Dissection of the effects induced by $\text{VEGF}_{165}$, $\text{VEGF}_{121}$ and Semaphorin 3A in endothelial cells

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III. ABSTRACT

The development of new blood vessels is a complex and highly regulated process that requires the coordinated action of different growth factors. Vascular Endothelial Growth Factor (VEGF) is a powerful inducer of angiogenesis, acting through the metabolic activation, proliferation and migration of endothelial cells.

The major angiogenic member of the VEGF family is VEGF-A, a gene which is transcribed to give rise to at least 4 major splicing variants, of 206, 189, 165 and 121 amino acids in humans. The angiogenic effect of the most abundant isoform (VEGF<sub>165</sub>) is basically mediated by its interaction with VEGFR2 (KDR) and VEGFR1 (Flt-1) as well as with the co-receptor Neupropilin-1 (NP-1). The shortest isoform (VEGF<sub>121</sub>) binds VEGFR1 and VEGFR2 only, but not NP-1. NP-1, together with PlexinA1, also acts as a receptor of Semaphorin 3A (Sema3A), a factor also involved in vascular patterning, besides its very well known role in axonal guidance.

The aim of this project has been the detailed analysis of the peculiarity/specificity of the effect of VEGF<sub>165</sub>, VEGF<sub>121</sub> and Sema3A in endothelial cells.

We first exploited an Adeno Associated Virus (AAV)-based gene delivery to unravel the in vivo effect of these cytokines. In particular, we observed that the prolonged expression of the main isoform of VEGF, VEGF<sub>165</sub>, acted as a powerful inducer of angiogenesis, stimulating the development of both a larger capillary network and the formation of an impressive new set of arterioles. Most surprisingly, an unexpected effect of VEGF<sub>165</sub> was also the recruitment to the sites of its expression, of a vast set of mononuclear cells. These cells derived from the bone marrow and expressed a broad set of monocytic markers (CD45+, CD11b+); their presence correlated with the formation of arterioles and the maturation of the VEGF-induced vasculature. Strikingly, VEGF<sub>121</sub> (the shorter form unable to bind NP-1) was, on the contrary, unable to induce maturation of the newly formed capillaries to mature arteries and to recruit cells, an observation that suggested that these monocytes might be essential in blood vessel maturation. We found that cell recruitment both in vitro and in vivo strictly depends on NP-1 and that Sema3A, a NP-1 activator, acts as a powerful recruiter of these cells. The expression of VEGF and Sema3A receptors by CD11b+ cells together with the VEGFR2 interaction with NP-1, indicate that these cells could be target of a peculiar VEGF and/or Sema3A induced signalling pathway.

Taken together, these results prompted us to develop an in vitro model to unravel the signalling features elicited by the VEGF<sub>165</sub>, VEGF<sub>121</sub> and Sema3A on endothelial cells. In
particular, we wanted to dissect these pathways through a proteomic approach involving the detection of the phosphoproteome of endothelial cells expressing specific receptor after treatment with the various ligands of interest.

Due to the large amount of growth factors required for this kind of experimentation, we first established a Baculovirus-based system to generate the recombinant factors. For this purpose, we engineered suitable plasmids encoding VEGF\textsubscript{165}, VEGF\textsubscript{121} and Sema3A endowed of a cleavable histidine tag at the N-terminus. Once the recombinant protein expression conditions were set up, we moved to optimize protein purification by affinity chromatography, together with the removal of the tag. The functionality of baculovirus-expressed VEGF\textsubscript{165} and VEGF\textsubscript{121} was ascertained by the analysis of VEGF receptor phosphorylation, as well as proliferation assays on endothelial cells; a cell contraction assay confirmed that the recombinant Sema3A produced was as active as protein expressed in mammalian cells.

To dissect the differential signalling induced by each of the recombinant factors, we explored their transduction pathways in endothelial cell lines expressing the main receptors (KDR and NP1). We set up the lysis conditions and bi-dimensional SDS-PAGE, together with the optimal phosho-proteome staining. The findings obtained, together with phosho-enrichment approaches, strongly supported that peculiar signalling pathways exist in endothelial cells, selectively triggered by VEGF\textsubscript{165}, VEGF\textsubscript{121} and Sema3A, as demonstrated by the differential pattern of phosphorylated proteins induced by the recombinant proteins. Furthermore, we first demonstrated a close similarity in the phosho-tyrosine proteome induced by VEGF\textsubscript{165} and Sema3A, at least in the cellular system examined.
IV. PUBLICATIONS


**Bone marrow cells recruited through the neuropilin-1 receptor promote arterial formation at the sites of adult neoangiogenesis in mice.**
J Clin Invest. 2008 Jun;118(6):2062-75

Zentilin L, Tafuro S, Zacchigna S, Arsic N, Pattarini L, Sinigaglia M, Giacca M.

**Bone marrow mononuclear cells are recruited to the sites of VEGF-induced neovascularization but are not incorporated into the newly formed vessels**
Blood. 2006 May 1;107(9):3546-54
V. ABBREVIATIONS

2DE               two dimensional gel electrophoresis
EC                endothelial cell
Flk-1            Fetal liver kinase 1, mouse VEGFR2
Flt-1            Fms like kinase 1, VEGFR1
h                hours
KDR              Kinase insert Domain Receptor, human VEGFR2
IEF              isoelectric focusing
NP                Neuropilin
PAE              porcine aortic endothelial cell
pI                isoelectric point
MI                myocardial infarction
MS                mass spectrometry
MW                molecular weight
Sema              Semaphorin
VEGF             Vascular Endothelial Growth Factor
VEGFR            Vascular Endothelial Growth Factor Receptor
VI. INTRODUCTION

VI.1. THE ANGIOGENIC PROCESS

The development of the vascular system is one of the earliest events in organogenesis. During early stages of embryo development, cells of mesodermal origin differentiate into haemangioblasts, progenitors of both hematopoietic and endothelial lineages, giving rise to blood vessels. In the following steps of the differentiation process, haemangioblasts produce angioblasts, whose aggregation results in formation of blood islands. Fusion of blood islands results in appearance of the primary blood vascular plexus, consisting of fine capillaries formed by endothelial cells (ECs). Notably, even at this early stage, known as vasculogenesis, capillaries acquire arterial or venous character, as a proof that the cell specificity is genetically programmed (Avraamides et al., 2008; Coultas et al., 2005; Pugh and Ratcliffe, 2003).

During the angiogenesis phase, the vascular plexus progressively expands by means of capillary branching and remodelling into a highly organized and stereotyped vascular network of larger vessels ramifying into smaller ones. Angiogenesis begins from local destruction of the wall of pre-existing blood vessels, activation of EC proliferation and migration. Forming endothelial-cell channels become then covered by pericytes and smooth muscle cells, which provide strength and allow regulation of vessel perfusion, a process termed arteriogenesis. After birth, angiogenesis still contributes to organ growth but, during adulthood, most blood vessels remain quiescent and angiogenesis occurs only in the cycling ovary and in the placenta during pregnancy. However, ECs retain their remarkable ability of dividing rapidly in response to a physiological stimulus, such as hypoxia and inflammation (Carmeliet, 2000).

When vessel growth is deregulated, it has a major impact on our health and contributes to the pathogenesis of many disorders. Indeed, a long list of pathologies is characterized or caused by excessive angiogenesis. Historically, the best known are cancer, psoriasis, arthritis and blindness, but many additional common disorders such as obesity, asthma, atherosclerosis and infectious diseases are included, and the list is still growing. Several congenital or inherited diseases are also caused by abnormal vascular remodelling. In addition, insufficient vessel growth and abnormal vessel regression not only cause heart and brain ischemia, but can also lead to neurodegeneration, hypertension, pre-eclampsia, respiratory distress, osteoporosis and other disorders (Bhushan et al., 1999; Chen et al.,
VI.2. MOLECULES INVOLVED IN THE ANGIOGENIC PROCESS

Angiogenesis is a fundamental physiological process during both embryogenesis and adulthood, requiring the coordinated action of a variety of growth factors and cell-adhesion molecules in both endothelial and mural cells. In recent decades, extensive studies have revealed a variety of angiogenic factors and their receptors (including molecules involved in vessel development and vascular cell specification -vascular endothelial growth factor (VEGF)-VEGFRs, Angiopoietin-Tie, Delta-Notch- as well as axon guidance clues -Ephrin-EphRs, and Semaphorin-Plexins and Neuropilin-) to be the major regulators of angiogenesis in vertebrates (Bussolino et al., 2006; Guttman-Raviv et al., 2006; Kuijper et al., 2007; Sainson and Harris, 2008; Siekmann et al., 2008) (Figure 1).

Figure 1. Formation of a functional circulation from endothelial progenitors.
a. Vascular progenitors appear in response to basic fibroblast growth factor bFGF and bone morphogenetic protein 4 (BMP4) in the posterior primitive streak (PPS) as vascular endothelial growth receptor2 (VEGFR2)/Flik-1-positive mesodermal cells. b. Flik-1-positive cells in the primitive streak give rise to both blood and endothelium (haemangioblasts), but are restricted to haematopoietic or angiogenic fate after emigrating into extra-embryonic sites (extra-embryonic ectoderm (EXE), yolk sac and allantois) and intraembryonic sites (embryonic ectoderm (EEC)). c. In the yolk sac, these progenitors aggregate into endothelial-lined blood islands that then fuse to generate a primary capillary plexus. d. The primary capillary plexus undergoes remodelling along with intra-embryonic vessels to form a mature circulation (g). e. Intra-embryonic angioblasts migrate along distinct pathways before (f) aggregating directly into the dorsal aorta or cardinal vein, without a plexus intermediate. g. The primary vessels (capillary plexus, dorsal aorta and cardinal vein) then remodel, together with the extraembryonic plexus, to form a mature vasculature, which along with VEGF and Notch involves the angiopoietins and Tie receptors4. h. Mural cells (pericytes and smooth-muscle cells) proliferate and differentiate in response to transforming growth factor- (TGF-β) signalling, and are recruited to vessels by platelet-derived growth factor (PDGF) secreted by endothelial cells. Ang, angiopoietin; Eph, Eph receptor family; Shh, sonic hedgehog; Np, Neuropilin (Coultas et al., 2005).

VI.2.1. VEGF family

Vascularogenesis and angiogenesis. The vascular endothelial growth factor family is considered one of the key players in the regulation of this process: as a matter of fact, VEGF-A initiates vessel formation and also triggers a chain of molecular and cellular events that ultimately lead to a mature vascular network. In brief, CD31+CD31+ VEGF receptor (VEGFR)-2-positive angioblasts form a vascular plexus that gives rise to the dorsal aorta, the cardinal vein and the embryonic stems of yolk sac arteries and veins. Sprouting angiogenesis is presumably facilitated by hypoxia, which up-regulates the expression of a number of genes involved in vessel formation, patterning and maturation, including nitric oxide synthase, VEGF, and angiopoietin-2 (Pugh and Ratcliffe, 2003). Existing vessels dilate in response to nitric oxide, a product of nitric oxide synthase, and become leaky in response to VEGF. The basement membrane and ECM dissolve in response to activation of proteases, such as matrix metalloproteinase (MMP) 2, MMP3 and MMP9, and suppression of protease inhibitors, such as tissue inhibitor of metalloproteinase-2 (Chen et al., 2005; Ferrara, 2005; Hoeben et al., 2004; Holmes et al., 2007).
VI.2.1.1. VEGF family members and isoforms

VEGF-A is the founding member of the VPF/VEGF family and was originally discovered on the basis of its potent ability to enhance vascular permeability. Other VPF/VEGF family members share a common VEGF homology domain and include VEGF-B, -C, and -D, as well as a VEGF homolog identified in the genome of the parapoxvirus Orf and the Placental Growth Factor (PIGF) (Coulta et al., 2005; Ferrara, 2005) (Figure 2).

![Figure 2. VEGF family member receptors and their biological effects.](image)

Each VEGF ligand (-A, -B, -C, -D, -E and PLGF) binds in a specific manner to three receptor tyrosine kinases, VEGFR1, -2, and -3. VEGFR1 is expressed on haematopoietic stem cells, macrophages and monocytes as well as on the vascular endothelium. VEGFR2 is expressed mainly on both vascular and
lymphatic endothelium, whereas expression of VEGFR-3 is generally restricted to lymphatic endothelium (Holmes et al., 2007).

**VEGF-A (ligand of VEGFR1 and 2, NP-1 and 2).** VEGF-A is the most comprehensively studied member of the VEGF family and it is considered one of the prime movers of physiological and pathological angiogenesis, by means of different mechanisms: mice lacking even one copy of the VEGF-A gene are embryonically lethal (Claesson-Welsh, 2003; Coultas et al., 2005; Ferrara, 2005; Hoeben et al., 2004; Karamysheva, 2008). A well-documented *in vitro* activity of VEGF family members is the ability to promote growth of vascular ECs derived from arteries, veins and lymphatics. The presence of VEGF receptors on non-ECs and studies in VEGF-mutant mice have accounted a role for this cytokine as an essential signalling factor in non-ECs, too: VEGF-A shows indeed a peculiar role in the hematopoietic repopulation, osteogenesis, skeletal muscle regeneration and neuronal survival, only to name a few (Arsic et al., 2004; Ferrara, 2005; Zelzer et al., 2002). Structurally, VEGF-A is a highly conserved, disulfide-bonded dimeric glycoprotein: it shares low but significant sequence homology with PDGF, and like PDGF has cysteine residues that form integral inter- and intrachain bonds. Crystal structure reveals that the two chains comprising VEGF-A are assembled in an antiparallel conformation, with receptor binding sites at either end: upon treatment with reducing agents, VEGF-A completely loses its biological activity (Coultas et al., 2005; Ferrara, 2005; Hoeben et al., 2004). The human VEGF-A is encoded by a single gene located in the short arm of chromosome 6; its genomic structure comprises eight exons separated by seven introns. Alternative exon splicing was initially shown to result in the generation of four different isoforms (VEGF_{121}, VEGF_{165}, VEGF_{189}, VEGF_{206}), coding for respectively 121, 165, 189 and 206 amino acids, after signal sequence cleavage. Less frequent splice variants have been also reported, such as VEGF_{145}, VEGF_{148} and VEGF_{183} (Ferrara, 2005) (Figure 3).
**Figure 3. VEGF-A major isoforms.**

VEGF-A gene is composed by eight exons encoding different structural motifs. Alternative mRNA splicing leads to the production of several different isoforms characterized by different amino-acid (aa) number. In humans, VEGF<sub>165</sub> is the most predominant form (Cross et al., 2003).

*VEGF<sub>165</sub> (ligand of VEGFR1 and 2 and NP-1 and 2).* VEGF<sub>165</sub> is the predominant isoform and lacks the residues encoded by exon 6. Although being a secreted molecule, a significant fraction of it remains bound to the cell surface and the extracellular matrix: these features make this isoform able to have both long- and short-range effects. Its action is mediated by its binding to the VEGF receptor 1 and 2, as well as Neuropilin1 and 2 (NP). As a matter of fact, native VEGF is a heparin-binding homodimeric glycoprotein of 45 kDa and exerts properties closely corresponding to those of VEGF<sub>165</sub> (Coultas et al., 2005; Ferrara, 2005; Hoeben et al., 2004). VEGF<sub>165</sub> has both immediate and late effects on vascular endothelium, underling significant roles of this molecule in inflammation and other pathological circumstances. *In vivo*, vasodilatation occurs primarily on muscular arteries and it arises mainly from the VEGF-A-induced synthesis of nitric oxide by ECs. As far as the second immediate effect is concerned, VEGF<sub>165</sub> was originally discovered as one of the most potent vascular permeability agents. VEGF<sub>165</sub> is known to extensively reprogram the endothelial-specific gene expression program, resulting in the increased
production of several proteins: molecules involved in blood clotting, fibrinolysis, proteases, glucose transporters, NO synthase, mitogens, anti-apoptotic factors and adhesion molecules will follow the final angiogenic outcome (Coutts et al., 2005; Gliki et al., 2002; Hoeben et al., 2004; Lohela et al., 2009). VEGF_{165} is a survival factor for the endothelium: in vitro studies revealed a fundamental role of the activation of the phosphatidylinositol (PI)-3 kinase–Akt (v-Akt murine thymoma viral oncogene homolog) pathway as well as the anti-apoptotic proteins Bcl-2 and survivin. In vivo the pro-survival VEGF action seems to be developmentally regulated and coverage by pericytes has been proposed to be one of the key events resulting in loss of VEGF vessel dependence: as a matter of fact, VEGF stimulation seems to be needed only at the early stages of vessel formation (Coutts et al., 2005; Ferrara, 2005).

**VEGF_{121} and VEGF_{189/206} (ligands of VEGFR1 and 2).** VEGF_{121} lacks the residues encoded by exons 6 and 7, thus lacking the capability to bind Neuropilins (NPs) and heparin. While VEGF_{121} is produced as a freely diffusible molecule, VEGF_{189} and VEGF_{206} bind to heparin and to the extracellular matrix with high affinity. The extracellular matrix-bound isoforms may be released in a diffusible form by plasmin cleavage at the C terminus, which generates a bioactive fragment (Coutts et al., 2005; Ferrara, 2005; Hoeben et al., 2004). The biological significance of the heparin-binding VEGF isoforms is also emphasized by the findings that 50% of the mice expressing exclusively either VEGF_{120} (mouse VEGF protein is shorter by one amino acid) or VEGF_{188} die shortly after delivery, with severe vascular anomalies, whereas the remainder die within two weeks (Gu et al., 2003; Zelzer et al., 2002). Taking into consideration the need of ECs for both long- and short-range guidance cues for directional migration, studies on EC filopodia suggest a main role for VEGF_{120} in the former and VEGF_{188} in the latter (Claesson-Welsh, 2003; Ellis and Hicklin, 2008; Hoeben et al., 2004).

**VEGF-B (ligand of VEGFR1).** VEGF-B is abundantly expressed in the adult myocardium, skeletal muscle and pancreas as a selective ligand for VEGFR1. In mouse embryonal tissues, high expression is seen in the developing heart, brown fat, muscle and spinal cord. VEGF-B null mice have smaller hearts, dysfunctional coronary vasculature, and impaired recovery from cardiac ischemia. Alternative splicing of the VEGF-B gene gives rise to two isoforms. Both VEGF-B_{167} and VEGF-B_{186} are produced as disulfide-linked homodimers; when co-expressed, they may influence the bioavailability of the VEGF-A molecule, through the generation of VEGF-B/A heterodimers (Ferrara, 2005). While VEGF-B_{167} binds NP-1, VEGF-B_{186} requires limited proteolysis to interact with the
Introduction

membrane molecule; notably, VEGF-B_{167} is likely to promote a signalling feature regardless the presence of VEGFRs, maybe involving Neuropilin interacting protein (Cai and Reed, 1999; Hoeben et al., 2004). Through the activation of VEGFR1, VEGF-B induces a weak EC proliferation response and the activation of Plasminogen Activator Inhibitor 1, thus probably avoiding an excessive matrix proteolysis (Hoeben et al., 2004).

**VEGF-C and D (ligands of VEGFR2 and 3).** VEGF-C is mainly expressed in the adult heart, placenta, ovary, small intestine and thyroid gland, whereas in embryonic tissue expression occurs in the sprouting points from embryonic veins of lymphatic vessels. VEGF-C is secreted as a disulfide-bonded homodimer that is proteolytically processed from a precursor polypeptide; the fully processed molecule is a non-covalently bound dimer (Ferrara, 2005). VEGF-D is found in the developing as well as in the adult lung, in the mature heart, skeletal muscle, colon and small intestine. Together with VEGF-C, VEGF-D defines a subfamily of lymphangiogenic cytokines sharing a close similarity to VEGF homology domains. They act through the activation of VEGFR2 and VEGFR3 and can also induce angiogenesis and enhance vascular permeability under appropriate circumstances (Ferrara, 2005).

**VEGF-E (ligand of VEGFR2).** VEGF-E has only ~20% of amino acid identity with VEGF and it is encoded by a parapox virus leading to exanthemous disease (Orf-virus). This molecule seems to be involved in the process of pathological angiogenesis in virus-infected lesions: actually the lesions caused by an Orf virus are highly vascularised and oedematous, with an increased number of vessels which arise from the proliferation of ECs and contains extensive inflammatory infiltrates composed by several cell types (Ferrara, 2005).

**PIGF (ligand of VEGFR1).** As its name implies, PIGF was originally discovered in the placenta: this molecule is not highly expressed in normal embryonic or adult tissues, but is expressed by many tumours and in other cases of pathological angiogenesis, where it is thought to supplement and potentiate VEGF-A effect (Ferrara, 2005).

VI.2.1.2. **VEGF-A-induced signalling pathways**

The members of the VEGF family act through their interaction with cognate canonical and non-canonical receptors on the surface of both endothelial and non-ECs. Functional inhibitors of VEGF and VEGFRs, such as anti-VEGF neutralizing antibodies and kinase activity inhibitors, have recently been approved as drugs to treat patients with colorectal, lung, renal and liver cancers, enforcing the importance of unravelling the complete scenario of the VEGFR downstream signalling, not only in the angiogenic process, but also
in physiological and pathological conditions (Claesson-Welsh, 2003; Ellis and Hicklin, 2008; Ferrara, 2005; Gerber et al., 1998; Murakami et al., 2008).

**Signalling mediated by VEGFR1 (receptor for VEGF-A, B and PlGF).** Although Fms-Like Tyrosine kinase (Flt) 1 was the first tyrosine kinase receptor (RTK) to be identified to bind VEGF-A (de Vries et al., 1992), the precise function of this molecule is still under debate: it seems to promote different effects depending on the developmental stage or the cell type analyzed. Upon high affinity ligand binding, the receptor undergoes a weak autophosphorylation on tyrosine residues: Tyr213 within VEGFR1 is a major binding site for PI3-K, Nck, and Src homology 2-containing tyrosine phosphatase SHP-2 (Cross et al., 2003; Hoeben et al., 2004; Karamysheva, 2008). VEGFR1 is expressed in vascular endothelial cells as well as in monocytes/macrophages and its signalling is involved in the migration of macrophages toward VEGF-A: it is worth noting that the kinase domain of VEGFR1 seems to be needed for macrophage recruitment and vascular leakage promoting lymphangiogenesis as well as angiogenesis, at least in the model analysed by Murakami and colleagues (Murakami et al., 2008). However, it is worth noting that the viability of mice models expressing VEGFR1 lacking the TK domain as well as the presence of soluble form of Fms-Like Tyrosine kinase imply a negative role in vascular development for VEGFR1, maybe by trapping VEGF-A via its ligand-binding domain in the embryonal stages (Coulats et al., 2005; Ferrara, 2005; Murakami et al., 2008). Further studies using retroviral vectors expressing a small interfering RNAs targeting either the VEGFR1 gene or the VEGFR2 gene, also suggest a role for the receptor 1 in the maintenance of endothelial integrity, through the modulation of the VEGF/Akt signalling (Claesson-Welsh, 2003; Ito et al., 1998; Murakami et al., 2008; Nishi et al., 2008). VEGF-B, selective VEGFR1 ligand, is not only dispensable in development and health, but also in most conditions of pathological angiogenesis, except for the ischemic myocardium: it is able to stimulate angiogenesis primarily in this tissue and promotes compensatory hypertrophy of the remote myocardium after myocardial infarction (Li et al., 2008). Adenoviral gene therapy approach in both normoxic and ischemic myocardium suggest a further involvement of NP-1 and G-protein mediated signalling in the angiogenetic effect observed in vivo (Lahteenvuor, 2009).

**Signalling mediated by VEGFR2 (receptor for VEGF-A, C, D and E).** The tyrosine kinase receptor named Kinase insert Domain Region (KDR) in human and Foetal liver kinase 1 (Flk-1) in mouse, seems to be the major mediator of the VEGF-induced biological effects: as a matter of fact, VEGFR2 null mice die in uterus because of lack in
vasculogenesis. The signalling feature induced upon VEGFR2 triggering has been analysed mainly using VEGF_{165} as a ligand. Exploiting the peculiar binding capability of different VEGF isoforms, Kawamura and colleagues analyse VEGF_{165} and VEGF_{121} signalling features in cells expressing VEGFR2 together with the co-receptor NP-1, thus observing a VEGF_{165} only p38 activation involved in cellular migration; studies of the signalling pathway induced are actually still far to be comprehensive (Kawamura et al., 2008). The phospho-peptide analysis of VEGF-A stimulated mammalian cells over expressing KDR revealed that the major phosphorylation sites are \text{tyr}_{951} and \text{tyr}_{966} in the kinase-insert domain, \text{tyr}_{1034} and \text{tyr}_{1069} within the kinase domain, and \text{tyr}_{1172} and \text{tyr}_{1214} in the C-terminal tail of the receptor (Takahashi et al., 2001). Phosphorylation of specific tyrosine residues in the receptor creates a consensus sequence for the recruitment of specific intracellular proteins, via their Src homology 2 (SH2) domains. These include Grb2, VEGF receptor-associated protein (also called T-cell-specific adapter molecule-VRAP/ TSAd) to phospho-\text{tyr}_{951}, phospholipase C-\gamma (PLC-\gamma), Serine/threonine-protein kinase (Sck) and Shb to phospho-\text{tyr}_{1175}, the adaptor proteins Nck and the protein tyrosine phosphatases SHP1 and SHP2 to phospho-\text{tyr}_{1214}; the human low-molecular-weight phosphotyrosine phosphatase HCPTPA has been also identified as a negative regulator of VEGFR2 signalling (Claesson-Welsh, 2003; Cross et al., 2003; Karamysheva, 2008). Moreover, the phosphorylation in serine and the ubiquitination in the carboxyl terminus of the VEGFR2 are probably involved in the VEGFR2 endosomal sorting and lysosomal degradation of the receptor–ligand complexes (Holmes et al., 2007)(Figure 4).
Figure 4. VEGFR2 signalling pathway.

VEGF binding to the receptor induces its dimerization and autophosphorylation. Activation of intracellular signalling cascades results in proliferation, migration, survival and increased permeability. Several intracellular proteins bind to specific phosphorylated tyrosine residues in the VEGFR2, via their SH2 domains, leading to the phosphorylation and activation of these proteins. Binding of PLC-γ to pY1175 results in the hydrolysis of PIP2. The produced DAG activates PKC, while IP3 induces the release of intracellular calcium (Ca²⁺). Entry of extracellular calcium, through specific channels, is also important for the activation of certain proteins. VRAP/TSAd binds to pY951 and forms a complex with Src. Binding of Nck to pY1214
results in activation of Cdc42 and p38MAPK. Binding of Shb to pY1175 regulates activation of FAK and PI3K. Abbreviations: BAD, Bel-2 associated death promoter; cPLA2, cytosolic phospholipase A2; DAG, sn-1,2-diacylglycerol; eNOS, endothelial nitric oxide synthase; Erk 1/2, extracellular regulated kinases 1 and 2; FAK, focal adhesion kinase; Gab1, Grb2-associated binder-1; HSP27, heat-shock protein 27; IP3, inositol (1,4,5)-triphosphate; MAPKAP 2/3, MAPK-activating protein kinases 2 and 3; MEK, MAPK/Erk kinase; NO, nitric oxide; p38 MAPK, p38 mitogen-activated protein kinase; PI3K, phosphoinositide 3-kinase; PGI2, prostacyclin; PIP2, phosphatidylinositol (4,5)-bisphosphate; PIP3, phosphatidylinositol (3,4,5)-triphosphate; PKB, protein kinase B; PKC, protein kinase C; PLC-γ, phospholipase C-γ; Src, Src-like protein; VEGF, vascular endothelial growth factor; VEGFR2, VEGF receptor 2; VRAP, VEGFR associated protein/TSAδ T-cell-specific adaptor molecule (Holmes et al., 2007)

**Proliferation.** A major mitogenic signalling mechanism for VEGF is the PLC-γ pathway, recruited to Tyr801 and Tyr1175 within VEGFR2, thus resulting in mobilization of intracellular Ca2+ and PKC activation (Gliki et al., 2002). PKC mediates activation of Erk 1/2 via Raf-1 and MEK and this pathway is a major mediator of both mitogenesis and cPLA2 activation, leading to generation of COX-derived prostanoids (PGs) (Lamalice et al., 2006; Shibuya, 2008).

**Survival.** VEGF-dependent EC survival is mainly mediated via PI3K-dependent PKCδ serine phosphorylation, thus leading to the activation of Akt, the anti-apoptotic serine-threonine kinase acting on molecules like BAD and Caspase 9 (Cross et al., 2003; Gerber et al., 1998; Gliki et al., 2002). Notably, beta-catenin increases KDR phosphorylation and promote its association with PI3K, thus suggesting an involvement of GSK3β signalling in Akt activation (Skurk et al., 2005).

**Permeability.** eNOS activation occurs through both Akt-dependent, Ca2+ independent, mechanisms, as well as through Ca2+ signalling: the resulted NO generation leads to an increase in permeability and probably contributes to the migration phenotype observed. As a matter of fact, NO activates the serine/threonine phosphatase calcineurin, leading to activation of the transcription factor NFAT (nuclear factor of activated T-cells) and induction of COX-2, which mediates long-term prostanoid production. NO and PG are suggested to be involved in several biological effects of VEGF, including angiogenesis, increased vasopermeability, hypotension and local arterioprotection (Cross et al., 2003; Hoeben et al., 2004).

**Migration.** Several lines of evidence clearly demonstrate a key role for the focal adhesion kinase FAK in the cytoskeletal reorganization as the observation that FAK inhibition prevented VEGF-A-induced actin reorganization: FAK phosphorylation at Tyr397 is sustained by Src Kinase and binds the p85 subunit of PI3K, providing a local source of PIP3 for the initiation of downstream signals mediated by PI3K activation. PIP3 leads to the activation of the small molecular-weight, GTP binding protein Rac, and results in lamellipodia/membrane ruffles and cell motility (Soga et al., 2001). Unlike many other
RTKs, the VEGFR2 does not contain the pY–X–X–M sequence recognised by the SH2 domain of the p85 regulatory subunit of PI3K. However, the Gab1 adaptor protein couples VEGFR2 activation to PI3K and cellular migration. Gab1 contains a binding site for the p85 subunit of PI3K and also binds to the VEGFR2, although the exact binding site in the receptor remains obscure (Holmes et al., 2007; Laramee et al., 2007). Recent evidences highlight also the Grb2-dependent Gab2 tyrosine phosphorylation upon VEGFR2 triggering; although Gab2 apparently do not bind the receptor, it interacts with signalling molecules including PI3K and SHP2, thus suggesting non-redundant activities of Gab1 and Gab2 (Caron et al., 2009). PI3K mediated Akt activation leads to phosphorylation of the actin-binding protein girdin, and eNOS activation; the integration with PLC-γ signalling and the Nck mediated fyn kinase and p38 activation results in actin stress fibers formation and cell migration (Claesson-Welsh, 2003; Ferrara, 2005; Holmes et al., 2007; Kitamura et al., 2008). With respect to actin depolymerisation, VEGF treatment induce the phosphorylation of LIM kinase and its target cofilin, an actin depolymerisation factor through a mechanism involving both PI3K as well as the rho-activated kinase (ROCK) (Gong et al., 2004).

**Neuropilins (NPs).** See below.

**Integrins.** See below.

### VI.2.1.3. Roles of VEGF-A in disease

VEGF-A is a pleiotropic cytokine exerting a fundamental role both in vasculogenesis as well as in physiological and pathological angiogenesis. Vessel growth deregulation has a major impact on human health and contributes to the pathogenesis of many disorder, some of which quite unexpected. Indeed a long list of disorders is characterized or caused by insufficient vessel growth and abnormal vessel regression. The normal physiology of several organs can be affected: the circulatory, nervous, gastrointestinal and reproductive systems are specifically involved in heart and brain ischemia, Chron disease and pre-eclampsia, only to name a few. On the other hand, cancer, psoriasis, arthritis and blindness, as well as obesity, asthma, and atherosclerosis are deeply studied as caused by abnormal vascular remodelling or excessive angiogenesis (Bhushan et al., 1999; Chen et al., 2005; Gliki et al., 2002; Guttmann-Raviv et al., 2006; Hoeben et al., 2004; Shibuya, 2008). As a matter of fact, most **tumours** display up-regulated VEGF mRNA levels and VEGF protein production, preferentially the VEGF-A$_{165}$ isoform. In most cancers, VEGF$_{165}$ is generally positively correlated with cancer progression and decreased survival rate; several studies have shown that combining anti-VEGF treatment (blocking its binding to VEGF receptors)
with chemotherapy or radiotherapy results in a greater anti-tumour effect than either treatment alone (Ellis and Hicklin, 2008; Ferrara, 2005; Pan et al., 2007a). Rheumatoid arthritis is an autoimmune, polyarticular disease characterized by the proliferation of the synovial lining cells and a massive cellular infiltration of the synovia with leucocytes: VEGF₁₆₅, VEGFR2 and Neuropilin-1 up-regulation closely correlate with the synovial angiogenesis observed. The increase in microvessel density is also observed in the non-inflammatory, degenerative osteoarthritis: strikingly, the VEGF₁₂₁ isoform is constitutively expressed in both conditions (Ikeda et al., 2000).

Diabete mellitus is an endocrine disease characterized by a generalized microangiopathy particularly affecting the retina, kidneys, nervous and vascular system; VEGF has emerged as a key mediator of intraocular neovascularisation and play a major role in the early stages of diabetic retinopathy (Duh and Aiello, 1999). Psoriasis is a common chronic skin disease characterized by recurrent erythematous skin plaques with epidermal hyperplasia, a variable inflammatory cells and a dermally derived VEGF-induced neovascularisation (Bhushan et al., 1999).

VEGF-A response is probably necessary for the myocardium preservation and limitation of the hypoxic cellular destruction in the case of cardiovascular ischemia. Regrettably, in this pathology as well as in the peripheral vascular disease, the natural compensatory angiogenesis is not always sufficient, maybe due to an insufficient pro-angiogenetic cytokines (as VEGF-A) production or an inadequate response to them. Despite major advances in re-vascularization techniques, the design of a safe and effective therapeutic angiogenesis is still an intriguing and unresolved task (Hoeben et al., 2004; Nussbaum et al., 2007; Rissanen and Yla-Herttuala, 2007; Tirziu and Simons, 2005).

VI.2.2. Angiopoietins

VI.2.2.1. Angiopoietins (1 and 2) (ligands of Tie 1 and 2)

Vessel stabilization. Tie is a tyrosine kinase family with immunoglobulin and epidermal growth factor homology domains and represents a novel class of receptor tyrosine kinases predominately expressed by vascular ECs. The orphan receptor Tiel1 is still the less studied member, despite of the genetic studies indicating a major role for this molecule in blood vessel restructuring, polarity, maturation and maintenance: notably, a constitutively active Tie2 results in Tiel1 phosphorylation (Laflamme et al., 2007; Loughna and Sato, 2001). Angiopoietins 1 and 2 are produced by mural and ECs respectively and they exert opposite functions acting on the same receptor Tie2: Ang2 does not induce phosphorylation of Tie2,
but instead blocks Ang1-mediated Tie2 receptor activation, thus destabilizing vessels, ultimately leading to vessel regression (Loughna and Sato, 2001). There is a strong link between angiopoietins and VEGF-A protein. As a matter of fact, in the presence of VEGF, Ang2 facilitates vascular sprouting anastomose, resulting in vascular loops and networks. On the contrary VEGF-induced vasculature is stabilized by Ang1 presumably favouring the communication between endothelium and mural cells (Arsic et al., 2003; Augustin et al., 2009; Ferrara, 2005). Recently, Lee and colleagues showed an Ang2-Tie2-dependent down-regulation of VEGF expression through the modulation of the transcription factor subunit Hypoxia Inducible Factor 1 α (Lee et al., 2008).

VI.2.3. Deltalike

VI.2.3.1. Deltalike (Dll4) (ligand of Notch 1-4)

Arterial-venous EC fate. Genetic analysis revealed the presence of four different receptor (Notch1-4) bound by five ligands (Dll1, 3, 4 and Jag1 and 2): of the two mammalian Notch receptors expressed in vascular endothelium, Notch1 is broadly expressed in diverse cell types, whereas Notch4 is preferentially expressed in ECs. Compelling evidences from the study of human diseases (like the Alagille syndrome and cerebral autosomal-dominant arteriopathy with subcortical infarcts and leukoencephalopathy) as well as of knockout animals, suggests an involvement of the Notch pathway in the arterial-venous fate of differentiating endothelial progenitors. As a matter of fact, well-known anatomical and physiological differences distinguish, among mature vessels, arteries and veins. Not only do they differ in the blood pressure they sustain and in the thickness of their smooth muscle cells coat, but these cells also have a distinct identity and origin. For instance, smooth muscle cells surrounding some thoracic vessels are derived from the neural crest, whereas coronary arteries are covered by cells arising both from epicardium and from mesenchyme. Genetic studies offered insight into the signals controlling arterial and venous identities of ECs: Dll4 seems to act in an autocrine manner on the arterial endothelium, through the receptor Notch1 and 4 (Coults et al., 2005; Ferrara, 2005; Shibuya, 2008). Actually, either abrogation or forced activation of Notch signalling disrupts blood vessel development, suggesting that this signalling pathway needs to be finely tuned to trigger proper angiogenesis. Notably, Zhang and colleagues demonstrate a role for Dll4-Notch4 signalling also in the VEGF-induced differentiation of human Mesenchimal Stem Cells towards an arterial or venous EC (Zhang et al., 2008).
Modulation of EC biology. The Notch pathway acts as a modulator of VEGF signalling: the VEGF-dependent up-regulation of Dll4 acts as a negative feedback that block EC proliferation through the transcriptional inhibition of VEGFR2 and NP-1, as well as in the increased expression of the decoy receptor VEGFR1. The Notch-dependent expression of the anti-apoptotic protein Bcl-2 and VEGFR3 results in a pro-survival effect on ECs (Coulteras et al., 2005; Jain, 2003; Sainson and Harris, 2008; Shawber et al., 2007).

VI.2.4.  **Ephrins**

VI.2.4.1.  Ephrin (EphA1, B1 and 2) (ligands of Ephrin receptors (EphA2, B2, 3 and B4))

Arterial-venous EC fate. The Eph receptor tyrosine kinases comprise the largest known family of growth factor receptors, and use the similarly numerous Ephrins as their ligands. The Ephrins are unlike ligands for other receptor tyrosine kinases in that they must be tethered to the membrane to activate their Eph receptors. Although initially characterized in the nervous system, knockout animal studies suggested key roles for EphrinB2 and its EphB4 receptor during vascular development. In particular EphB2-EphB4 are likely to be specific markers of venous versus arterial EC: detected on subsets of developing blood vessels before the onset of circulation, EphrinB2 is a peculiar feature of arteries, whereas, its receptor EphB4 of veins Ephrin-B2 knockout mice display defects in angiogenesis by both arteries and veins in the capillary networks of the head and yolk sac as well as in myocardial trabeculation (Kuijper et al., 2007).

Angiogenesis and modulation of EC biology As a matter of fact tumour vasculature is indeed characterized by an increase in EphA1 and its receptor EphA2 levels and recent evidences suggest an involvement of this signalling in the regulation of lung vessel permeability. The requirement for Ephrin signalling is indeed proved by the inhibition of capillary tube-like formations by human umbilical vein ECs (HUVECs) upon expression of a dominant negative form of EphA2 (Kuijper et al., 2007). Thus, it is not surprising the down-regulation of EphA1 expression in resting ECs (Cheresh and Stupack, 2008). Mice lacking the expression of Platelet-EC adhesion molecule-1 unravelled a potential interaction with EphB2-EphB4 and eNOS in the survival, migration, and functional organization of EC during retinal vascular development and angiogenesis (Dimaio et al., 2008).
VI.2.5.  **Semaphorins**

VI.2.5.1.  **Semaphorins (Sema3, 4, 5, 6) (ligands of Plexins A and B, Neuropilin 1 and 2)**

**Modulation of EC biology.** Semaphorins are implicated in the regulation of many biological processes, such as neural development and organ morphogenesis in the embryo, immune response, angiogenesis and invasive tumour growth in the adult. Semaphorin signalling affects cytoskeletal remodelling and Integrin-dependent adhesion, consequently affecting cell migration; however, it has also been implicated in the regulation of cell proliferation, apoptosis and, recently, in cell differentiation. Notably, Neuropilin1 (NP-1), a transmembrane protein which modulates VEGF signalling in ECs, acts in conjunction with Plexin A1 as a receptor for Semaphorin 3A (Sema3A), a factor that is also involved in vascular patterning and regulation of tumour growth (see below) (Casazza et al., 2007; Franco and Tamagnone, 2008; Staton et al., 2007; Tamagnone and Comoglio, 2004).

VI.2.5.2.  **Semaphorin family members**

The Semaphorins are aptly named, drawing analogy with the system of flags and lights that is used in rail and maritime communication for the direction of movement. The Semaphorin family is indeed a large family of secreted and membrane bound molecules, which have been originally identified for their ability to affect axon steering, fasciculation and branching in developing nervous system (Huber et al., 2003). Semaphorins are actually also implicated in the regulation of many biological processes mainly involving cell morphology and adhesion remodelling, such as neural development and organ morphogenesis in the embryo, immune response, angiogenesis and invasive tumour growth in the adult (Acevedo et al., 2008; Bagnard et al., 2001; Bashaw, 2004; Catalano et al., 2006; Gu et al., 2005; Guttmann-Raviv et al., 2006; Kruger et al., 2005).

The Semaphorin family contains 21 vertebrate genes and eight additional genes that are found in invertebrates. Sema proteins are defined by the presence at their NH₂-terminal ends of the SEMA domain, a ~ 400 amino acid conserved region that folds into a seven-blade β-propeller, structurally similar to the α Integrin β-propeller domain; interestingly this domain is also found in Plexins and in the receptor tyrosine kinase Met and Ron (Bussolino et al., 2006; Kruger et al., 2005). Based on the similarity of their COOH-terminal sequences downstream the SEMA domain and their phylogenetic relations, Semaphorins are divided into eight classes. Except for the viral sema (V- Sema), all other molecules display a 60 amino acid long Plexin-Semaphorin-Integrin (PSI) domain. While
trombospondin domain presence is a peculiar feature of class 5 sema, and a single immunoglobulin (Ig)-like domain is only found in Semaphorin classes 2, 3, 4 and 7. The cellular localization as a transmembrane or a glycosylphosphatidylinositol (GPI) linked protein is a characteristic of classes 1, 4, 5, 6 and 7 and is involved in the bidirectional signalling observed (Franco and Tamagnone, 2008; Zhou et al., 2008) (Figure 5).

**Figure 5. Semaphorin classification.**

There are eight classes of Semaphorins and four types of Plexin. Class 1 and 2 Semaphorins and PlexinA and PlexinB are found in invertebrates. Class 3–7 Semaphorins are found in vertebrates. The last group includes Semaphorins encoded by viruses. In vertebrates there are four A-type Plexins, three B-type, one C-type and one D-type. Arrows indicate binding interactions detected between Semaphorins and Plexins. Labels on the arrows indicate which specific Semaphorin has been shown to interact with which Plexin. Blue labels indicate the necessity for Neuropilin1 (dark blue) or Neuropilin2 (light blue) as co-receptors. The domains of each molecule are drawn in the picture. PSI = Plexin, Semaphorin and Integrin, Ig = immunoglobulin (Ig), thrombospondin domains. IPT =Ig-like, Plexins and transcription factors, GAP= GTPase-activating protein (Kruger et al., 2005).
In the following paragraphs each class of Semaphorins will be analysed and the biological feature of the main ligand-receptor complexes unravelled in detail.

**Class 1 and 2 Semaphorins.** Class 1 and 2 Semaphorins are molecules exclusively expressed in invertebrates and have been extensively characterized in Drosophila Melanogaster.  

_Sema1a and b (ligands of PlexinA); Sema2a (ligand of PlexinB)._ PlexinA is the receptor for the transmembrane Sema1a and 1b proteins, whereas PlexinB binds the secreted Sema2a. The expression patterns of these molecules are relatively non-overlapping and the establishment of the tibial sensory projection within the limb bud relies on the combinatorial action of both Semaphorins. Notably, PlexinA1 associates with receptor tyrosine kinase-like transmembrane protein Off-track and the guanylyl cyclase Gyc76C to fully transduce Sema1a signalling (Kruger et al., 2005).

**Class 3 Semaphorins.** The secreted Sema3s have been described mainly as guidance cues on a great number of axon subsets in order to prevent their aberrant growth during the development of neuronal connectivity. The observed congruence between the peripheral nervous and the cardiovascular systems strongly supports these molecules as key players involved in the modulation of angioblast migration, blood vessel branching and vascular patterning: as a matter of fact, Sema3s are considered one of the guidance cues shared between these two systems; moreover their receptors are expressed in EC (Castellani and Rougon, 2002; Geretti et al., 2008; Gu et al., 2003; Tamagnone and Comoglio, 2004). Furthermore, PlexinD1, Sema3C, Sema3A and NP-1 mediated Sema3 signalling is fundamental for heart development and the formation of the main outflow tracts (Kruger et al., 2005; Zhou et al., 2008). Class 3 Semaphorins consist of seven (Sema3A to Sema3G) secreted vertebrate disulfide-bound homodimers exerting an additional C–terminal short basic domain. Neuropilin (NP)-1 and -2 are vertebrate transmembrane glycoproteins acting as key components of Sema3 receptors (Bussolino et al., 2006; Gu et al., 2003; Guttmann-Raviv et al., 2006; Tamagnone and Comoglio, 2004).

_Sema3A (ligand of NP-1); Sema3F (ligand of NP-1 and 2)._ Sema3F acts mainly as a NP-2 agonist and has been shown to act as a tumour suppressor in different cancer cell lines: this molecule can inhibit cell migration, angiogenesis and lymphangiogenesis, being maybe under the control of p53. Whereas, Sema3A (discovered as Collapsin as an inducer of growth cone collapse of vertebrate sensory neurons) exerts controversial effects in different tumour models: as a matter of fact Sema3A is expressed in several tumours and
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inhibits T cell mediated response, but it also negatively affects tumour angiogenesis as well as the growth of a subset of tumour cell lines (Kigel et al., 2008; Neufeld and Kessler, 2008; Tran et al., 2007). Sema3A has actually a major role in the development of cortical connectivity, guiding axons from their initial sprouting point out of the cortical plate of the cerebral cortex, through the corpus callosum and during their movement to subcortical targets in the internal capsule and at the entry of the spinal cord (Bechara et al., 2008; Castellani et al., 2000; Gu et al., 2003). As a matter of fact, Sema3A can repel growth cones while it simultaneously stimulates dendrites outgrowth. Notably, Sema3A is able to inhibit VEGF-induced EC growth and VEGF-induced angiogenesis, but the reverse situation has also been described in neuronal cell types, suggesting a neurovascular regulation based on the balanced action of these molecules (Bagnard et al., 2001; Miao et al., 1999). Furthermore, Sema3A and 3F are likely to modulate in a synergistic manner EC adhesion and migration during angiogenesis, as well as EC apoptosis (Acevedo et al., 2008; Bielenberg and Klagsbrun, 2007; Guttmann-Raviv et al., 2007). Notably, all the vascular defects observed in Sema3A−/− embryos resemble at least in part the phenotype of EphB2 or EphB4 mutant mice (Serini and Bussolino, 2004).

Sema3B (ligand of NP-1 and 2). Sema3B is an inducer of tumour cell line apoptosis: it has been discovered as a tumour suppressor molecule, together with Sema3F, by investigation of homozygous deletion occurring in a subset of cancers (Kruger et al., 2005; Neufeld and Kessler, 2008).

Sema3C (ligand of NP-1 and 2). Sema3C has been shown to play a key role in heart development, to promote angiogenesis and to modulate the immune system. Its aberrant expression in recurrent squamous cell carcinoma patients and in metastatic lung carcinoma suggests a positive role for this molecule in tumour development (Kruger et al., 2005; Neufeld and Kessler, 2008).

Sema3D (ligand of NP-1 and 2). Zebrafish models, in which there are four NPs, unravelled a role for Sema3D in axon migration; Neurepilins (zebrafish NP-1a and 2b) contribution probably defines Sema3D repulsive and attractive phenotypes observed (Tran et al., 2007). Kigel and colleagues recently demonstrate a novel role for Sema3D in repelling HUVECs migration and in inhibiting tumourigenesis in vivo (Kigel et al., 2008)

Sema3E (ligand of PlexinD1). Sema3E is the only class 3 Semaphorin able to directly interact with Plexins and its action probably limits angiogenic sprouting, thus defining areas of vascular exclusion in a PlexinD1-dependent manner (Gu et al., 2005; Zhou et al., 2008). In the developing nervous system, PlexinD1 triggering by Sema3E attracts or repels
specific axons clusters and controls the formation of select forebrain projections. NP-1 interaction with PlexinD1 is also detected prior Sema3E engagement, thus regressing Sema3E-induced axon repulsion into attraction (Chauvet et al., 2007; Zhou et al., 2008). Recent pieces of evidence showed Sema3E-dependent primary ECs repulsion as well as anti-tumourigenesis (Kigel et al., 2008).

**Sema3G (ligand of NP-2).** Through the binding to NP-2, Sema3G induces the repulsion of sympathetic axons, but not dorsal root ganglion ones. Recent lines of evidences suggest this molecule as an anti-tumourigenic molecule in vivo and a significant prognostic molecule of gliomas (Karayan-Tapon et al., 2008; Kigel et al., 2008).

**Class 4 Semaphorins.** Class 4 Semaphorins are the largest group of transmembrane Semaphorins, including Sema4A-4D, 4F and 4G. Most of the Sema4s have potential PDZ-domain interaction motifs, involved in the organization of the membrane structure called “postsynaptic density”, a higher-order protein structure present in postsynaptic membranes that functions to concentrate neurotransmitter receptors. In the immune system, Sema4s are likely to modulate the activation state, migration and survival of lymphoid cells, even if they do exert also different effects on non-lymphoid cells through the binding to PlexinBs and D1. Bidirectional signalling for class 4 Semaphorins in the nervous system and immune system is probable; yet it is unknown if these interactions are regulated by binding to Plexins (Kruger et al., 2005; Zhou et al., 2008).

**Sema4A (ligand of PlexinD1).** Sema4A binds its high affinity receptor PlexinD1, thus inhibiting VEGF mediated EC migration and proliferation in vitro and angiogenesis in vivo. Sema4A acts indeed by suppressing Rac-GTP-dependent cytoskeletal rearrangements, Akt activation and Integrin-mediated cell adhesion. Moreover, Sema4A signal in neurons results in a Rho kinase-dependent growth cone collapse.

**Sema4D (ligand of CD72, PlexinB1 and B2); Sema4A (ligand of Tim-2).** Through the binding to the low affinity immune receptors these two molecules acts in an opposite manner modulating B-cell homeostasis and B-cell mediated immunity. As a matter of fact, Sema4A induce the phosphorylation of Tim-2, whereas CD72 is de-phosphorylated upon Sema4D treatment, thus dissociating B-cell receptor complex.

Several lines of evidence suggest that in ECs, Sema4-induced formation of focal adhesion complexes and Integrin-mediated adhesion is mediated by Rho activation. As a matter of fact, Sema4D, also called CD100, is the first example of pro-angiogenic Semaphorins: by activating PlexinB, Sema4D triggers epithelial cells toward an invasive growth phenotype as well as primary ECs towards a pro angiogenic phenotype by the engagement of the
hepatocyte growth factor/scatter factor receptor, Met (Conrotto et al., 2005; Giordano et al., 2002). Recent evidences strongly support a further role for Sema4D in brain development and neuronal migration via PlexinB1-Met activation (Giacobini et al., 2008). Moreover, breast carcinoma cells expressing ErbB2, but not Met, migrate upon Sema4D exposure in a Rho-dependent mechanism (Neufeld and Kessler, 2008).

**Class 5 Semaphorins.** Class 5 Semaphorins comprise two transmembrane molecules, Sema5A and 5B, identified both in vertebrates and in invertebrates and are characterized by a unique extracellular domain containing seven thrombospondin type-1 repeats, in addition to the SEMA domain. There is no a clear correlation between Sema5B and Plexins, but that it does not bind any of PlexinBs; several lines of evidence suggest a role for Sema5B in the viability of renal carcinoma cells (Hirota et al., 2006).

**Sema5A (ligand of PlexinB3).** Sema5A can inhibit neurite outgrowth and induce growth cone collapse as well as activation of the intracellular signalling of Met, through the binding to PlexinB3 in a way similar to the one induced by Sema4D. The observed fibroblast cell collapse occurs through PlexinB3 activation, whereas EC migration upon Sema5A treatment is PlexinB3-Met-dependent (Artigiani et al., 2004; Kruger et al., 2005). The impairment of cardinal veins ramification in Sema5A−/− and Sema5A mRNA localization in the mesoderm surrounding the cranial vessels in wild type embryos suggest a possible role for Sema5A in the vascular branching control in a region-restricted manner (Bussolino et al., 2006; Fiore et al., 2005).

**Class 6 Semaphorins** Class 6 Semaphorins are membrane associated proteins, which act through the activation of PlexinA in a Neuropilin independent manner. A characteristic of these molecules is the reverse signalling, achieved through the binding of the tyrosine kinase Abl to Sema6D, Src to Sema6B and the Ena/Vasp-like protein (EVL) to Sema6A; actually the intracellular events induced by the engagement of different Sema6-PlexinA interactions are still to be unravelled in detail. The complex signalling feature of these molecules is indeed involved in axonal targeting, as well as in cardiac development, and probably in muscle maturation (Zhou et al., 2008).

**Sema6A (ligand of PlexinA1, A2 and A4).** In spite of the lack of information concerning the effects of full-length Sema6A on tumour progression, the extracellular domain of this molecule seems to affect negatively EC migration and tumour angiogenesis (Neufeld and Kessler, 2008). Prislei and colleagues recently demonstrate a correlation between Sema6A and class III tubulin in A2780 cells suggesting a novel function in the cytoskeleton composition and microtubule dynamics (Prislei et al., 2008).
**Sema6C and D (ligands of PlexinA1).** These molecules are involved in the formation of lamina-associated axon projection: Sema6A regulates the hippocampus, while the C and D isoforms modulate the sensory axons in spinal cord (Zhou et al., 2008). Sema6D down-regulation as well as the reverse signalling observed in Sema6D expressing cells clearly demonstrate its fundamental role in heart morphogenesis: Sema6D Knockdown results in thin myocardia resembling the one observed in PlexinA1−/− mice (Tran et al., 2007). During cardiac development, Sema6D attracts or repels ECs in the cardiac tubes depending on the expression pattern of PlexinA1; this Semaphorin has been involved in cardiac morphogenesis by means of distinct biological activities exerted through its receptor, PlexinA1, which formed complexes with Off-track (a receptor with a kinase domain but no associated kinase activity) and VEGFR2 in adjacent regions of the cardiac tube. The VEGFR2-PlexinA1 crosstalk has been shown also in different cell lines, like the asbestos-related malignant malignant pleural mesothelioma cells in which Sema6D can actually trigger VEGFR2 activation through a PlexinA1-dependent mechanism (Catalano et al., 2009; Toyofuku et al., 2004).

**Class 7 A and viral Semaphorins.** Sema7A is the vertebrate homologue of the two viral Semaphorins SemaVA and VB.

**Sema7A (ligand of PlexinC1 and Integrinsβ1).** This Semaphorin modulates T-cell mediated immune response, as well as the peripheral and central axon growth. Sema7A seems to affect both negatively (via PlexinC1) and positively (via Integrins) melanocyte adhesion and morphology. The proper axon tract formation in vivo needs the Sema7A-Integrinsβ1 mediated axon growth. Furthermore transformed growth factor β1 (TGFβ1)-induced pulmonary fibrosis is enhanced by Sema7A, through a still unknown mechanism (Zhou et al., 2008).

**VI.2.5.3. Sema3-induced signalling pathway**

Given phenotypic differences due to the genetic background used, different knockout models strongly define a fundamental role for Sema3s and their receptors in the vascular development and tumoural angiogenesis, therefore failing in defining a clear-cut correlation among Sema3s, NPs and Plexins (Gu et al., 2005; Serini and Bussolino, 2004; Serini et al., 2003). Although the exact mechanism by which the difference in responsiveness is achieved is still unknown, high cyclic nucleotides (cAMP or cGMP) levels seem to correlate with attraction and vice versa (Bashaw, 2004; Huber et al., 2003; Kruger et al., 2005). Actually, Sema3A and Sema3B need also tyrosine kinase activity to exert their activity in neurons and the biological context can modulate their response.
through the activation of mainly cell adhesion molecules as Integrins and RTKs (Bechara et al., 2008; Bussolino et al., 2006; Castellani and Rougon, 2002; Falk et al., 2005; Sasaki et al., 2002). Castellani and colleagues demonstrated the need for the cell adhesion molecule of the immunoglobulin superfamily L1 in Sema3A-induced response in neurons (Castellani et al., 2000). Notably, Serini and colleagues demonstrated the autocrine loop of Sema3 chemorepellents as a fundamental element in the vascular morphogenesis, through the inhibition of Integrin mediated adhesion of EC to the extracellular matrix (Serini et al., 2003). The hypothesis of a cross-talk between Semaphorins and tyrosine kinase receptors is supported by pieces of evidence demonstrating that PlexinA1 interacts with Off-track protein, as well as with VEGFR2, which can be phosphorylated upon Sema6D activated PlexinA1 in malignant mesothelioma cells and (Catalano et al., 2009; Toyofuku et al., 2004). The molecular final tuning of Sema3-induced signalling is obtained through the activation of several membrane molecules: receptors for Sema3s comprise binding subunits, the Neuropilin1 and 2 (NP), coupled with signalling co-receptors, the PlexinsA and D, which mediate the activation of different molecules, like Rho GTPases, CRMPs, mitogen-activated protein kinase (MAPK), PI3K and Src kinase families as well as the induction of redox reactions (Bashaw, 2004; Bussolino et al., 2006; Castellani and Rougon, 2002; Franco and Tamagnone, 2008; Negishi et al., 2005).

**PlexinA.** All the four PlexinA members do not possess a peculiar tissue distribution. They are able to confer a morphological “contraction” in COS cells when co-expressed with NP-1 upon Sema3A treatment, but the exact mechanism through which activation of PlexinA by Sema3A leads to the actin cytoskeleton collapse it is still far from being deeply understood (Togashi et al., 2006). Animal models deficient for PlexinA3 and A4 define the PlexinA3 requirement for Sema3A to inhibit branching of cortical neurons and the selective modulation of specific hippocampal fibres and pyramidal axons, while the complex PlexinA3–NP-2 as the main Sema3F receptor in vivo in sensory and sympathetic neurons (Yaron et al., 2005). Moreover, several Plexin interactors, like the molecule interacting with CasL proteins (MICAL) and Ran bone morphogenetic protein (Ran BMP), can be also considered as a potential link for Plexin-A receptors to retrograde transport and microtubule function in axonal guidance and cytoskeletal rearrangement (Togashi et al., 2006; Zhou et al., 2008).

**Signalling mediated by PlexinA1.** As well as the other members of the Plexin family, PlexinAs directly interact with small intracellular GTPases and their GAP activity is regulated by a RacGEF factor (FERM, RhoGEF and pleckstrin domain protein 2, FARP2).
Upon PlexinA1 engagement, RAC1 is activated, thus enhancing the binding of the small GTPase Rnd1 to Plexin and the activation of p21 activating (PAK) and ROCK kinases: the LIM kinase is then phosphorylated in Thr-508 and acts on its substrate coflin (Aizawa et al., 2001). However, activated PlexinA1 can also sequester RAC1, triggering the dephosphorylation of the actin-degrading enzyme coflin and final actin depolymerisation (Bussolino et al., 2006; Casazza et al., 2007; Franco and Tamagnone, 2008). Rnd1-Plexin interaction stimulates the Plexin GAP action on R-RAS, thus leading to the inactivation of phosphatidylinositol-3-OH kinase (PI3K) and consequently of Akt. The inhibition of β1 Integrin signalling is the result of the combined inhibition of PI3K and of the type 1 phosphatidylinositol phosphate kinase (PIPKIγ661) by FARP2 (Franco and Tamagnone, 2008; Toyofuku et al., 2005; Zhou et al., 2008).
Fes/Fps is a non-receptor-type tyrosine kinase that directly binds to the cytoplasmic region of PlexinA1. In the resting state, Neuropilin directly associates with PlexinA1 and blocks its binding to Fes/Fps. Sema3A binding to Neuropilin allows Fes/Fps to associate with and phosphorylate PlexinA1 as well as CRMP2/CRAM complex (Aizawa et al., 2001; Negishi et al., 2005). As a matter of fact this event, together with Cdk5 and GSK3β activation may participate in Sema3A signalling through regulation of microtubule dynamics by phosphorylation of CRMP2 (Cole et al., 2006).(Figure 6).

![Figure 6. Sema3 signalling pathway in neuronal cells.](image)

PlexinAs are receptors for Sema3s and Sema6s. Sema3s (red) mediate diverse biological functions including axon guidance, cardiovascular development and immune function. PlexinAs (blue) are receptors for Sema3s Sema3s bind Neuropilins (Npn-1 or Npn-2) with high affinity to assemble a Npn–PlexinA receptor complex. This receptor complex requires IgCAMs (light purple) for specific repulsive or attractive axon guidance events. Sema3 binding to the Npn–PlexinA complex promotes FARP2 (plum) dissociation from PlexinA. Dissociated FARP2 activates Rac1 (magenta), which facilitates the Rnd1 (white)–PlexinA association and drives PIPK1β661 (light sky blue)-mediated Integrin (orange) inhibition. Active Rac1 also might facilitate the sequential activation of PAK (tan), LIMK1 (deep pink) and coflin (orange) to control actin dynamics. Rnd1–
Introduction

PlexinA interactions stimulate PlexinA RasGAP activity, which suppresses R-Ras (turquoise) and inactivates PI3K (bisque) signalling. Sema3s can also inhibit PI3K through PTEN (dark golden). PI3K inactivation inhibits Integrin-mediated adhesion signalling and induces the sequential inhibition of Akt (light green), activation of GSK-3b (cyan) and inactivation of CRMP2 (purple). Phosphorylation of CRMP2 by GSK-3b relies on a Cdk5 (light salmon) and Fyn (yellow)-dependent priming phosphorylation. CRMP2 has been proposed to regulate microtubule dynamics and endocytosis. Unlike other Sema3s, Sema3E directly binds PlexinD1 to induce endothelial cell (EC) repulsion and controls intersomatic vessel patterning through unidentified cytosolic mechanisms. Sema3E also acts as an axon repellent or attractant through PlexinD1 (Zhou et al., 2008).

**PlexinD1.** PlexinD1 can be detected at low levels in different adult tissues like heart, placenta, lung, kidney, thymus, liver and brain and can be directly bound to Sema3E only, modulating axon branching and cell migration (Chauvet et al., 2007). Notably, PlexinD1 knockout mice show defects in the cardiac outflow tract patterning, which probably requires Sema3A and Sema3C signalling involving also NP-1 and NP-2 containing complexes. Very little is known about the intracellular events responsible for the observed phenotypes (Gu et al., 2005; Zhou et al., 2008). NP-1 actually co-clustered with the Semaphorin receptor PlexinD1 in Sema3C treated ECs. The interaction is less stable than with VEGFR2, likely reflecting differences between the signalling mechanisms of NP-1 as either VEGFR2 or PlexinD1 co-receptor (Salikhova et al., 2008).

**Neuropilins.** Neuropilin1 (NP-1) and 2 (NP-2) are 130-140 KDa single spanning transmembrane glycoproteins; soluble-splicing isoforms have also been detected (Chen et al., 2005; Guttmann-Raviv et al., 2006; Staton et al., 2007). Their tissue specificity does not overlap: during embryonic development, arteries produce NP-1 protein, while NP-2 is initially expressed in veins and later in lymphatics: mice lacking both Neuropilins display a phenotype similar to the one observed in VEGFR2 knockout animals. They only share a 40 % identity and show a large extracellular region split into 3 domains. The first domain is composed by two “a domain” repeats (a1a2), mainly responsible for the binding of NP-1 to Sema3A and homologous to C1r and C1s complement component; the second domain is composed by “b domain repeats (b1b2)”, major binding site for VEGF<sub>165</sub> and homologous to coagulation factors CF V/VIII; finally, the “meprin, A5, μ domain” (MAM) is needed for Sema3A signalling, most likely being involved in receptor dimerization (Bussolino et al., 2006; Chen et al., 2005; Geretti et al., 2008; Gu et al., 2003; Takahashi et al., 1999).

**Neuropilin1.** Soker identified as a specific receptor of VEGF<sub>165</sub> NP-1, a molecule that binds the collapsin-Semaphorin family and is implicated in neuronal axon guidance; actually this molecule is thought to convey VEGF<sub>165</sub> and Sema3A signals *in vivo* during cardiovascular and neural development (Gu et al., 2003; Miao et al., 1999; Soker et al., 1998). Although NP-1 and VEGFR2 co-cluster in quiescent ECs, they need their ligand to
be bound to each other; the short 40 amino acid cytoplasmic region of NPs, which apparently lacks any known signalling motif, supported in the past the role for NPs as mere co-receptors, involved in the modulation of the signal triggered by neighbouring transmembrane molecules (Geretti et al., 2008; Tamagnone and Comoglio, 2004). Strikingly, the analysis of primary ECs expressing a chimeric receptor bearing the extracellular domain of NP-1 fused with the cytoplasmic region of the epidermal growth factor receptor, suggested NP-1 as a potential signalling inducer through its three C-terminal amino acids (SEA) (Wang et al., 2003). These residues are fully conserved during evolution and interact with the PDZ (PSD-95/Dlg/ZO-1) domain of a NP interacting protein (NIP) involved in vesicular trafficking, thus maybe regulating both VEGF and Sema signalling (Naccache et al., 2006). The knockdown of this interactor in zebrafish revealed a phenotype highly resembling the NP-1 knockdown, thus suggesting the involvement of both molecules in the same pathway (Geretti et al., 2008; Kawamura et al., 2008; Lee et al., 2002; Wang et al., 2006). Recent evidences demonstrated the internalization of NP-1 strictly dependent on NIP and ligand specific: VEGF<sub>165</sub> induces clathrin-dependent endocytosis, whereas Sema3C induces lipid raft (Salikhova et al., 2008). Valdembri and colleagues recently suggested a NP-1-NIP role totally independent from VEGF and Sema3A binding: through its cytoplasmic domain, NP-1 is likely to stimulate the spreading of ECs on fibronectin through the NIP-dependent internalization of the Integrin α<sub>β</sub> (Valdembri et al., 2009)

**Integrins.** These heterodimeric transmembrane proteins are constituted by unique combinations of single α and β subunits, giving rise to at least 22 different receptors with peculiar and overlapping specificity for several extracellular matrix components. A part from the very well known role in supporting cell adhesion and migration, strong evidences support Integrins as key tuner of growth factor signalling, wound healing, angiogenesis, heart hypertrophy and cancer cell metastatic phenotype (Avraamides et al., 2008; Brancaccio et al., 2006; Bussolino et al., 2006). At the cytoplasmic face of the plasma membrane, Integrins interact with actin filaments indirectly via interaction with several proteins, like talin, vinculin, α-actinin, paxillin, filamin, zyxin, VASP, p130CAS, Src, FAK and melusin just to name a few. As a matter of fact, structural and signalling proteins are finely integrated in a complex machinery that mediates Integrin–actin association. Talin and vinculin are indeed key structural elements, while the other signalling molecules trigger intracellular events including the activation of MAP, focal adhesion, Src and PI3 kinases as well as GTPases (Avraamides et al., 2008; Brancaccio et al., 2006; Eliceiri,
2001). Since many of these effectors are also target of several growth factors, it is not surprising that the suitable extracellular matrix background strongly enhance the signalling elicited by platelet-derived, epidermal and vascular endothelial growth factors (Eliceiri, 2001; Soga et al., 2001). The coordinated action of Integrins and growth factor receptors is likely to transduce mechanical stimuli into chemical signals via intracellular signalling pathways: the crosstalk is likely to act through direct binding to the receptor, like Integrrin$\alpha_\beta_3$ to VEGFR2 and Integrrin$\beta_1$ to NP-1, or at the intracellular effectors level, like in the case of $\alpha_\beta_3$ and VEGFR2 (Avraamides et al., 2008; Eliceiri, 2001; Fukasawa et al., 2007; Soga et al., 2001). The GTPase R-Ras is thought to be one of the common targets of growth factor and Semaphorins. Both in neurons and in ECs, Sema3A reduces the level of active $\beta_1$ Integrins and the stimulation of $\beta_1$ counteracts Sema3A function (Serini et al., 2003; Toyofuku et al., 2005).

VI.2.5.4. Role of Sema3 in disease

Besides its very well known role in axonal guidance, class 3 Semaphorins are involved in angiogenesis, lymphangiogenesis and tumour growth. Their receptors, expressed on endothelial and tumour cells, are likely to be responsible of the inhibitory action of Sema3s in these cellular systems (Chen et al., 2005; Guttmann-Raviv et al., 2006; Guttmann-Raviv et al., 2007; Neufeld and Kessler, 2008; Soker et al., 1998). Tumour expression of VEGF$_{165}$ and class 3 Semaphorin may actually regulate the degree of cancer angiogenesis and progression: several evidences support the high VEGF$_{165}$-Sema3A ratio as a poor prognostic value in several tumour models and NP-1 inhibitory antibodies exert an additive anti-tumourigenic effect when administrated in combination with anti-VEGF therapies (Chen et al., 2005; Geretti et al., 2008; Pan et al., 2007a; Staton et al., 2007). Notably, the p53 loss of function in tumour cells results in Sema3F down-regulation and induction of pathological angiogenesis (Futamura et al., 2007). All the class 3 Semaphorins can be cleaved by furin-like pro-protein convertases, but only the NP independent Sema3E acquires opposite properties once processed: the full length protein is actually an anti-angiogenic molecule, whereas the cleaved one is a metastasis inducer (Neufeld and Kessler, 2008). Furthermore, Sema3A is likely to contribute to tumour cells escape from immune surveillance, through the inhibition of primary T-cells proliferation and cytokine production (Catalano et al., 2006).

The finding that a cell adhesion molecule of the immunoglobulin superfamily L1 contributes to Sema3A signalling suggests an involvement of this Semaphorin in the development of a severe neurological syndrome named CRASH (Corpus callosum
hypoplasia Retardation, Adducted thumbs, Spastic paraplegia and Hydrocephalus) (Castellani et al., 2000). This syndrome is caused by mutations in the L1 gene: although it remains to be defined the precise Sema3A contribution in its pathogenesis, a particular mutation abrogates the ability of L1 to interact with NP-1, indicating that patients with this mutation express less active Sema3A receptors (Yaron and Zheng, 2007).

Different animal models of epilepsy, deriving from kainic acid injection, correlate the pathology outcome with a peculiar reduction in the hippocampal expression of different members of class 3 Semaphorins, namely Sema3A, Sema3C and Sema3F. Knockout mice models seem to support a protective role from seizures of Sema3F, being the KO more prone to seizures than the wild-type animals, even in the absence of neuronal injury (Barnes et al., 2003). In order to avoid the developmental defects observed in the Sema3F knockout mouse, inducible conditional knockout animals are needed to unravel direct functional evidences supporting this hypothesis. Interestingly, 10–20 % of individuals with the fragile X syndrome also develop epilepsy and the mRNA encoding Sema3F is a target of the fragile X mental retardation protein and it is has been decreased in fragile X syndrome patient cells, thus supporting the Sema3F involvement in both diseases (Yaron and Zheng, 2007). In the injured nerve, the expression of Sema3A and other members of class 3 Semaphorins are up-regulated, probably restricting peripheral nerve regeneration and anatomical plasticity (Yaron and Zheng, 2007).

VI.3. INDUCTION OF THERAPEUTIC ANGIOGENESIS

In 1971, Folkman and colleagues demonstrated for the first time that tumour development was dependent on neo-vascularisation, probably involving angiogenic growth factors (Folkman, 1971). The unravelling of the molecular mechanisms occurring in the physiological vasculogenesis and angiogenesis results in the classification of two classes of diseases: the first in which the inhibition of the pathological angiogenesis could cure a particular disease or delay its progression (as take place in retinopathies and in tumours); the second one characterized by insufficient angiogenesis (as observed in atherosclerosis and in ischemic diseases). Individuals with coronary and peripheral artery disease are currently treated with drugs, such as 3-hydroxy-3-methyl-glutaryl-CoA reductase inhibitors (‘statins’), vasodilators and antiplatelet agents. Actually a large number of patients, suffering from disabling symptoms due to severe progression of these pathologies, still wait for a therapy more effective than coronary artery bypass grafting, percutaneous catheter-based interventions and balloon angioplasty (Ellis and Hicklin,
2008; Ferrara, 2005; Hoeben et al., 2004; Jacobs, 2007; Karamysheva, 2008). In this perspective, therapeutic angiogenesis aims at safely and efficiently recreating the natural process whereby new blood vessels are formed to supply oxygen and replenish tissues that have been damaged by underperfusion and ischemia (Chen et al., 2005; Guttmann-Raviv et al., 2006; Holmes et al., 2007; Jacobs, 2007). Once identified the optimal therapeutic factor or combination of molecules, the therapeutic approach aims to the arteriogenesis induction by means of an accumulation of angiogenic factors locally, in a certain therapeutic concentration for a certain period in an isolated ischemic tissue area, and with a minimal overflow of molecules into non-ischemic tissues, to avoid the pathological angiogenesis occurrence. Most of the performed trials actually succeeded in accomplishing various secondary endpoints, but still failed in achieving a statistically significant improvement in exercise performance, as requested by the Food and Drug Administration (Annex and Simons, 2005; Jacobs, 2007; Simons, 2005; Tirziu and Simons, 2005).

VI.3.1. Recombinant protein administration

Evidences that members of the fibroblast growth factor (FGF) family, VEGFs, hepatocyte and platelet derived growth factor (HGF, PDGF), as well as granulocyte colony-stimulating factor (G-CSF), stimulate angiogenesis in vivo, suggest a therapeutic potential for these molecules. Several clinical studies have been focused on the intravenous and intracoronary delivery of these factors as unique molecules or as a cocktail composed of protein exerting complementary effects (Annex and Simons, 2005; Jacobs, 2007); among these, are worth noting two clinical studies aiming to the cure of myocardial failure and peripheral artery disease, namely the VEGF in ischemia for vascular angiogenesis (VIVA) and therapeutic angiogenesis with recombinant EGF-2 for intermittent claudication (TRAFFIC) trials (Henry et al., 2003; Lederman et al., 2002). In spite of the little beneficial effects observed, the absence of a statistically significant improvement in the vast majority of the studies ultimately led to the widespread belief that protein therapy, especially with a single agent, is not a viable option to treat ischemic cardiovascular disease (Annex and Simons, 2005; Coulitas et al., 2005; Jacobs, 2007; Jain, 2003).

Actually, several trials are still ongoing and results of the European cooperative acute stroke study III (ECASS III) trial, involving intravenous injection recombinant tissue plasminogen activator within 3h of the onset of acute ischemic stroke, actually revealed modest good outcome in terms of safety and efficacy (Rothwell, 2009). The very fast pharmacokinetics of recombinant proteins infused in the circulation lead to high concentrations of the molecule for a very short period of time, thus inducing in some rh
basic FGF or rhVEGF treated animal models a systemic hypotension. To overcome this issue, different drug administration routes have been taken into consideration, as the development of scaffolds to extend the drug half-life or the direct injection into the ischemic tissue (Annex and Simons, 2005; Coultas et al., 2005; Horowitz et al., 1997; Jacobs, 2007; Jain, 2003) (Figure 7).

<table>
<thead>
<tr>
<th>Protein</th>
<th>Trial type</th>
<th>N</th>
<th>Delivery</th>
<th>Results/comments</th>
</tr>
</thead>
<tbody>
<tr>
<td>FGF1</td>
<td>Phase 1, OL</td>
<td>20</td>
<td>IM injection</td>
<td>Safe</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Captivity blast at injection site</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Reduced ischemic zone size</td>
</tr>
<tr>
<td>FGF2</td>
<td>Phase I/II DBR</td>
<td>24</td>
<td>Heparin-alginate</td>
<td>Effect sustained at 3 years</td>
</tr>
<tr>
<td>FGF2</td>
<td>Phase 1, OL</td>
<td>52</td>
<td>IC infusion</td>
<td>Hypotension at high dosages</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Improved symptoms</td>
</tr>
<tr>
<td>FGF3</td>
<td>Phase 1, OL</td>
<td>30</td>
<td>IC infusion</td>
<td>Hypotension at high dosages</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Dilatation of epicardial coronaries</td>
</tr>
<tr>
<td>FGF2</td>
<td>Phase II, DBR</td>
<td>337</td>
<td>IC infusion</td>
<td>Safe/No effect on ETT or SPECT</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Short-term improvement in symptoms</td>
</tr>
<tr>
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<td></td>
<td></td>
<td></td>
<td>compared to placebo</td>
</tr>
<tr>
<td>VEGF-A165</td>
<td>Phase 1, OL</td>
<td>15</td>
<td>IC infusion</td>
<td>Hypotension at low dosages</td>
</tr>
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<td></td>
<td></td>
<td></td>
<td></td>
<td>Reduced SPECT defect size</td>
</tr>
<tr>
<td>VEGF-A165</td>
<td>Phase 1, OL</td>
<td>14</td>
<td>IV infusion</td>
<td>Safe</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>No clear effects</td>
</tr>
<tr>
<td>VEGF-A165</td>
<td>Phase II, DBR</td>
<td>165</td>
<td>IC+IV infusion</td>
<td>No improvement in ETT, symptoms,</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>or SPECT compared to controls</td>
</tr>
<tr>
<td>GM-CSF</td>
<td>Phase 1/II, DBR</td>
<td>21</td>
<td>IC+IV 2 wk infusion</td>
<td>Improved collateral flow index in the GM-CSF group</td>
</tr>
</tbody>
</table>

Figure 7. Protein gene therapy clinical trials.

In this table are summarised the main clinical trials involving the use of recombinant protein. N. number of patients enrolled in the trial; DBR= Dynamic balancing randomization, is a tree-based method allowing different levels of imbalance in different strata which ensures a balance for each level of prognostic risk factors (conditional balance) whilst at the same time preserving randomness. OL= type of clinical trial in which both the researchers and participants know which treatment is being administered; EET= Exercise Evaluation Team; SPECT= Single photon emission computed tomography; IC= intracoronaric; IV= intravenous; IM= intramuscular (Annex and Simons, 2005)

VI.3.2. Gene and Cell based Therapies for heart disease

Cardiac repair is an exciting novel therapeutic concept. Through cellular therapies, the concept of “regenerating” heart muscle and vascular tissue has revolutionized the approach of treating heart disease. In the heart, cellular repair strategies can include direct transplantation of cells into damaged environments (Cell therapy) and therapies that prompt the heart to regenerate damaged tissues (Gene therapy) (Tirzii and Simons, 2005).
VI.3.2.1. Cell therapy

The first approach, the so called “CELL THERAPY” focuses on repopulation of the injured myocardium by transplantation of healthy cells. Several different cell types, including skeletal myoblasts, c-kit+/Sca-1+/lin- bone marrow (BM) cells, BM mesenchymal stem cells (MSCs), BM “side-population” (SP) cells, endothelial progenitor cells (EPCs), and cardiac stem cells (CSCs), have been utilized in an effort to achieve cardiac reconstitution and restoration of function following myocardial infarction (MI) (Jackson et al., 2001; Kawamoto et al., 2001; Nussbaum et al., 2007; Orlic, 2003; Srivastava and Ivey, 2006).

Actually the little beneficial effect observed is likely due to a paracrine effect, rather than to the replacement of the necrotic tissue by transplanted cells. Failure to produce new myocardial fibers in clinically relevant numbers was attributed to cell death occurring after engraftment and inability of engrafted myoblasts to differentiate and integrate within the host myocardium; hence, electromechanical coupling is not likely to occur after in vivo grafting (Arnesen et al., 2007).

Until recently the heart has been considered a post-mitotic organ. This dogma has been challenged by recent findings of populations of stem cells or cardiac progenitor cells (CPCs) either resident in the mammalian heart or recruited from the circulation from which new myocytes can be derived. These clusters contain Lin-, Sca-1+, c-Kit+ and more recently isl-1+ cells and many of these express markers for cell proliferation and transcription factors associated with early cardiac development like GATA-4 and Nkx2.5.

When isolated, these cells show in vitro properties of stem cells: they are self-renewing, clonogenic and multipotent, giving rise to myocytes, smooth muscle, and vascular cells. Furthermore, when these cells are injected into the border zone of an MI, they are capable of forming a large area of new myocardium, containing CPC derived myocytes and blood vessels (Jackson et al., 2001; Orlic, 2003; Srivastava and Ivey, 2006).

These evidences suggest the existence of a lineage commitment of stem cells towards becoming cardioblast and then cardiomyocytes in the heart.

The identification and isolation of bona fide cardiac progenitors raises immediately the question if the damaged myocardium is really enable the re-enter in the cell-cycle and regenerate; if this is the case, great effort has to be taken to identify the key molecules able to trigger the mitotic switch.

The observed partial remuscularization of rat myocardial infarcts by human embryonic stem cell-derived cardiomyocytes together with the development of technologies to
reprogram differentiated human fibroblasts into pluripotent stem cells encourage their study to better unravel a possible therapeutic application (Laflamme et al., 2007; Mohamadnejad and Swenson, 2008).

VI.3.2.2. Gene Therapy

Gene therapy is a viable alternative to conventional therapies in coronary artery diseases and heart failure. The use of the gene transfer approach, besides offering nowadays an innovative tool to the treatment of several diseases, may and must be considered of basic importance to investigate the physiological role of several molecules in the proper cellular and subcellular environment.

The characteristics of each gene therapy approach are depicted in Figure 8.
Vector | Advantages | Disadvantages
--- | --- | ---
Naked plasmid DNA | Easy to produce safe | Very low transduction efficiency
Adenovirus | High transduction efficiency | Inflammation with high doses
Relatively high transgene capacity | | Transparent expression
Easy to produce in high titers | | Transparent expression
Transduces quiescent cells | | Transparent expression
Trojan for multiple cells | | Transparent expression
Adeno-associated virus (AAV-1, -2, 5, 6, 8, 9) | Long-term gene expression | Limited transgene capacity
Moderate immune response | | Difficult to produce in large quantities
Transduces quiescent cells | | Difficult to produce in large quantities
High tropism for skeletal muscle (AAV-1, -6) | | Difficult to produce in large quantities
and myocardium (AAV-9 and -9) | | Difficult to produce in large quantities
Wild type does not cause disease in humans | | Difficult to produce in large quantities
Lentivirus | Long-term gene expression | Non-specific integration
Transduces quiescent cells | | Low transduction efficiency
Relatively high transgene capacity | | Limited tropism
Low immune response | | Difficult to produce in large quantities
Retrovirus | Long-term gene expression | Non-specific integration
Relatively easy to produce | | Transduces only dividing cells
Low immune response | | Limited tropism
Herpes simplex-virus (HSV-1) | High transduction efficiency | Transduces only dividing cells
High transgene capacity | | Cytotoxicity
Trojan for neuronal cells | | Limited tropism
Epstein-Barr-virus | High transduction efficiency | Transduces only dividing cells
High transgene capacity | | Difficult to produce in large quantities
Extraduenosomal replication
Baculovirus | High transgene capacity | Transient expression
Easy to produce in high titers | | Inflammation with high doses
Rapid construction of recombinant baculoviruses | | Limited tropism
Wild type does not cause disease in humans
Antisense oligonucleotides | Easy to produce | Limited efficacy
siRNA | More potent than antisense approach | High gene transfer efficiency required
| | High gene transfer efficiency required

Abbreviation: siRNA, small interfering RNA.

Figure 8. Gene therapy approaches.

Here are summarized the main characteristics of all gene therapy approaches. siRNA= small interfering RNA (Rissinan and Yla-Herttuuala, 2007).

More than 20 cardiovascular gene therapy trials have been initiated worldwide. Most of these trials involve gene transfer of angiogenic factors to ischemic hearts in an attempt to promote new vessel formation in patients with coronary-artery disease (Giacca, 2007; Grines et al., 2002; Hedman et al., 2009; Jazwa et al., 2007; Rissinan and Yla-Herttuuala, 2007; Tirziu and Simons, 2005; Vincent et al., 2007).
Although a number of issues regarding the best candidate gene as well as the most suitable gene delivery system are still unresolved, myocardial gene transfer has been carried out either by direct intramyocardial injection or by intracoronary instillation of vector containing solutions (Figure 9).
<table>
<thead>
<tr>
<th>Trial</th>
<th>Therapeutic target application</th>
<th>Therapeutic agent</th>
<th>Administration</th>
<th>Control treatment</th>
<th>n</th>
<th>Primary endpoint</th>
<th>Results</th>
</tr>
</thead>
<tbody>
<tr>
<td>KAT</td>
<td>Therapeutic angiogenesis in CAD (CCS class II–III)</td>
<td>AdVEGF&lt;sub&gt;si&lt;/sub&gt; or plasmid/ liposome/ VEGF&lt;sub&gt;si&lt;/sub&gt;</td>
<td>Intracoronary injection at the angioplasty site</td>
<td>Ringer’s lactate</td>
<td>103</td>
<td>Improved myocardial perfusion at 6 months</td>
<td>Positive (adenovirus group only)</td>
</tr>
<tr>
<td>REVASC trial</td>
<td>Therapeutic angiogenesis in CAD (CCS II–IV)</td>
<td>AdVEGF&lt;sub&gt;si&lt;/sub&gt;</td>
<td>Intramyocardial injection via mini-thoracotomy</td>
<td>Best medical treatment (no placebo treatment)</td>
<td>67</td>
<td>Time to 1 mm ST-segment depression on ETT at 26 weeks</td>
<td>Positive</td>
</tr>
<tr>
<td>Euroinject one trial</td>
<td>Therapeutic angiogenesis in CAD (CCS III–IV)</td>
<td>Naked VEGF&lt;sub&gt;si&lt;/sub&gt; Plasmid</td>
<td>Percutaneous Intramyocardial injections</td>
<td>Placebo plasmid</td>
<td>74</td>
<td>Improved myocardial perfusion at 3 months</td>
<td>Negative</td>
</tr>
<tr>
<td>Genais</td>
<td>Therapeutic angiogenesis in CAD (CCS III–IV)</td>
<td>Naked VEGF-2 (VEGF-C) plasmid</td>
<td>Percutaneous Intramyocardial injections</td>
<td>Vehicle</td>
<td>295 (404 planned)</td>
<td>ETT at 3 months</td>
<td>Negative at interim analysis, stopped</td>
</tr>
<tr>
<td>Northern</td>
<td>Therapeutic angiogenesis in CAD (CCS III–IV)</td>
<td>AdVEGF&lt;sub&gt;ni&lt;/sub&gt;</td>
<td>Intramyocardial injections</td>
<td>Vehicle</td>
<td>120 (planned)</td>
<td>Change in myocardial perfusion in stress/rest at 12 weeks</td>
<td>Ongoing</td>
</tr>
<tr>
<td>NOVA</td>
<td>Therapeutic angiogenesis in CAD (CCS II–IV)</td>
<td>AdVEGF&lt;sub&gt;ni&lt;/sub&gt;</td>
<td>Intramyocardial injections</td>
<td>Vehicle</td>
<td>129 (planned)</td>
<td>ETT at 26 weeks</td>
<td>Stopped</td>
</tr>
<tr>
<td>AGENT-2</td>
<td>Therapeutic angiogenesis in CAD (CCS II–IV)</td>
<td>AdFGF-4</td>
<td>Intramyocardial injection</td>
<td>Vehicle</td>
<td>52</td>
<td>SPECT at 8 weeks</td>
<td>Positive</td>
</tr>
<tr>
<td>AGENT-3</td>
<td>Therapeutic angiogenesis in CAD (CCS II–IV)</td>
<td>AdFGF-4</td>
<td>Intramyocardial injection</td>
<td>Vehicle</td>
<td>416</td>
<td>ETT at 12 weeks</td>
<td>Negative (subgroup of &gt;55 yr with CCS III–IV positive)</td>
</tr>
<tr>
<td>AGENT-4</td>
<td>Therapeutic angiogenesis in CAD (CCS II–IV)</td>
<td>AdFGF-4</td>
<td>Intramyocardial injection</td>
<td>Vehicle</td>
<td>116</td>
<td>ETT at 12 weeks</td>
<td>Negative</td>
</tr>
<tr>
<td>AWARE</td>
<td>Therapeutic angiogenesis in CAD (CCS III–IV)</td>
<td>AdFGF-4</td>
<td>Intramyocardial injection</td>
<td>Vehicle</td>
<td>300 (women)</td>
<td>ETT at 6 months</td>
<td>Ongoing</td>
</tr>
<tr>
<td>Italics</td>
<td>In-stent restenosis in CAD</td>
<td>Anti-sense oligonucleotide against c-myc</td>
<td>Local delivery after stent implantation</td>
<td>Saline</td>
<td>85</td>
<td>% neointimal volume obstruction at 6 months</td>
<td>Negative</td>
</tr>
<tr>
<td>Prevent IV</td>
<td>Vein graft failure in CAD</td>
<td>edifoligide (an E2F transcription factor decoy)</td>
<td>Ex vivo pressure-mediated delivery</td>
<td>Buffered saline</td>
<td>2,400</td>
<td>Death or vein graft stenosis of &gt;75% at 12–18 months</td>
<td>Negative</td>
</tr>
<tr>
<td>ISIS</td>
<td>Familial hypercholesterolemia</td>
<td>ISIS 301012 (antisense oligonucleotide inhibitor of apoB)</td>
<td>Intravenous followed by weekly subcutaneous injections for 4 weeks</td>
<td>Unknown</td>
<td>36</td>
<td>Percent reduction in LDL-cholesterol from baseline to 12 weeks</td>
<td>Positive (35% reduction of LDL-cholesterol at 39 days)</td>
</tr>
</tbody>
</table>

Figure 9. Clinical trials to treat coronary artery disease.

Abbreviations: ABI, ankle brachial index (the ratio of the blood pressure in the lower legs to the blood pressure in the arms) CCS<sup>+</sup> case control system; Ad, adenovirus; CLI, critical limb ischemic; FGF, fibroblast growth factor; HIF-1α, hypoxia inducible factor-1α; HGF, hepatocyte growth factor; PAD, peripheral arterial disease; PWT, peak walking time; vascular endothelial growth factor; EET<sup>=</sup> Exercise Evaluation Team; "A measure of the efficacy in relation to the study protocol-defined primary or secondary endpoint (Rissanen and Ylä-Herttuala, 2007).

An interesting field to which the gene transfer approach has been applied is either
mobilization of progenitor or stem cells to the damaged area or stimulation of a regenerative program within the organ (Minatoguchi et al., 2004). Recent studies have suggested that bone-marrow-derived primitive stem cells or circulating precursors can be recruited to the injured heart, differentiate into endothelial cells and even lead to myocardial regeneration (Askari et al., 2003). It is worth noting that different cell types have been used since several laboratories characterized endothelial progenitor cells through different protein expression profiles (Arnesen et al., 2007). Although encouraging, the results should be cautiously interpreted due to the controversial outcome observed, the small sample sizes and the absence of a concurrent control group, thus indicative of the absolute need for additional adequately powered, randomized, double-blinded trials (Jacobs, 2007; Jain, 2003; Tirziu and Simons, 2005).

In addition, there is now accumulating evidence that the heart contains resident stem cells that can be induced to develop into cardiac muscle and vascular tissues (Arnesen et al., 2007; Matsuura et al., 2004). These cardiac progenitors would be recruited to repair the infarcted myocardium by direct delivery of several cytokines that potentially stimulate myocardial healing and repair in the setting of MI.

**Gene Transfer To The Myocardium**

Studies on the regulation of gene expression in the myocardium have relied heavily on transfection into primary cells, in particular neonatal cardiac myocytes and, to a more limited extent, adult myocytes.

Direct transfer *Plasmid DNA* has distinct advantages and limitations. It can achieve prolonged transgene expression, but DNA uptake is poor (cardiomyocyte transduction rates: 0.1% at the injection site) (Jazwa et al., 2007; Lin et al., 1990).

A critical requirement for gene therapy protocols is the ability to achieve control of the foreign gene expression appropriately in the host. Specific promoter elements can be used to regulate transgene expression in response to physiological stress (Rissanen and Yla-Herttuala, 2007).

The gene delivery system more commonly used in ongoing phase I / II trials are viral vectors, in particular *retroviruses* from mouse and human origin.

The life cycle of the retrovirus is now well characterized (Yi et al., 2005). Following infection into its host, the viral RNA is reverse-transcribed into a double-stranded DNA molecule that then becomes integrated into the cellular genome. By exploitation of this stable integration into the host genome following infection, long-term expression of the
recombinant gene is achieved. However, integration of retroviral sequences is dependent on cell division, thus they may be of little use for the myocardium. Retroviral protocols are actually limited by the relatively low viral titres that can be obtained, by the limited size of foreign sequence they can accommodate and by the potential for oncogenicity and insertional mutagenesis (Cavazzana-Calvo et al., 2000; Yi et al., 2005).

Unlike retroviruses and plasmid DNA gene transfer, unique properties of adenoviral vectors include an exceptionally high efficiency of infection and the ability to accommodate large fragments of foreign DNA (up to 8 kb). Their transduction efficiency is at least an order of magnitude higher than that observed with plasmid DNA, tissue penetration is vastly improved and transgene expression is proportionately (Woo et al., 1998).

Of particular importance for gene transfer to the myocardium is the property to efficiently infect both proliferating and terminally differentiated cells. In contrast with retroviruses, Ad can be prepared at much higher stock concentrations; furthermore, the adenoviral genome remains episomal, so that the potential for oncogenesis and insertional mutagenesis is avoided (Giordano et al., 1996; Grines et al., 2002; Hedman et al., 2009; Rissanen and Yla-Herttuala, 2007).

However, there are several limitations to the use of the Ad, mainly related to the stimulus of humoral response in the host, activation of neutralizing antibodies, inflammation and elimination of both vector and host cell (Giacca, 2007; Jazwa et al., 2007).

Because of the inflammatory responses associated with infection by Ad and other viral vectors, attention has been paid to the potential for using viruses defective for viral genes. Adeno-associated virus is an example of such a defective virus able to pack exogenous genes into its viral coat (Cervelli et al., 2008). This non-pathogenic human parvovirus differs from Ad in that it generally integrates into a specific site in the genome of the host cell (Chr 19q13.4) and may provide longer-term transgene expression (Cervelli et al., 2008). Actually viral vector long-term gene expression is likely to be achieved thanks to the AAV ability to transduce non-dividing cells (like myocytes and neurons) without the immune response induction; the AAV genome seems not to be integrated, but rather persists in an episomal form (Giacca, 2007; Jazwa et al., 2007).

Because of its high infectivity and low immunogenicity, AAV gene transfer has shown great potential for the cardiovascular applications (Giacca, 2007). Therapeutic angiogenesis by delivery of genes coding for pro-angiogenic growth factors VEGF, fibroblast growth factor (FGF) and hepatocyte growth factor (HGF) has been shown to

40
promote neo-vascularization and functional recovery of ischemic myocardium in several animal models (Giordano et al., 1996; Su et al., 2000). Despite the promising findings of these small-scale trials, there are several outstanding issues related to the safety and sustainability of the approach, in light of recent evidences that constitutive over-expression of VEGF in mouse heart led to intramural angiomas, followed by heart failure and death (Celletti et al., 2001). These observations underscore the necessity for regulated expression of pro-angiogenic factors. Such a strategy may require the incorporation of promoter sequences such as hypoxia-sensitive responsive elements capable of rendering expression of the therapeutic transgene subservient to the pathophysiological changes in myocardial oxygen tension.

The use of the AAV for a basic research finalized to clarify the physiological mechanisms regulating the normal cellular functions is a pre-requisite to understand what’s wrong in a defined pathological contest. At this purpose, the use of AAV to genetically modify small animals without any inflammatory response and with a prolonged expression of the transgene is an innovative tool aside to knockout and transgenic animals. Therefore, the unique tropism of AAV for skeletal and cardiac muscles makes them particularly appealing for their use in a neo-angiogenetic therapy or myocardial tissue regeneration.

In particular, the inducing neo-angiogenesis capacity in ischemic muscle of AAV coding for VEGF and AngI and the unique property to induce the muscular regeneration in the transduced environment have been demonstrated (Arsic et al., 2004; Arsic et al., 2003). The convergence of gene transfer and genomic technology will help to reveal the function of new genes and to uncover new roles for previously known or unknown genes, thus leading to the discovery of new therapeutic targets.

**Use of AAV to enhance the recruitment of progenitor cells and their homing in damaged heart.**

The use of AAV vectors in a cell therapy approach of treatment of ischemic myocardium leads to appealing therapeutical perspectives in the contest of precursors mobilization from bone marrow and enhancement of their homing in the injured tissue.

The number of EPCs can be increased after application of several chemokines and cytokines, which include G-CSF, stromal derived growth factor (SDF-1), leukemia inhibitory factor (LIF), insulin-like growth factor (IGF-1), erythropoietin (EPO), and VEGF (Hiasa et al., 2004; Minatoguchi et al., 2004; Musaro et al., 2004).
In addition to their role in expanding and mobilizing bone marrow-derived progenitor cells, they may have direct myocardial-protective effects, reasonably through a paracrine mechanism.

G-CSF plays a critical role in regulation of proliferation, differentiation, and survival of myeloid progenitor cells. G-CSF also causes a marked increase in the release of hematopoietic stem cells into the peripheral blood circulation, a process termed mobilization. Recently, G-CSF has been reported to stimulate healing and repair, to improve cardiac function, and to reduce mortality after acute MI (Minatoguchi et al., 2004; Orlic, 2003). Actually, the mechanism by which G-CSF ameliorates cardiac dysfunction is not fully understood (Orlic, 2003).

EPO is important for erythrocyte survival and differentiation; it has the ability to maintain vascular auto-regulation and attenuating primary (apoptotic) and secondary (inflammatory) causes of cell death. In a rat model of infarction, EPO reduces cardiac myocyte loss by 50%, sufficient to normalize hemodynamic function after reperfusion. The hematopoietic growth factor EPO has been found to mediate repair and regeneration after brain and spinal cord injury, including the recruitment of stem cells into the region of damage (Kawamoto et al., 2001). IGF-1 is a cytokine that can enhance nuclear phospho-Akt and telomerase, thus delaying cardiomyocyte aging and death, and it can improve stem cell homing, healing, and regeneration of the injured muscle (Musaro et al., 2004).

SDF-1 is a chemokine considered to play an important role in the trafficking and survival of hematopoietic, endothelial progenitors and mesenchymal stem cells. Locally delivered SDF-1 raises the vasculogenesis and subsequently contributes to ischemic neo-vascularization in vivo by enhancing endothelial progenitor cell (EPC) recruitment in ischemic tissues (Askari et al., 2003; Kawamoto et al., 2001). Since SDF-1 has also been shown to mediate the recruitment of circulating progenitors for smooth muscle cells, its role in vascular remodeling may be even more universal.

Gene transfer to the target ischemic tissue of angiogenetic chemokines might be another effective strategy for therapeutic neo-vascularization. Thus, intramuscular or intramyocardial VEGF gene transfer has been shown to mobilize EPCs in case of ischemia or coronary disease (Kalka et al., 2000).

The widespread belief in the field is that these differently characterized progenitor cells are likely the same cellular population observed in diverse differentiation states. Anyway further biochemical studies together with randomized, double blind trials are needed to better define the identity and role of the progenitor population.
Taken together, cytokine-mediated regenerative therapy may evolve to be a novel therapeutic strategy for MI.

VI.4. IN VITRO APPROACHES TO STUDY ANGIOGENESIS

When deregulated, the angiogenetic process contributes to several malignant, ischemic, inflammatory, infectious and immune disorders. Molecular insights into these processes are being continuously generated to understand the steps and mechanisms involved in the process to offer new therapeutic opportunities. As a matter of fact the behaviour of the main players of angiogenesis, such as endothelial, muscle cells and pericyte, is driven by peculiar signalling pathways induced by the environmental conditions. Many studies are analysing the activation/inhibition of a few subset of mRNA or proteins, depending mainly on the prevailing signalling model and knowledge, but the correct interpretation of the role of peculiar molecules is strongly affected by redundancy and compensation mechanisms still unknown. It is now a widespread belief in the field that a broader and unbiased method of assaying molecular changes is needed to better understand the signalling feature of cells. For the last decade, governments and industry have sponsored several ‘-ome’ projects that specify the complete contents of DNA/ RNA/ protein within human and other organisms. Based on this global census, the major challenges in therapy are to understand first the molecular wiring connecting the different molecular components, and second to identify the points in which the wiring is disrupted in clinical diseases, to develop effective therapies to induce safe angiogenesis.

VI.4.1. Molecular fingerprint of the endothelium

Being main players of blood vessel development during embryogenesis as well as in the adult life, endothelial cells have more interesting roles than just acting as channels for blood circulation. They are the main sources and target of several paracrine signals, thus promoting stem cell development and organ formation. In addition, the signals that are primarily used to promote endothelial development might also act directly on other cell types, reinforcing the complex interplay between the vasculature and surrounding tissues (Coulta et al., 2005). Thus, the generation of a molecular fingerprint of endothelial cells in resting and activation condition could add meaningful new pieces of evidence, thus allowing the design of better therapies.
VI.4.1.1. Transcriptional profile

The success of the genome sequencing projects is continuously fuelling the development of methodologies to analyze the overall profile of gene expression. Different endothelial cell lines have been analysed looking for either a subset of transcripts of interest or a global transcriptome profile. A quantitative approach of polymerase chain reaction (Real time-PCR) revealed the modulation in the expression of 49 protease and protease inhibitors in human saphenous vein smooth muscle cells, endothelial cells, and monocyte-derived macrophages upon different treatments (Shi and Dolganov, 2006). Global transcriptome profiles have been instead obtained thanks to the microarray Affymetrix GeneChips and serial analysis of gene expression (SAGE) techniques. By exploiting the first approach, transcriptional profile of endothelial cells, like the primary human umbilical venous endothelial cells (HUVEC), has been widely assessed to unravel the protein expression induction profile in different conditions, such as VEGF treatment (Abe and Sato, 2001; Mellberg et al., 2009). Mammary epithelial cell transcriptome has also been evaluated upon infection with an adenoviral vectors carrying a constitutively active form of MEK1 (MAPK, Erk1) (Grill et al., 2004). Whereas Chu and colleagues analysed human aortic endothelial cells by exploiting the unbiased genome wide analysis of gene expression via the SAGE technique: the generation of short cDNA-derived gene tags from the overall population of mRNA molecules is followed by their concatamerization and cloning into plasmid vectors, thus allowing their sequencing to generate quantitative catalogues of expressed genes of resting cells as well as cells subjected to laminar shear stress or hypoxic environment (Chu and Peters, 2008; Ning et al., 2004). The huge limitation of these studies is the weak correlation between protein and mRNA levels, with correlation coefficients around of 0.4 for highly expressed mRNA and 0.1 for poorly transcribed genes (Resing, 2002).

VI.4.1.2. Proteome profile

Proteomics strongly differs from genomics in terms of both complexity and dynamic variability; the detection of mRNA levels to infer protein expression could be misleading because the regulatory mechanisms are different and often uncoupled. Several approaches are continuously developed to address the issue of the total proteome of different cell types/tissues in different physiological and pathological conditions (Mann and Jensen, 2003; Resing, 2002; Unwin et al., 2006; Zheng et al., 2005). The detailed description of the main proteomic approaches exploited is depicted in the following paragraph.
VI.5. PROTEOMIC APPROACHES

VI.5.1. Proteome analysis

VI.5.1.1. Protein profiling microarrays
Microarrays allow the parallel analysis of multiple proteins in a high throughput, miniaturized format for global protein screening. Actually, these techniques are nowadays used mainly for the screening of prognostic markers in clinics, because 1) the analysis is predetermined and defined protein targets are selected a priori, and 2) it still need to overwhelm issues of cross-reactivity, reproducibility, high background levels and reduced binding activity of molecules immobilized on the array (Unwin et al., 2006). In the forward-phase arrays, the presence and/or the relative amount of a group of proteins of interest are assessed thanks to an array of molecules immobilized on a slide. Antibodies or aptameres act as capture molecules: upon probing with cell lysates or serum, the protein binding is evaluated through direct labelling or indirect detection. This method allows the assessment of proteins of interest in biological sample. The reverse-phase arrays are used for the simultaneous analysis for a single analyte of multiple samples in a western blot type approach. A complex sample, like a cell lysates or serum is immobilized on the array and then processed for the detection of the molecule of interest (Unwin et al., 2006).

VI.5.1.2. Two dimensional PAGE
The technique of two dimensional gel polyacrylamide gel electrophoresis was first described by O’Farrell in 1975 (O'Farrell, 1975). As a matter of fact in the first dimension, isoelectric focusing (IEF), proteins are resolved according to their overall electric charge: immobilized pH gradient gels allow the “focusing” of the protein species depending on their isoelectric point (pI). The second dimension of separation is represented by a sodium dodecyl sulphate PAGE; every single spot visualized in the final gel contains proteins having a defined molecular weight (MW) and pI (Resing, 2002). The molecules are then detected and relatively quantified thanks to chemical stains, chosen considering sensitivity, reproducibility and compatibility to downstream methods used for protein characterization. The change of molecular mass and pI upon particular protein modification is easily detectable through 2D gel separation and the relative quantification can be achieved thanks to computer-based gel comparison; nevertheless, reproducibility and background issues often affect negatively the statistically significance of the results achievable. Taken into account the possible dynamic range of abundance of proteins in a biological sample can be of $10^6$, another major limitation of 2D gel electrophoresis is its ability to resolve no more
than 1000 proteins, thus obtaining the main visualization of the most abundant and idrophilic species, since highly idrophobic proteins are poorly solubilised before separation. To enrich the sample in membrane bound molecules together with reducing sample complexity, several lysis conditions have been developed coupled with affinity based protein purification strategies. The fractioned material is then separated through one or two dimensional electrophoresis.

**Tandem MS and MS/MS protein identification.** The most significant breakthrough in proteomics is the mass spectrometry identification of gel separated proteins, which extends analysis far beyond the mere protein visualization. Proteins are first degraded into peptides by sequence specific proteases such as trypsin. Peptides are preferred for MS analysis, since proteins cannot easily be eluted from gels without detergents like SDS (which are detrimental to mass spectrometry) and because large proteins are usually heterogeneous and hence hardly unambiguously identify in a sequence database (Resing, 2002; Steen and Mann, 2004; Unwin et al., 2006).

Mass spectrometers consist of a series of components: 1) a means of the peptide mixture introduction; 2) an ionization mechanism (electrospray ionization -ESI- or matrix-assisted laser desorption/ionization- MALDI); 3) a section to separate, select, and fragment peptides (quadrupole MS, time of flight –TOF MS- or quadrupole ion trap); 4) a mass analyzer with a vacuum system, and 5) an ion detector (Resing, 2002; Steen and Mann, 2004) (Figure 10).

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**Figure 10. Mass spectrometry analysis.**

A protein population is prepared from a biological source and the last step in protein purification is often SDS–PAGE. The gel lane that is obtained is cut into several slices, which are then in-gel digested. Numerous different enzymes and/or chemicals are available for this step, but usually trypsin is the method of choice. The generated peptide mixture is separated on- or off-line using single or multiple dimensions of peptide separation. Peptides are then ionized by electrospray ionization (depicted) or matrix-assisted laser desorption/ionization (MALDI) and can be analysed by various different mass spectrometers. Finally, the peptide-sequencing data that are obtained from the mass spectra are searched against protein databases using one of a number of database-searching programmes. Examples of the reagents or techniques that can be used at each step of this type of experiment are shown beneath each arrow. 2D, two-dimensional; FTICR, Fourier-transform ion cyclotron resonance; HPLC, high-performance liquid chromatography (Steen and Mann, 2004).
1-2) In **LC-ESI ionization**, peptides enter the mass spectrometer by elution from a microscale, capillary, high-performance liquid chromatography (HPLC) column that is directly coupled to the mass spectrometer. When a peptide arrives at the end of the column, it flows through a needle; at the needle tip the liquid is vaporized and the peptide is subsequently ionized by the action of a strong electrical potential (electrospray ionization, ESI). In **MALDI ionization**, co-precipitate of light absorbing matrix (usually K-cyano-4-hydroxycinnamic acid or dihydroxy benzoic acid) and the peptide solution is first put on a microplate and then it is irradiated with a short pulse of UV light in the vacuum. Some of the released peptides are ionized by attachment of protons and are accelerated in a strong electric field.

3-5) Once within the vacuum system, the peptides are guided and manipulated by electrical fields that aid in determining their mass. The three main types of mass spectrometers used in proteomics are **quadrupole MS**, quadrupole **ion traps**, and time of flight (TOF MS). Each of these differs in how they ascertain the mass or mass/charge ratio (m/z) of the peptides. After determining the peak centroids and calibrating the spectrum, for example on trypsin auto-digestion products, a set of highly accurate peptide masses is obtained. Each instrument generates a mass spectrum, which is a recording of the signal intensity of the ion at each value of the m/z scale (Dalton units per charge). Once the m/z values and peak intensities are generated, the “peptide mass fingerprint” results in the identification of peptides primary structure. The protein is identified by comparison of the resulting “mass fingerprint” to corresponding mass predictions contained in a database. In a second step, if the mass fingerprinting is not sufficient for identification, peptides are processed in the tandem MS approach, in which two MS step are performed. After peptide mass fingerprinting, a particular peptide ion is isolated, energy is imparted by collision with an inert gas (such as nitrogen molecules or argon or helium atoms), and this energy causes the peptide to break apart. This process, often referred to as “collision-induced dissociation”, results in the production of a tandem MS, MS/MS, or MS² spectrum. The final peptide fragmentation pattern is then used to identify the protein in a database, even on the basis of one or a few peptide sequences (Mann and Jensen, 2003; Steen and Mann, 2004).

Another important consideration for 2DE experiments is throughput. A 2DE experiment typically takes 3 to 5 days to complete; although gels can be run simultaneously in batches, the process is still very variable, time consuming and technically demanding. Lastly, the MS analysis of each spot has been automated robotically, but may still lack sufficient
throughput and robustness to meet the needs of large-scale comprehensive profiling (Mann and Jensen, 2003; Resing, 2002; Steen and Mann, 2004).

VI.5.2. **Relatively quantitation proteomics**

Unfortunately, in spite of the revolutionary impact of the mass spectrometric identification methods, mass spectrometric analysis does not allow protein quantification, due to the extreme variability of peptide signals in the mass spectrometer. Due to differences in amino acid sequence, two peptides obtained from the same protein are likely to be ionized with different efficiency, thus resulting in different spectra. Indeed, the same peptide can results in different spectra when analyzed twice; slight differences such as the age, condition and position of the electrospray needle, as well as the presence of co-eluting peptides and solvent composition can affect the ionization of the same peptide. To overcome the high variability in the sample preparation, protein digestion and mass spectrometer analysis, several methods have been developed (Steen and Mann, 2004).

In the **stable isotope labelling** chemically identical but isotopically distinct tags are added to the peptides to allow the comparison. The differentially modified target residue will not affect the overall ionization procedure of the peptides and relative quantification will be achieved by comparing the signal intensities of peptides with a distinct isotope-induced mass disparity. The labelling procedure can be achieved through the stable isotopically labelling of the essential amino acids in culture (SILAC), the use of heavy water while performing the protein digestion (as occurs in the $^{18}\text{O}$ incorporation, that modify all the residues at their C-terminal), or the chemically modification of the tryptic peptides (as occurs in the isotope coded affinity tag, ICAT, in $^{13}\text{C}^{15}\text{N}$ guanidination and dimethyl labelling) (Mann and Jensen, 2003; Unwin et al., 2006)

(a) As a matter of fact SILAC technique is a suitable method for quantitative analysis as well as for functional studies: different cell populations are grown in medium containing isotopically some essential amino acids as leucine, arginine and lysine. Because the cell proteome is completely labelled at the start of the experiment, protein synthesis and degradation do not affect the measured signal and relative peak intensities are a direct measure of the relative protein amount. The need for isotopically labelled medium as well as at least five population doublings to reach isotopic equilibrium is the main disadvantage, thus restricting only to cell lines the possible system to study (Blagoev et al., 2003; Blagoev et al., 2004; Dengjel et al., 2007; Mann and Jensen, 2003). The more feasible approaches are the ones in which the digested peptides are modified before MS analysis. Among these, in ICAT
procedure, cysteine residues are chemically labelled with a high or light isotope together with a biotin tag to allow affinity purification. An easier and alternative approach is the dimethyl labelling, which use formaldehyde to rapidly induce the reductive amination of the ε amino group of lysines. Although labelling techniques differ in the amino acid target and in the kind of modification induced, the overall key feature is the possibility to mix at least two samples before performing the MS analysis, thus strongly decreasing the variability among different samples. The complexity of the final sample is actually also resulting in a very complex final spectra obtained, thus leading to a difficult data interpretation (Hsu et al., 2003; Unwin et al., 2006).

(b) **Isobaric tags for relative and absolute quantifications** (iTRAQ) technique incorporate up to four mass tags that label N-terminus all the free amines of the digested peptides. Being each tag isobaric, the same peptides from each sample, even if differently tagged, appear as a single peak in the peptide fingerprinting. However, upon fragmentation, each tag results in a specific reporter ion at a certain m/z ratio; this approach allows a higher sensitivity, but it does not solve experimental variability issues and needs the carry out of tandem MS on all the peptides to achieve quantification (Unwin et al., 2006).

(c) **Differential MS** is a label-free relative quantification based on normalization strategies that enable comparison of signal strength of the same peptide in two distinct sample runs (Unwin et al., 2006). (Figure 11).
Figure 11. Relative quantitation in mass spectrometry.

(a) Stable isotope labelling can be used for relative quantification. Various methods exist for incorporating the ‘heavy’ and ‘light’ isotopes. Although being chemically identical, isotopes enable peptides from each sample to be discriminated and relatively quantified in the mass spectrometer by a comparison of the intensities of peaks that elute from a liquid chromatography system at the same time. (b) iTRAQ tags have identical overall mass and can react with all peptides via free amine groups. Here, each chemically identical peptide from the four samples appears at the same mass in an MS experiment. Colour coding in the stacked bars represents the fact that the isobarically tagged peptide has come from four different samples. In MS mode, there is no means of distinguishing from which of the four samples the peptides were derived. On fragmentation, however, specific reporter ions are released that differ in mass owing to differential isotope usage. The ratios between these ions are thus representative of the relative expression of that peptide or protein. (c) Label-free relative quantification involves analysing samples in separate experimental runs in series. Peptides are separated by liquid chromatography and analysed by MS. A peptide-specific elution profile is then generated, which shows how much of the peptide was detected throughout the experimental
Absolute quantitation proteomics. In case relative quantification seems not sufficient to address the issue, absolute quantification is needed. Among the techniques now available, they are worth noting the following ones. In AQUA (Absolute quantification) and QCAT of techniques, the quantification is achieved namely through the introduction of a precise concentration of a stable isotope- labelled synthetic peptide of interest or through the design and construction of an artificial gene encoding a concatenation of tryptic peptides of interest (QCAT protein) (Unwin et al., 2006).

VI.5.3. Analysis of protein modification

The one gene-one protein concept is clearly overwhelmed by the presence of different proteins due to alternative splicing and to a huge variety of protein modifications, including glycosylation, phosphorylation, ubiquitination, oxidation, alkylation and biotinylation, just to name a few. Several approaches are continuously developed to enable a detailed investigation of proteome and post-translational regulation (Blagoev et al., 2004; Dengjel et al., 2007; Mann and Jensen, 2003; Resing, 2002; Unwin et al., 2006).

VI.5.3.1. Functional microarrays

Functional Microarrays have been developed to assess biological properties like enzyme activity and substrate specificity, even though they present the same disadvantages and problems of the protein profiling microarrays. Activity based protein profiling (ABPP) is a functional proteomic technology in which the probe contains a reactive group to specifically bind only the active enzyme and a tag to visualize the interaction, thus allowing to monitor the enzyme activity directly and not its mere presence. To assess the phosphorylation pattern of different cells, protein microarrays have been developed in which different phospho-tyrosine binding domains or antibodies against phosphorylated residues have been immobilized or coupled to protein profiling microarrays. Through this approach, specific phosphorylated tyrosine residues of ErbB receptors have been identified, as well as a possible involvement of NP-1 in the VEGF<sub>165</sub>-induced p38 MAPK phosphorylation (Kawamura et al., 2008; Unwin et al., 2006).

VI.5.3.2. MS identification of affinity enriched proteins

Phosphorylation is a widespread post-translational modification regulating a large numbers of biological processes such as signal transduction, protein interaction and function. It has
been estimated that around 30% of the proteome in a given mammalian cell at some point during their expression are phosphorylated and a wide variety of MS based methodologies aim at their characterization exploiting the mass increment of 80 Da per phosphate group in combination with phosphatase treatment. Taken into account that the group is likely to affect peptide ionization leading to a decreased signal intensity for phosphorylated peptides in the presence of non-phosphorylated species, several approaches have been developed to enrich or selectively stain the modified molecules. Cell metabolic labelling with $^{32}$P orthophosphate provides a comprehensive and direct detection of phosphorylated species; in spite of obvious safety disadvantages, it has been used to assess different signalling features, such as the one triggered by the transforming growth factor -β in human breast epithelial cells (Stasyk et al., 2005). Immobilized metal affinity chromatography (IMAC) is a safe approach to enrich a protein tryptic digest in phospho-peptides; methylation of the extracts is performed to reduce non-specific binding of acidic non-phosphorylated species. Although different affinity resins are available, the aspecific binding of non-phosphorylated species is still the main issue that needs to be solved. Specific antibodies against phospho-proteins/peptides are also used to perform selective immunoprecipitation to enrich the sample prior the MS analysis. Since anti-phospho-serine/threonine antibodies are still not suitable for large scale immunoprecipitation approaches, the vast majority of the publications aimed at the detection of phospho-tyrosine species to partially describe the phosphorylation status. Taken into account that only 0, 1% of all protein phosphorylation occur on tyrosine residues, several alternative and sensitive approaches have been developed to assess changes in phosphorylation patterns. Several antibodies are available and different groups successfully adopted this technique alone, or in combination with metabolic labelling (SILAC) or phospho-peptide enrichment (IMAC) to enrich the sample in phosphorylated species (Gronborg et al., 2002; Rush et al., 2005; Salomon et al., 2003). Selective phospho-protein visualization followed by MS identification has been also achieved thanks to the phospho-tyrosine detection through western blot or phospho-specific staining together with the in gel digestion of the spots of interest from another identical 2D gel (Kim et al., 2007; Mann and Jensen, 2003).

VI.5.4. Analysis of protein interactors

The specific protein-protein interaction can result in the activation, inhibition or modulation of almost all the biological functions occurring in cells. A useful method to study protein-protein interactions is affinity purification using a bait molecule, and it is generally confined to confirm suspected interaction through western blots. Actually,
several protein purification strategies have been developed to achieve the identification of the overall protein population interacting with the protein of interest of a multi-protein complex with a defined function. Tags like the FLAG peptide allows the selective and efficient immunoprecipitation of the bait of interest followed by the identification of novel protein interactors; the following MS analysis can be performed only on visualized bands of interest or on the tryptic digest of the overall immunoprecipitated sample, resulting in a more complex spectra, but resulting in a more comprehensive list of protein (Bish and Myers, 2007). As a matter of fact, the double affinity purifications allow the efficient selection and release from the affinity column under mild conditions. In the tandem affinity purification (TAP) this aim is achieved through the addition of the calmodulin-binding peptide and protein A separated by a site of cleavage for the Tobacco Etch Virus protease to the bait protein of interest. In the affinity purification coupled with mass spectrometry (AP-MS) approach, high throughput systems allow the generation of stable cell lines expressing a huge number of baits that are then double affinity purified to detect peculiar interactors (Rigaut et al., 1999; Wepf et al., 2009). The combined approach in which SILAC metabolic labelling has been used together with fusion protein GST-SH2 domain of the Grb2 adaptor, allows the detection of functional protein interaction of epidermal growth factor receptor (Blagoev et al., 2003). Although these approaches managed to aim major protein interactors, the weak nature of many protein-protein binding is still a hard issue to be faced, especially in the case of membrane and idrophobic proteins.
VII. AIM OF THE STUDY

In this thesis, the peculiar features of molecules binding related receptors of the VEGFR family are analysed in details. In particular, the most abundant angiogenic isoform of VEGF-A, VEGF165, whose action is mainly mediated by its interaction with VEGFR2 (KDR), VEGFR1 (Flt-1) as well as with the co-receptor Neuropilin-1 (NP-1), has been compared to a known NP-1 activator, Sema3A (involved in vascular patterning, besides its very well known role in axonal guidance) and to VEGF121, the shorter isoform which is able to bind to VEGFR1 and VEGFR2 only.

- *In vivo* analysis of skeletal muscles, in which VEGFs overexpression over time in adult mice have been achieved by Adeno Associated Viral vector transduction, unravelled the recruitment of a myeloid population as an exclusive feature of VEGF165 and Sema3A. The molecular characterization of the expression pattern of the different VEGFRs isoforms together with their interaction with NP-1 have been performed, to eventually define this myeloid population as a possible specific target cell type for VEGFs and/or Sema3A peculiar signalling.

- In order to produce huge amounts of recombinant ligands for the signal transduction related studies, we improved the baculovirus-based recombinant proteins production technique. In particular, we engineered a novel vector able to induce the secretion of tagged proteins, leaving just a glycine residue upstream the coding sequence of interest, thus possibly not affecting at all the biochemical features of the target molecule. The efficacy of the recombinant ligands has been validated *in vitro*.

- We used the “in house” produced recombinant ligands to investigate the signalling pathway downstream the VEGFRs in endothelial cells, using different proteomic approaches. Taking into consideration that VEGFs signalling is mediated by tyrosine kinase receptors, the dissection of the very early events in the induced signalling pathway have been investigated in porcine aortic endothelial cells stable transfected with KDR and NP-1.
MATERIAL AND METHODS

VII.1. CELL CULTURE AND REAGENTS

VII.1.1.1. Cell lines

*Parental porcine aortic endothelial cells (PAE) expressing KDR and NP-1* were kindly provided by Dr. M. Klagsbrun (Department of Surgery and Pathology, Vascular Biology Program, Children's Hospital, Boston, MA) and were grown in F12 medium (GIBCO) containing 10% FCS (GIBCO).

*Human Umbilical Vein Endothelial primary Cells* (HUVEC) (CLonza) were grown on 30 mg/ml vitrogen-coated dishes using the EGM-MV Bullet Kit (5% foetal bovine serum [FBS] in EBM with 12 mg/ml bovine brain extract, 1 mg/ml hydrocortisone, and 1 mg/ml GA-1000 (CLonza).

*CV-1 (simian) in Origin, and carrying the SV40 genetic material cells* (COS7) were grown in DMEM (Dulbecco’s Modified Eagle Medium) (GIBCO) supplemented with 10% FCS. *Sf9* insect cells (B82501, Invitrogen) and *Mimic Sf9 cells* (12552014, Invitrogen), were grown at 27°C in SF900 II (GIBCO)

VII.1.1.2. Kit and reagents

*Antibodies*. Western blot have been generally performed by incubating the primary antibodies in a 1:1000 dilution in TBS Tween 0.2 %, milk 5 %. Secondary antibodies (DAKO) have been used in a 1:3000 dilution and the detection has been performed by ECL (32106, Pierce).

Western blot/FACS: anti-CD11b (101207, Biolegend), anti-CD45 (553081, Biolegend) anti–NP-1 (sc-7239; Santa Cruz Biotechnology Inc./ AF566, R&D), anti-NP-2 (AF567, R&D), anti–Flk-1 (sc-504; Santa Cruz Biotechnology Inc./ 555308, Biolegend), anti–Flt-1 (c-17; Santa Cruz Biotechnology Inc./), and anti-Hsp70 (SPA-815; StressGen), anti- VEGF (sc-152, Santa Cruz Biotechnology Inc.), anti-Plexin-A1 (sc-10139, Santa Cruz Biotechnology Inc.) and biotin anti-Gr-1 (553124, BD).

Anti phospho-tyrosine antibodies used have been used at a 1:10000 dilution in TBS Tween 0.2 % BSA 5 %: PY99 (sc-7020, Santa Cruz Biotechnology Inc.), PY20 (309301, Biolegend), conjugated PY20 (A 4720, SIGMA Aldrich), conjugated PY100 (9419, Cell Signaling), Anti 6xHis (552565, BD, Pharmingen).

Anti phospho-serine antibody has been used at a 1:3000 dilution in TBS Tween 0.2 % BSA 5 % (P3430, SIGMA Aldrich).
**Vectors.** pFastBacHT (10584-027, Invitrogen), pFasBacI (10360-014, Invitrogen), pAcGP67 (gentle gift of Prof. Claude Lazure, PhD, University of Montreal, Montreal, Quebec, Canada), pAAV-VEGF$_{165}$, pAAV-VEGF$_{121}$, pAAV-Sema3A.

**Enzymes.** Polymerase Restriction enzymes BamHI (R0136, New England Biolabs), EcoRI (R0101, New England Biolabs), PvuII (R3151, New England Biolabs), Smal (R0141, New England Biolabs); His-AcTEV protease (12575-015, Invitrogen); N-glycosidase PNGase F (P0704S, New England Biolabs), Pfu DNA polymerase (M7741, Promega)

**Reagents.** Coomassie Brilliant Blue G-250 (20279, Pierce), Coomassie Brilliant Blue R-250 Dye (20278, Pierce), Silver nitrate (S6506, SIGMA Aldrich)

**VII.2. CHARACTERIZATION OF THE RECRUITED MYELOID CELL POPULATION**

**VII.2.1.1. Western blot**  
CD11b+ cells were isolated from total BM (extracted from tibiae and femurs of BALB/c mice) using CD11b magnetic cell separation system beads (Miltenyi Biotec) and cultured in RPMI 1640 supplemented with 10% high-quality FBS (GIBCO). Immunoprecipitation was performed starting with 2 × 10$^6$ CD11b+ cells from BALB/c mice stimulated with 50 ng/ml rhVEGF$_{165}$ (30 minutes on ice, followed by 7 minutes at 37°C) and lysed in a buffer containing 150 mM NaCl, 20 mM Tris, pH 7.5, 1% Triton X-100, protease inhibitor (tablets; Roche) and phosphatase inhibitors (10 mM NaF, 1 mM Na3VO4). For each point, 1.4 mg total proteins were immunoprecipitated using anti–NP-1 (sc-7239; Santa Cruz Biotechnology Inc.). Proteins were resolved by 8% SDS-PAGE and visualized with specific antibodies.

**VII.2.1.2. FACS analysis**  
Cell suspension has been incubated with primary/secondary antibodies at a dilution 1:100 for 1-2h at 4°C and finally analysed at the Becton Dickinson FACSCalibur flow cytometer.

**VII.2.1.3. AP ligand-binding assay**  
CD11b+ cells from BALB/c mice were seeded on poly-lysine-coated slides, fixed with 70% ethanol, and incubated overnight with supernatants from HEK293 cells overexpressing VEGF$_{165}$-AP, VEGF$_{121}$-AP, Sema3A-AP, and AP-tag, the last as a negative control. After additional fixation in 60 % acetone, 4 % PFA, and 40 mM HEPES, pH 7.0, a colouring reaction was developed by using NBT/BCIP tablets (Roche)
VII.3. BACULOVIRUS MEDIATED PROTEIN PRODUCTION

PCR primers:
to mutagenise pFastBacHT:
Fwd: GGATCCCAGCTGAAAAATACAGGTTTTCGCTT;
Rv: ACCGAAAACCTGATTTTTCAGCTGGCCATGGGATCC;
Fwd1: GGTGATGGTAGTACGCCCTTTAAGGTTTCGGACCAGAGCTCCG;
Rv1: CCGATCTCGGTCGCCAAACCCCTAAGGGGTCTACTACCATCACC);
to clone gp67:
Fwd: CCCGCTTAAGATGCTACTAGATAATCAG;
Rv: TAGCTTAAGCGCAAGGAGAATG;
to clone VEGF165 and VEGF121:
Fwd: CGATCCGGGCCGCCCATGGCAGAGGGAGGAGG;
Rv: CGATAAGCTGATATCGAATTCTGCTGACCTGCG;
to clone Sema3A:
Fwd: CGATCCGGGAAGTATCAGAATAGGAAGAC;
Rv: CGATGAATTCTCAAGCACCTGCTGGGTCCTC.

The insertion of the gp67 secretion signal has been performed through AflIII restriction enzyme digestion. Cloning of genes of interest has been performed by insertion of the coding sequence but the secretion signal into the pFastBacHTgp67 through PvuII/Smal-EcoRI digestion. The resulting construct reconstitutes the TEV protease recognition site and allows the secretion of the tagged recombinant protein; upon cleavage, the native protein will present only an additional glycine upstream its wild type amino acid sequence. The Bac-to-Bac Baculovirus Expression System (10359-016, Invitrogen) datasheet has been followed in detail: briefly, the site-specific transposition of an expression cassette into a baculovirus shuttle vector (bacmid) propagated in E. coli allows the production of viral particles from transfected Sf9 cells. The flowchart of the Bac-to-Bac Baculovirus Expression System is drawn in the Figure 12.
The oligonucleotides exploited to validate the correct recombination have been the Bacmid primer Forward (GGTGCAGGCCCTCTTCGCTATTAC) and Reverse (GAGCGCAACGAATTAATGTGAGT). The correct recombinant bacmid has been purified by Midiprep assay (11451-010, Marligen) and transfected in insect Sf9 cells through the commercial lipid Cellfectin (10362-010, Invitrogen). Virus has been amplified by infecting a higher number of cells and then the viral titre has been measured by an alternative method to viral plaque analysis. The measurement is based on the fact that, at MOI 1, the cells are supposed to duplicate once and then to stop growing. Serial dilution of
viral preparation are incubated with a constant amount of Sf9 cells, and the growth curve pattern examination reveal the viral concentration correspondent to MOI1, in which the viral particles are as numerous as the cell.

Protein purification of the untagged VEGF_{165} has been performed by using FPLC (AKTA FPLC, Amersham Bioscience); the cation exchange chromatography has been performed by using the column Mono S HR 5/5 (17-0547-01, Amersham Biosciences) with buffer A (20 mM MES 150 mM KCl) and buffer B (20 mM MES 1 M KCl). Gel filtration has been performed in the FPLC (AKTA FPLC, Amersham Bioscience) by using the column Superdex 75 (54800-U, Amersham Biosciences) with PBS.

As far as His-tagged protein purification is concerned, TALON resin (PT1320-1, Clontech Laboratories) has been used following in detail the datasheet indication. In brief, medium containing the recombinant protein has been incubated with the resin (1ml of resin/50ml of medium), already equilibrated in the medium, for 3 h at 4°C. The resin has been washed twice in high stringency conditions (20 mM Tris pH 7.4, 12.5 mM Imidazole, 500 mM KCl); One the column has been poured to an empty column, 20 bed volume of less stringent buffer (20 mM Tris pH 7.4, 12.5 mM Imidazole, 100 mM KCI) have been used until the Absorbance of the flowthrough at 280 nm was around 0. Eluted fractions with an high concentration of Imidazole (20 mM Tris pH 7.4, 120 mM Imidazole, 100 mM KCI) have been collected and load on a SDS-PAGE followed by coomassie staining and western blot analysis using an anti His antibody. The fractions containing the protein of interest have been dialysed (128 350, Spectra/Por, cut-off 8 KDa) and subjected to TEV protease incubation at 16 °C for 30 min. A second purification by TALON resin released the untagged recombinant protein, which has been concentrated in PBS, quantified and stored at -80 °C in 0.1 % of BSA.

**VII.3.1. Analysis of recombinant protein function**

**VII.3.1.1. Proliferation assay**

The blue dye Resazurin (1 mg/ml, R7017, SIGMA Aldrich) salt has been added to the cell culture medium: its reduction to resosufin (pink and highly fluorescent) allows the detection and measurement of parameter of cell proliferation and cytotoxicity at luminometer (PerkinElmer envision multimode plate reader)
VII.3.1.2.  COS7 cell contraction

Semaphorin-Induced Morphological Effects on COS-7 have been evaluated by transfecting COS7 cells with 2 μg of pBluescript PlexiA1 and pBluescript NP-1 by the lipofectamine method. After 48 hours, cells were stimulated with 100 nM commercial Sema3A, recombinant Sema3A or left untreated. An immunofluorescence staining using a NP-1 antibody and Alexa Fluor anti-goat 488 (A21467, Invitrogen, dilution 1:100) revealed the cell contraction in stimulated cells. Images were taken at 100x magnitude using the Zeiss LSM510 confocal microscope equipped with: Axiovert 100M reverse microscope, Ar 488, 514 nm and HeNe 543 nm lasers, LSM510 software 3.2 release.

VII.4.  PHOSPHO-PROTEOME DETECTION

VII.4.1.1.  Phospho-specific staining

*PhosP-tag* (FP1401, Perkin Elmer) and *ProQ Diamond* (P33300, Molecular Probes) staining have been used following the manufacturer’s instructions. The measurements and subsequent analyses have been performed reading samples at the expected wavelenght using the Versadoc 3000 (BioRad).

**Gel analysis.** *PDQuest* is robust and nimble software linked to Versadoc 3000 that allows stained gel analysis. The selection of the faintest spot, the smallest spot (usually the same spot as the faintest spot), and the largest spot cluster gel images on the screen is needed for the automated detection and matching tool. Among all the spots indentified, the overall pattern has been manually checked; this procedure has been performed for the different gels.

The software creates a theoretical gel, in which are drawn all the spots detected in both control and the gel of interest; then a colour is assigned to each spot according to its feature. Grey colour is assigned to the spots present in both the gels, while differential stains reveal peculiar features of spots present in each single gel.

VII.5.  PHOSPHO-PROTEOME ENRICHMENT

VII.5.1.1.  Anion exchange chromatography

Mustang Q membrane (Pall, MSTG25Q6) has been used following the outlines suggested in the datasheet. The equilibration and binding buffer is 25mM Tris pH 8.0 supplemented with increasing amount of NaCl to reach the 0.5M final concentration.
VII.5.1.2.  Phospho-tyrosine immunoprecipitation

Taken into account that 1 μl of conjugate antibody could bind 0,5 μg of tyrosine-phosphorylated proteins, 10-60 μl of Monoclonal anti-phosphotyrosine-coupled agarose (PY20, A 4720, SIGMA Aldrich) have been incubated with 5-20 mg of total protein lysates obtained from PAE KDR NP-1 differentially treated. Lysates have been incubated for 3 hours at 4°C shaking. Beads were washed three times with 1 ml of complete lysis buffer and three times with PBS, pH 7.4 to completely remove the detergents that would interfere with the following Mass spectrometer identification.

Buffer that have been tested are modified RIPA buffer (Tris HCl 50 mM pH 7.4, NaCl 150 mM, Na desoxycholate 0.25 %, NP-40 1 %, EDTA 5 mM, EGTA 5mM, MgCl2 5 mM, NaF 1 mM, Na3VO4 1 mM, Beta GlycerolPhosphate 25 mM, NP40 1 %, PMSF 1 mM) and a milder buffer (HEPES 50 mM pH 7.4, NaCl 100 mM, EDTA 5 mM, EGTA 5 mM, MgCl2 5 mM, NaF 1 mM, Na3VO4 1 mM, Beta GlycerolPhosphate 25 mM, NP40 1 %, PMSF 1 mM)

VII.6. PROTEIN SEPARATION TECHNIQUES

VII.6.1.1. Two dimensional gel electrophoresis

The 2D gel electrophoresis has been performed by using the lysis buffer containing 8M Urea 2% CHAPS, Benzonase (SIGMA), Ser and tyr phosphatase inhibitor (SIGMA). Cells have been lysed with two steps of freeze and thaw before adding 1% IPG buffer, 50mM DTT (to reduce S-S bonds), and Bromophenol Blue (to monitor the IsoElectricFocusing progress) for the first dimension. The solution has been then applied to the strip holder to rehydrate the immobilised 4-7 pH gradient gel strip (163-2001, BioRad) for the IEF process (18h RT rehydratation). The first dimension run has been performed at the Protean IEF cell (BioRad) (500 V 2 h step, 2 h 1000 V gradient, 2 h 4000 V gradient, 8000 V 50000 Vh step). The strip has been then equilibrated in SDS equilibration buffer (50 mM Tris.HCl pH 8.8, 6 M Urea, 30 % Glycerol to reduce electroendosmosis and improve protein transfer, SDS 2 %) + 5 mg / ml DTT 15’ (to preserve the fully reduced state of denaturated and un-alkylated proteins). The following alkylation of thiol groups adding to the equilibration buffer 25 mg/ml of iodoacetamide (to prevent protein reoxidation during electrophoresis). The 2nd dimension has been performed on SDS-PAGE on 12% acrylamide.

VII.7. MASS SPECTROMETRY IDENTIFICATION
VII.7.1.1. In gel digestion and peptide extraction

After cutting the bands/spots of interest in very small pieces of around 1 mm², the samples have been completely destained and then extensively washed in ultrapure water. Subsequent dehydratation and rehydratation steps have been performed thanks to the use of methanol, diAmmonium Phosphate and the SpeedVac, together with reduction and alkylation reaction when necessary. The dehydrated sample has been finally rehydratated in the digestion basic buffer (5 μg/ ml trypsin in 50 mM Ammonium Bicarbonate buffer pH 8.0 containing 10 % Acetonitrile) for 4-24 hours at 37 °C. The tryptic peptides have been collected and further extracted by sonicating in 5% Formic Acid (~20-50ul) to cover gel pieces. The combined supernatants have been dried in the SpeedVac and resuspended in 50 % Acetonitrile/ 0.1 % trifluoracetic acid or matrix solution and analyzed by mass spectrometry (Applied Biosystems 4800 MALDI TOF/TOF).

VII.7.1.2. Digestion on beads

The immunoprecipitated samples have been extensively washed in the complete lysis buffer containing a non ionic detergent as Igepal CA-630 (NP-40). A Glycerol step has been performed to better remove aspecific interactors. In details, the resuspended beads have been gently overlaid on 200 μl of 20 % glycerol containing lysis buffer at the bottom of a clean centrifuge tube; the pelleting of the beads by centrifugation removed all the aspecific binders. The final three washes have been performed in PBS buffer to remove all possible detergents present during the immunoprecipitation. The beads have been then covered with 20 mM Ammonium Bicarbonate in which 100 ng of trypsin enzyme has been added. Taking advantage from the fact that native antibody is more resistant to proteolysis that cytosolic proteins, 3-6 h of digestion allowed to collect the peptide mixture. The supernatant can be reduced and alkylated at this time, another aliquot of trypsin can be added and the digestion continued, or the supernatant can be acidified by the addition of acetic acid or TFA to 0.1 %. Automated peptide separation through high performance liquid chromatography according to their idrophobic properties is coupled with the sequential spotting on a MALDI plate (Probot Ultimate 3000, LC Packings) that is then processed in the Mass spectrometer.
VIII. RESULTS AND DISCUSSION

VIII.1. IN VIVO ANALYSES

In the Molecular Medicine laboratory, we optimized the production of a series of Adeno Associated Viral Vectors (AAV) to induce the expression of several anti- or pro-angiogenic factors in vitro and in vivo, aiming at dissecting the mechanisms underlying the formation and maintenance of new blood vessels during physiological and pathological angiogenesis (Arsic et al., 2004; Arsic et al., 2003; Giacca, 2007; Zacchigna et al., 2005; Zacchigna et al., 2008; Zentilin et al., 2006).

VIII.1.1.1. VEGF165 and Sema3A recruit bone marrow cells

We observed that the prolonged expression of the main isoform of VEGF, VEGF165, acted as a powerful inducer of angiogenesis, stimulating the development both of a large capillary network and the formation of an impressive set of new arterioles (Arsic et al., 2003). Most surprisingly, an unexpected effect of VEGF165 was also the recruitment, to the sites of its expression, of a vast set of mononuclear cells. These cells were derived from the bone marrow and expressed broad monocytic markers (CD45+, CD11b+); their presence correlated with the formation of arterioles as well as the maturation of the VEGF-induced vasculature. It is worth noting that we did not detect incorporation of these cells in the newly formed vasculature, or only as a very rare event (Zentilin et al., 2006). Strikingly, the shortest VEGF isoform (VEGF121) was, at the same time, unable neither to induce maturation of the newly formed capillaries into mature arteries nor to recruit cells: this observation first suggested that these bone marrow monocytes might be essential in blood vessel maturation. Since the major difference between VEGF165 and VEGF121 (besides the property of the former to bind polyanions) is the interaction with NP-1, we hypothesized that the major determinant for the recruitment of the bone marrow cells was the engagement of this receptor. Indeed, we found that cell recruitment strictly depends on NP-1 and that Sema3A, a NP-1 activator, acts as a powerful recruiter of these cells in vitro and in vivo (Zacchigna et al., 2008).
VIII.1.1.2. Molecular characterization of bone marrow recruited cells

Since NP-1 usually acts through the formation of membrane heterodimers with other receptors, we sought to determine the involvement of possible NP-1 partners in CD11b+ cell migration. Consistent with the real-time RT-PCR and immunofluorescence results, we first purified mouse primary CD11b+ cells from the bone marrow by the use of magnetic beads: FACS analysis confirmed the efficient purification of a population double positive for the panleucocitic marker CD45 as well as the monocytic marker CD11b (panel A and B of Figure 13).
Results and Discussion
Figure 13. FACS analysis of primary CD11b+ cells purified from mice bone marrow.
Results and Discussion

A. Bone marrow cells were sorted according to CD11b expression by the use of magnetic beads and labelled with an anti-CD45 antibody. This surface marker was found in nearly half of the CD11b- fraction (left plot), and in all the CD11b+ cells (right plot). B. Sorted CD11b+ cells were double labelled for CD11b and CD45, to show that all the cells co-express both markers. C. CD11b+ cells were differently labelled to assess the expression of NP-1, NP-2, Plexin-A1, Flt-1, Flk-1 and Gr-1, as indicated in each panel.

The examination of the purified cell population actually revealed the expression VEGFRs as well as PlexinA1 and the myeloid marker Gr-1. The presence of these receptors of interest has also been confirmed by western blot; endothelial cells have been used as a positive control for CD11b positive and negative cell population analysis (panel A of Figure 14). As a matter of fact, only abundant levels of NP-1 and mouse VEGFR1 have been detected in the total lysates, while immunoprecipitation experiments did confirm the binding of both Flk-1 and Flt-1 to NP-1, with the signal being even stronger for Flk-1 than for Flt-1. Thus, we concluded that Flk-1, even if expressed at very low levels, is tightly associated to NP-1 in CD11b+ cell (Panel B of Figure 14)
Figure 14. Molecular characterization of CD11b+ bone marrow cells.

A. Western blotting for the main VEGF receptors in CD11b+ and CD11b- cells purified from the bone marrow of AAV-VEGF<sub>165</sub>-injected mice. As a control, PAE-KN cells (PAE KDR NP-1) were used in the anti-Flt-1 and anti-NP1 blotting, whereas HUVEC were used for Flk-1 and NP-2 blottings. Anti-Hsp70 antibodies were used as a loading control. B. Co-immunoprecipitation of NP-1 in CD11b+ cells. Whole cell lysates (WCL) were immunoprecipitated (IP) using anti-NP1, anti-Flk-1 or anti-Flt-1, as indicated; the immunoprecipitates were then examined by Western Blotting (WB) using anti-NP-1 antibody. Input in the
WCL represents 2% of the amount used in the Co-IP experiment. PAE-KN: porcine aortic endothelial cells, stably transfected to overexpress KDR and NP-1, were used as a positive control.

Binding assay of recombinant alkaline phosphatase fused ligand actually defined only VEGF$_{165}$ and Sema3A as CD11b+ cell binders, consistent with a main role of the NP-1 molecule (Figure 15).

![Image](image-url)

Figure 15. Sema3A and VEGF$_{165}$ bind CD11b+ cell membrane.

Cell supernatants containing fusion proteins between AP and VEGF165, VEGF121, or Sema3A were added to CD11b+ cells. Binding was detected upon addition of VEGF$_{165}$-AP and Sema3A-AP, but not VEGF$_{121}$-AP. AP-tag only was used as a negative control. Original magnification, ×20.

These results clearly support the existence of a differential signalling feature of the VEGF isoforms and of some similarities between Sema3A and VEGF$_{165}$-induced phenotype.
Besides the property of binding to heparin, the main difference that distinguishes VEGF\textsubscript{165} and VEGF\textsubscript{121} is the ability of the former to interact with NP-1, at least at physiological concentrations and in the in vivo conditions used in our study.

The high expression level of NP-1 in purified BM CD11b\textsuperscript{+} cells, which has been confirmed from the analysis of the infiltrating cells into the sites of neo-angiogenesis in vivo, suggests the existence of a peculiar signalling pathway in this cell population induced by NP-1 interactors. The further characterization of this myeloid population did strongly support this hypothesis: as a matter of fact, VEGFR1 and 2 are present indeed on their cell surface and co-immunoprecipitation experiments are in agreement with the role of VEGFRs-NP-1 complexes as mediators of the observed VEGF\textsubscript{165} and Sema3A-induced migration (Zacchigna et al., 2008).

It is worth noting that VEGF\textsubscript{121} was not even able to bind to CD11b\textsuperscript{+} cell membrane, in contrast with previous findings in which the factor was used at very high concentrations (Pan et al., 2007b), nor to induce their migration, thus indicating that the engagement of NP-1 is necessary for both events (Zacchigna et al., 2008).

We were really fascinated in tackling the issue of dissecting the signalling feature induced by VEGF\textsubscript{165} and Sema3A in this myeloid population to unravel the precise molecular mechanisms through which the peculiar in vivo effect acts; in this perspective, VEGF\textsubscript{121} could have been considered as a negative control to better define NP-1 role in the process.

Taking into consideration that VEGF signalling is mediated by tyrosine kinase receptors, the dissection of the very early events in the induced intracellular pathway has been tackled through the analysis of the comprehensive phospho-proteome.

Considering technical issues of phospho-proteome related studies, as the need of large quantities of proteins to be analyzed as well as a reproducible methodology, we decided to focus our analyses onto a more amenable model to study, such as endothelial cells. Although we demonstrated that the soluble factors induce a direct recruitment of bone marrow cells, it is very likely that the early events observed in vivo upon VEGF and Sema3A sustained expression are also due to the induced effect on resident cells. The final phenotype of bone marrow cells observed in vivo is reasonably closely super imposable to the behaviour of known target of VEGFs and Sema3A, such as endothelial cells.

We decided to focus our studies on porcine aortic endothelial cells, since they do express Plexins, but not any of the VEGFRs and they are widely exploited as a model to identify the peculiar role of single membrane molecules in an endothelial environment (Kawamura et al., 2008; Miao et al., 1999; Ratcliffe et al., 2002). In particular we investigated the
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effects VEGF\textsubscript{165} and Sema3A in stable cell transfectants with VEGFR2 and NP-1, while VEGF\textsubscript{121} has been used as a control, since it is unable to engage the NP-1 receptor and to recruit bone marrow cells \textit{in vivo}. 

VIII.2. BACULOVIRUS MEDIATED PROTEIN PRODUCTION

Since this kind of studies in proteomics requires abundant amount of recombinant factors to be used in the stimulation experiments, we decided to set up “in house” the production of the recombinant proteins of interest. We initially considered different protein production approaches in several host cells, from bacteria to eukaryotic cells. Since the native pattern of post-translational modifications is needed for the molecules of our interest in order to maintain optimal functions, we decided to use the Bac-to-Bac Baculovirus Expression System (Luckow et al., 1993).

As an initial approach, we decided to produce an untagged form of the VEGF$_{165}$ isoform. The subcloning of the VEGF$_{165}$ coding sequence into the pFastBac1 expression plasmid has been followed by the transformation into the DH10Bac competent E.Coli cells. This strain contains a baculovirus shuttle vector (bacmid) which allows its recombination with the pFastBac expression plasmid. The purified recombinant bacmid has been transfected into Sf9 cells and the first viral stock has been collected 96 h post transfection. The viral preparation has been amplified and checked to determine the viral titre; finally the optimal viral amount (Multiplicity Of Infection, MOI) and timing conditions for protein production have been fixed. Since we were able to obtain a satisfying protein expression only in presence of a 5% foetal bovine serum in the culture medium of Sf9 cells, we needed to purify the secreted recombinant protein, through cation exchange chromatography followed by a gel filtration step procedure (Figure 16).
Figure 16. Gel filtration purification of VEGF<sub>165</sub>.

After performing liquid chromatography, the second step of gel filtration allows the exhaustive separation of BSA (having a MW around 60 KDa) and VEGF<sub>165</sub> (having a MW around 21 KDa). Collected fractions are loaded on a 12% SDS-PAGE and subjected to coomassie stain; the sample containing the protein of interest are collected, dialysed and concentrated before being aliquoted and stored at -80°C.

In spite of the purity of the recombinant protein, this procedure was abandoned due to stacking of the chromatography column upon scaling-up, for the presence of foetal bovine serum in the medium. We then preferred to move to a more effective, suitable and flexible strategy: the construction of a plasmid for the secretion of a tagged protein whose tag can be cleaved to release the wild type recombinant isoform.

VIII.2.1. pFastBacHTgp67 construction

To overcome issue of protein purification and its large amount to be produced, we based our cloning strategy onto two vectors already available: pAcGP67, containing the most effective baculovirus-encoded secretion signal and pFastBacHT, to clone the gene of interest in frame with a cleavable N-terminal 6xHis tag. The cloning procedure is summarized in Figure 17.
Figure 17. pFastBacHTgp67 construction.

PCR-based mutagenesis has been employed to remove the first ATG codon from the commercial pFastBacHT. The same technique has been used to add two additional restriction sites in pFastBacHT to allow the insertion of the secretion signal (an AflIII site) and to remove undesired sequences between the Tobacco Etch Virus protease cleavage site and the coding sequence of the gene of interest (PvuII site). The insertion of the secretion signal of the acidic glycoprotein gp67 of the Autographa californica nuclear polyhedrosis virus upstream the 6xHis tag of pFastBacHT modified vector have been performed thanks to an AflIII digestion; the correct insertion have been screened by restriction enzymes digestion and sequencing.

The resulting construct allows the secretion of the 6xHis tagged recombinant protein; upon cleavage, the native protein will only have an additional glycine upstream its wild type amino acid sequence.

VIII.2.2. **Protein production setting up**

We then set up the production of tagged recombinant VEGF_{165} protein.
The **recombination with the bacmid** of the plasmids upon transformation into the DH10Bac competent E.Coli cells were confirmed by PCR (Figure 18).

![Bacmid clones](image)

**Figure 18. VEGF<sub>165</sub> bacmid recombination validation.**

VEGF<sub>165</sub> coding sequence integration has been validated through PCR with primers annealing on the bacmid sequences spanning the site of integration. A further PCR in which one primer has been substituted by another annealing to the VEGF coding sequence define the positive clones used in the following steps.

The first **baculovirus production** was performed upon transfection of the purified recombinant bacmid in Sf9 cells and virus containing medium has been collected 96 h post transfection. Protein production was assessed by Western blot, before viral amplification and optimal viral titre detection.

To **optimize protein production conditions**, several variables were evaluated. First of all serial dilution of the viral stock has been used to define the optimal **Multiplicity of Infection** (MOI, number of viral particles for cell) and the **optimal timing** for the infection: as a matter of fact, the optimal conditions were obtained MOI 10 72 h after infection (Figure 19).
Since VEGF\textsubscript{165} is a glycosylated protein, we tested a Sf9 derivative cell line (Mimic Sf9), modified to stably express a variety of mammalian glycosyl-transferases in order to produce more “mammalian-like” proteins in baculovirus expression systems. The characterization revealed that Mimic Sf9 cells produce less VEGF\textsubscript{165} of a higher molecular weight and the treatment with N-Glycosidase F, also known as PNGase F, confirmed indeed that the differences observed were due to peculiar glycosylation (Figure 20). Although VEGF produced in Mimic Sf9 actually seemed to be more stable than the one produced in Sf9, we decided to use Sf9 for the large scale protein production, taken into account the minor amount of protein produced by Mimic Sf9.
Figure 20. Mimic Sf9 produce less VEGF165 of a higher MW due to glycosilation.

A. Mimic Sf9 and Sf9 produced VEGF165 upon MOI 10 viral infection, has been collected at different time points and ran on a SDS-PAGE to be checked by western blot anti VEGF. Mimic Sf9 produce less amount of protein that present a higher MW in time. B. Mimic and Sf9 VEGF165 obtained at MOI10 72 h post infection has been processed to the N-Glycosidase F (PNGase F) oligosaccharides cleavage. Western blot against 6xHis tag on the 12% acrylamide resolved samples clearly indicate that cleaved proteins exert similar molecular weight.

**Protein purification** has been performed based on an IMAC technique in which the reversible interaction between histidine chains and immobilized cobalt ions allows the final
release of recombinant polyhistidine-tagged proteins. Coomassie stained SDS-PAGE and Western blots are necessary to detect the eluted fractions containing the protein of interest. The dialysis of the samples of interest is necessary before the Tobacco Etch Virus protease cleavage; different incubation conditions have been evaluated to finally identify the optimal cleavage settings: 30 min at 16°C. The use of a His-tagged version of the protease allows the purification of the untagged protein only (Figure 21).

![Diagram of protein purification process]

**Figure 21. His-tag is efficiently removed upon AcTEV protease incubation.**

Purified His-VEGF165 has been incubated with TEV protease at 16 °C for different period of time. SDS-PAGE followed by silver staining has been performed. Native VEGF has been resolved from His-AcTEV protease thanks to a second purification step. Samples containing the protein of interest are collected, dialysed and concentrated before being aliquoted and stored at -80 °C.
VEGF₁₂₁ and Sema3A have been produced in a similar manner (Figure 22).

Figure 22. VEGF₁₂₁ and Sema3A purification.
Recombinant His-VEGF₁₂₁ and His-Sema3A have been produced as described for His-VEGF₁₆₅. Upon the tag removal, native VEGF₁₂₁ and Sema3A have been resolved from His-AcTEV protease thanks to a second His purification step. Samples have been visualized by silver staining on a 12 % SDS-PAGE for VEGF₁₂₁ (Panel A) and 8 % SDS-PAGE for Sema3A (Panel B). Fractions containing the protein of interest (unbound proteins in the figure) are collected, dialysed and concentrated before being aliquoted and stored at -80 °C.

The characterization of the recombinant VEGF, has been performed to seek for its capability to induce VEGFR2 phosphorylation, endothelial cell proliferation and to compare its activity to the commercially available one (Panel A and B of Figure 23).

The Sema3A functionality has been tested by transfecting PlexinA1 and NP-1 in simian COS7 cells: the recombinant protein was as effective as the commercial counterpart in inducing cytoskeletal rearrangements leading to cell contraction (Panel C of Figure 23).
Figure 23. Functional validation of recombinant proteins.

A. VEGFR2 phosphorylation pattern. PAE KDR NP-1 have been differentially treated with the commercial or the baculovirus produced protein (home made in the figure). Lysates have been resolved in 8% acrylamide gel and subjected to p-tyr (phospho tyrosine) western blot followed by VEGFR2 western blot. Similar results have been obtained for VEGF164 isoform. B. Proliferation assay on endothelial cells (HUVEC and PAE KDR NP-1). Cells have been treated with the recombinant factors for 4 days before Alamar blue proliferation assay. T-test values define as significant the proliferation phenotype measured for both the VEGFs. C. Sema3A “contraction assay”. 200 ng/ml of recombinant Sema3A, baculovirus produced one (home made in the figure) have been applied to COS7 stable transfected with PlexinA1 and NP-1. The immunofluorescence against NP-1 allows the identification of the “contracted” cells.

Here we efficiently provide a novel useful tool to produce huge amount of recombinant ligands by recombinant baculovirus production. The novel vector pFastBacHTgp67 allows the secretion of recombinant molecules finally characterized by no additional amino acids but a glycine upstream their coding sequence. This system will be thus suitable for the production of proteins finally almost identical to their native counterpart.

VIII.3. PHOSPHO-PROTEOME DETECTION

Cell signalling mechanisms transmit intracellular signals via post-translational protein modifications, most importantly through reversible protein phosphorylation, at least in mammalian cells. The aim of the in vitro section of the studies herein described is the
comparison of the signalling pathways elicited by the different VEGF family members (in particular, VEGF$_{165}$ and VEGF$_{121}$) and Sema3A on endothelial cells, by a combination of biochemical and proteomics approaches.

To better unravel the peculiar signalling feature induced by the different ligands we decided to analyze porcine aortic endothelial cells (PAE), stable transfected with the major VEGF receptor, the human KDR and NP-1. This system has been considered the most suitable for our purposes since the simultaneous presence of specific receptors which can be stimulated by ligands with peculiar binding capability can offer a way to unravel the specific role of membrane molecules in the phosphoproteome observed in each condition.

This milestone entailed the following tasks:

- Setting up of 2D gel electrophoresis
- Phospho-proteome visualization
- Phospho-protein enrichment

**VIII.3.1. Setting up of 2D gel electrophoresis**

**Protein separation.** Two-dimensional electrophoresis (2-D electrophoresis) is a powerful and widely used method for the analysis of protein extracts from cells, tissues, or other biological samples. This technique resolves protein according to two independent properties in two steps: the first-dimension step, isoelectric focusing (IEF), separates proteins according to their isoelectric points (pI); the second-dimension step, SDS-polyacrylamide gel electrophoresis (SDS-PAGE), separates proteins according to their molecular weights. Each spot on the resulting two-dimensional array corresponds to a single protein species in the sample. Thousands of different proteins can thus be separated, and information such as the protein pI, the apparent molecular weight, and the amount of each protein is obtained (O'Farrell, 1975).

The experimental sequence for 2D electrophoresis has been evaluated in detail to set up the best final condition to separate phosphorylated species.

**Sample preparation** is one of the more critical steps in the procedure, because appropriate sample solubilisation, protein denaturation and reduction are essential for good 2D results. We initially decided testing the simplest procedure, such as to lyse the cells in the buffer used for the first dimension: 8 M Urea and 1 M Thiourea to solubilise and denature proteins, 2% CHAPS to solubilise idrophobic proteins and minimize protein aggregation.

The initial protocol, in which standard conditions have been used, actually resulted not suitable for the endothelial cells studied since the separation obtained was very bad.
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We spent quite a lot of time in performing several trials to understand which step was the main responsible for the bad separation: we change several variables, like different focusing conditions or different lysates preparation. Finally, we realized that the critical step was indeed the sample preparation. Protein precipitation in TriChloroacetic Acid before the separation was very effective in strongly reducing the lysate complexity, thus resulting in a nice 2D gel separation, in spite of the depletion of a significant protein amount, due to the difficulty in solubilising the precipitated material (Figure 24).
**Figure 24. 2D gel of TCA precipitated protein lysate.**

The gel has been produced by separating the protein extract obtained from 1mg of PAE KDR NP-1 cells in 3-10 non linear pH gradient 16x18 cm gel upon TCA precipitation.

**IPG strip rehydration and IsoElectricFocusing:** As a first global and general approach we used immobilized, non linear 3-10 pH gradient to better resolve the middle pH range.
The rehydration was made at 20 °C for 12 h. The first dimension separation has been performed at low current with a gradually increasing voltage. Upon testing several IEF conditions and different immobilized pH gradient strips, we decided to increase the rehydration incubation time to 18 h and to use narrow pH gradient (pH 4-7), to better resolve the proteins.

**SDS-PAGE:** Figure 25 shows a representative 2D gel separation of 0.3 mg of PAE KDR NP-1 protein lysates in a pH 4-7 linear gradient of 7 cm. It clearly shows some “trains of spots”, namely proteins with similar MW, but different pI, likely representing differently modified variants of the same protein. Taken into account that phosphorylation affects the pI of a protein, we can conclude that the final 2D gel protocol can potentially nicely resolve differently modified species of interest.

![MW pH 4 to pH 7](image)

**Figure 25.** 2D gel with the final protocol.
The gel has been produced by separating the lysate obtained from 0.3 mg of PAE KDR NP-1 cells in 4-7 pH gradient 8.6 x 6.8 cm gel following the final protocol.

**Protein visualization.** To understand which staining technique was the most sensitive and the more compatible with mass spectrometry, we performed a BSA amount titration of some of the most sensitive staining procedures commercially available. After testing *Colloidal Coomassie* (1 ng sensitive, MS compatible) and *Blue silver* (1 ng sensitive, MS compatible), the final choice has been the *Shevchenko modified silver stain* (0.5 ng sensitive, MS compatible) (Candiano et al., 2004; Shevchenko et al., 1996) (Figure 26).

![BSA (ng) Colloidal coomassie Blue Silver Shevchenko modified Silver stain](image)

**Figure 26. Shevchenko modified silver staining is the staining method of choice.**

Decreasing amount of BSA have been resolved in SDS-PAGE followed by the three staining procedure. In gel digestion of the detected bands defined the sensitivity of 1 ng for the Colloidal Coomassie, 1 ng for Blue Silver and 0.5ng for Shevchenko modified Silver stain.

**VIII.3.2. Phospho-proteome visualization**

**Treatment conditions.** Once defined the suitable separation and visualization approaches, we moved to the optimization of the treatment conditions.

As a matter of fact, the phosphorylation of the VEGF tyrosine kinase receptor is the first event that triggers all the VEGF-dependent downstream signalling features that finally lead to the observed phenotypes. Thus, we treated starved PAE KDR NP-1 with different VEGF concentrations for different times, to finally state the optimal stimulation conditions as 50 ng/ ml of recombinant factor for 7 minutes at 37 °C (Figure 27)
Figure 27. Optimal timing condition definition.

PAE KDR NP-1 have been treated for different period of time at 37 °C with the recombinant ligands. Protein lysate obtained from different samples have been resolved in 8 % SDS-PAGE and then subjected to Western Blot anti-phospho-tyrosine. The membrane has been then stripped and reprobed against VEGFR2. Immunoprecipitation of phospho-tyrosine protein followed by western blot against VEGFR2 ended with comparable results.

As far as *Sema3A* treatment is concerned, we decided to maintain the same condition used for VEGFs stimulation. Although there are evidences that Sema3A needs the tyrosine kinase activity to exert its own effect, there are no clear evidences for specific phosphorylated targets in endothelial cells. Furthermore, we decided to treat endothelial cells with 200 ng/ ml of recombinant factor, the same concentration used to induce COS7 cell contraction.

**Phospho-protein specific staining.** At this point we initially decided to test commercially available staining to detect phosphorylated species, in order to identify possible spots of interest, then isolated and identified through mass spectrometry.

We first assess the *Pro-Q Diamond phospho-protein gel staining* on control and VEGF_{165}treated PAE KDR NP-1, but unluckily any phospho-specific signal was detected. We then tested the *Phos-tag 540 Phospho-protein Gel Stain*. This reagent is characterized by a new family of phospho-protein-selective dyes that selectively bind to the phospho- monoester residues of phospho-serine, phospho-threonine and phosphotyrosine residues via a charge-based coordination of chelated Zn^{2+} cations. The chelator is readily coupled to
a variety of fluorophores, offering the ability to match spectral properties of a particular
stain with available fluorescent gel imaging platforms. Equal amounts of control, VEGF_{121}
and VEGF_{165} treated PAE KDR NP-1 lysates have been resolved in a pH 4-7, 7cm 2D gel
and then analyzed using the robust and nimble software PDQuest (Figure 28 and Figure
29).

![Figure 28. Phospho-proteome staining of differently treated PAE KDR NP-1.](image)

PAE KDR NP-1 were left untreated or incubated with VEGFs for 7 min at 37 °C. Total protein lysate has
been then resolved in 2D gel electrophoresis and subjected to phospho-proteome staining. These gels are
representative of three independent experiments.
Figure 29. Phospho-stain of VEGFs treated PAE KDR NP-1.

A. Equal amount of PAE KDR NP-1 lysates obtained from control and VEGF_{165} and VEGF_{121} treatment have been resolved in 2D gel electrophoresis and then subjected to the phospho-specific staining. B. The same gels have been subsequently stained for the total proteome by Silver staining.

The merge of the two gels obtained from VEGF_{121} and VEGF_{165} treated samples created an “integrated” 2D gel in which are present all the spots the software identified in one or both the gels in analysis (Figure 30).
Figure 30. 2D gel comparison of VEGF$_{165}$ and VEGF$_{121}$ phospho-stained gel.
Once the spots identified by the software have been manually validated, an ideal gel has been created by the software through the merge of VEGF\textsubscript{165} and VEGF\textsubscript{121} treated sample gels (depicted in Figure 29). A qualitative analysis has been then performed and colour assigned. Grey = common proteins to both treatment. Blue = VEGF\textsubscript{165} only phospho-protein. Red= VEGF\textsubscript{121} only phospho-protein. The black spots correspond to common saturated spots.

The program further confers a particular colour depending on the spot nature: the grey/black ones are common to both samples, the red ones are uniquely present in the VEGF\textsubscript{121}-stimulated sample, while the blue ones are only in VEGF\textsubscript{165} one. This qualitative analysis, representative of three independent experiments, has been followed by the isolation of the spots of interest and the “in gel” digestion, to proceed for protein identification through mass spectrometry. Unfortunately, using this approach, we were not able to identify any protein, probably because of the very small amount present in the spot of interest.

The only difference between the two VEGF (121 or 165) isoforms is the presence of the seventh exon in the latter one. Several pieces of evidence aim to assess whether the properties of the shorter product, VEGF\textsubscript{121}, can be just explained as a decrease in the strength of the induced signalling pathway or as a result of peculiar features. As a matter of fact, mice expressing only the shorter VEGF\textsubscript{121} isoform die shortly after birth with deficit in the distribution of endothelial cells and impaired myocardial angiogenesis and skeletogenesis, thus indicating the incapability of VEGF\textsubscript{121} of substituting VEGF\textsubscript{165} \textit{in vivo}. Moreover, studies on VEGF\textsuperscript{Ac} and VEGF\textsuperscript{Ah} embryos suggest a tight dose-dependent regulation of embryonic vessel development by the two VEGF isoforms (Coultais et al., 2005; Ferrara, 2005; Zelzer et al., 2002).

The analysis of the phospho-proteome profile of endothelial cells expressing VEGFR2 and NP-1 has been proven as a very powerful system to tackle this issue. The hypothesis of a weaker strength in the signalling induced by the shorter isoform would be strengthened by the identification of all the VEGF\textsubscript{121}-induced phospho-proteins as a subgroup of VEGF\textsubscript{165}-induced ones, while different outcomes would support the alternative hypothesis.

The outcome of the phospho-protein staining was very satisfactory: using this approach we identified significant differences as well as similarities between the different VEGF isoforms analyzed, thus implying differential phosphorylation pattern in treated cells. In first instance, the sharing of the vast majority of phosphorylated species between the two treatments (highlighted in grey in Figure 30) did confirm the common features observed \textit{in vitro} and \textit{in vivo}. Moreover, VEGF\textsubscript{121}-induced a phosphorylation pattern of proteins (in red in the Figure 30), completely different from the VEGF\textsubscript{165} and control sample, thus strongly
supporting an independent role for the shorter isoform in mediating a peculiar signalling. It is not surprising that the only VEGF165-induced phosphorylation events (highlighted in blue in the Figure 30) are exceeding the number of VEGF121 only events, thus enforcing the already known main role on endothelial cells of the longer isoform.

A recent work suggested the binding of VEGF121 to NP-1 at very high concentration of the ligand, but the in vivo findings do not support an in vivo role of this findings (Pan et al., 2007b; Zacchigna et al., 2008). We can speculate that these results support an additional role for NP-1, acting not as mere co-receptor, but as an integrated player in the complex signal triggered by neighbouring transmembrane molecules or maybe by itself alone.

Once qualitatively defined an independent signalling for VEGF121 in angiogenesis, as well as a unique function for NP-1 in the VEGF165-induced phenotype, the identification and characterization of proteins differentially regulated by these ligands would have been the next step to fully tackle the molecular mechanisms of interest.

Unfortunately, we didn’t succeed in any of the protein characterization using the mass spectrometer, in spite of the MS compatibility of the modified Shevchenko silver staining procedure. Possible causes could have been the low efficiency of the sample digestion and extraction from the silver stained gels, together with the too long time consuming procedures to stain and isolate the spots of interest, prior to the MS analysis.

A possible future evolution of the phosphoproteome detection could be exploiting very recently combined staining procedures, like the one in which silver has been integrated with ethyl violet and zincon (Jin et al., 2008).

**Western Blot.** As a matter of fact the phosphorylation on different amino acids exerts peculiar functions, thus leading to the activation or inhibition of target proteins. The ratio of phosphorylation on phospho-serine: phospho-threonine: phospho-tyrosine is 1800:200:1 in vertebrates, thus leading to the need of a complex coordinated action of different kinases to finally affect the cellular metabolism and behaviour.

Several studies showed the use of antibodies for the detection or enrichment of phosphorylated species, with the final aim to unravel the phospho-proteome profile of particular systems (Blagoev et al., 2004; Dengjel et al., 2007; Gronborg et al., 2002; Rush et al., 2005; Zheng et al., 2005). Therefore, we tried to optimize the western blot technique on 2D gels, in order to detect differential phosphorylated species. Of course, one dimensional gel electrophoresis would have been easier to handle and more reproducible,
but we anyway abandoned this possibility, due to the fact that co-migrating proteins in one dimension would affect their unambiguous MS identification.

After stimulation, treated and control cell lysates were separated by 2D electrophoresis and both tyrosine and serine/threonine phosphorylated proteins were detected by Western Blot using specific antibodies. A parallel experiment using identical samples was performed to stain the total proteome and to isolate the spots of interest, thus leading to in-gel trypsin digestion and MS identified using both MALDI-TOF mass fingerprinting and ESI peptide sequencing.

Although we tested different conditions to reduce background and obtain reproducible western blotting results (Figure 31), the comparison between the total proteome staining and the antibody-specific signal was not possible.
Figure 31. 2D western blot visualization of phosphorylated.

Total lysate of PAE KDR NP-1 starved (left side) or treated with VEGF_{165} have been resolved in 2D gel electrophoresis. Blotted sample on a membrane have been subjected to western blot against phospho-serine residues (Panel A), or phospho-tyrosine residues (Panel B).
We then tried a technique established by Luque-Garcia and colleagues to directly cut and identify proteins from the nitrocellulose membrane used for the western blots after the ECL detection (Luque-García et al., 2008), but this approach failed, as well as the use of PVDF membranes.

Although the very intriguing procedure, further improvement are actually needed to reach a feasible and amenable protocols to extract and identify proteins from a membrane already subjected to western blot.

VIII.4. PHOSPHO-PROTEOME ENRICHMENT

Whichever would have been the method to analyze the samples, we also wanted to assess whether phospho-protein enrichment could be an alternative or complementary approach suitable for the identification of the protein of interest.

VIII.4.1. Anion exchange chromatography

To tackle this issue, we initially exploited an anion exchange chromatography (Mustang Q membrane (Pall)) to enrich the sample in tyrosine, serine and threonine phosphorylated species. Protein lysates from both control, and VEGF_{165} cells were applied to a membrane constituted by a modified version of polyethersulfone. Eluted fractions by increasing salt concentration have been analysed through western blot against phosphorylated species.

Due to the poor protein loading capacity (less than 0.2 mg of total protein lysates) and to the aspecific binding to the column of molecules in which negatively charged amino acids were highly represented, this approach did not result in an effective phospho-protein enrichment.

VIII.4.2. Immunoprecipitation

*Immunoprecipitation followed by gel visualization*. Immunoprecipitation is a common approach widely used to purify proteins of interest together with their interactors. In the case of phosphorylation, there are no available good antibodies against the phospho-serine and threonine residues for immunoprecipitation, so we decided to investigate the phosphorylation on tyrosine residues.

First of all we set up the optimal immunoprecipitation conditions, trying several lysis buffers and kind of antibodies. In particular, we tested lysis buffer as a modified RIPA, as well as a milder one in which the only detergent was 1% NP-40. Different antibodies have
been tested, namely the clone PY100 (Cell signalling), PY20 (SIGMA) and PY99 (Santa Cruz). The more efficient lysis exerted by the RIPA buffer actually resulted in a higher background when compared to the immunoprecipitation performed in the milder one (Figure 32).

![Mild buffer](image1)

![RIPA buffer](image2)

**Figure 32. Optimization of the immunoprecipitation against phospho-tyrosine.**

A modified version of RIPA buffer as well as a milder buffer has been tested together with three antibodies. Three immunoprecipitation using three antibodies against phospho-tyrosine containing proteins (PY100, PY99; PY20) have been performed. Samples have been resolved in 4-20 % gradient gel blotted against phosphotyrosine (antibody PY20). The high level background observed in RIPA lysed samples as well as the comparison between different antibodies efficacy define the PY20 antibody in the mild buffer as the condition of choice. C= untreated PAE KDR NP-1; V= VEGF_{165} treated PAE KDR NP-1

Once optimized the immunoprecipitation step, we performed serial experiments in which PAE KDR NP-1 were differentially treated with the 3 ligands: taken into account that 1 µl of conjugate antibody could bind 0.5 µg of phospho-tyrosine proteins, 10-50 µl of PY20 has been incubated with 5-20 mg of total protein lysates obtained from PAE KDR NP-1 differentially treated.

To better resolve immunoprecipitated species, we separated them onto 4-20% gradient 16x 18 cm gels. Although the high starting protein amount, we anyway used the silver staining to visualize the enriched proteins, probably because of the small absolute amount of
phospho-tyrosine proteins together with the not so efficient PY20 immunoprecipitation; arrows indicate band enriched upon treatment (Figure 33)
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Figure 33. Immunoprecipitation of phospho-tyrosine proteins.

PAE KDR NP-1 has been differentially treated with VEGF_{165}, VEGF_{121} and Sema3A for 7 min. Immunoprecipitation against phosphorylated proteins in tyrosine has been performed and resolved in a homemade 4-20% gradient gel (Panel A and B represent namely the upper and lower part of the same gel). Arrows indicate bands of interest, increased upon one or more ligand exposure. All the highlighted proteins have been in gel digested and processed by mass spectrometry.

These results were reproducible and satisfying: several interesting bands could be identified and strikingly many similarities were detected between VEGF_{165} and Sema3A treatments. These findings clearly indicate that these ligands, in spite of their very different nature, are significantly overlapping in the phospho-tyrosine pattern induced in endothelial cells. It is also worth noting that most of the bands present in both VEGF_{165} and Sema3A are actually missing in the VEGF_{121} treated sample (bands n. 1, 3, 5 and 8). Since the only common biochemical feature between VEGF_{165} and Sema3A is their ability to bind NP-1, this result supports an independent role of this membrane molecule in the induction of a peculiar signalling feature.

On the other hand, endothelial cells treated with VEGF_{121}, which is unable to bind NP-1, appears to be characterised by a very weak phosphorylation pattern on tyrosine, as only one protein is specifically phosphorylated upon this treatment (band n. 6). Upon comparison with the 2D gel image in which phospho-serine, phospho-threonine and phospho-tyrosine are highlighted, we can speculate that there could be actually
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concordance in the position of one of the phosphorylated spot and the band visualized by PY20 immunoprecipitation, thus enforcing the significance of this outcome.

Protein immunoprecipitation followed by MS identification. The previous approaches succeeded in demonstrating the presence of a differential phospho-tyrosine profile in PAE KDR NP-1 upon different treatments. The unsuccessful identification through mass spectrometry analysis of any “in gel” digested proteins was probably due to the low amount of protein present in the band/spot and to the poor elution form silver stained gels.

Keeping this issue in mind, we decided to modify the final approach, avoiding the gel separation step and directly proceeding to the digestion of the on bead-bound proteins. This approach implies the removal of all the detergents in the immunoprecipitation washes, to allow the trypsin digestion to occur directly on the beads. Considering that native antibodies are more resistant to proteolysis than cytosolic molecules, all the proteins containing the epitope of interest (phospho-tyrosine) and their interactors would be digested. The complex tryptic sample is then split according to the idrophobic properties of each peptide and spotted into a MALDI plate. Two different procedures have been performed: in the first case the samples have been either differentially labelled and mixed before the liquid chromatography separation; in the other one each sample has been individually analysed to obtain simpler spectra (Bish and Myers, 2007). Samples have been analysed at the Mass Spectrometry Core Facility operational at the ICGEB, Trieste (in collaboration with Mike Myers).

We initially applied this approach on control or VEGF_{165} treated PAE KDR NP-1: the two samples have been differentially labelled and then mixed to be analysed at the mass spectrometer. Although the procedure was successful to analyse and identify hundreds of proteins, the final obtained spectra were characterized by a significantly high background signal. Analyzing the peptides obtained from our samples, a huge amount of nucleic acid binding proteins and cytoskeletal component resulted as major components.

To obtain simpler MALDI spectra, we then moved to the individual analysis of the control and VEGF_{165} treated samples. Although this approach did not completely solved issues of aspecific binders and strength of phospho-proteins interaction with the antibody, the spectra outcome was improved and more specific.

We repeated several times this procedure and the list of proteins selectively phosphorylated on tyrosine residues upon VEGF_{165} treatment are represented in figure (Figure 34).
Results and Discussion

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Figure 34. VEGF165 only phospho-proteins in tyrosine.

VEGF165 treated and untreated PAE KDR NP-1 have been collected and enriched in tyrosine phosphorylated protein by immunoprecipitation. The table shown above draws the common molecules of three independent experiments. Proteins have been identified according to Entrez Gene (NCBI); the score is a value that depends on the amount of peptides matching the same protein and the possibility of false positive results: higher is the score, lower is the probability of mis-identification. In yellow are highlighted proteins of interest.

The list comprises cytoplasmic proteins part of the cytoskeleton as well as some
intracellular mediator of signalling pathway. It is interesting the presence of PLC-γ, SHC-transforming protein 1 (Sck), tyrosine protein kinase CSK, Src family members Fyn and Yes, heat shock protein 90, Breast cancer anti-estrogen resistance 3, vimentin and tensin 1, highlighted in yellow in Figure 34.

We were quite satisfied by these results, since we were able to nicely identify some already known target of phosphorylation upon VEGF treatment, namely SHC, Vimentin, CSK, YES, PLC-γ and hsp 90 (Cross et al., 2003; Duval et al., 2007; Ratcliffe et al., 2002; Werdich and Penn, 2005). Cytoskeletal reorganization is an event triggered by VEGF to in endothelial cells that involves the modification of a significant number of cytoskeletal components; the involvement and the relevance of each finding will be unravelled in detail in the following discussion.

Several systems have been exploited to enrich a sample in phosphorylated species, using both chromatography and immunoprecipitation: tyrosine immunoprecipitation has indeed been demonstrated here as a very powerful technique. Figure 33 shows the separation of differently treated endothelial cells resolved in a gradient gel. The resulting data clearly depict a qualitative analysis of PAE KDR NP-1 behaviour upon VEGF-induced phosphorylation. This tool efficiently succeeded in defining, for the first time, an active role for Sema3A in the induction of a tyrosine phosphorylation profile. In spite of its known inhibitory role in “guidance”, the presence of phosphorylated species upon Sema3A treatment is in agreement with the interference in the biological effect induced by this ligand in neuronal cells upon tyrosine kinase inhibitor treatment (Franco and Tamagnone, 2008).

The conserved tyrosine residues in the cytoplasmic tail of Plexin family members, some of them being in close proximity of regulatory regions, together with pieces of evidence that plexins might become phosphorylated when over-expressed, support Plexins as possible target of tyrosine kinases. Moreover, the presence of dominant negative mutants of ErbB2, Met and Fyn lacking kinase activity results in strongly reduced phosphorylation of associate Plexins (Franco and Tamagnone, 2008; Giordano et al., 2002; Sasaki et al., 2002).

VEGFR2 association with PlexinA1 has been actually shown in cell transfection assays and Sema6D presence has been demonstrated to increase KDR level of phosphorylation upon VEGF to treatment in outgrowing cells from the cono-truncal mice cardiac segment (Toyofuku et al., 2004). Although we can not exclude a Sema3A-dependent effect on
VEGFR2 signalling due to the presence of Plexins on PAE cell membrane, we never detect the phosphorylated KDR receptor just upon Sema3A treatment, at least in our in our cell model.

Moreover, we were really intrigued by the similarity between VEGF_{165} and Sema3A-induced phosphorylation pattern on tyrosine. The findings that VEGF_{165} and Sema3A shared the induction of phosphorylation on peculiar protein species strongly supports the similar \textit{in vivo} findings and they can be reasonably responsible for the early events detected in AAV-VEGF_{165} and AAV-Sema3A transduced skeletal muscles (Zacchigna et al., 2008; Zentilin et al., 2006). This result is extremely relevant, since it is the first biochemical demonstration that the triggering of tyrosine phosphorylation of specific proteins can be one of the common molecular features between two ligands, which just shared their binding to NP-1. We can thus speculate that NP-1 may be shared as a co-receptor between VEGFR and Plexins, thus leading to an integrated signalling network that finally leads to the phosphorylation outcomes we detected, at least in our cell system; the different level of expression of endogenous plexins compared to KDR and NP-1 has to be anyway taken into consideration. Further membrane bound signalling molecules could take part in the phosho-proteome observed as single or integrated players in the VEGFR2-NP-1 triggered signalling pathway.

Furthermore, the immunoprecipitation of protein lysates of endothelial cells expressing only VEGFR2 would be the best system to demonstrate that the observed pattern is actually dependent on NP-1. Upon binding of Sema3A, neuropilins couple to plexins to regulate chemorepulsion of axons. The three C-terminal amino acid residues of NP-1 and NP-2, SEA, may associate with the PDZ-containing protein GIPC/synectin that interacts with RGS-GAIP, a GTPase activating protein for Goi subunits. Whether VEGF binding to NP-1 leads to VEGFR-2-dependent activation of this pathway has not been yet settled (Cai and Reed, 1999; Chen et al., 2005; Gu et al., 2003). The NP-1 relevance in the induction of tyrosine phosphorylation of peculiar proteins has been already underlined: in particular Kawamura and colleagues exploited an antibodies array to compare VEGF_{165} and VEGF_{121} signalling feature in order to define p38 as a specific downstream target of NP-1. It is worth noting that one of the common features between VEGF_{165} and Sema3A phospho-tyrosine immunoprecipitation is a protein band characterized by a molecular weight comparable with p38.

Although Sema3A-induced main phenotype (cell contraction, axon repulsion) is totally different from the cellular consequences induced by VEGF_{165} stimulation (proliferation,
migration), we can speculate that the shared phosphorylated protein species we detected upon each of the treatments could be early molecular targets in the signal transduction pathways downstream both Sema3A and VEGF_{165}, able to mediate the early cellular events both in cell contraction /axon repulsion and in cell proliferation/migration. VEGF_{121} did induce the selective phosphorylation in tyrosine of a smaller group of proteins when compared to the longer isoform, thus supporting its definition as a weaker proliferation inducer, and one protein only is uniquely phosphorylated by VEGF_{121}. This phenotype is in agreement with the outcome of phospho-specific staining and it contributes to the definition of the shorter VEGF isoform as an individual player triggering a peculiar phospho-proteome pattern.

It is worth noting that the only peculiar VEGF_{121} immunoprecipitated protein has the same MW of one of the spots observed in the stained 2D gel. Further pieces of information are given by the VEGF_{121}-induced spots absent in the immunoprecipitated samples: these molecules are likely to be actually phosphorylated in serine or threonine residues.

As a matter of fact VEGF-induced phospho-tyrosine modifications in porcine endothelial cells is quite fainter in comparison with the phosphorylation pattern observed in studied any cancer cell line. Actually a consistent number of signalling studies have been performed in tumour models, where the cellular signalling machinery is pushed toward a transformed phenotype and many phosphorylation mechanisms are actually constitutively activated.

Porcine aortic endothelial cells are a cell model that responds to VEGFs by means of proliferation and migration, but the phosphorylation pattern observed is weaker when compared to other systems of study. This is not due to a poor amount of signalling complexes on the membrane, since even the transfection of VEGFR2 is still unable to improve the induced phosphorylation pattern.

As far as protein identification is concerned, the technology we used has been proven to be successful in the characterization of the immunoprecipitated species: as a matter of fact finding of known VEGF target of phosphorylation clearly indicates the feasibility of this procedure. The SH2 domains present in adaptors like SCK and PLC-γ are responsible of their recruitment to phosphorylated VEGFR2 (Claesson-Welsh, 2003; Holmes et al., 2007). Phosphorylation of Src family kinases Fyn and Yes induces an integrated signalling feature resulting in the migrational phenotype observed (Duval et al., 2007; Lamalice et al., 2006; Werdich and Penn, 2005). Furthermore, hsp90 observed phosphorylation in the
immunoprecipitated samples suggest the involvement in our system of the already known nitrix oxide-dependent permeability (Duval et al., 2007).

Several pieces of evidence indicate the involvement of cytoskeletal rearrangement upon VEGF treatment. Tensin1 is a SH2 containing focal adhesion molecule that binds to actin filaments and is a negative regulator of cell migration. Tensin phosphorylation modulates cell adhesion to extracellular matrix; its modification upon VEGF treatment can be considered as a signalling event involved in the induced migrational phenotype.

Breast Cancer Antiestrogen Resistance 3 (BCAR3) is a GDP exchange factor whose expression has been observed in HUVE cells (Vervoort et al., 2007). The activation by BCAR3 of Rac, p21 activated kinase (PAK1), and cyclin D could be view a relevant result considering the vimentin phosphorylation by PAK1: this kinase is known to be recruited to focal adhesions upon VEGF treatment, where it interferes with vimentin capability of forming 10 nm filaments (Goto et al., 2002; Stoletov et al., 2004).

Clearly, the absence of a comprehensive database of porcine proteome in MASCOT software did interfere in obtaining straightforward and unique results, even if the presence of exhaustive databases of proteome of other organisms did strongly help in identifying the porcine homologues.
IX. CONCLUSIONS

The development of new blood vessels is a complex and highly regulated process that requires the coordinated action of different growth factors. VEGF-A is a powerful inducer of angiogenesis acting through the metabolic activation, proliferation and migration of endothelial cells. Different VEGF-A isoforms bind to different receptors. The biological effects of VEGF_{165} are basically mediated by VEGFR2 (KDR); actually the signal is finely tuned. VEGF_{165} binds to KDR, VEGFR1 and their co-receptor NP-1, its isoform VEGF_{121} acts only through KDR and VEGFR1. Finally, NP-1, in conjunction with PlexinA1, is the receptor for Sema3A, a factor involved in axonal guidance and in vascular patterning. The resulting signalling network gives rise to the final angiogenetic effect.

In this thesis we investigated and compare the effect of the main VEGF-A isoform, VEGF_{165}, to the shorter VEGF_{121} and to the NP-1 activator Sema3A to finally report:

1) The *in vivo* observation integrated with the molecular characterization of the myeloid population exclusively recruited by VEGF_{165} and Sema3A strongly support the engagement of NP-1 as a molecular mechanism common to these ligands.

2) The cellular system of porcine aortic endothelial cells has been explored by optimizing several phospho-proteome techniques. Although no novel key players of the VEGF-specific signalling have been identified, our findings demonstrate for the first time that Sema3A can induce the phosphorylation of peculiar species, at least in the cellular model analysed. We also demonstrated the existence of common molecular features in the tyrosine phosphorylation pattern induced by VEGF_{165} and Sema3A. Furthermore, VEGF_{121} was shown to trigger a peculiar phosphorylation pattern; among the proteins phosphorylated only after VEGF_{121} incubation, only one is likely to be modified in tyrosine. Supplementary experimental approaches have to be anyway exploited in the future to unravel the identity of the molecular mediators responsible for the observed induced effect.
X. BIBLIOGRAPHY


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