFR2 binds to and activates its own promoter *in vivo* in VEGF-activated ECs. Moreover, we observed that this mechanism is blocked by treating ECs with Bevacizumab or Sunitinib.

4.3. RESULTS

VEGFR2 NUCLEAR INTERNALIZATION IS A DYNAMIC PROCESS THAT IS CORRELATED WITH TRANSCRIPTIONAL ACTIVITY IN ECs

In order to investigate the functional relevance of nuclear VEGFR2 we established an EC model of VEGFR2 overexpression (EC VEGFR2 IRES GFP) using a lentiviral infection approach. Positively transduced cells were sorted using GFP expression and VEGFR2 overexpression was confirmed by both immunofluorescence and confocal microscopy (Figure 1A) and western-blot analysis (Figure 2A, first panel). By confocal analysis we found that VEGFR2 expression was increased both in the cytoplasm and nucleus of EC VEGFR2 IRES GFP, when compared to untransduced EC or EC transduced with a control vector (EC IRES GFP), (Figure 1A). Our data suggest that ECs overexpressing VEGFR2 results in high levels of this protein that are not degraded and also accumulate in the nucleus.

We decided to further investigate the nuclear internalization of VEGFR2 by performing photobleaching studies on living ECs to measure the nuclear turnover of GFP tagged VEGFR2. First, we photobleached VEGFR2-GFP in the whole cell nucleus and then quantified the nuclear fluorescence recovery by time-lapse imaging. According to our results, a full recovery of the fluorescence signal was observed within 100 s (Figure 1B, upper panel and 1C), suggesting a rapid turnover of VEGFR2-GFP between the cytoplasm and the nucleus.

Interestingly, we also found that a single point mutation at tyrosine residue Y951 of the VEGFR2 results in a slower turnover rate compared to ECs expressing the wild-type protein, with fluorescence intensity not recovering to its pre-photobleaching baseline within the duration of FRAP experiment.

ECs expressing mutations in other tyrosine residues (such as Y1059, Y1054 and Y996) were evaluated and presented similar recovery kinetics following bleaching when compared to VEGFR2-GFP cells (Figure 1C).

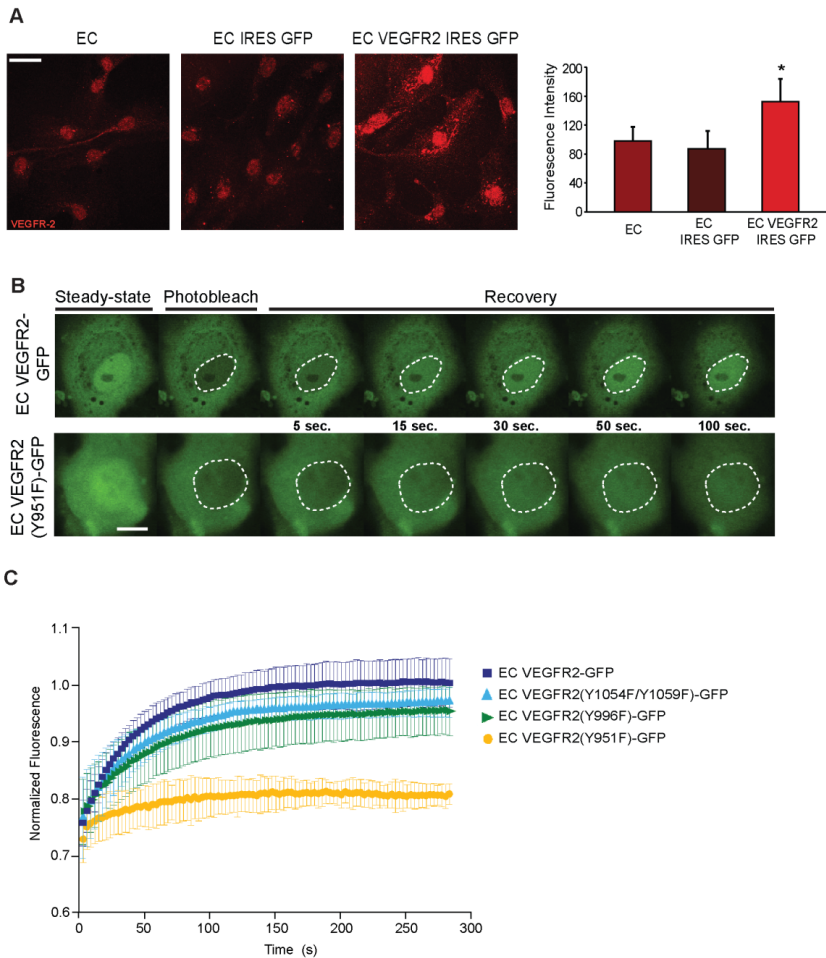


Figure 1. VEGFR2 nuclear translocation is a rapid process that is affected by the VEGFR2 tyrosine Y951.

(A) EC, EC IRES GFP and EC VEGFR2 IRES GFP were cultured in growing media and VEGFR2 overexpression was analysed by immunofluorescence. Cells were stained with a rabbit anti-human VEGFR2 antibody (Alexa 594). Results shown are representative z-projections of at least three independent experiments. Scale bar: 20 μ m. Right panel shows mean fluorescence intensity of VEGFR2 in the cell nucleus. * $P < 0.0001$. (B and C) FRAP analysis was performed in EC VEGFR2-GFP and mutants EC VEGFR2(Y1054F/Y1059F)-GFP, EC VEGFR2(Y996F)-GFP and EC VEGFR2(Y951F)-GFP. Fluorescence signal of the entire nucleus was photobleached with a single 488-nm high intensity laser pulse and subsequent fluorescence recovery was recorded for 280 s. (B) Selected images of VEGFR2-GFP protein in EC VEGFR2-GFP (upper panel) and EC VEGFR2(Y951F)-GFP (lower panel) before bleaching (steady-state) and at the indicated intervals post-bleaching (from 5 to 100 s). White circles indicate the bleached region. (C) Fluorescence intensity in the bleached region was measured every 5 s for 280 s and normalized for the initial intensity. Data show results obtained in three independent experiments, with at least ten different cells analysed in each case. Error bars represent standard deviation (SD).

Taken together, our results suggest that the translocation of VEGFR2 from the cytoplasm to the nucleus is a rapid and dynamic process in which the tyrosine residue Y951 plays an important role.

Because VEGFR2 signaling promotes cell proliferation and survival, we tested if increased levels of VEGFR2 modified the levels of nuclear proteins involved in these processes. We observed increased levels of Cyclin A, p65 (NF κ B) and Sp1 in the nucleus of EC VEGFR2 IRES GFP, compared to control EC IRES GFP (Figure 2A). The expression of YY1 was not significantly altered in the same cells (Figure 2A). Since some of these nuclear proteins are transcription factors (TFs), we evaluated whether their nuclear expression levels were mirrored by a change in their DNA binding activities using EMSA assays. We found a p65 (NF κ B) increased DNA binding activity in the VEGFR2 overexpressing cells, compared to control EC IRES GFP (Figure 2B, left panel), which is consistent with the increased protein levels in the nucleus (Figure 2A). Interestingly, the DNA binding activity of YY1 was also increased in ECs overexpressing VEGFR2 (Figure 2B, right panel). These results indicate that binding activities of several TFs are increased in ECs expressing VEGFR2, suggesting an enhanced transcriptional activity in these cells.

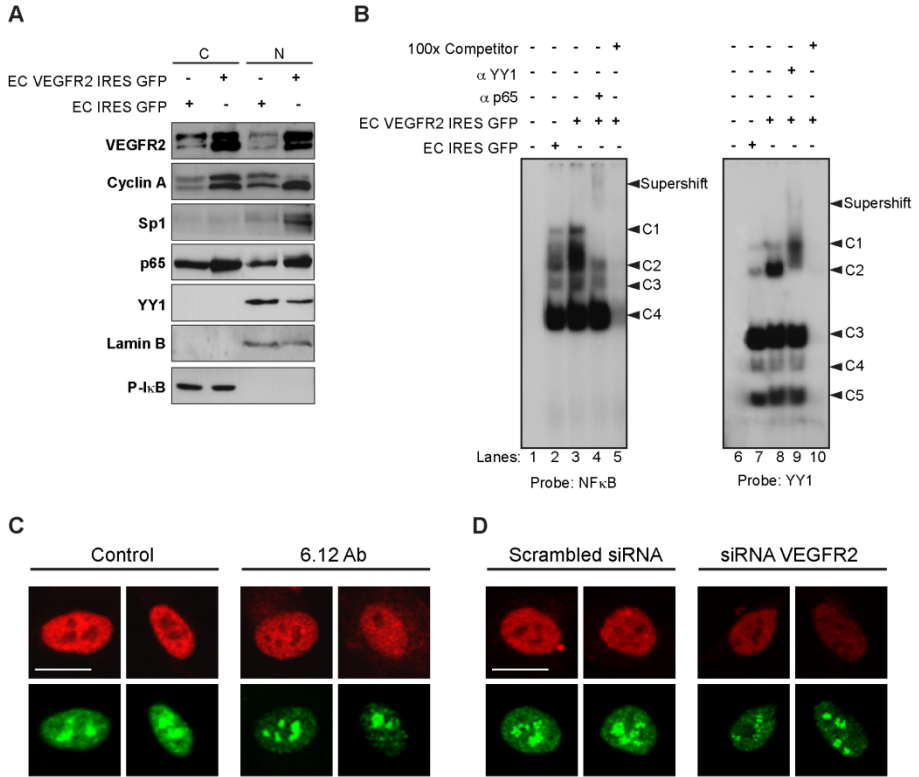


Figure 2. VEGFR2 nuclear internalization levels correlate with transcriptional activity of ECs.

(A) Cytoplasmic (C) and nuclear extracts (N) from EC IRES GFP and EC VEGFR2 IRES GFP were analysed by Western blot with antibodies against VEGFR2, Cyclin A, Sp1, p65, YY1. P-IkB and Lamin B were used as cytoplasmic and nuclear controls, respectively. (B) Nuclear extracts from EC IRES GFP (lanes 2,7) and EC VEGFR2 IRES GFP (lanes 3,8) were incubated with NFκB (left panel, lanes 2 and 3) or YY1 (right panel, lanes 7 and 8) radiolabeled probes. Four NFκB (C1-C4) or five YY1 complexes (C1-C5) are indicated with black arrows. Specific anti-p65 (lane 4) or anti-YY1 (lane 9) were introduced in the binding reaction to analyse the appearance of a supershift complex (as indicated in both panels) in EC VEGFR2 IRES GFP cells. Using the same cells, a competitive assay using 100x excess of cold probe of NFκB (lane 5) or YY1 (lane 10) was performed. Control lanes 1 and 6 contain only the radiolabeled probes. (C) ECs were cultured in growing media, treated or not with 6.12 Ab for 1 h and incubated with 5-FU for 15 min. (D) ECs were cultured in growing media and transfected with scrambled siRNA or VEGFR2 siRNA. ECs were incubated with 5-FU for 15 min, 24 h post-transfection. (C and D) Cells were fixed, sequentially labeled, on the same slide with a rabbit anti-human VEGFR2 (Alexa 594) and a mouse anti-human BrdU antibody (Alexa 488) and analysed by confocal microscopy. Results shown are representative z-projections of three independent experiments. Scale bar: 20 μm.

For that reason, we decided to test if the levels of transcription in ECs were also altered when the nuclear accumulation of VEGFR2 was experimentally reduced. For this, we took advantage of our previous observation that a neutralizing antibody against VEGFR1 (6.12 Ab) decreases VEGFR2 levels in the nucleus [12]. Using a 5-fluorouracil (5-FU) incorporation assay, we observed that the levels of transcription were decreased after 6.12 Ab treatment, compared to control cells (Figure 2C). Similar results were obtained when the levels of VEGFR2 were reduced by using the siRNA technology (Figure 2D). As shown in Figure S1A, a pool of VEGFR2 siRNA oligos used in our experiments effectively abrogated the VEGFR2 expression as assessed by qRT-PCR (by 70% compared with the scrambled siRNA oligos). Taken together, these results suggest that the levels of EC transcription could be correlated with the nuclear accumulation of VEGFR2.

NUCLEAR VEGFR2 INTERACTS WITH THE TRANSCRIPTION FACTOR SP1 IN THE NUCLEUS OF ECs

The above results suggest that the nuclear levels of VEGFR2 could modulate cell transcription and we decided to investigate the specific role of VEGFR2 in this process. We asked if VEGFR2 interacts with nuclear proteins and if it modulates cell transcription. To address this question, VEGFR2 was immunoprecipitated from EC nuclear extracts and the proteins that directly or indirectly interacted with VEGFR2 were identified by Mass Spectrometry (MS) analysis (Figure 3A and 3B). Approximately 310 proteins were identified with high confidence with the Mascot algorithm. Proteins with a Mascot score greater than 200 were listed in Table S1. Analysis of these possible partners with Ingenuity Pathway Analysis (IPA) identified 95 proteins in 22 enriched categories for biological functions as represented in Figure 3B.

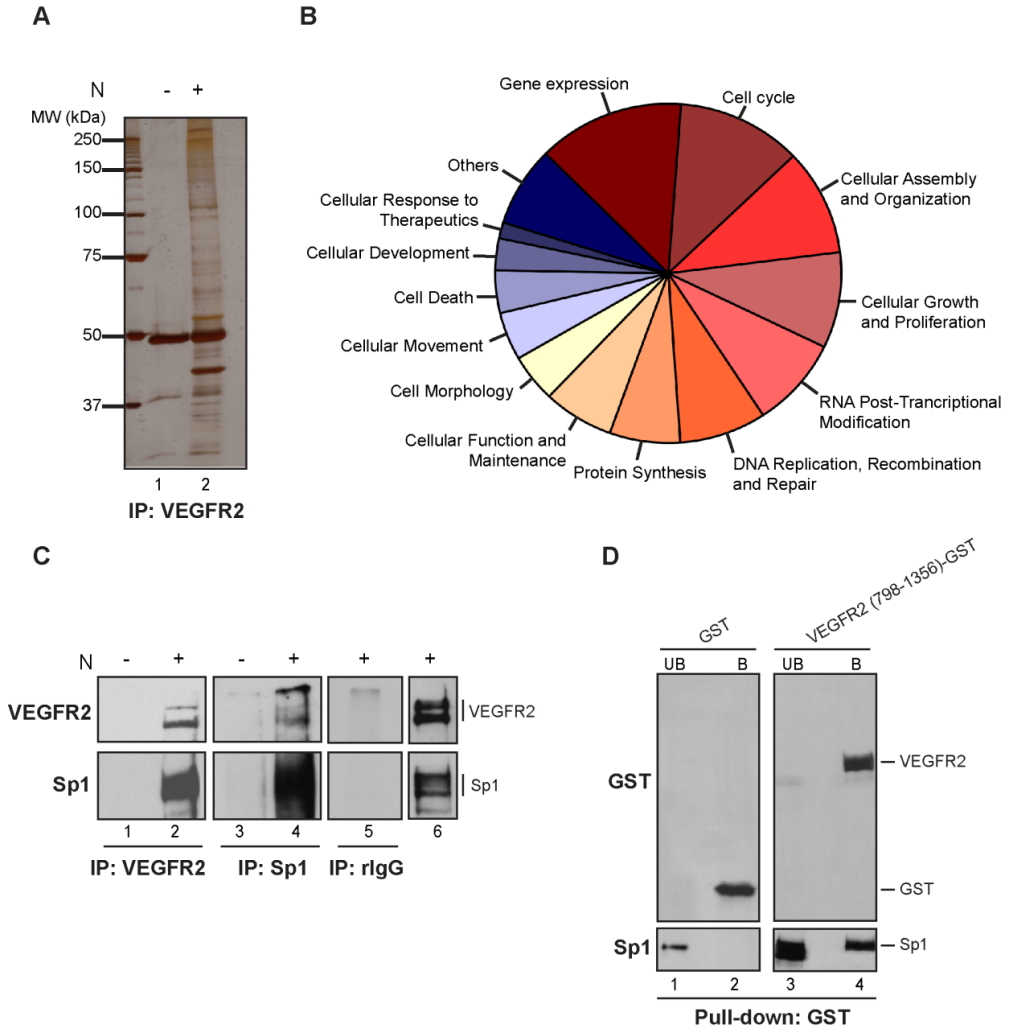


Figure 3. Nuclear VEGFR2 interacts with the transcription factor Sp1 in the nucleus of ECs.

(A) Immunoprecipitation (IP) of 1 mg EC nuclear extract with anti-human VEGFR2 was performed and resolved in 8% SDS-PAGE, following silver staining (lane 2). VEGFR2 antibody plus beads (without N) were used as negative control for immunoprecipitation (lane 1). The protein marker is shown as molecular weight (MW) in thousands. (B) Representation of the mass spectrometry analysis of the nuclear VEGFR2 IP, showing the categories for the different biological functions of the identified proteins ($p < 0.05$). (C) Immunoprecipitation (IP) of EC nuclear extracts (N) was conducted with the VEGFR2 (lane 2), Sp1 (lane 4) and rabbit IgG (rlgG-lane 5) antibodies followed by VEGFR2 (upper panel) or Sp1 (lower panel) immunoblotting. VEGFR2 (lane 1) or Sp1 (lane 3) antibodies plus beads (without N) were used as negative controls for immunoprecipitation. Non-immunoprecipitated nuclear cell extract (lane 6) was also included in the experiment. (D) Pull-down assay: Sp1 protein fused to a HA tag was incubated with GST alone (lanes 1 and 2) or VEGFR2 (789-1356)-GST (lanes 3 and 4). GST-unbound (UB) (lanes 1 and 3) and bound (B) fractions (lanes 2 and 4) were loaded and analyzed with GST (upper panel) and Sp1 (lower panel) antibodies.

Interestingly, proteins involved in gene expression are among the most abundant, suggesting that nuclear VEGFR2 could interact with proteins involved in gene transcription.

In order to confirm the data obtained by MS, we performed western-blot on nuclear VEGFR2 immunoprecipitates. We confirmed an interaction between VEGFR2 and Sp1 (Figure 3C). Interestingly, Sp1 is a transcription factor that regulates multiple genes important to angiogenesis. The antibody against Sp1 does not cross-react with other members of the Sp family, indicating a specific interaction between VEGFR2 and Sp1. Furthermore, we performed pull-down assays using purified proteins and our results suggest an interaction between Sp1 and the region containing amino acids 789 to 1356 of VEGFR2 (Figure 3D).

NUCLEAR VEGFR2 BINDS TO AND ACTIVATES THE VEGFR2 PROXIMAL PROMOTER IN ECs

Since it was previously shown that Sp1 is involved in the transcriptional regulation of *VEGFR2* gene [13,14] and because our results suggest a nuclear interaction between Sp1 and VEGFR2 (Figure 3C and 3D), we hypothesized that VEGFR2 could be involved in the regulation of its own transcription. In order to answer to this question, we decided to investigate if VEGFR2 could bind to its own promoter. Quantitative ChIP assays were performed on ECs, cultured in growing media. We chose a region of the human *VEGFR2* proximal promoter that comprises five Sp1 binding sites between -300/+1 relative to the transcription start site (Figure 4A). We observed that Sp1 binds to the *VEGFR2* proximal promoter (4.2 fold \pm 0.14 increase relative to the control IgG), (Figure 4B), which was consistent with previous reports [13,15]. Interestingly, we observed a 13.8 fold \pm 0.55 increase in binding of VEGFR2 relative to the negative control IgG (Figure 4B). As expected, when ECs were transfected with Sp1 siRNA in order to reduce its expression, the binding of Sp1 to the *VEGFR2* promoter was significantly decreased (Figure 4C). Moreover, the binding of VEGFR2 to its own promoter was abrogated in ECs

transfected with VEGFR2 siRNA (Figure 4C). Note that in both transfections, the expression of *Sp1* and *VEGFR2* were downregulated approximately 70% compared with that of scrambled siRNA-transfected ECs (Figure S1). Curiously, we also found that the binding of Sp1 to *VEGFR2* promoter was significantly increased when the expression of VEGFR2 was downregulated by siRNA. In contrast, the binding of VEGFR2 to its own promoter was reduced in ECs transfected with Sp1 siRNA (Figure 4C).

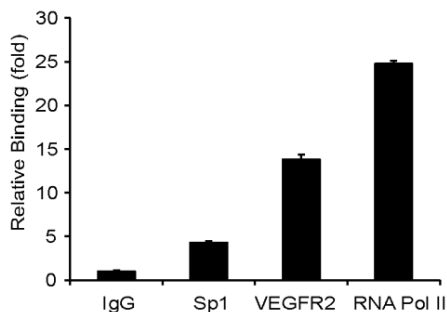
In order to confirm that VEGFR2 binds to its own promoter, we performed EMSA assays using as a probe the same region of the *VEGFR2* proximal promoter analyzed by ChIP. We identified four complexes (C1-C4) with distinct electrophoretic mobilities (Figure 4D, lane 2), which did not form when an excess of cold probe was introduced in the reaction (Figure 4D, lane 4), establishing their specificity.

A

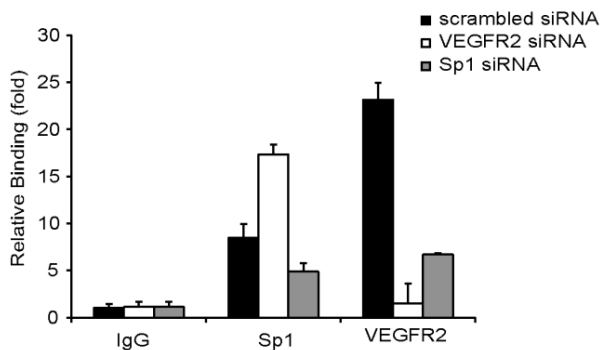
Human VEGFR2 promoter

-354 GTGAGGGAGCGCTGCTCTTCGCAGCGCTCCTGGTGATGCTCCCCAAATTTTCGGGGACCGG
 -294 CAACGAT TAAATCTTGGAGTTGCTCAGCGCCCGTTACCGAGTACTTTTTATTACACCA
 -234 GAAACAAAGTTGTTGCTCTGGGATGTTCTCTCCTGGCGACTTGGGGCCAGCGCAGTCC
 -174 AGTTGTGTGGGAAATGGGGAGATGTAATGGGCTTGGGGAGCTGGAGATCGCCGCCGGG
 -114 TACCCGGGTGAGGGGCCGGGCTGGCCGCACGGGAGGCCCTCCTCCGCTCCGGCCCGGC
 -54 CCGCATGGCCCCGCTCCGCGCTCTAGAGTTTCGGCACCGCTCCCACCCTGCCTGAG
 Sp1 Sp1 Sp1

B



C



D

100X Competitor - - - +
 ID VEGFR2 - - + -
 Nuclear extract - + - +

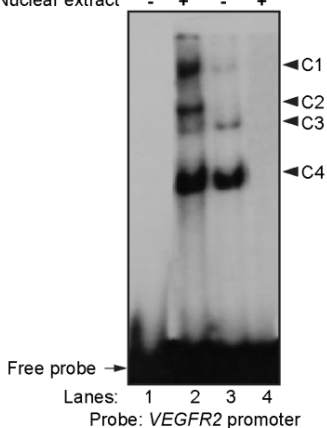


Figure 4. Nuclear VEGFR2 binds to the VEGFR2 proximal promoter in ECs.

(A) Sequence of the human *VEGFR2* proximal promoter (retrieved from Ensemble database accession number: ENSG00000128052) and underlined of the putative Sp1 binding sites. The transcription start site is indicated in gray. (B) ChIP assays of the *VEGFR2* proximal promoter were performed using ECs cultured in growing media. Antibodies against VEGFR2 and Sp1 were used. Normal rabbit/mouse IgG were used as control. Also, an antibody for RNA Pol II was used to test the promoter activity. All values are relative to control IgG background and normalized to an intergenic region. Data are mean \pm s.e.m. of triplicates and represents three independent experiments. (C) ChIP assays of the *VEGFR2* proximal promoter were performed in ECs 24 h post-transfection of scrambled siRNA or VEGFR2 siRNA or Sp1 siRNA. Antibodies against VEGFR2 and Sp1 were used. Normal rabbit IgG was used as control. Values are relative to control IgG background and normalized to an intergenic region. Data are mean \pm s.e.m. of triplicates and represents three independent experiments. (D) EMSA analysis of the *VEGFR2* promoter with EC nuclear extracts (lane 2) or VEGFR2 immunodepleted (ID VEGFR2) extract (lane 3) was conducted. Four complexes (C1-C4) are indicated with black arrows. A competitive assay using 100x excess of cold probe of VEGFR2 promoter was conducted (lane 4) using EC nuclear extract. Control lane 1 contains only the radiolabeled probe.

To evaluate the presence of VEGFR2 in the shifted complexes, we first tried a supershift assay using an antibody against VEGFR2, which failed to produce any change in the mobility of the shifted complexes (data not shown). As the VEGFR2 antibodies were active in immunoprecipitation experiments, we used an immunodepletion approach to evaluate the presence of VEGFR2 in the shifted complexes. Using these VEGFR2-immunodepleted nuclear extracts in the EMSA assays we observed an absence of the C2 complex and a strong reduction in the intensity of the C1 complex while the C3 and C4 complexes were not significantly altered (Figure 4D, lane 3). An IgG-depleted control extract did not affect the intensity of these complexes (Figure S2B). Simultaneously, the VEGFR2 depletion in the protein extracts was confirmed by western-blot (Figure S2A). These results are consistent with the presence of VEGFR2 in the C1 and C2 complexes. A similar experiment performed using Sp1-depleted extract showed a decrease in the intensity of C1 and C2 complexes (Figure S2B), suggesting that Sp1 and VEGFR2 are present in the same DNA/protein complexes.

Finally, we investigated the ability of the nuclear VEGFR2 to transcriptionally activate its own promoter using luciferase reporter assays.

For these experiments we used the 3T3 VEGFR2-GFP cells, which constitutively express VEGFR2, and compared to control 3T3 GFP cells that do not express VEGFR2 [12]. Transfection of a pGL3 control vector alone did not produce significant differences in luciferase activities in both cell lines (Figure 5A).

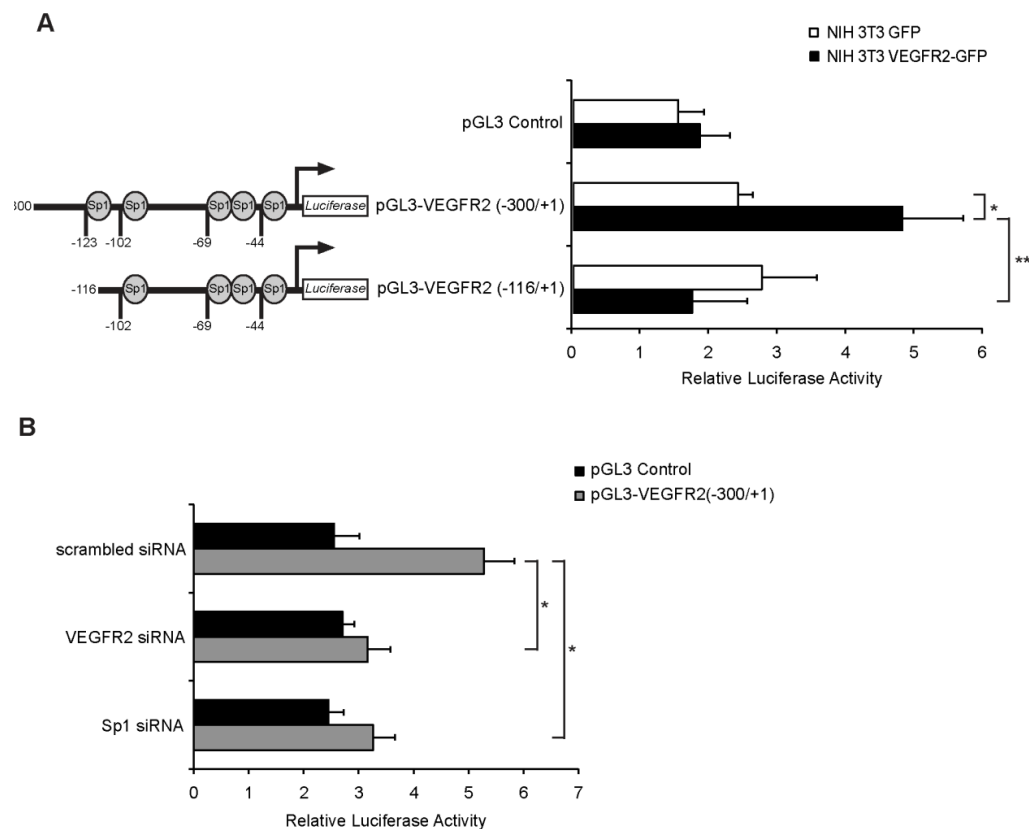


Figure 5. Nuclear VEGFR2 activates the human VEGFR2 proximal promoter.

(A) NIH 3T3 GFP and NIH 3T3 VEGFR2-GFP were transfected with pGL3 control or pGL3 VEGFR2 (-300/+1) or pGL3 VEGFR2 (-116/+1). The β -gal plasmid was co-transfected as a control. Promoter activities were measured with luciferase activity normalized to β -gal. The results are expressed as the relative luciferase activities. Data are mean \pm s.e.m. of relative luciferase activities from four independent experiments, each performed in triplicate. * p = 0.007; ** p = 0.001. (B) NIH 3T3 VEGFR2-GFP were transfected with scrambled siRNA, VEGFR2 siRNA or Sp1 siRNA. At 48 h post-transfection the relative luciferase activity of pGL3 control or pGL3 VEGFR2 (-300/+1) was measured. Data are mean \pm s.e.m. of relative luciferase activities from three independent experiments, each performed in triplicate, (* p = 0.003).

However, when we transfected a construct including the VEGFR2 proximal promoter spanning -300/+1, we observed a significantly higher luciferase

activity in the VEGFR2-GFP cells (4.8 fold \pm 0.88, $p=0.007$) compared to control cells (2.4 fold \pm 0.22), (Figure 5A). Specifically, we confirmed a reduction in luciferase activity in VEGFR2-GFP cells co-transfected with VEGFR2 siRNA when compared to scrambled siRNA-co-transfected cells (Figure 5B). Moreover, no increased luciferase activity over basal levels (1.74 fold \pm 0.8) was observed in the 3T3 VEGFR2-GFP cells, when transfected with a reporter construct containing a shorter fragment (-116/+1) of the *VEGFR2* promoter (Figure 5A). These results suggest that VEGFR2 is indeed able to activate transcription from its own promoter and that this activation requires the region between -300/-116 relative to the transcription start site.

Consistent with our previous findings where we found a decrease of the binding of VEGFR2 to its own promoter when the expression of Sp1 was reduced by siRNA (Figure 4C), we also observed a significant decrease of the luciferase activity in VEGFR2-GFP cells co-transfected with Sp1 siRNA (Figure 5B).

Taken together, these data strongly suggest a previously unrecognized function of nuclear VEGFR2 as a possible transcription factor involved in the regulation of its own transcription.

VEGFR2 BINDING TO ITS OWN PROMOTER IS DEPENDENT OF VEGFR2 ACTIVATION

To further analyze the possible functional relevance of the VEGFR2 binding to its own promoter, we took advantage of our finding that VEGFR2 nuclear translocation requires activation by VEGF [12]. We did not observe DNA binding of VEGFR2 (0.2 fold \pm 0.03) when ECs were cultured under basal medium (without supplements or serum), which is consistent with the absence of nuclear VEGFR2 in these culture conditions [12]. Also, the Sp1 binding was negligible under these conditions (2.3 fold \pm 0.13), (Figure 6A).

However, after 30 min of VEGF stimulation, we observed a strong increase in binding of VEGFR2 (159.6 fold \pm 5.21) to its own promoter (Figure 6A). In the same cells, we failed to observe an increment of Sp1 binding (1.03 fold \pm 0.17, relative to control IgG), (Figure 6A).

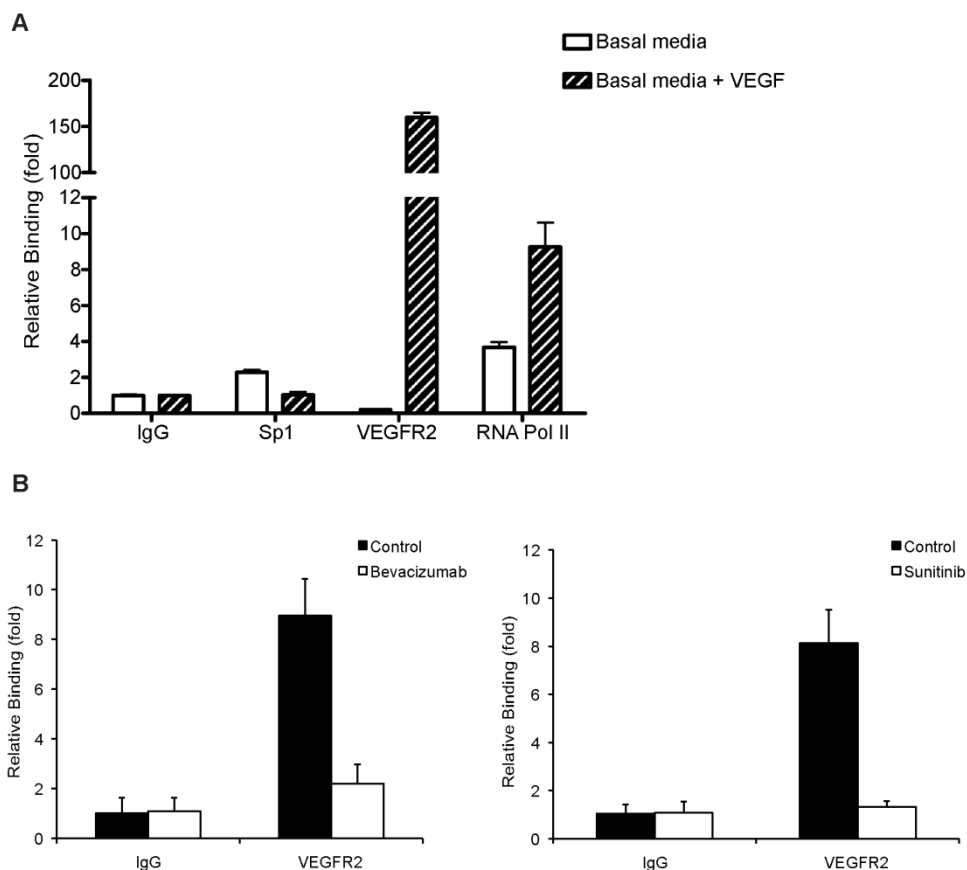


Figure 6. VEGFR2 binding to its own promoter is dependent of VEGFR2 activation.

ChIP assays of the *VEGFR2* proximal promoter were performed using (A) ECs cultured in basal medium for 48 h, and were left unstimulated or stimulated with VEGF (20 ng/ml) for 30 min. Antibodies against VEGFR2 and Sp1 were used. Normal rabbit/mouse IgG were used as control. Also, an antibody for RNA Pol II was used to test the promoter activity. Values are relative to control IgG background and normalized to an intergenic region. Data are mean \pm s.e.m. of triplicates and represents three independent experiments. (B) ECs cultured in growing media were left untreated or treated with 0.5 mg/ml Bevacizumab (left panel) or 0.1 μ M Sunitinib (right panel) for 16 h. In the Sunitinib experiments, DMSO was used as vehicle. ChIP values are relative to control IgG and normalized to an intergenic region. Data are mean \pm s.e.m. of triplicates and represents three independent experiments.

These results demonstrate that VEGFR2 activation by VEGF is crucial for VEGFR2 binding to its own promoter *in vivo*, suggesting that VEGFR2, as a nuclear protein, could be involved in amplifying the angiogenic response. To further explore this idea, we treated ECs with two anti-angiogenic agents,

which block the VEGFR2 activation, in order to evaluate their effect on VEGFR2 binding to its own promoter. Our results showed that both Bevacizumab, a monoclonal antibody against VEGF and Sunitinib, a VEGFR2 tyrosine kinase inhibitor, led to a strong reduction of the binding of VEGFR2 to its own promoter, as estimated by quantitative ChIP (Figure 6B).

These results were relatively specific to VEGFR2 inhibitors as no effect on VEGFR2 binding was detected when ECs were treated with Iressa, an epidermal growth factor receptor-tyrosine kinase inhibitor (Figure S3).

4.4. DISCUSSION

The classical view of signaling through VEGFR2 considers that the membrane receptor is phosphorylated upon ligand binding, activating intracellular signaling cascades that regulate a wide range of biological outcomes, including cellular survival, proliferation, migration and differentiation [2]. Until recently, internalization of VEGFR2 was thought to be the pathway for downregulation of the signaling through receptor degradation. Consistent with this, it was found that VEGFR2 is ubiquitinated by c-Cbl [16] and that activated PKC marks the receptor for internalization and degradation [17]. However, increasing evidence indicates that internalized VEGFR2 may also have signaling activity. For instance, it has been shown that phosphorylated VEGFR2 can be internalized in a VE-cadherin-dependent manner to endosomal compartments, retaining activation of signaling pathways and sustaining cell proliferation and migration, rather than receptor degradation [18]. Recently, it was shown that ephrinB2 activation controls VEGFR2 internalization, which is required for activation and downstream signaling of the receptor during vascular sprouting in physiological and pathological conditions [19,20]. We also demonstrated that the nuclear internalization of VEGFR2 is required for endothelial recovery following injury [12]. Finally, we and others found constitutive nuclear localization of VEGFR2 in proliferative tumor cells, suggesting that this protein may be involved in nuclear molecular mechanisms that contribute to tumor progression [7,8,9,10,11].

Taken together, these different studies suggest that the intracellular trafficking of VEGFR2 is linked to its signaling activity that contributes to the amplification of the angiogenic response.

In the present report we have shown in living ECs that VEGFR2 rapidly translocates to the nucleus and the VEGFR2 tyrosine residue Y951 plays an important role in this dynamic process. The role that VEGFR2 might play in the nucleus has remained undisclosed. Here, we show for the first time to our knowledge that nuclear VEGFR2 has transcriptional activity. In particular, we show that VEGFR2 binds to its own promoter in VEGFR2-activated ECs *in vivo* and that VEGFR2 can activate transcription from this promoter in reporter assays. These findings suggest that VEGFR2 might participate in the positive feedback regulation of its own expression. This is consistent with previous reports showing that VEGF binding to membrane VEGFR2 results in increased levels of VEGFR2 gene transcription and protein expression [21]. Similarly, it was observed that mechanoactivation produces translocation of VEGFR2 to the nucleus [22], which is accompanied by an upregulation of the VEGFR2 gene transcription [23].

Our results now indicate that this increase in VEGFR2 expression depends, at least in part on VEGFR2 transcriptional activity. Our EMSA data revealed the existence of different VEGFR2 containing complexes (C1 and C2 complexes) with different mobilities when bound to the promoter, suggesting that VEGFR2 interacts with additional molecules when bound to DNA. Our MS profiling data seems to support this idea. In particular, Sp1 stands out as one of the VEGFR2-interacting proteins in the nucleus of ECs. This is consistent with previous data showing that Sp1 is implicated in the transcriptional regulation of genes important to angiogenesis, including, VEGF and VEGFR2 [13,14,24]. These two molecules interact, as shown by co-immunoprecipitation experiments. Pull-down assay experiments using purified proteins further confirmed that the interaction between VEGFR2 and Sp1 is direct. Our EMSA data using Sp1 or VEGFR2 depleted extracts also indicate that Sp1 and VEGFR2 are present in the same protein-DNA complexes. Interestingly, the

-300/+1 bp region of the VEGFR2 promoter, identified as one of the key elements for the regulation of VEGFR2 expression [14,15,25,26], contains five Sp1 binding sites and is able to bind both Sp1 and VEGFR2. Our reporter assays show that the region between -300/-116 relative to transcription start site is essential to confer the VEGFR2 promoter VEGFR2-dependent transcriptional activity. Moreover, our results indicate that VEGFR2 is essential for the activity of its own promoter since cells that do not express VEGFR2 have significantly lower levels of the VEGFR2 promoter activity compared to VEGFR2-expressing cells. Accordingly, the levels of the VEGFR2 promoter activity observed in VEGFR2-expressing cells are significantly decreased if the expression of VEGFR2 is reduced by siRNA. However, at the moment we do not know if VEGFR2 binds to a consensus DNA sequence and the nature of this sequence. Clearly, identification of other transcriptional targets of VEGFR2 will help to address this issue. Also, understanding the mechanism of VEGFR2 transcriptional activity will require the complete identification of the molecules interacting with VEGFR2 at the promoters/enhancers. In ECs, binding of VEGFR2 to DNA requires VEGF-activation, since this binding cannot be detected in ECs cultured in the absence of this growth factor. Moreover, ECs treated with anti-angiogenic agents that block VEGFR2 activation present negligible levels of VEGFR2 DNA binding activity. This is consistent with the finding that the nuclear translocation of the receptor is dependent of VEGF activation [12], and further supports the idea that nuclear translocation/transcriptional activity of VEGFR2 is an integral part of the signaling mediated by this receptor.

While in ECs VEGFR2 nuclear translocation and consequently its DNA binding depends on VEGFR2 activation by VEGF, it has been reported that some tumor cells present constitutive nuclear localization of VEGFR2. If these tumor cells also present constitutive VEGFR2 transcriptional activity, this could result in resistance to anti-VEGF therapies. Several anti-VEGF strategies have been developed, including neutralizing antibodies to VEGF such as Bevacizumab [5] or tyrosine kinase inhibitors such as Sunitinib [6]. Their goal is to block the

VEGF signaling mediated by the membrane receptor VEGFR2. However, these anti-angiogenic inhibitors are not effective in all tumors, and the benefit even for responding patients is usually modest, indicating that mechanisms of intrinsic and evasive resistance to anti-VEGF therapy do exist [27]. Therefore, the analysis of VEGFR2 transcriptional activity in those tumors and the identification of the target genes will surely help to better understand its role in tumor angiogenesis and to devise novel therapeutic approaches.

In conclusion, our findings provide novel insights into the role of VEGFR2 as a nuclear protein. Here, we demonstrate that in VEGF-activated ECs, nuclear VEGFR2 may act as a transcription factor by binding to and activating its own promoter. By this mechanism nuclear VEGFR2 could be involved in amplifying the angiogenic response.

4.5. MATERIALS AND METHODS

REAGENTS

VEGF (20 ng/ml) was purchased from Sigma-Aldrich, USA. Bevacizumab (Avastin®, Genentech/ Roche, USA), (0.5 mg/ml) was provided by the Oncology Service of Santa Maria Hospital. Sunitinib (0.1 μ M) was provided by Pfizer International, USA. Iressa (0.1 μ M) was purchased from Tocris Bioscience, UK. 5-fluorouracil (5-FU, 2 mM) was purchased from Sigma-Aldrich, USA.

CELL CULTURE

Primary Human Umbilical Vein Endothelial Cells (HUVEC referred as ECs) were kindly provided by Dr Shahin Rafii (Cornell University Medical College, New York, USA). ECs, passage 4-8, were cultured in 0.02 % gelatin-coated dishes in growing endothelial medium (basal EBM-2 medium supplemented with EGM-2 singlequots, BBE and 5 % of Fetal Bovine Serum (FBS)) as provided by the manufacturers (Lonza, USA). In basal media experiments, upon reaching confluence ECs were cultured in basal EBM-2 medium for 48 h. HEK-293 T cells were cultured in DMEM (Invitrogen Corporation, USA)

supplemented with 10 % FBS. NIH 3T3 GFP and NIH 3T3 VEGFR2-GFP cells were described and characterized in [12] and were cultured in DMEM supplemented with 10 % FBS and 800 $\mu\text{g/ml}$ Neomycin-G418 (Invitrogen Corporation, USA).

GENERATION OF LENTIVIRAL VECTORS EXPRESSING VEGFR2

The lentiviral vector FU IRES GFP was generated by replacing the GFP of FUGW with the IRES GFP from pIRES GFP (Stratagene Inc., USA). FU-VEGFR2 IRES GFP was generated by releasing full length VEGFR2 from pSP73-VEGFR2 using *KpnI*/*XhoI* restriction sites and cloned in FU-IRES GFP using the *Bam*HI restriction site by blunt-end cloning. All constructs were confirmed by DNA sequencing.

LENTIVIRAL PRODUCTION

Lentiviral particles were obtained with the transfection of HEK-293T cells using a standard calcium phosphate precipitation protocol. HEK-293T cells (≈ 50 % confluent) were transfected with the lentiviral vector plasmids FU-IRES GFP or FU-VEGFR2 IRES GFP together with the HIV-1 packaging vector Delta 8.9 and the VSV-g envelope glycoprotein. The viral supernatants were collected 60 h post-transfection and filtered through a 0.45 μm pore size filter. ECs were seeded at 7.5×10^4 cells (12 well plate) 24 h before transduction and then exposed to 500 μl of virus supernatant (supplemented with polybrene to a final concentration of 4 $\mu\text{g/ml}$). 72 h post-infection the GFP positive cells were sorted by FACS Aria (Becton, Dickinson and Company, USA). ECs expressing FU-IRES GFP were named EC IRES GFP; ECs expressing FU-VEGFR2 IRES GFP were named EC VEGFR2 IRES GFP.

IMMUNOFLUORESCENCE AND CONFOCAL MICROSCOPY

ECs were cultured on gelatin-coated glass coverslips. The cells were fixed in 1% (v/v) formaldehyde/ PBS, for 10 min, at 4 °C and washed in PBS. After permeabilization with 0.1% (v/v) Triton X-100 plus 5% (v/v) normal serum, cells were incubated in different conditions with the following antibodies:

VEGFR2 (Santa Cruz Biotechnology, USA) at 4 °C, overnight, followed by incubation with Alexa Fluor 594 (Molecular Probes, Invitrogen Corporation, USA) for an additional hour, at room temperature or BrdU (Sigma-Aldrich, USA), for 30 min, at room temperature, followed by incubation with Alexa Fluor 488 (Molecular Probes, Invitrogen Corporation, USA) for additional 30 min, at room temperature. The samples were mounted in Vectashield (Vector Laboratories, USA) and analyzed by confocal microscopy. Sets of optical sections of 5 µm intervals along the Z-axis (from bottom to top of cells) were acquired on a Zeiss LSM 510 META (Carl Zeiss, Germany) inverted laser scanning confocal microscope using a PlanApochromat 63x/1.4 oil immersion objective. Alexa Fluor 488 and GFP fluorescence were detected using the 488 nm line of an Ar laser (45 mW nominal output) and a BP 505-550 nm filter. Alexa Fluor 594 fluorescence was detected using a 594 nm HeNe laser (2 mW nominal output) and a LP 615 nm filter. Potential bleed-through from the different fluorophores was avoided by performing sequential multi-track/frame imaging sequences. Z-projections were obtained using ImageJ (<http://rsbweb.nih.gov/ij/>).

5-FU INCORPORATION

ECs were cultured in growing media on gelatin-coated glass coverslips, treated with 6.12 Ab (1 µg/ml) and scrambled or VEGFR2 siRNA following incubation with 5-FU (2 mM) for 15 min. The cells were fixed, permeabilized, and sequentially labeled for BrdU and VEGFR2 according to the immunofluorescence protocol described above.

RNA INTERFERENCE

SMART pool siRNA targeting human *VEGFR2* or human *Sp1* and non-targeting pool siRNA (scrambled siRNA) were purchased from Dharmacon (UK). Knockdown of *VEGFR2* or *Sp1* was performed according to the manufacturer's recommendation. Briefly, ECs were transfected with VEGFR2, Sp1 or scrambled siRNA (50 nM) using the Dharmafect 4 reagent

(Dharmacon, UK). After 24 h, cells were used in qRT-PCR, ChIP, 5FU-incorporation or luciferase assays.

CELL FRACTIONATION AND WESTERN BLOT ANALYSIS

Nuclear and cytoplasmic protein extracts were prepared as described [12]. Equal amounts of protein extracts were separated by SDS-PAGE, transferred to nitrocellulose and probed with antibodies against VEGFR2, P-I κ B (both from Cell Signaling Technology Inc., USA), Cyclin A, Sp1, p65, YY1, Lamin B (all from Santa Cruz Biotechnology, USA)

IMMUNOPRECIPITATION

Nuclear extracts were pre-cleared with 25 μ l of protein G-Sepharose beads (Sigma-Aldrich, USA). Nuclear protein supernatants were incubated with antibodies against VEGFR2 (Cell Signaling Technology Inc., USA) or Sp1 (Santa Cruz Biotechnology, USA) and rabbit control IgG (Santa Cruz Biotechnology, USA) overnight, at 4 °C, and incubated with protein G-Sepharose beads for an additional hour, at 4 °C. Beads were washed once in a lysis buffer containing 500 mM NaCl and twice in a lysis buffer containing 150 mM NaCl. Beads were resuspended in SDS loading buffer and boiled for 5 min. Samples were separated by SDS-PAGE followed by Western blot analysis or silver staining for mass spectrometry analysis.

PULL-DOWN ASSAYS

The GST fusion protein containing amino acids between 789-1356 of human VEGFR2 (VEGFR2 (789-1356)-GST) was purchased from Sigma-Aldrich, USA. The Sp1 protein fused to a HA-tag (Sp1-HA) was obtained from Enzo Life Sciences, USA. For pull-down assays, 3 μ g of Sp1-HA were incubated with glutathione-sepharose 4B beads (GE Healthcare, USA) for 1 h, at 4 °C, in binding buffer containing 50 mM Tris-HCl pH7.5, 150 mM NaCl, 10% Glycerol, 1% NP-40, 1 mM orthovanadate and complete protease inhibitors. The beads were spin down and the pre-cleared supernatant was incubated with 3 μ g of purified GST or VEGFR2 (789-1356)-GST proteins overnight, at 4 °C. Peptide

complexes were recovered with 20 μ l of glutathione-sepharose beads for 1 h, at 4 °C. The supernatants were kept as the unbound fractions (UB) and the beads were washed eight times in the binding buffer. Protein were eluted from the beads in reducing laemmli's buffer, resolved by SDS-PAGE, transferred to nitrocellulose and analyzed by Western blot with the indicated antibodies.

MASS SPECTROMETRY

1D SDS-PAGE gel lanes were cut into 2 mm slices using an automatic gel slicer and subjected to in-gel reduction with dithiothreitol, alkylation with iodoacetamide and digestion with trypsin (Promega Corporation, USA, sequencing grade), essentially as described [28]. Nanoflow LC-MS/MS was performed on an 1100 series capillary LC system (Agilent Technologies, USA) coupled to an LTQ linear ion trap mass spectrometer (Thermo Fisher Scientific Inc) operating in positive mode and equipped with a nanospray source. Peptide mixtures were trapped on a ReproSil C18 reversed phase column (Dr Maisch GmbH; column dimensions 1.5 cm \times 100 μ m, packed in-house) at a flow rate of 8 μ l/min. Peptide separation was performed on ReproSil C18 reversed phase column (Dr Maisch GmbH; column dimensions 15 cm \times 50 μ m, packed in-house) using a linear gradient from 0 to 80 % B (A = 0.1 % formic acid; B = 80 % (v/v) acetonitrile, 0.1 % formic acid) in 70 min and at a constant flow rate of 200 nl/min using a splitter. The column eluent was directly sprayed into the ESI source of the mass spectrometer. Mass spectra were acquired in continuum mode; fragmentation of the peptides was performed in data-dependent mode. Peak lists were automatically created from raw data files using the Mascot Distiller software (version 2.1; MatrixScience). The Mascot search algorithm (version 2.2, MatrixScience) was used for searching against the SwissProt database (release SwissProt_54.8.fasta; taxonomy: mammalian). The peptide tolerance was typically set to 2 Da and the fragment ion tolerance was set to 0.8 Da. A maximum number of 2 missed cleavages by trypsin were allowed and carbamidomethylated cysteine and oxidized methionine were set as fixed and

variable modifications, respectively. The Mascot score cut-off value for a positive protein hit was set to 60. Individual peptide MS/MS spectra with Mascot scores below 40 were checked manually and either interpreted as valid identifications or discarded. Typical contaminants, also present in immunoprecipitations using beads coated with pre-immune serum or antibodies directed against irrelevant proteins were omitted from the table. The proteins identified were further analyzed with the Ingenuity Pathway Analysis Software (Ingenuity Systems, Inc., USA) and clustered according to their involvement in different biological functions. The enriched categories obtained were represented according to their *p*-value.

CHROMATIN IMMUNOPRECIPITATION (CHIP)

5×10^8 ECs were fixed with 1 % formaldehyde for 10 min at 37 °C and subjected to quantitative ChIP analysis as previously described [29]. Briefly, 5 µg of the specific antibodies were pre-bound overnight, at 4 °C, to protein G-Dynal magnetic beads (Invitrogen Corporation, USA), added to the diluted sonicated chromatin (4x 20 s, 50% output in Soniprep 150, Sanyo) and immunoprecipitated overnight, at 4 °C. Antibodies used were as follows: VEGFR2 (Cell Signaling Technology, Inc., USA), Sp1 (Santa Cruz Biotechnology, USA), RNA Polymerase II (Covance, USA) and Rabbit/Mouse IgG (Santa Cruz Biotechnology, USA).

The magnetic bead-chromatin complexes were collected and washed in RIPA buffer (containing 50 mM HEPES pH 7.6, 1 mM EDTA, 0.7 % Na deoxycholate, 1 % NP-40, 500 mM LiCl). Chromatin-protein complexes were further washed in 1x TE buffer, eluted from beads in 1 % SDS, 100 mM NaHCO₃ and heated overnight at 65 °C to reverse the formaldehyde cross-linking. DNA fragments were purified with a QIAquick Spin Kit (QIAGEN, Germany). The DNA amount of *VEGFR2* gene immunoprecipitated was quantified by RT-PCR using primers designed for the amplification of the *VEGFR2* proximal promoter (-300/-159 relative to the transcription start site). The primers used were as follows: 5' CCGGCAAGCGATTAAATCTTGGAG 3' (sense) and 5' TTTCCCCACACAACACTGGACTGC 3' (antisense). Additionally

were used primers for the amplification of an intergenic region in chromosome 10 as described [30]. The PCR reaction mixture used was as follows for a 25 μ l total volume: 1x SybrGreen (Applied Biosystems, USA), 100 nM of each primer, and 2.5 μ l of each ChIP DNA sample (input 1:10). All reactions were performed and analysed as triplicates using a Fast 7500 Real time PCR (Applied Biosystems, USA). The results were normalized based on the $\Delta\Delta$ Ct method as previously described [29,30,31]. Briefly, the threshold cycles (Ct) from total input samples were subtracted from the Ct of the IgG control and from the experimental IP (VEGFR2, Sp1 and RNA Pol II). The fold difference between the corrected value for the total input and corrected experimental IP value was calculated as $2^{\Delta\Delta$ Ct. The fold difference over background obtained for *VEGFR2* promoter was further normalized to the value obtained for the intergenic region.

QUANTITATIVE RT-PCR

Total RNA and cDNA were prepared and quantitative RT-PCR (qRT-PCR) was performed as described [12]. *VEGFR2* primer sequences were as follows: 5' ATTCTCCCCCGCATCA 3' (sense) and 5' GCTCGTTGGCGCACTCTT 3' (antisense). *Sp1* primer sequences were as follows: 5' TCGGATGAGCTACAG AGGCACAAA 3' (sense) and 5' AAAGTGCCACACTCAGAGCTACA 3' (antisense).

ELECTROFORETIC MOBILITY SHIFT ASSAYS (EMSA) AND IMMUNODEPLETING EMSA (IDEMSA)

The oligonucleotides including consensus recognition sequence for TFs YY1, NF κ B and Sp1 are derived from Transcruz gel shift oligonucleotides (SantaCruz Biotechnology, USA). A DNA probe comprising the same region of the *VEGFR2* promoter (-300/-159) amplified in ChIP assays was also used. EMSA were performed following standard methodology as described [9]. Probes were labeled with γ -ATP 32P (Perkin Elmer, USA) and incubated for 20 min at room temperature with 10 μ g of EC nuclear extracts in a binding buffer (containing 10 mM HEPES, 4% Ficoll, 70 nM NaCl, 2 mM DTT,

100 µg/mL bovine serum albumin and 0.01% NP40). For competition assays, a 100-fold molar excess of unlabeled probe was incubated in the binding reaction. For supershift assays, 1 µg of the p65 or YY1 antibodies were added to the binding reaction for an additional 30 min at room temperature. DNA-protein complexes were resolved in 5 % non-denaturing polyacrilamide gels. IDEMSA were performed as described [32]. Briefly, 30 µg of EC nuclear extracts were depleted of VEGFR2 by immunoprecipitation with anti-human VEGFR2 antibody for 1 h followed by incubation with sepharose beads for an additional hour, at 4 °C. After centrifugation, 10 µg of the VEGFR2 immunodepleted supernatant were used for each reaction of EMSA/Supershift as described above.

LUCIFERASE REPORTER ASSAYS

The proximal promoter of *VEGFR2* gene was amplified from genomic DNA by PCR and cloned into a pGL3-promoter firefly luciferase vector (Promega Corporation, USA). Briefly, the sequence from -300/+1 of the *VEGFR2* proximal promoter was amplified using oligonucleotides with flanking restriction enzyme sites 5' *Xho*I and 3' *Bgl*II. The primers sequences were as follows: FW: 5' AAGCTCTCGAGGGTTAATTAAGACCGCAAGCGATTAAATCTTGGAG 3'; RV: 5' AGATCTTTAGATCTGTAGCAGGGTGGGAGCTGGTGCCGA 3'. A deletion fragment of the region from -116/+1 bp of the *VEGFR2* promoter was obtained using the same procedure. The primer sequence was as follows: FW: 5' AGCTCTCGAGGGTTAATTAAGGTACCCGGGTGAGGGGCCGGGCT 3'. All constructs were confirmed by DNA sequencing. For luciferase reporter gene assays, NIH 3T3 GFP and NIH 3T3 VEGFR2-GFP expressing cells growing in 24-well plates were co-transfected with 400 ng of pGL3 control or pGL3 VEGFR2 (-300/+1) or pGL3 VEGFR2 (-116/+1) and 40 ng of the pCMV-βgal (Clontech Laboratories, Inc., USA) using the Fugene 6 transfection reagent (Roche Applied Science, USA). 24 h post-transfection, cells were harvested and luciferase activity was measured using Luciferase Assay Reagent (Promega Corporation, USA). β-galactosidase activity was

measured using Trophic Reagent (Applied Biosystems, USA). Results were normalized by dividing the luciferase activity values for β -galactosidase activity values and represented as relative luciferase activity. Data are mean \pm s.e.m. of triplicates and represents three independent experiments.

STATISTICAL ANALYSIS

Data are represented as the mean \pm s.e.m., and statistical analysis was performed with Student's *t* test. A *p* value < 0.05 was considered statistically significant.

4.6. ACKNOWLEDGMENTS

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4.8. SUPPLEMENTARY RESULTS

VEGFR2 AND SP1 KNOCKDOWN USING siRNA

We experimentally reduced the expression of VEGFR2 and Sp1 using siRNA technology. A pool of VEGFR2 siRNA oligos effectively abrogated the *VEGFR2* expression by 70% compared with the scrambled siRNA, in ECs as evaluated by qRT-PCR (Figure S1A). Similar results were obtained using a pool of Sp1 siRNA oligos and compared to scrambled siRNA (Figure S1B).

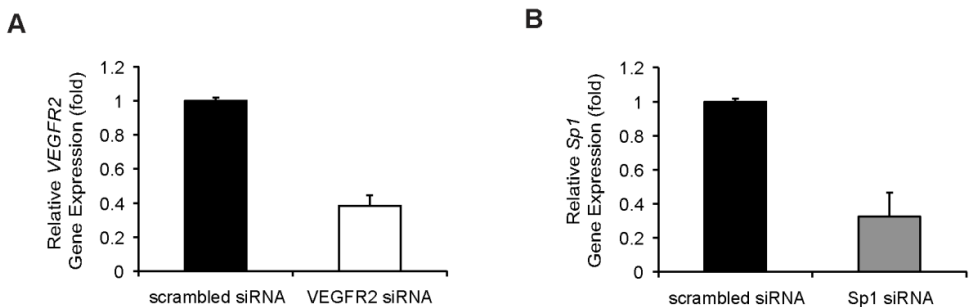


Figure S1. *VEGFR2* and *Sp1* relative gene expression is decreased after *VEGFR2* or *Sp1* siRNA transfection.

ECs were transfected with scrambled siRNA, VEGFR2 siRNA or Sp1 siRNA and 24 h later the (A) *VEGFR2* or (B) *Sp1* mRNA was quantified by qRT-PCR. Data are mean \pm SD and represents the fold change in *VEGFR2* or *Sp1* gene expression relative to the internal calibrator (scrambled siRNA) in triplicates measurements and are representative of three independent experiments.

VEGFR2 AND SP1 ARE PRESENT IN THE SAME PROTEIN/DNA COMPLEXES

We have observed in EMSA assays that VEGFR2-immunodepleted nuclear extracts failed to form the C2 complex and showed a decreased intensity in C1 complex (please see Figure 4D). These results confirmed the presence of VEGFR2 in the DNA/protein complexes established with the *VEGFR2* promoter region analyzed in the EMSA experiments. The VEGFR2 depletion was confirmed by Western-blot analysis (Figure S2A).

Furthermore, we confirmed that these results were specific, since the same experiment using IgG immunodepleted extracts had no effect in the intensity of the complexes observed (Figure S2B). Also, we observed that Sp1-depleted extracts showed a decreased intensity in the complexes C1 and C2,

confirming that both VEGFR2 and Sp1 are present in the same DNA/ protein complexes in the promoter region of *VEGFR2* analyzed (Figure S2B).

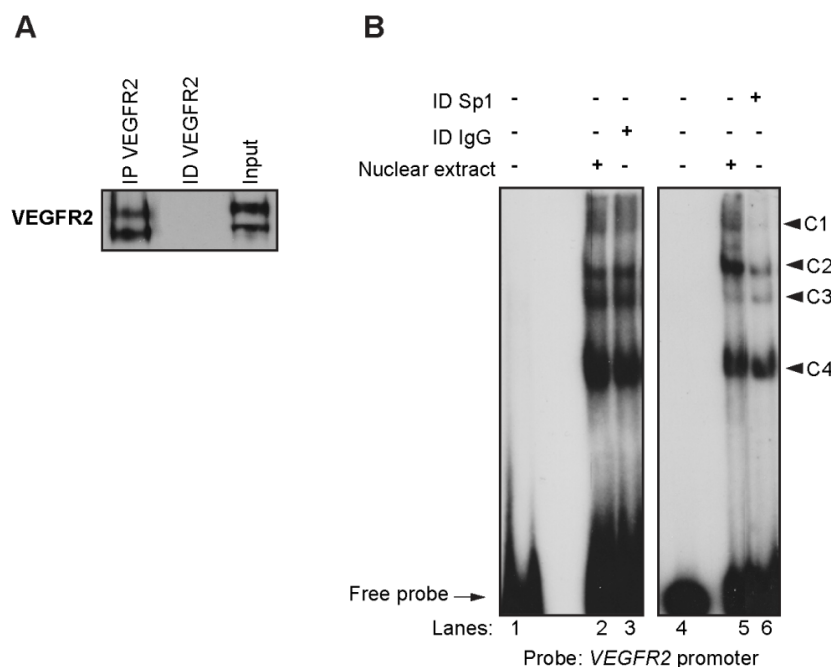


Figure S2. Sp1 and VEGFR2 are present in the same protein/DNA complexes.

(A) Immunoprecipitation (IP) of 30 μ g EC nuclear extract with anti-human VEGFR2 were analyzed by Western-blot. The immunodepleted extract (ID VEGFR2) and Input were also included as control. (B) EMSA analysis of the VEGFR2 promoter with IgG-immunodepleted (ID Rabbit IgG) (lane 3) or Sp1-immunodepleted (ID Sp1) extracts were conducted. As a positive control EC nuclear extracts (lane 2, 5) were also evaluated. Four complexes (C1-C4) are indicated with black arrows. Control lanes 1 and 4 contain only the radiolabeled probe.

VEGFR2 BINDING TO ITS OWN PROMOTER IS INDEPENDENT OF EGFR ACTIVATION

Our previous results showed that VEGFR2 activation by VEGF is crucial for VEGFR2 binding to its own promoter in vivo. We showed that both Bevacizumab, and Sunitinib, led to a strong reduction of the binding of VEGFR2 to its own promoter, further supporting this idea. To further validate the specific effect of the VEGFR2 inhibitors on the VEGFR2 DNA binding, we tested the effect of an epidermal growth factor receptor-tyrosine kinase inhibitor, Iressa in ECs, by quantitative ChIP. Our results showed no difference in the DNA binding of VEGFR2 to its own promoter in the presence or

absence of Iressa further supporting the specific effect of the VEGFR2 inhibitors used previously (Figure S3).

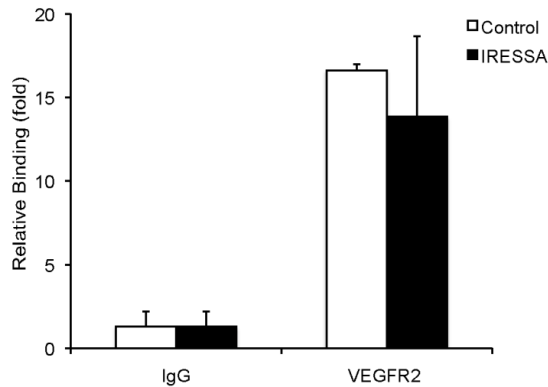


Figure S3. VEGFR2 binding to its own promoter is independent of EGFR activation.

ChIP assays of the *VEGFR2* proximal promoter were performed using ECs cultured in growing media and treated or not with 0.1 μ M Iressa for 16 h. Ethanol was used as vehicle in the control cells. ChIP values are relative to control IgG background and normalized to an intergenic region. Data are mean \pm s.e.m. of triplicates and represents three independent experiments.

VEGFR2 PUTATIVE INTERACTIONS IN THE NUCLEUS OF ECs

The proteins identified by MS as putative interacting partners with nuclear VEGFR2 with a Mascot score greater than 200 are listed in Table S1 (Appendix A). The most significant results analyzed above (Figure 3B) with IPA software refer to proteins involved in gene expression such as, Transcription intermediary factor 1 β (TRIM28), Nucleolysin TIAR (TIAL1), TATA element modulatory factor (TMF1), Myb-binding protein 1A (MYBBP1A) or AT-rich interactive domain-containing protein 4A (ARID4A), among others. Other proteins involved in DNA replication, recombination, and repair such as, Breast cancer type 1 susceptibility protein (BRCA1) and Serine-protein kinase ATM (ATM), were also identified. In the future these results will be confirmed by western blot on VEGFR2 immunoprecipitates.

Our results also suggest the involvement of VEGFR2 in RNA post-transcriptional modifications as putative interactions with splicing factors U2AF35 and U2AF65 were identified in our MS analysis. However, we were unable to confirm an interaction between VEGFR2 and these factors in

VEGFR2 immunoprecipitates (Figure S4A). Also, the presence of VEGFR2 was not observed in U2AF65 immunoprecipitates (Figure S4B).

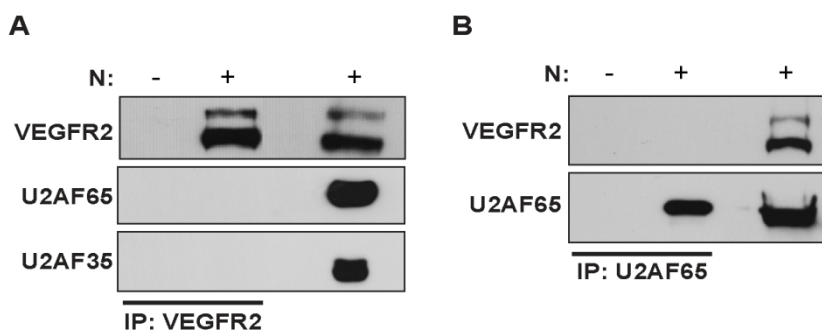


Figure S4. VEGFR2 does not interact with U2AF35 and U2AF65.

(A) Immunoprecipitation (IP) of 1 mg EC nuclear extract was conducted with anti-human VEGFR2 followed by western blotting using the anti-human VEGFR2, anti-human U2AF65 (Sigma-Aldrich) and anti-U2AF35 (ProteinTech Group, Inc., USA) antibodies. VEGFR2 antibody plus beads (without N) were used as negative control for immunoprecipitation. 100 μ g of non-immunoprecipitated EC nuclear extract were included as control. (B) Immunoprecipitation (IP) of 1 mg EC nuclear extract was conducted with anti-human U2AF65 followed by western blotting using the anti-human VEGFR2 and anti-U2AF65. U2AF65 antibody plus beads (without N) were used as negative control for immunoprecipitation. 100 μ g of non-immunoprecipitated EC nuclear extract (Input) were included as control.

VEGFR2 NUCLEAR LEVELS ARE ASSOCIATED WITH DIFFERENTIAL TRANSCRIPTIONAL PATTERNS

We showed that EC transcription levels correlate with VEGFR2 nuclear accumulation (please see Figure 2C and 2D). Based on these results, we investigated if VEGFR2 nuclear accumulation could also be correlated with changes in the DNA binding activity of transcription factors (TFs) involved in cell growth.

With this objective, ECs were treated with 6.12 Ab or KDRi, since we showed that ECs cultured in the presence of these agents present decreased VEGFR2 nuclear levels compared to untreated cells. The respective nuclear cell extracts were used to screen the profile activities of several TFs by performing a “TranSignal Protein/DNA Array Cell Growth” (Panomics, USA). Briefly, the nuclear extracts were incubated, in a binding buffer, with a set of biotinylated probes comprising DNA binding consensus sequences for the TFs analyzed

(Figure S5A, left panel), allowing the formation of DNA/protein complexes. Next, the DNA/protein complexes formed were purified from the free probes and hybridized to the TranSignal array membrane. The results were detected by HRP-based chemiluminescence according to the manufacturer's instructions and quantified using ImageJ software (NIH, USA). The results were expressed as a ratio between 6.12Ab or KDRi and control. We observed that the majority of the TFs analyzed, such as c-Myb, CREB, E2F1, EGR, FKHR, YY1, NF κ B, pax-2 and pax-3, showed a significantly increased DNA binding activity (> 2 fold), in cells treated with 6.12 Ab or KDRi (Figure S5B). Surprisingly, these results do not reflect the results obtained with the 5-FU incorporation assay, suggesting that in the same culture conditions, these TFs are differently modulated compared to the general transcription reduction observed before. To confirm the results obtained from our TranSignal array, we performed EMSA, using the same nuclear extracts (Figure S5C, S5D and S5E). Briefly, we analyzed the DNA binding activity of CREB (Figure S5C), YY1 (Figure S5D) or NF κ B (Figure S5E) using a DNA radiolabeled probe comprising the consensus binding sequences of each of these TFs incubated with the nuclear extracts obtained above.

We observed a slight increase in the DNA binding of CREB, YY1 and NF κ B in cells treated with 6.12 Ab (Figure S5C, S5D and S5E). We were unable to observe a significant change in the DNA binding activity for these TFs in cells treated with KDRi (Figure S5C, D and E). Taken together, the results obtained with the EMSA do not confirm the results obtained with the TranSignal array. Since we used the same nuclear extracts, we expected to obtain similar results. Following the loss of consistency in the results, both technical approaches should be repeated using other nuclear extracts. However, both approaches imply significant costs. Moreover, the results obtained here are the consequence of ECs treated with agents that may have undesired effects in other cell targets. Therefore, the apparent contradictory results may also be due to indirect effects in unidentified molecular targets, making it difficult to interpret the results. For these reasons, we developed a different approach to

modulate the VEGFR2 nuclear accumulation in ECs. ECs overexpressing VEGFR2 and several tyrosine point mutants were generated.

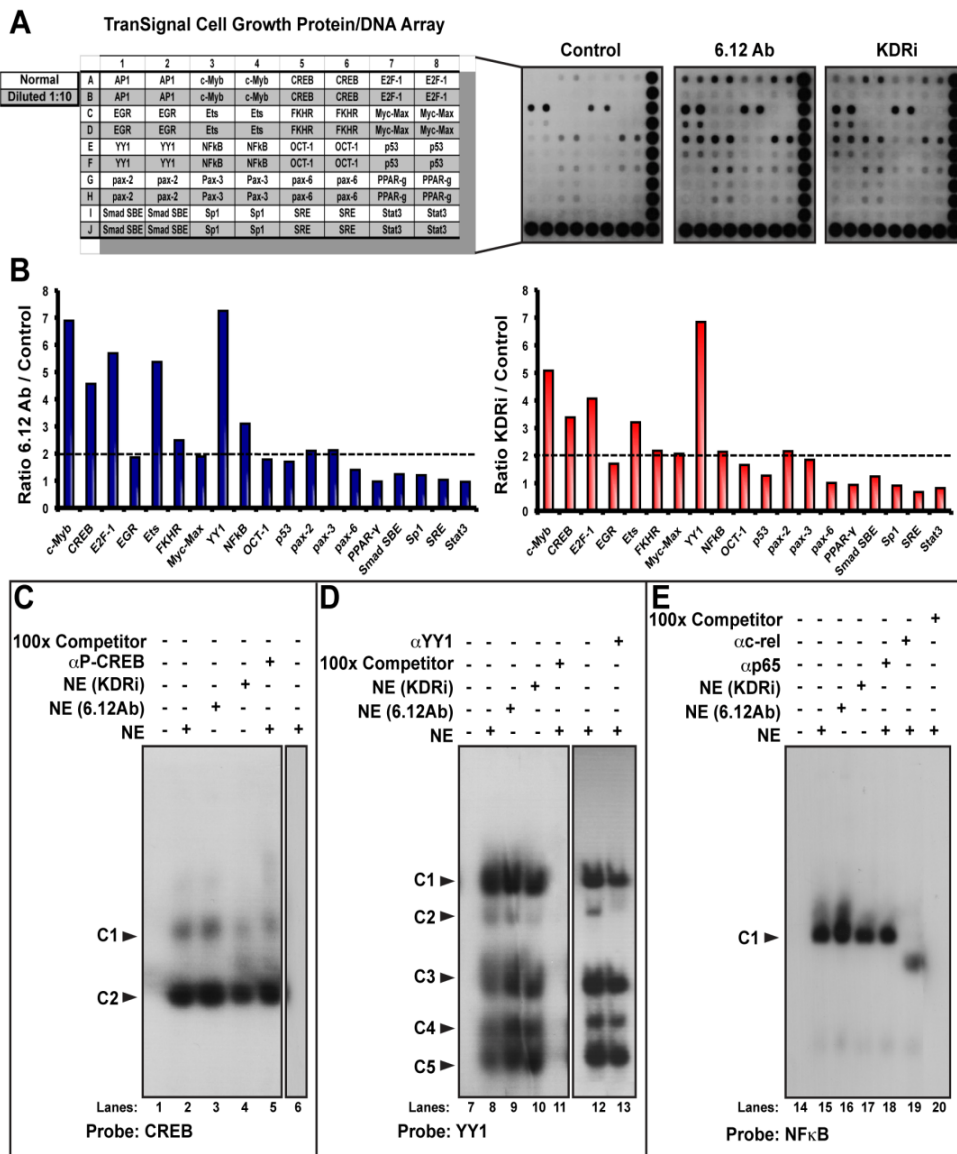


Figure S5. The activity and expression of several transcription factors (TFs) is modulated with the nuclear accumulation of VEGFR2.

Nuclear extracts were prepared from ECs cultured in growing media conditions and treated with 6.12 Ab (1 $\mu\text{g/ml}$) or KDRi (70 nM) for 24 h or left untreated (control). 25 μg of nuclear extract from each condition were used for the “TranSignal Protein/DNA Array Cell Growth” (Panomics, USA). (A) Schematic diagram of the array membrane (left panel). Each spot contains the specific DNA consensus binding sequence for the TF indicated and in duplicate. The grey spots for each indicated TF contain the same DNA sequence diluted 1:10. The grey columns on the right and bottom sides are spotted with biotinylated DNA and were used for alignment of the membranes (Adapted from manufacturer protocol). The TFs activities profiles for the experimental conditions tested – “Control”, “6.12 Ab” and “KDRi” at 15 s exposure (right panel). (B) The intensity of each spot in the three membranes was quantified using Image J and normalized for the background intensity. The TF DNA binding activities obtained for the three conditions were compared and represented as the ratio between 6.12 Ab and control (left panel) or ratio between KDRi and control (right panel). (C-E) The DNA binding activity of CREB (C), YY1 (D) or NF κ B (E) was confirmed by EMSA experiments using 10 μg of the same EC nuclear extracts used in the array. Nuclear extracts from control (lanes 2,8,15), 6.12 Ab (lanes 3,9,16) or KDRi (lanes 4,10,17) were incubated with CREB (C), NF κ B (D) or YY1 (E) radiolabeled probes. Two CREB complexes (C1-C2), five YY1 complexes (C1-C5) and two NF κ B (C1-C2) are indicated with black arrows. Specific anti-P-CREB (lane 5), anti-YY1 (lane 13), anti-p65 (lane 18) or anti-c-rel (lane 19) were introduced in the binding reaction to analyze the appearance of a supershift complex. A competitive assay using 100x excess of cold probe of CREB (lane 6), YY1 (lane 11) or NF κ B (lane 20) was performed. Control lanes 1 and 6 contain only the radiolabeled probes.

VEGFR2 DNA BINDING IN ECs IS NOT POTENTIATED DURING HYPOXIA

We have described above the VEGFR2 nuclear translocation and DNA binding activity upon VEGF stimulation. Since VEGF expression is upregulated during hypoxia [1], we tested if in these conditions, the VEGFR2 DNA binding is potentiated. Our hypothesis is that the increased VEGF expression could result in increased VEGFR2 activation and consequently, increased VEGFR2 DNA binding activity. Accordingly, we analyzed by quantitative CHIP the VEGFR2 DNA binding activity of ECs cultured in growing media conditions and treated with cobalt chloride (CoCl₂- chemically mimetizes hypoxia by stabilization and activation of HIF proteins), (Figure S6). We observed that under hypoxic conditions, the VEGFR2 DNA binding was not significantly increased, when compared to control normoxic conditions (Normoxia – 4.81 ± 0.84 vs Hypoxia – 5.14 ± 0.62).

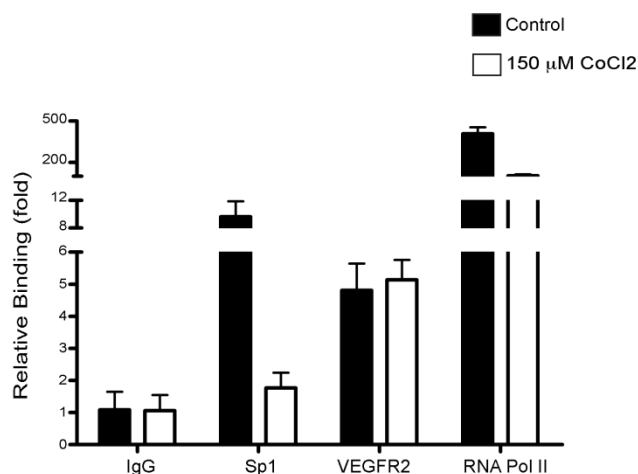


Figure S6. VEGFR2 DNA binding is not significantly increased during hypoxia.

ChIP assays of the *VEGFR2* proximal promoter under hypoxic conditions were performed using ECs cultured in growing media and left untreated or treated with 150 μ M CoCl₂ for 36 h. Antibodies against VEGFR2 and Sp1 were used. Normal rabbit/mouse IgG were used as control. Also, an antibody against human RNA Polymerase II was used to test the promoter activity. All values are relative to control IgG background and normalized to an intergenic region. Data are mean \pm s.e.m. of triplicates and represents three independent experiments

Our results also showed that Sp1 DNA binding was decreased in hypoxia when compared to normoxia culture conditions (Normoxia – 9.62 ± 2.25 vs Hypoxia – 1.76 ± 0.48). However, to confirm these results, it would be required to measure the VEGF levels after CoCl₂ treatment. A 4 fold increased *VEGF* expression was reported with 150 μ M CoCl₂ [2]. It would also be interesting to analyze *VEGFR1* expression in the same culture conditions since it was reported that not only *VEGF* but also *VEGFR1* gene expression is directly upregulated in ECs exposed to hypoxia [3]. This suggests that although the VEGF levels might be increased under these hypoxic conditions, and therefore an increased VEGFR2 activation/nuclear translocation is expected, VEGFR1 levels might also be increased, suggesting that VEGF sequestration and consequently a lower VEGF accessibility to VEGFR2 might occur. It has not been identified a HIF regulatory element in the *VEGFR2* promoter region [3], and the transcriptional *VEGFR2* expression was not changed in

ECs exposed to hypoxia [3]. Therefore, a direct hypoxic-mediated effect in *VEGFR2* expression was not expected.

4.9. SUPPLEMENTARY REFERENCES

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CHAPTER V

GENERAL DISCUSSION

The canonical mode of action of receptor tyrosine kinases (RTKs) considers that the activation of these receptors by specific ligands at the cell surface leads to the modulation of signal transduction cascades that elicit a variety of biological functions [1]. In this study, we show that the biological function of VEGFR2 might involve a novel activity that requires its internalization to the nucleus, in addition to this canonical mechanism. Our results strongly suggest that VEGFR2 might act as a transcription factor and that it is involved in the regulation of its own transcription in a possible positive feedback loop that might be important for maintaining VEGFR2 signaling in activated cells, amplifying the angiogenic response.

Whether nuclear VEGFR2 is also involved in the transcriptional regulation of other genes remains to be determined. Our MS results suggest that potential VEGFR2 partners in the nucleus are proteins involved in the regulation of gene expression and DNA repair, among others. Also, the expression or transcription factor activity of different nuclear proteins was increased in ECs over-expressing VEGFR2, which correlated with the increased levels of VEGFR2, further suggesting that the transcriptional activity of VEGFR2 might be extended beyond its own regulation.

Additionally, several studies have documented the presence of VEGFR2 or its ligand VEGF not only at cell surface but also in the nucleus. In particular, it was observed the nuclear internalization of VEGF in bovine adrenal cortex ECs following *in vitro* wounding [2]. Also, nuclear VEGFR2 was observed in normal and neoplastic renal tissues [3,4], in the nucleus of MCF-7, HeLa and HL60 cells [4] and in bovine and rat EC [5,6]. Similar findings were reported in studies showing that VEGFR2 is constitutively phosphorylated and located at the nucleus of VEGF-producing leukemias [7,8]. These results further suggest a specific and important role for VEGF/VEGFR2 in the nucleus.

Our findings suggest a dual mechanism for the biological activity of this receptor. One of them involves the canonical signaling pathway represented by the triggering of signal transduction cascades upon activation of VEGFR2 by VEGF at the membrane [1]. The second mechanism would be mediated by

the internalization of the activated receptor and the regulation of gene expression. The balance between these two activities is probably required during physiological and pathological events.

The recognition of VEGFR2 as a membrane protein, playing a critical role in tumor angiogenesis, has led to the development of different anti-angiogenic strategies [9]. However, the existence of mechanisms for evasive resistance to anti-VEGF therapy has been demonstrated [10,11]. In particular, it was shown that an anti-VEGFR2 blocking antibody (DC101 Ab, ImClone) promotes tumor re-growth in mice, after an initial period of growth repression [10] and an invasive phenotype remained augmented after termination of therapy, contributing to the evasive resistance to the anti-angiogenic therapy [11].

However, we must consider that in tumor cells, VEGFR2 therapy inhibits the signaling mediated by the membrane VEGFR2 [12]. The effects in the nuclear translocation of VEGFR2 were not studied. Because we have shown that a neutralizing antibody against VEGFR2 (IMC-1C11 Ab, ImClone) failed to block VEGFR2 nuclear translocation, the effect of these therapies on the nuclear activity of VEGFR2 must now be considered.

Based on these data, we may hypothesize that the nuclear role of this angiogenic factor may be involved in the resistance to anti-VEGF therapy.

In addition to VEGFR2, other RTKs might share this dual signaling mechanism. Evidence has been collected in the last years showing the presence of several RTKs, such as EGFR, FGFR in the cell nucleus and that these receptors also have transcriptional activity [13,14,15], as we describe here for VEGFR2. Interestingly, it was shown that cells with acquired resistance to Cetuximab (a neutralizing antibody against epidermal growth factor receptor (EGFR), [16]) have a constitutive translocation of EGFR to the nucleus [17]. This suggests that the nuclear expression of EGFR may be a critical determinant for resistance to therapy [17].

INTERACTION WITH VEGFR1 IS IMPORTANT FOR THE NUCLEAR INTERNALIZATION OF VEGFR2

Several studies have suggested different roles for VEGFR1 and VEGFR2 [18]. In particular, the affinity of VEGF is higher for VEGFR1 than for VEGFR2 [9], but in contrast to VEGFR1, VEGFR2 has a potent tyrosine kinase activity and is considered the major mediator of the signaling responses induced by VEGF [9]. Based on the biochemical and genetic data it was proposed that VEGFR1 could be a negative regulator of the VEGF activity, acting as a “decoy” receptor to sequester VEGF, thus rendering it less available for interacting with VEGFR2 [19].

We now show that VEGFR1 is also important for VEGFR2 internalization (Figure 1). This is supported by our results showing that neutralizing agents against VEGFR1 efficiently blocked VEGFR2 nuclear translocation. Furthermore, we showed that activation of VEGFR1 alone, through the use of PlGF, results in VEGFR2 phosphorylation by a mechanism inhibited by the 6.12Ab and KDRI.

The interaction between the two receptors induces VEGFR2 phosphorylation [20], which, as will be discussed below, is important for the internalization process. The ability of activated VEGFR1 to induce intermolecular trans-phosphorylation of VEGFR2, increasing VEGFR2 phosphorylation, has been previously reported [20] and is consistent with the functional interaction between the two receptors. However, while this interaction seems to be clear [20], the mechanism for the trans-activation mediated by VEGFR1 remains to be fully explained. Autiero and colleagues [20] reported two alternative crosstalk mechanisms between the two VEGF receptors. One of them is the trans-activation of VEGFR2 homodimers by VEGFR1 homodimers [20], which suggests that this crosstalk may not be direct. Indeed, it was previously suggested that intermediate signaling molecules such as PI3K, PKC or MAPK might be involved in this process, either by stimulating intrinsic kinase activity of VEGFR2 or by suppressing an associated tyrosine phosphatase [5,20].

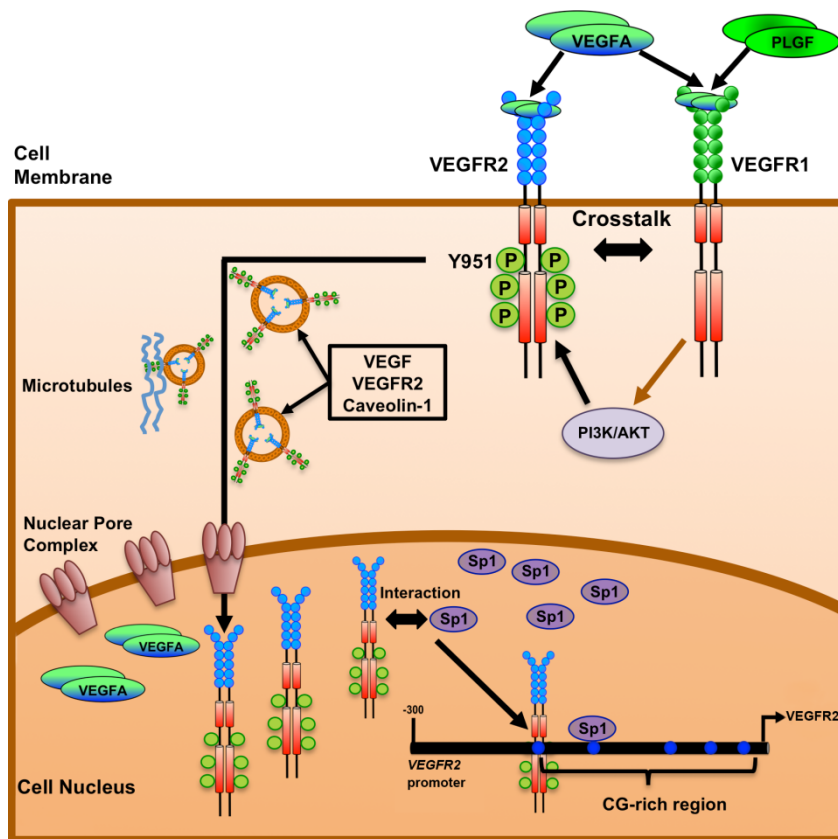


Figure 1. Schematic representation of the VEGF-VEGFR2 nuclear internalization in ECs.

In the presence of VEGF, both VEGF and VEGFR2 are internalized to the cell nucleus in a VEGFR1-dependent process. This mechanism requires the activation of PI3K pathway and involves a caveolin-1 mediated endocytosis and the microtubules. In the nucleus, VEGFR2 interacts with Sp1 transcription factor and binds to an Sp1-responsive region in the *VEGFR2* proximal promoter.

Consistent with this indirect mechanism, we showed that treatment with a PI3K inhibitor blocked VEGFR2 internalization, suggesting that PI3K might mediate the VEGFR2-trans-phosphorylation induced by VEGFR1, which is required for the VEGFR2 nuclear internalization process (Figure 1). The second crosstalk mechanism proposed suggests that PLGF/VEGF heterodimers or VEGF homodimers can induce the formation of VEGFR1/VEGFR2 heterodimers in which the trans-phosphorylation of each monomer was observed [20], which is consistent with a more direct trans-activation mechanism. In our study we cannot exclude the contribution of both crosstalk mechanisms to the internalization of VEGFR2. However, if the

activation of VEGFR2 internalization requires heterodimerization with VEGFR1, the physical interaction between the two receptors must be then lost because in all the culture conditions tested, VEGFR1 was always located at the cell surface.

The requirement of the VEGFR crosstalk for VEGFR2 nuclear internalization seems to be in contradiction with the analysis of mutant mice carrying a truncated VEGFR1 that lacks the tyrosine kinase domain. *Vegfr1^{TK-/-}* mice are viable and fertile and do not exhibit deficiencies in vascular development, indicating that the VEGFR1 tyrosine kinase activity is dispensable for embryonic angiogenesis [21]. However, it was described that growth of VEGF/PLGF-dependent-Lewis lung carcinomas (LCC), was retarded in *Vegfr1^{TK-/-}* mice compared to wild-type mice [22]. This finding could indicate that the functional crosstalk between the two VEGF receptors and consequently, VEGFR2 nuclear translocation might be required under certain pathological circumstances, such as tumor growth. In the future, it would be important to analyze the subcellular localization of VEGFR2 in the blood vessels associated with these tumors to test if the retarded tumor growth is correlated with a decreased nuclear internalization of the receptor. This could further suggest the involvement of the nuclear activity of VEGFR2 in the evasive resistant mechanisms against anti-angiogenic therapy.

VEGFR2 PHOSPHORYLATION – THE DRIVING FORCE FOR NUCLEAR TRANSLOCATION

The VEGF or VEGFR1-mediated activation of VEGFR2 induces the phosphorylation of several tyrosine residues in the intracellular region that are required for the functional activity of VEGFR2 [18,20]. We showed that tyrosine phosphorylation of VEGFR2 is also essential for the internalization process (Figure 1). Several findings described in this work support this idea. In particular, we showed that VEGFR2 mutants with deleted kinase and C-terminal tail domains have impaired nuclear internalization. Furthermore, Bevacizumab or Sunitinib, two anti-angiogenic drugs that inhibit VEGFR2 phosphorylation, blocked the binding of VEGFR2 to its own promoter. This

result is consistent with the requirement of tyrosine phosphorylation for VEGFR2 internalization and its subsequent transcriptional activity.

Although, both activation of signal transduction pathways and nuclear internalization require the phosphorylation of VEGFR2, molecular analyses indicate that the tyrosine residues involved are different. Our experiments showed that internalization of VEGFR2 requires phosphorylation of Y951 located in the kinase-insert domain (Figure 1). Conversely, the tyrosine residues Y1054 and Y1059 located in the kinase catalytic domain, which have been described as required for maximal VEGFR2 kinase activity *in vitro* [23], seemed to be dispensable for the internalization process. Interestingly, it has been reported that a tyrosine kinase-inactive VEGFR2 can be phosphorylated by VEGFR1 in tyrosine residues other than Y1054/1059 [20]. As internalization of VEGFR2 is dependent of VEGFR1-mediated phosphorylation, this observation further supports that the activation of these residues is not required for the internalization process.

Although the importance of the activation of tyrosine Y951 residue has not been evaluated in animal models, it has been shown that in sprouting embryonic body (EB) vessels the phosphorylation of tyrosine Y951 is enhanced compared to resting vessels, suggesting that activation of this residue might be required for EC migration mediated by VEGFR2 [24]. These observations are consistent with our data showing that ECs expressing VEGFR2(Y951/996F)-GFP mutant have a decreased migratory response compared to control ECs expressing VEGFR2(WT)-GFP. Since the phosphorylation in Y951 seems to be associated with the nuclear internalization of VEGFR2, these results might indicate that the nuclear activity of this receptor is probably associated with EC migratory properties. Indeed, this observation is consistent with our results showing that following wounding *in vitro*, there is a rapid nuclear internalization of VEGF and VEGFR2 in ECs at the wound edges that is not observed in ECs far from the wound. Furthermore, the blockage of the VEGFR1-mediated VEGFR2 nuclear translocation led to a delayed EC recovery following injury. This effect correlates with a lack of

internalization of VEGF and VEGFR2, further suggesting a functional relevance for the nuclear translocation of VEGF and VEGFR2.

Genetic studies in mice expressing the VEGFR2 (Y951F) mutant will be useful to evaluate the requirement of phosphorylating tyrosine Y951 for the nuclear internalization of VEGFR2 and for the biological activity of this receptor.

VEGF AND VEGFR2 – FROM THE MEMBRANE TO THE NUCLEUS

The mechanism by which an integral membrane protein translocates to the nucleus remains largely unanswered. Different mechanisms have been proposed to explain the nuclear transport of other membrane RTKs [15]. The best-studied case is the nuclear translocation and activity of EGFR. The most accepted proposed mechanism suggests that the route for the nuclear translocation of EGFR involves a clathrin-mediated endocytosis and the retrograde transport after endocytosis through membrane compartments such as the Golgi apparatus and the endoplasmic reticulum (ER) [15].

In the case of VEGFR2, a number of observations indicate that nuclear translocation might involve a caveolae-mediated endocytosis mechanism. First, VEGFR2 seems to interact with components of this endocytic pathway. We have shown that the VEGF-VEGFR2 complex interacts with caveolin-1 in the cytoskeletal fraction of ECs (Figure 1). Other reports showed that VEGFR2 colocalizes in the perinuclear area with both caveolin-1 and with dynamin-2, a GTPase involved in receptor-mediated endocytosis [25]. In addition, it was recently reported that dynamin-2 inhibitors blocked the VEGF-induced internalization of VEGFR2, resulting in decreased tip EC filopodial extensions [26]. Furthermore, we have shown that drugs inhibiting microtubule polymerization, such as nocodazole, blocked the internalization of the VEGF-VEGFR2 complex. Interestingly, caveolae are transported from the membrane to intracellular organelles along microtubules [27], and dynamin-2 seems to require interactions with functional microtubules to stimulate its GTPase activity and promote vesicular transport [28,29,30,31]. In this context, our finding that VEGFR2 fails to internalize upon microtubule depolymerization is

consistent with the involvement of caveolae in the internalization of this receptor (Figure 1).

It has been suggested that the internalization of VEGFR2 could also be mediated by clathrin-dependent endocytosis at cell junctions, in the absence of VE-Cadherin [32]. Importantly, even through this pathway VEGFR2 retains its signaling activity when internalized to intracellular endosomes [32]. Clathrin-dependent internalization has been described for EGFR, where it seems to be associated with receptor turnover, either by recycling it to the membrane or for targeting it for degradation in a c-Cbl-dependent manner [33]. We cannot exclude that both clathrin and caveolar-dependent endocytosis might be responsible for the VEGFR2 internalization in ECs. Indeed, a similar c-Cbl dependent mechanism has been described for VEGFR2 [34], although it remains controversial since other reports suggested that c-Cbl is not involved in VEGFR2 degradation [35]. Consistent with the second report, we were unable to observe an interaction between VEGFR2 and c-Cbl in ECs.

In addition to its detachment from the cell membrane, the nuclear translocation of VEGFR2 requires the receptor to be transported from the cytoplasmic vesicles into the nuclear compartment. The mechanism for this transportation is not clear but seems to require the nuclear pore complex (NPC), (Figure 1). Our finding that drugs inhibiting the NPC induced accumulation of VEGF and VEGFR2 in the perinuclear region supports this conclusion. Furthermore, our FRAP analysis of the nuclear internalization dynamics of VEGFR2-GFP in ECs also suggested an active transport of VEGFR2 from the cytoplasm to the nucleus, which is consistent with the involvement of the NPC in this process. The typical mechanism for the import of proteins through the NPC is mediated by the binding of the protein nuclear localization signals (NLS) to importin α and β to form a complex that interacts with the NPC so that the protein can enter the nucleoplasm [36].

However, the presence of NLS in the VEGFR2 sequence was neither described in the literature nor identified in the bioinformatics analyses performed by us. The proposed model for FGFR1 internalization could

suggest a solution for this apparent paradox. FGFR1, which is also devoid of a NLS sequence, is chaperoned to the nucleus by its ligand FGF2 that harbors NLS sequences, inducing their nuclear translocation in an importin β -dependent manner [37]. VEGF has five potential NLS sequences in the C-terminal region [2] and it is possible that the VEGF-NLS sequences drive the complex VEGF-VEGFR2 through the NPC to the nucleus. Consistent with this hypothesis, we found that VEGF and VEGFR2 are transported together as a complex. Further analysis of interactions between VEGF and VEGFR2 with importins and their contribution to this process will be required to fully understand the internalization process. Additionally, experiments using a VEGFR2 construct fused to NLS sequences could further illustrate the contribution of VEGF in this internalization process.

The subcellular localization of the VEGF-VEGFR2 complex, including the presence of both proteins in the nucleus, is a dynamic process. This is supported by the fact that during a time course of 7 h after VEGF stimulation, the levels of nuclear VEGFR2 protein undergo variations. Moreover, after performing a wound, our results also suggest that the VEGF/VEGFR2 nuclear internalization is associated with a dynamic process according to a physiological response. VEGF and VEGFR2 are rapidly internalized in ECs close to the wound edge (as early as 15 min post-wounding) and decreases upon EC recovery. Taken together, these results support the existence of a dynamic process that controls the levels of VEGF and VEGFR2 in the nuclear compartment.

VEGF-VEGFR2 complexes are also actively exported from the nucleus back to the cytoplasm/cell surface. The majority of the proteins shuttling from the nucleus to the cytoplasm use a nuclear export signal (NES)-CRM1 receptor dependent pathway [38], although the involvement of alternative mechanisms for nuclear export processes has been appreciated [39,40]. Our finding that VEGF is arrested in the nucleus upon leptomycin B treatment suggests that this molecule exit the nucleus following the classical export receptor-mediated mechanism [38]. Further studies will be required to identify possible NES

sequences in VEGF, as well as the recruitment of an export receptor such as CRM1. Interestingly, our results suggest that VEGFR2 is exported by a different mechanism, as its exit from the nucleus is not affected by leptomycin B.

An alternative export mechanism has been described for Smad3, which is mediated by exportin 4 and requires Ran GTPase as a co-factor [40].

Exportin 4, a member of the importin-family was described to recognize complex protein motifs in Smad3, resembling an export signal distinct from the well-characterized exportin 1 (CRM1)-dependent NES [40]. Whether this alternative nuclear export mechanism or another still not identified system is involved in the shuttling of VEGFR2 from the nucleus to the cytoplasm remains to be determined. The mapping of potential export motifs in the VEGFR2 sequence could help to elucidate the mechanisms that mediate the nuclear-cytoplasmic transport of the receptor.

A major question that remains to be answered is how these transmembrane receptors can be released from the membranes of the intracellular organelles or vesicles and be transported to the nucleus. This was investigated for FGFR1. In this case, it was shown that this receptor has an atypical transmembrane domain, which interacts with pp90 ribosomal S6 kinase 1 mediating its release from the membrane [41]. We do not expect a similar mechanism for VEGFR2 since this receptor has a typical hydrophobic transmembrane domain [1].

Another important question that remains unanswered is whether VEGF itself plays a role in the nucleus or if it is only required to mediate the internalization of VEGFR2. We have shown that the VEGF-VEGFR2 interaction during the internalization process is consistent with its contribution to this dynamic mechanism. However, in the nucleus this interaction is lost and their nucleo-cytoplasmic export seems to require different pathways. A screening of the proteins that might interact with VEGF in the nucleus will be a first step to elucidate to this problem.

VEGFR2 IN THE NUCLEUS – A POTENTIAL TRANSCRIPTION FACTOR

The function and relevance of VEGFR2 in the nuclear compartment of ECs was our main focus after defining the molecular basis of the internalization process. Our results clearly show that VEGFR2 has transcriptional activity, thus opening the possibility for an involvement of VEGFR2 in the regulation of a pro-angiogenic response in ECs at the transcriptional level. In this work we were able to establish that VEGFR2 binds and activates its own promoter (Figure 1). Our finding that the binding of VEGFR2 to its proximal promoter is dramatically increased in ECs cultured in the presence of VEGF further supports that this binding is directly linked to the activation of the receptor by VEGF. This is consistent with a functional relevance for this process.

VEGFR2 regulation of its promoter apparently involves interactions with the Sp1, a transcription factor that has been described as a major regulator of *VEGFR2* expression in ECs [42]. In particular, we have shown that Sp1 directly interacts with the intracellular domain of VEGFR2 (789-1356 aa) by pull-down assays using purified proteins. Furthermore, we have shown that VEGFR2 binds to the Sp1-responsive region of the *VEGFR2* promoter, suggesting a close interplay between Sp1 and VEGFR2 in the modulation of the *VEGFR2* expression (Figure 1). This interaction was further supported by our EMSA experiments showing that VEGFR2 and Sp1 are present in the same protein-DNA complexes in the *VEGFR2* promoter.

Different studies focused on the role of Sp1 in angiogenesis showed that several of its target genes (e.g., *VEGFR2*, *VEGF* and *Sp1*) were upregulated in human pancreatic tumors, suggesting its involvement in tumor progression and metastasis [43,44]. In particular, it was shown that the combined treatment with bevacizumab (a neutralizing antibody against VEGF) and mithramycin A (an Sp1 inhibitor), suppressed more efficiently the expression of these angiogenic factors both *in vitro* and in tumors growing in nude mice [44]. Since we observed that treatment with Bevacizumab or Sunitinib blocked the VEGF-induced VEGFR2 DNA binding, we can speculate that the Sp1-mediated expression of at least *VEGFR2* gene is influenced by nuclear

VEGFR2. In this context, it will be important to better understand the interactions between VEGFR2 and Sp1 in the control of *VEGFR2* expression. For instance, it will be important to understand whether both VEGFR2 and Sp1 must simultaneously interact with the promoter to elicit upregulation of *VEGFR2* expression or if any of the two factors can activate the promoter in the absence of the other. Clearly, experiments of sequential chromatin immunoprecipitations (ChIP-Re ChIP), using consecutively the VEGFR2 and Sp1 antibodies will help to elucidate these issues.

Having shown that VEGFR2 regulates its own transcription, it is now important to know if the receptor is involved in the regulation of other genes and/or if it is directly involved in the regulation of different cellular responses in ECs. This is one of the most important future perspectives of this work and implicates novel lines of research. The identification of other VEGFR2 transcriptional targets will be extremely important to further understand the role of this receptor in the nucleus. The use of ChIP coupled with high-throughput sequencing analysis (ChIP-seq), [45] will allow us to identify the genome wide profiling of VEGFR2-DNA interactome and therefore contribute to the identification of genes regulated by VEGFR2. A good approach for these experiments could be using ECs cultured in the presence of VEGF, since in these conditions we could identify the most relevant genes involved in the angiogenic response that are regulated by VEGFR2. In these conditions we could combine the VEGFR2 DNA-binding activity profile obtained by ChIP-Seq with a full transcriptome analysis using RNA-Seq, thereby defining not only the gene targets regulated by VEGFR2 but also their biological outcome. However, this approach would also identify genes regulated by members of the VEGF pathway independently of nuclear VEGFR2, requiring further validation of the selected targets.

Different aspects of this new molecular pathway remain to be defined. For instance, it is not clear if VEGFR2 binds directly to a consensus sequence in the *VEGFR2* promoter or if Sp1 or other nuclear protein mediates the observed VEGFR2 DNA binding activity. We cannot exclude that VEGFR2 binds directly to DNA and that the nuclear effects observed are directly

mediated by VEGFR2. The analysis of the binding profiles obtained in ChIP-Seq could help us to identify a consensus-binding pattern of VEGFR2. If the sequencing profiling of the VEGFR2 binding coincides with known binding sequences for other transcription factors, such as Sp1 or others, it could indicate that VEGFR2 is associated with these transcription factors and not directly bound to DNA. Conversely, a conserved VEGFR2 binding pattern throughout the genome without overlapping with other established transcription factor binding sequences could indicate that VEGFR2 binds directly to DNA and could be a good approach to identify its consensus binding sequence.

Another useful approach to identify a potential DNA binding sequence for VEGFR2 is an *in vitro* PCR-based approach named cyclic amplification and selection of targets (CASTing), [46]. However, this technique requires the use of purified proteins, which could be experimentally challenging in the case of full-length VEGFR2, since this is an insoluble protein comprising a hydrophobic transmembrane domain, making its purification extremely difficult.

CONCLUDING REMARKS

The findings compiled in this thesis can have a great impact in the vascular biology field and contribute to a better understanding of the molecular mechanisms involved in angiogenesis and to reveal new mechanisms of resistance to anti-angiogenic therapies.

The nuclear translocation of VEGFR2 and the regulation of its own expression impact the current model of vessel sprouting. This model predicts that ECs, exposed to the highest VEGF levels, gain a competitive advantage to acquire a tip-cell fate and position in the sprout [47]. It was also recently shown that this effect is mediated by elevated VEGFR2 levels that promote an upregulation of *DLL4* levels [48]. The elevated VEGFR2 levels in cells exposed to higher levels of VEGF could be explained by our findings indicating that VEGFR2 is involved in the regulation of its own transcription by a possible VEGFR2 positive feedback loop. Therefore, we can speculate that *in vivo*, the higher levels of VEGFR2 observed at tip cells might be regulated by the

nuclear activity of the receptor in these cells. Consistent with this idea, it was recently shown that the internalization of VEGFR2 is required for VEGF-induced tip cell filopodial extension *in vivo* [26]. Elevated levels of DLL4 in tip cells activate Notch signaling in neighboring cells that produces a reduction of VEGF-mediated signaling, which results in the downregulation of DLL4 and VEGFR2 expression [47,48,49,50,51,52,53]. As a consequence these cells acquire stalk cell fate [48]. This is consistent with our wound healing assays *in vitro* where increased levels of nuclear VEGF-VEGFR2 were only observed in ECs close to wound edges early after injury, suggesting an increase in VEGFR2 levels after wounding and its normalization upon recovery.

In the context of tumor angiogenesis, the nuclear internalization of VEGFR2 and its involvement in the regulation of its own transcription might also have a great impact, since it has been reported that some tumor cells can have a constitutive nuclear localization of VEGFR2 [4,7,8], mediated by autocrine loops of VEGF and VEGFR2 [7]. Therefore, it would be important to analyse the effects of the anti-angiogenic agents, such as Bevacizumab and Sunitinib, in the nuclear internalization and transcriptional activity of VEGFR2 in tumor cells, as a constitutive VEGFR2 transcriptional activity could be implicated in the mechanisms of resistance to anti-VEGF therapies.

Overall, the novel biological activity of VEGFR2 discovered during this work will contribute for a better understanding of the molecular mechanisms of angiogenesis and possibly help the improvement and future design of novel therapeutic approaches.

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APPENDIX A

Appendix A – Table 1. VEGFR-2-interacting proteins as identified by MS analysis. Proteins listed according to their Mascot score.

Hit	Mascot S.	Original ID	Description	Ensembl ID	kDa	Cover (%)	unique/ total peptides
1	4887	MYH9_HUMAN	Myosin-9 - Homo sapiens (Human)	ENSG00000100345	227.6	43.7	76/138
2	1951	PLEC1_HUMAN	Plectin-1 - Homo sapiens (Human)	ENSG00000178209	533.4	11.8	41/44
3	1628	MYO1C_HUMAN	Myosin-1c - Homo sapiens (Human)	ENSG00000197879	118.8	26.6	23/42
4	1627	FIBB_HUMAN	Fibrinogen beta chain precursor - Homo sapiens (Human)	ENSG00000171564	56.6	45.8	25/27
5	1122	MYH10_HUMAN	Myosin-10 - Homo sapiens (Human)	ENSG00000133026	229.8	11.3	19/32
6	965	FIBA_HUMAN	Fibrinogen alpha chain precursor - Homo sapiens (Human)	ENSG00000171560	95.7	22.9	16/21
7	955	FIBG_HUMAN	Fibrinogen gamma chain precursor - Homo sapiens (Human)	ENSG00000171557	52.1	41.1	14/17
8	878	SFPQ_HUMAN	Splicing factor, proline- and glutamine-rich - Homo sapiens (Human)	ENSG00000116560	76.2	24.8	12/16
9	712	PABP1_HUMAN	Polyadenylate-binding protein 1 - Homo sapiens (Human)	ENSG00000070756	70.9	23.1	11/12
10	687	A26CA_HUMAN	ANKRD26-like family C member 1A - Homo sapiens (Human)	NA	122.9	12.1	10/44
11	674	TMOD3_HUMAN	Tropomodulin-3 - Homo sapiens (Human)	ENSG00000138594	39.7	34.1	9/9
12	642	RBM14_HUMAN	RNA-binding protein 14 - Homo sapiens (Human)	ENSG00000239306	69.6	15.8	9/10
13	634	CAZA1_HUMAN	F-actin-capping protein subunit alpha-1 - Homo sapiens (Human)	ENSG00000116489	33.1	48.3	8/9
14	622	HNRPL_HUMAN	Heterogeneous nuclear ribonucleoprotein L - Homo sapiens (Human)	ENSG00000104824	60.7	31.7	11/14
15	618	DREB_HUMAN	Drebrin - Homo sapiens (Human)	ENSG00000113758	71.8	15.6	7/19
16	595	MYH14_HUMAN	Myosin-14 - Homo sapiens (Human)	ENSG00000105357	228.9	7.7	13/25
17	504	K2C6C_HUMAN	Keratin, type II cytoskeletal 6C - Homo sapiens (Human)	ENSG00000170465	60.3	17.2	8/9
18	490	RAI14_HUMAN	Ankyrin-14 - Homo sapiens (Human)	ENSG00000039560	110.6	15.5	10/11
19	481	CYTSA_HUMAN	Cytospin-A - Homo sapiens (Human)	ENSG00000100014	124.9	15.8	11/11
20	464	DDX3X_HUMAN	ATP-dependent RNA helicase DDX3X - Homo sapiens (Human)	ENSG00000215301	73.6	20.8	9/10
21	457	RL7_HUMAN	60S ribosomal protein L7 - Homo sapiens (Human)	ENSG00000147604	29.3	29.8	10/11
22	420	FBRL_HUMAN	rRNA 28S;-O-methyltransferase fibrillarin - Homo sapiens (Human)	ENSG00000105202	33.9	30.5	7/11
23	418	RS3_HUMAN	40S ribosomal protein S3 - Homo sapiens (Human)	ENSG00000149273	26.8	34.2	6/6
24	394	HNRDL_HUMAN	Heterogeneous nuclear ribonucleoprotein D-like - Homo sapiens (Human)	ENSG00000152795	46.6	18.6	7/14
25	392	LUZP1_HUMAN	Leucine zipper protein 1 - Homo sapiens (Human)	ENSG00000169641	120.8	16.6	13/15
26	391	LIMA1_HUMAN	LIM domain and actin-binding protein 1 - Homo sapiens (Human)	ENSG00000050405	85.6	15.4	8/9
27	383	GELS_HUMAN	Gelsolin precursor - Homo sapiens (Human)	ENSG00000148180	86.0	10.9	5/8
28	380	K2C5_HUMAN	Keratin, type II cytoskeletal 5 - Homo sapiens (Human)	ENSG00000186081	62.6	15.4	8/17
29	366	GNAI2_HUMAN	Guanine nucleotide-binding protein G(i), alpha-2 - Homo sapiens (Human)	ENSG00000114353	41.0	23.4	6/6
30	356	EZRI_HUMAN	Ezrin - Homo sapiens (Human)	ENSG00000092820	69.5	13.1	7/7
31	342	DESM_HUMAN	Desmin - Homo sapiens (Human)	ENSG00000175084	53.6	16.4	8/18
32	340	CV028_HUMAN	UPF0027 protein C22orf28 - Homo sapiens (Human)	ENSG00000100220	55.7	15.0	6/8
33	298	PP1G_HUMAN	Serine/threonine-protein phosphatase PP1-gamma - Homo sapiens (Human)	ENSG00000186298	37.7	16.1	4/4
34	294	RL10A_HUMAN	60S ribosomal protein L10a - Homo sapiens (Human)	ENSG00000198755	25.0	30.4	6/6
35	292	5NTD_HUMAN	58S;-nucleotidase precursor - Homo sapiens (Human)	ENSG00000135318	63.9	14.1	5/6
36	266	RS4X_HUMAN	40S ribosomal protein S4, X isoform - Homo sapiens (Human)	ENSG00000198034	29.8	16.7	4/5
37	265	RRBP1_HUMAN	Ribosome-binding protein 1 - Homo sapiens (Human)	ENSG00000125844	152.8	5.8	5/6
38	265	K2C75_HUMAN	Keratin, type II cytoskeletal 75 - Homo sapiens (Human)	ENSG00000170454	59.8	13.2	6/22
39	264	K1109_HUMAN	Uncharacterized protein KIAA1109 - Homo sapiens (Human)	ENSG00000138688	559.2	5.5	15/29

APPENDIX A

Hit	Mascot S.	Original ID	Description	Ensembl ID	kDa	Cover (%)	unique/ total peptides
40	261	MBB1A_HUMAN	Myb-binding protein 1A - Homo sapiens (Human)	ENSG00000132382	149.7	6.9	6/6
41	257	SYNE2_HUMAN	Nesprin-2 - Homo sapiens (Human)	ENSG00000054654	801.8	3.8	16/18
42	251	BUB3_HUMAN	Mitotic checkpoint protein BUB3 - Homo sapiens (Human)	ENSG00000154473	37.6	11.0	3/3
43	235	PCBP2_HUMAN	Poly(rC)-binding protein 2 - Homo sapiens (Human)	ENSG00000197111	39.0	24.4	5/14
44	235	NIN_HUMAN	Ninein - Homo sapiens (Human)	ENSG00000100503	245.2	5.1	10/16
45	234	PABP4_HUMAN	Polyadenylate-binding protein 4 - Homo sapiens (Human)	ENSG00000090621	71.1	10.4	5/5
46	233	THIL_HUMAN	Acetyl-CoA acetyltransferase, mitochondrial precursor - Homo sapiens (Human)	ENSG00000075239	45.5	16.2	4/4
47	228	IGHG2_HUMAN	Ig gamma-2 chain C region - Homo sapiens (Human)	ENSG00000211893	36.5	17.8	3/4
48	223	AMPN_HUMAN	Aminopeptidase N - Homo sapiens (Human)	ENSG00000166825	109.9	9.2	7/8
49	221	RS2_HUMAN	40S ribosomal protein S2 - Homo sapiens (Human)	ENSG00000140988	31.6	15.4	4/5
50	215	JHD2B_HUMAN	JmjC domain- histone demethylation protein 2B - Homo sapiens (Human)	NA	193.2	7.8	9/12
51	210	BRE1A_HUMAN	E3 ubiquitin-protein ligase BRE1A - Homo sapiens (Human)	ENSG00000155827	114.2	8.5	8/9
52	203	DYH8_HUMAN	Dynein heavy chain 8, axonemal - Homo sapiens (Human)	ENSG00000124721	518.0	5.3	13/14

