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The role of two different isoforms of VEGF-A in human tumour angiogenesis, development, and prognosis

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The role of two different isoforms of VEGF-A in human tumour angiogenesis, development, and prognosis

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ABSTRACT

To grow beyond 1-2 mm in size, a tumour needs to establish an independent blood supply to sustain an ever-increasing demand for oxygen and nutrients. This requirement is fulfilled through the production of several pro-angiogenic growth factors, eventually leading to accelerated neo-vessel formation. While numerous pro-angiogenic factors have been characterized so far, Vascular Endothelial Growth Factor A, (VEGF-A) has been identified as a predominant regulator of tumour angiogenesis by facilitating endothelial cell (EC) migration, proliferation, tube formation, and survival. VEGF-A gene structure and function have been extensively elucidated over last decades. Notwithstanding, due to conflicting results both in experimental and clinical settings, VEGF-A contribution to tumour progression is still debated. Some controversies arise due to the fact that most of the studies have underestimated the impact of VEGF-A gene alternative splicing, which eventually gives rise to several splicing isoforms. In keeping with this possibility, we previously demonstrated that the two most abundant VEGF-A splicing isoforms ($VEGF_{165}$ and $VEGF_{121}$) have different biological effects *in vivo*. In particular, we showed that both isoforms are equally able to activate the local endothelium, eventually leading to capillary sprouting, but only the longer isoform, $VEGF_{165}$, is also able to promote full vessel maturation. This ability can be ascribed to the capacity of $VEGF_{165}$, but not $VEGF_{121}$, to recruit a peculiar population of accessory myeloid cells, which sustains vessel maturation. We termed these cells Nrp-1 Expressing Mononuclear cells (NEMs) as they express Neuropilin-1 (Nrp-1), a non-tyrosine kinase co-receptor crucially involved in their mobilization.

Hence, our data strongly point towards a differential contribution of VEGF-A splicing isoforms to tumour angiogenesis. Based on these findings, we investigated the impact of $VEGF_{165}$ and $VEGF_{121}$ on tumour angiogenesis and progression in human tumour samples.

To assess the relevance of VEGF-A splicing in the context of human malignancies, we first quantified the relative abundance of $VEGF_{165}$ and $VEGF_{121}$ in a set of colorectal cancer patients. The ratio of isoform expression ($VEGF_{165}/VEGF_{121}$) was analysed both in tumour mass and in a matched sample of healthy mucosa, harvested at least 10 cm far from the tumour site.

The VEGF isoform ratios were not statistically different in mucosa and tumour. Similarly, no correlation with Grading (G), Stage (T), or Vascular Extra-Parietal Invasion (IVEP) was detected. However, the $VEGF_{165}/VEGF_{121}$ ratio was significantly higher in

tumours derived from patients with metastatic disease, arguing for a facilitating role of $VEGF_{165}$ in hematogenous tumour dissemination.

Despite the absence of any obvious trend in VEGF-A splicing regulation, the $VEGF_{165}/VEGF_{121}$ ratio showed a significant degree of variability. We thus further analyzed a restricted group of patients who strongly up-regulated $VEGF_{165}$ ($VEGF_{165}^{high}$ patients). Interestingly, these patients showed reduced lymph node infiltration but improved vessel morphology.

In accordance with the above information, tumours that strongly up-regulated Semaphorin3A (Sema3A - known Nrp-1 ligand and NEM recruiter) showed improved vessel structure and better outcome (no metastasis, no lymph node infiltration and longer survival).

Taken together, these data suggest that two major Nrp-1 ligands, $VEGF_{165}$ (but not $VEGF_{121}$) and Sema3A, are able to enhance vessel maturation in tumours, possibly through the recruitment of NEMs. This evidence is consistent with the conclusion that improved tumour vascular function is detrimental for tumour growth, in concert with a number of recent reports highlighting the role of vessel normalization in tumour progression. According to this notion, high levels of either $VEGF_{165}$ or Sema3A correlated with better disease outcome, as evaluated by lymph node infiltration and overall survival. Of interest, however, up-regulation of $VEGF_{165}$ was not associated with a reduced metastatic spread (as Sema3A did), while, on the contrary, higher levels of $VEGF_{165}$ were found in patients with metastatic disease. Taken together, these data appear fully consistent with recently published information supporting the concept that the vascular normalization induced by anti-angiogenic therapies indeed elicits tumour cell spreading and metastasis.

Our additional studies on human specimens and cell culture identified hypoxia as a crucial regulator of VEGF splicing. Ongoing experiments to further elucidate the effect of other environmental factors on the regulation of VEGF splicing balance is in progress.

1. INTRODUCTION

1.1 Physiological and pathological aspects of angiogenesis

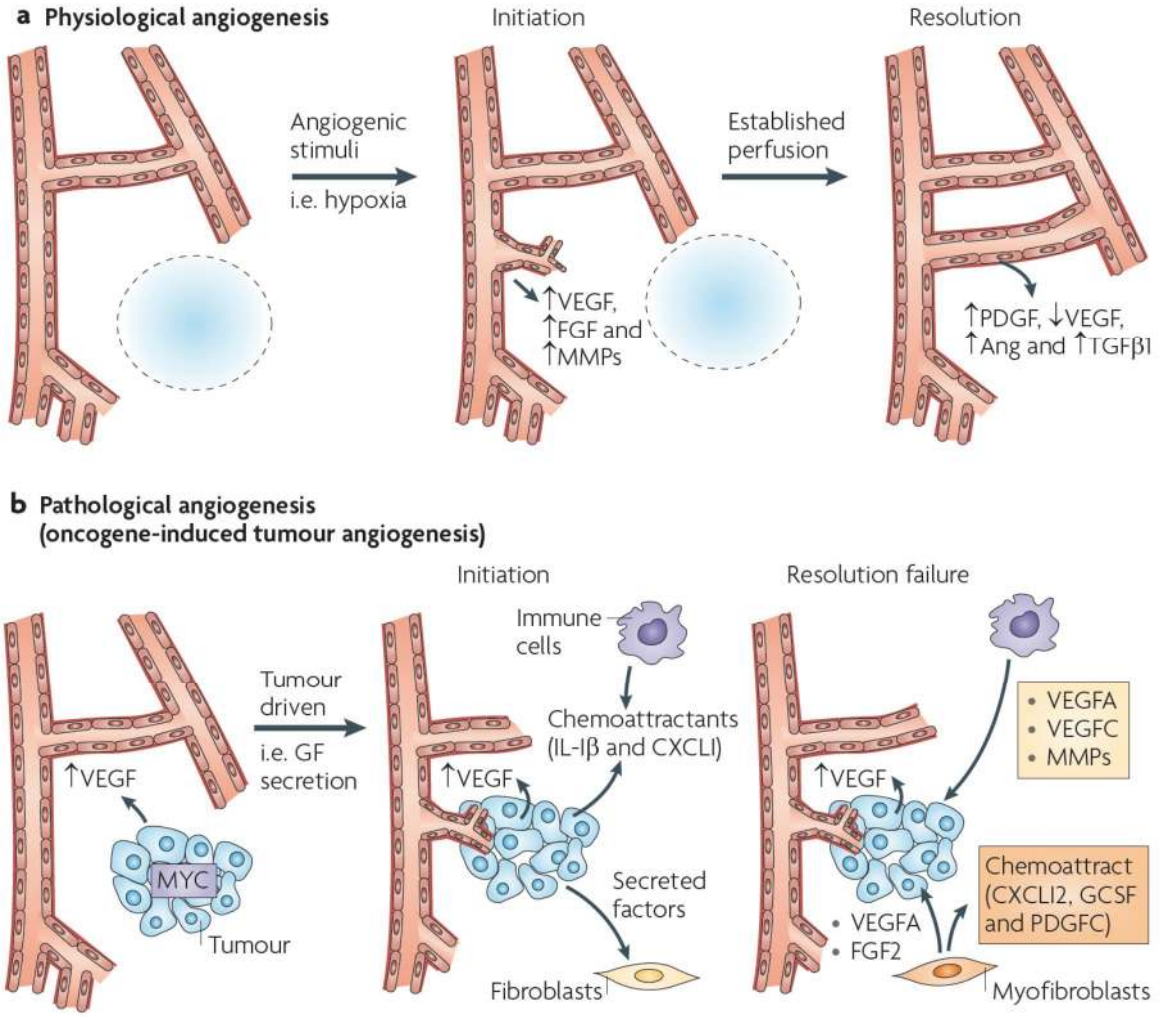
Angiogenesis is the process of creating new blood vessels from the pre-existing vasculature. It is an intricate but tightly orchestrated series of events, that always begins with the proliferation of resident endothelial cells (ECs), mainly triggered by the local production of secreted pro-angiogenic factors. Then, newly generated ECs organize in 3D structures which eventually evolve into mature vessel tubes. Full vessel maturation involves lumen formation and the coverage of ECs with mural cells (typically pericytes and smooth muscles cells), a key event in the stabilization of newly formed blood vessels. This highly organized process has been the focus of a large body of studies, specifically due to its importance in development, tissue maintenance and survival, as well as in a wide range of human diseases. During last three decades, intensive research has obtained a large body of knowledge regarding both activators and inhibitors of angiogenesis. Physiological angiogenesis, as a crucial process during embryonic development, happens in adulthood only during wound healing, skeletal growth, menstrual cycle (Smith, 2001; Torry and Torry, 1997), and pregnancy. It occurs also in a wide range of diseases including intraocular neovascular disorders, immunogenic rheumatoid arthritis, psoriasis, and tumourigenesis. In addition, angiogenesis plays an essential role in tumour growth, invasion, and metastasis (Folkman et al., 1989). Under both physiological and pathological angiogenesis, a cascade of highly coordinated cellular activities leads to the establishment of new blood vessels in response to increased requirement for oxygen and nutrients (Figure 1).

A tumour mass cannot grow beyond 2-3 mm^3 without angiogenesis. Indeed, when a tumour grows up to 1-2 mm in diameter, its demand for oxygen and nutrients exceeds the local supply. This results in a hypoxic microenvironment that consequently cooperates with other oncogenic stresses to induce angiogenesis. Neovascularization provides the primary tumour with additional supply of nutrients and oxygen and promotes the dissemination of the tumour cells to distant organs.

Most of the solid tumours do not have an intrinsic angiogenic capability, thus they traverse two phases of growth: an avascular phase followed by a vascular phase in which new capillaries penetrate the tumour resulting in its growth and progression. Since tumour progression occurs after extended periods of non-neovascularized tumour dormancy, the

existence of an “angiogenic switch” was speculated as a requirement for this to happen. This switch involves more than simple up-regulation of pro-angiogenic factors and seems to be the result of balance between positive and negative regulators. Tumour growth requires disruption of this balance. A wide range of factors contribute to this switch, including progenitor endothelial cells (PECs), crosstalk between angiogenic stimulators and their receptors, and the interplay between vasculogenesis and lymphangiogenesis.

Increased angiogenic capacity is mostly observed long before any morphological manifestation of neoplastic transformation (Brem et al., 1977; Gimbrone and Gullino, 1976a, b). In this regard, angiogenesis might be considered as an early marker of neoplastic transformation.



(Chung et al., 2010)

Figure 1. Physiological and pathological angiogenesis. a) During physiological angiogenesis, various stimuli (such as hypoxia) induce an organized sequence of events that constitutes the angiogenesis cascade. In the phase of initiation, an increase in growth factors such as VEGF-A and fibroblast growth factor (FGF) derived from endothelial cells leads to vessel destabilization and initiation of vessel sprouting and endothelial cell proliferation. Matrix metalloproteinases (MMPs) facilitate extracellular matrix (ECM) remodelling and increase the bioavailability of ECM-sequestered growth factors. When new vessels establish, blood perfusion takes place and, concomitantly, levels of VEGF decline; the resolution phase begins coincident with an increase in platelet-derived growth factor (PDGF), angiopoietins (Ang), and transforming growth factor-β1 expression responsible for the recruitment and subsequent stabilization of mural cells and vascular smooth muscle cells around the nascent vessel. **b)** Under pathological conditions, tumour- secreted VEGF-A triggers the angiogenesis cascade. Tumour cells lead to increased expression of soluble factors also responsible for activating resident fibroblasts and for infiltrating immune cells. Such stromal cell types are able to sustain the angiogenic process via the secretion of vascular growth- and inflammation-promoting elements. GDSF, granulocyte colony stimulating factor; IL-1β, interleukin-1β.

1.2 Colorectal Carcinoma

Cancer of the large bowel (also known as colorectal cancer) is one of the leading causes of cancer mortality worldwide. Colorectal cancer (CRC) is the third most frequently diagnosed cancer in men and women and the second cause of cancer-related death in the United States and other developed countries but occurs much less frequently in the developing world. It had been estimated that 141,210 men and women (71,850 men and 69,360 women) were diagnosed with and 49,380 men and women would die of colorectal carcinoma in 2011 (NCI's SEER Cancer Statistics Review). Despite all recent advances in the management of CRC, there is a strong need for more efficient and well-tolerated anti-cancer drugs. Colorectal carcinoma, similar to other sorts of cancers, requires angiogenesis to grow beyond few millimetres. The fact that tumour progression and metastasis is dependent on blood vessels makes angiogenesis a putative target for therapy. In this regard, one of the known pathways contributing to this process is the vascular endothelial growth factor (VEGF) and its receptors.

1.3 Vascular Endothelial Growth Factors

The VEGF gene family consists of various members, which include VEGF-A, -B, -C, -D, -E, PlGF, and snake venom-derived VEGFs (Dvorak, 2002; Hicklin and Ellis, 2005). The best characterized among all VEGF family members is VEGF-A. Vascular endothelial growth factor A or VEGF-A (commonly referred to as VEGF), is a potent mitogen involved in mitogenesis, angiogenesis, endothelial survival as well as the induction of hematopoiesis (Kowanetz and Ferrara, 2006). VEGF is encoded by a single gene located on chromosome 6 in humans and consists of eight exons (Harper and Bates, 2008). It plays a crucial role in the morphogenesis, differentiation, and stability of vessels by regulating the proliferation, migration and persistence of ECs (Lamalice et al., 2007). It also contributes to vasculogenesis and lymphangiogenesis during embryonic development (Shibuya and Claesson-Welsh, 2006).

The need for VEGF-A signalling during early vasculogenesis/angiogenesis has been emphasized by the observation that mice lacking a single VEGF-A allele die at about embryonic day 9.5 (Carmeliet et al., 1996; Ferrara et al., 1996), indicating a dose-dependent regulation of embryonic vessel development by VEGF. In 1989, Napoleone Ferrara purified this protein from bovine pituitary follicular cell conditioned media as a potent endothelial

mitogen (Ferrara and Henzel, 1989). Interestingly, the same polypeptide had been previously isolated as vascular permeability factor in 1983 (Senger et al., 1983). In fact, the cloning of VEGF was a great milestone in understanding the process of angiogenesis. Besides its multiple roles in several facets of vessel formation, both during development and in adulthood, it was more recently found to be an important trophic factor for a disparate variety of cell types, most notably neuronal cells (Carmeliet and Storkebaum, 2002).

Importantly, its role as a key mediator of tumour angiogenesis is now well established and has been long considered a therapeutically addressable bottle-neck for tumour growth. This has led to decades of debates on the efficacy of anti-VEGF cancer therapy that eventually came to an end with the approval of the first anti-angiogenic agent, bevacizumab, an anti-VEGF monoclonal antibody, by the U.S. Food and Drug Administration (FDA) in 2004, with an indication for the treatment of some types of refractory CRCs. A debate on the therapeutic usefulness of bevacizumab has been rejuvenated by recent discoveries shedding shadows on the clinical benefits of VEGF inhibitors, as discussed later in the text (Ebos et al., 2009; Paez-Ribes et al., 2009). In fact, most of the human cancer cells express VEGF-A, and its major receptor (VEGFR-2) is also highly expressed by ECs involved in tumour angiogenesis (Kerbel, 2008). Furthermore, VEGF-A is also secreted by tumour stromal cells such as fibroblasts, monocytes, and platelets (Kut et al., 2007).

All members of VEGF family have been shown to possess angiogenic capacity, although at dissimilar degree (Harper and Moses, 2006). VEGF-B, which only binds VEGF Receptor-1 (VEGFR-1), has been long considered non-angiogenic and a mere “tuning” factor for VEGF-A (Hirashima et al., 2003). VEGF-C and VEGF-D that bind mainly VEGF Receptor-3 are primarily lymphangiogenic factors (Alitalo et al., 2005). Conversely, the role of PlGF (Placental-derived Growth Factor) is far more ambiguous and apparently affects different aspects of angiogenesis (Bais et al., 2010; Luttun et al., 2002).

1.3.1 Structure of VEGF

VEGF-A, as the best-characterized member of the VEGF family, is a homodimeric glycoprotein comprised of two identical 23 KDa subunits. Among several alternatively spliced isoforms of human VEGF-A, $VEGF_{165}$, as a heparin-binding homodimeric glycoprotein of approximately 45 KDa (Leung et al., 1989), has been considered as the most abundant and mitogenic isoform of this group. Other important isoforms of VEGF-A, including $VEGF_{121}$, $VEGF_{189}$, and $VEGF_{206}$ originated from alternative splicing of VEGF-A

gene, which consists of eight exons (Neufeld et al., 1999). The longer VEGFs such as $VEGF_{189}$ and $VEGF_{206}$ bind heparin in the extracellular matrix, while the lack of basic residues in exon 6 and 7 in $VEGF_{121}$, results in the loss of extracellular matrix segregation (Houck et al., 1992). Indeed, a large body of evidence indicates that the heparin-binding VEGF-A isoforms play an essential role in the initiation of vascular branching (Gerhardt et al., 2003; Ruhrberg et al., 2002). In addition, $VEGF_{165}$, which lacks exon 6, exists in both bound and freely diffusible protein (Neufeld et al., 1999). Besides being variable in bioavailability and bioactivity, isoforms of VEGF show different receptor specificities. For instance, $VEGF_{165}$, via exon 7-encoded domains, binds to tyrosine kinase receptors VEGFR1/Flt1 and VEGFR2/Flk1, also neuropilin-1 (NRP-1) and NRP-2 (Ferrara et al., 2003; Olsson et al., 2006).

1.3.2 Alternatively Spliced Isoforms of the VEGF-A pre-mRNA

As briefly introduced in the previous Chapter, the VEGF mRNA undergoes alternative splicing to give rise to various isoforms (Houck et al., 1991). The VEGF gene consists of 8 exons and encompasses nearly 14 Kbp (Figure 2). Basal transcription is driven by a TATA-less promoter, specifically tuned by diverse stimuli, among which hypoxia is worth mentioning (Giacca, 2010). At least seven protein isoforms are produced by translation of various mRNAs subjected to alternative splicing of the native human VEGF-A pre-mRNA (Tischer et al., 1991) composed of 206, 189, 183, 165, 148, 145, and 121 amino acids (aa) after the removal of the signal peptide. All VEGF isoforms are secreted as covalently linked homodimers. The three most abundant isoforms detected so far in vivo are $VEGF_{121}$, $VEGF_{165}$, and $VEGF_{189}$.

All the mRNAs coding for the various isoforms contain exons 1 to 5 in their 5′ regions and are characterized by different combinations of exons 6, 7, and 8, or a portion of these, in their 3′ regions. In VEGF-A_{121}, exon 5 is spliced to the 6 aa-encoding exon 8. In the mRNA coding for VEGF-A_{165}, the whole exon 7 is included between exon 5 and exon 8, while in the case of $VEGF_{148}$, the 5′ part of exon 7 (7a) is spliced to exon 8, generating an open reading frame that terminates protein translation in exon 8 by the inclusion of an additional single amino acid, as schematically shown in Figure 2. The mRNA isoforms coding for VEGF-A_{206}, VEGF-A_{189} and VEGF-A_{183} include complete exon 6 (coding for 41 amino acids) or parts of exon 6 shorter at their C-terminus. The coding mRNA for the longest VEGF-A isoform (VEGF-A_{206}) contains whole exon 6, while mRNAs coding for VEGF-A_{189} and VEGF-A_{183}

are generated using alternative 5′ donor sites that reduce the coding region to 24 aa (exon 6a) and 18aa (exon 6a′) respectively. Only VEGF-A_{145} contains exon 6a inserted between exon 5 and 7. A 1881 bp-long 3′UTR ends the transcripts, containing multiple alternative polyadenylation signals. This region also includes a number of AU-rich elements (AREs) shown to be involved in the regulation of mRNA stability under hypoxic conditions (Paulding and Czyzyk-Krzeska, 2000).

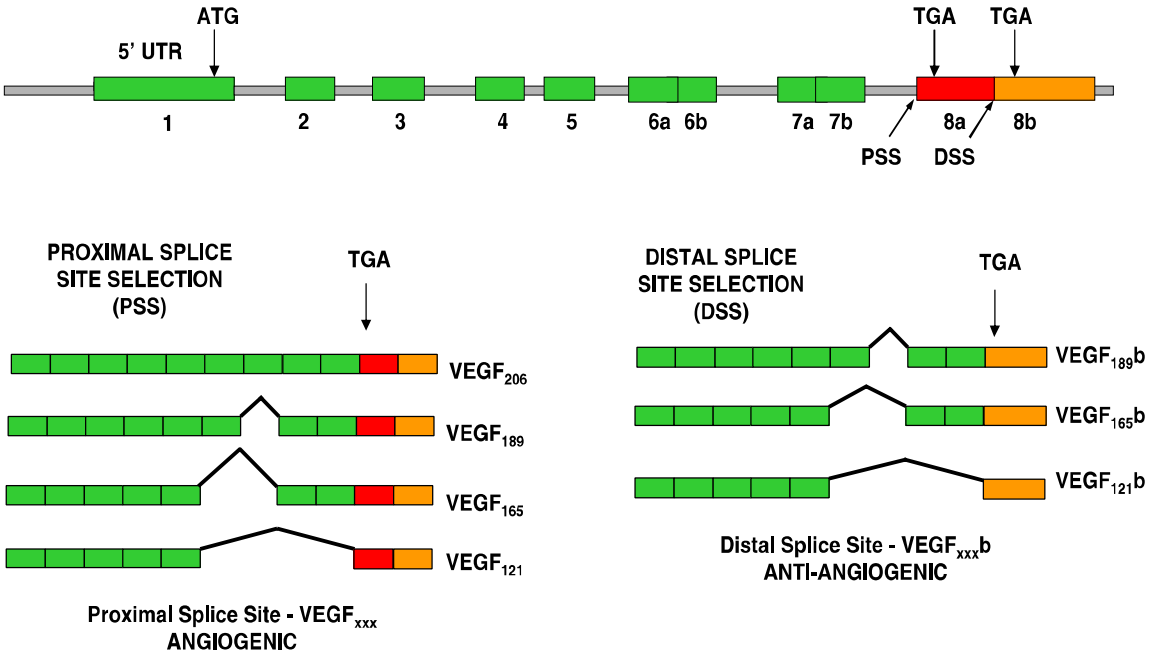
Additional variability is generated when a 3′ splice site distal to that typically used by exon 8 is utilized. This specifies a shorter exon (exon 8b), the 5′ of which codes for 6 amino acids different from classic exon 8. This distal splice site choice can occur combined with exon 6 or 7 inclusion or exclusion, resulting in the generation of a family of polypeptides differing from the above-mentioned isoforms by the last six amino acids. The most prominent of these polypeptides is VEGF-A_{165}b. Interestingly, it has been shown that the protein isoforms encoded by these exon 8b-containing mRNAs have inhibitory potential rather than stimulatory effects on the VEGF receptors (Harper and Bates, 2008).

The N-terminal portion of VEGF-A, encoded by exons 1-5, is sufficient to bind and activate the typical VEGF receptors. As a consequence, all the VEGF-A splicing isoforms can interact with VEGFR-2 and with a 10-fold higher affinity with VEGFR-1 (Ferrara et al., 2003). Instead, exons 6 and 7 at the C-terminus of the protein are essential in specifying its bioavailability and biodistribution. Particularly, the 24 aa- and 44 aa- stretches encoded by exons 6a and 7 are highly enriched in clusters of basic amino acids, creating the capacity to bind negatively charged heparin (heparin binding domains, HBDs). These HBDs determine the capacity to bind cell surface and extracellular matrix heparan sulfate proteoglycans (HSPGs) and thus crucially modulate their localization in the extracellular matrix. Various VEGF isoforms distribute in the environment of a VEGF-secreting cell, depending on the presence or absence of the heparin-binding domain (Ferrara et al., 2003). It has been suggested that the differential localization of VEGF isoforms in the extracellular matrix might regulate vascular branching pattern (Ruhrberg et al., 2002). VEGF-A_{121}, as the shortest isoform, lacks any HBD and is weakly acidic. According to these characteristics, it is freely diffusible. In contrast, VEGF-A_{189} and VEGF-A_{206} have two HBDs and are kept in the extracellular matrix and on the cell surface. VEGF-A_{165}, the most abundant isoform (Ferrara and Davis-Smyth, 1997), differs from VEGF-A_{121} only by the inclusion of exon 7. Its moderate affinity for heparin enables this isoform to be present as a soluble and a cell-bound factor (Krilleke et al., 2009). The functional significance of these HBDs remain elusive, but it

has been reasonably proposed that binding to the extracellular matrix allows the HBD-containing VEGF-A isoforms to form a concentration gradient that extends from the ischemic areas which is crucial for proper angiogenic stimulation (Oshikawa et al., 2010).

Differential effects of $VEGF_{165}$ and $VEGF_{121}$ isoforms were correlated with their differential capacity to recruit, to the sites of neoangiogenesis, a novel population of $CD11b^+$, $Gr\text{-}1^-$ circulating cells deriving from bone marrow and expressing the Nrp-1 receptor. It has been demonstrated that $VEGF_{165}$ acts as a potent chemoattractor for these Nrp-1 expressing mononuclear (NEM) cells, while $VEGF_{121}$ fails to do so. In addition, it has been observed that NEMs play an important role by secreting PDGF-B, TGF-beta and other chemokines active in promoting vessel maturation, which in turn results in a higher degree of α-SMA+/NG2+ mural cell coverage and decreased vascular leakiness (Zacchigna et al., 2008). Altogether, it is strongly suggested that VEGF-A_{121} is sufficient neither for normal vasculogenesis in the embryo development nor for adult angiogenesis and it is not only because of its incapability to determine a concentration gradient through HSPG binding, but also due to its failure in inducing peri-endothelial cell recruitment, vessel maturation and arterial specification through NEM recruitment via exon 7-mediated NRP-1 binding (Zacchigna et al., 2008). On the other hand, the regulatory role of the VEGF-A isoforms consisting of exon 8b requires further investigation. For instance, VEGF-A_{165}b differs from VEGF-A_{165} only in the C-terminal six amino acids, which gives this protein the ability of having only a weak stimulatory activity (Bates et al., 2002). VEGF-A_{165}b still binds VEGF receptors with equal affinity as other VEGF-A ligands, however is a poor ligand of VEGFR-2, specifying a distinct tyrosine phosphorylation pattern, and fails to bind HSPGs and to interact with NRP-1, in a similar way as in the case of VEGF-A_{121}. Therefore, VEGF-A_{165}b may act competitively with other VEGF isoforms and suppress their angiogenic activity (Woolard et al., 2004).

Collectively, it is now clear that different VEGF isoforms have dissimilar biological functions in vivo, and this is highly valuable to fully understand the elaborate activity of VEGF-A during physiological and pathological angiogenesis. Indeed, its angiogenic capacity has been long studied and is now well established, but the inability to translate experimental models to the clinic might suggest the existence of more complex, still obscure tuning of VEGF biological activity (Carmeliet and Jain, 2011). As discussed later in this thesis, VEGF alternative splicing might represent a poorly investigated mechanism partially able to justify such an unresolved complexity.



(Ladomery et al., 2007)

Figure 2. Exon structure of the VEGF gene.

1.3.3 Family of VEGF receptors

During the early 1990s, human VEGF receptors were structurally identified by cDNA cloning (Millauer et al., 1993; Pajusola et al., 1992; Shibuya et al., 1990; Terman et al., 1991). VEGF receptors were originally characterized on the surface of vascular endothelial cells, and for many years it was believed that their expression was restricted to endothelial and peri-endothelial compartment (Ferrara, 2002). It was subsequently shown that VEGFRs exist also on bone marrow-derived cells such as monocytes (Shen et al., 1993) and on a large variety of other cell types in the body. VEGF binds two receptor tyrosine kinases (RTKs), VEGFR-1 and VEGFR-2, which are highly homologues. VEGFR-1(Flt-1) and VEGFR-2 (Flk-1/KDR) both have seven Ig-like domains in the extracellular region, a single-transmembrane area, and a consensus tyrosine kinase sequence (Terman et al., 1991) interrupted by a kinase insert domain and are activated by ligand-triggered dimerization (Matthews et al., 1991). VEGFRs, upon ligand binding, dimerize and induce mitogen-activated protein kinase (MAPK), phosphoinositide-3 kinase and other pathways involved in the function of endothelial cells (Ferrara et al., 2003). In response to ligand binding, VEGF receptor tyrosine kinases activate a series of separate downstream signalling pathways (Kowanetz and Ferrara, 2006).

Similar to *Vegfa*$^{+}$/- mice, *Vegfr2*- null mice display early embryonic lethality and severe vascular defects (Shalaby et al., 1995), underscoring the pivotal role of this receptor in VEGF-A signalling. Whereas a large body of evidence suggests that VEGFR-2 is the key receptor for VEGF-A-induced endothelial cell responses such as mitogenesis and vascular permeability, the role of VEGFR-1 is more intricate (Shibuya, 2006). Through sequestration of VEGF-A, VEGFR-1 can adversely regulate VEGFR-2 signalling (Hiratsuka et al., 1998; Park et al., 1994). It can also promote monocyte migration (Barleon et al., 1996), endothelial cell secretion of proteases (Hiratsuka et al., 2002) and growth factors (LeCouter et al., 2003), the growth of specific cancer cells overexpressing this receptor (Lichtenberger et al., 2010; Wu et al., 2006), and sustain myocardial function after infarction and heart failure (Lahteenvuo et al., 2009; Zentilin et al., 2010), underpinning the limitations of the conventional outlook that put toward VEGFR-1 as a simple decoy receptor for VEGF-A. Another member of the same family of RTKs is VEGFR-3 (Flt-4), which is not a receptor for VEGF-A, but instead for VEGF-C and VEGF-D and mainly contributes to lymphangiogenesis (Alitalo et al., 2005).

1.3.3.1 VEGFR-1 (Flt-1)

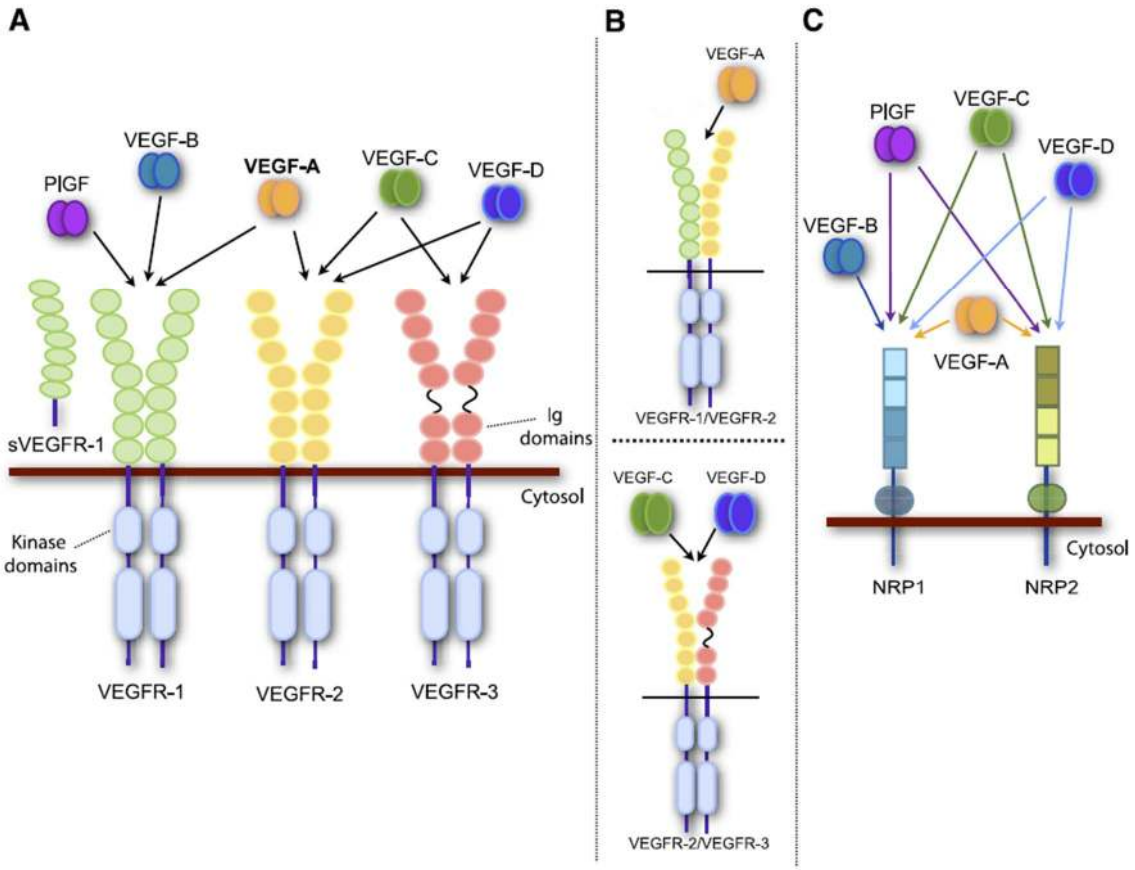
More than a decade ago, VEGFR-1 was the first receptor tyrosine kinase (RTK) identified as a VEGFR (de Vries et al., 1992). VEGFR-1 functions and signalling features may vary depending on the developmental stage and the cell type. Interestingly, expression of VEGFR-1 is up-regulated by hypoxia via a HIF-1-dependent mechanism (Gerber et al., 1997), suggesting an independent role in VEGF signalling under stress conditions.

VEGFR-1 binds different members of the VEGF family, namely VEGF-A, VEGF-B, and PlGF (Ferrara, 2004) (Figure 3). It has been long considered as a simple “decoy” receptor, able to weaken VEGF-A signalling, competing for its binding with VEGFR-2, which in turn was considered the proper VEGF-A receptor. In this respect, it has been shown that an alternatively spliced soluble form of VEGFR-1 (sFlt-1) is an inhibitor of VEGF activity, through the seizure of circulating VEGF-A (Hiratsuka et al., 1998). Indeed, Flt-1 has a strong affinity for its ligand; in detail, the ability of binding VEGF and PlGF has been referred to the second Ig-like domain of this receptor (Christinger et al., 2004). Despite this strong affinity, Flt-1 shows a weak tyrosine autophosphorylation activity in response to VEGF-A. However, it has been more recently demonstrated that VEGFR-1 can interact with different signal-transducing proteins and generate a mitogenic signal (Maru et al., 1998). In

addition, it has been suggested that a crucial function of VEGFR-1 signalling in the vascular endothelium is not the control of angiogenesis but, rather, the paracrine secretion of tissue-specific growth factors (Zhang et al., 2009).

1.3.3.2 VEGFR-2 (KDR, human; Flk-1, mouse)

VEGFR-2 has been primarily found on endothelial cells, where it mediates all major angiogenic effects of VEGF-A, but it has also been detected on a subset of multipotent human hematopoietic stem cells (HSCs) (Kabrun et al., 1997). During early embryogenesis, VEGFR-2 is highly expressed in vascular endothelial progenitors, whereas in later stages of vasculogenesis, VEGFR-2 expression decreases. However, under the conditions of pathological angiogenesis such as in tumours, its expression is upregulated (Matsumoto and Claesson-Welsh, 2001). Among the most important functions of VEGFR-2, are the stimulation of vascular endothelial cell survival and growth, in addition to the promotion of angiogenesis. Indeed, VEGFR-2 is the key mediator of the mitogenic, angiogenic, and permeability-enhancing roles of VEGF-A (Ferrara et al., 2003). The VEGFR-2 binding sites have been located in the second and third Ig-like domains. Dimerization and ligand-dependent tyrosine phosphorylation gives rise to its mitogenic, chemotactic signalling. Activation of VEGFR-2 results in endothelial cell growth through stimulation of the Raf-Mek-Erk pathway.



(Ruiz de Almodovar et al., 2009), modified

Figure. 3. Family members of VEGF and their receptors. A) Mammalian VEGF family members are VEGF-A, -B, -C, -D, and PlGF. Among them, VEGF-A binds to both VEGFR1 and VEGFR2, whereas VEGF-B and PlGF only bind to VEGFR-1. VEGF-C and VEGF-D can bind to VEGFR2 and VEGFR3. **B)** VEGF-A may also bind to heterodimeric receptor complexes formed by VEGFR1 and VEGFR2. Similarly, VEGF-C and –D are able to bind heterodimers formed by VEGFR2 and VEGFR3. **C)** Neuropilin-1 and -2 function as co-receptors for canonical VEGF-VEGFR complexes and regulate VEGF receptor activation and signalling. Some splicing isoforms of VEGF-A bind to both Nrp-1 and Nrp-2. Among family members of VEGF, VFGF-B only binds to Nrp-1.

1.4 Neuropilins

Neuropilins (NRPs) were initially discovered as neuronal receptors for class III semaphorins. There are two neuropilin genes, *nrp1* and *nrp2*. Neuropilins are 130-140 KDa, non-tyrosine kinase receptors and have a rather large extracellular domain divided into some subdomains, which act as ligand binding sites. Conversely, they consist of a very short transmembrane domain, and a short cytoplasmic domain (Neufeld and Kessler, 2008).

It was later demonstrated that NRP-1 has a role in vascular morphogenesis (Klagsbrun et al., 2002; Soker et al., 1998). They are expressed on at least tumour and endothelial cells and regulate angiogenesis by acting as receptors for members of the Sema3A and VEGF family (Bielenberg et al., 2006; Neufeld and Kessler, 2008). In general, NRP-1 and NRP-2 are single spanning transmembrane glycoproteins with a crucial role in the development of neuronal and vascular systems as receptors for members of class-3 Semaphorin (SEMA3s) family of axonal guidance factors and at the same time for members of the vascular endothelial growth factor family of angiogenesis factors. NRPs were basically shown to contribute to the process of axonal guidance but soon after, it became evident that these receptors were also involved in normal blood vessel formation, tumour angiogenesis, and tumour progression (Ellis, 2006).

NRP-1 is able to bind to $VEGF_{165}$, VEGF-B, VEGF-C, VEGF-D, VEGF-E, PlGF-2, HGF, SEMA3A, SEMA3B, and SEMA3C while NRP-2 ligands are $VEGF_{165}$, $VEGF_{145}$, VEGF-C, VEGF-D, HGF, SEMA3B, SEMA3C, and SEMA3F (Kolodkin et al., 1997; Sulpice et al., 2008; West et al., 2005). Since the cytoplasmic domain of NRP-1 is short, it is thought that its joining to the other signalling proteins is necessary for its bioactivity. The ligand binding domains of NRP consist of: (i) an A domain composed of two a-domain repeats or two complement binding (CUB) domains (a1a2), (ii) a B domain composed of two b-domain repeats or two coagulation factor V/VIII homology- like domains (b1b2), and (iii) a C domain or a meprin A5 (MAM) domain that is believed to be important for NRP dimerization and the neuropilins' interaction with other membrane receptors (Chen et al., 1997; Giger et al., 1998; He and Tessier-Lavigne, 1997) (Figure 4). The B domain of NRP is substantial and sufficient for binding of $VEGF_{165}$ to both NRP1 and NRP2 (Gu et al., 2002). However, for NRP-1, simultaneous presence of the A domain enhances $VEGF_{165}$ binding significantly. SEMA3s and $VEGF_{165}$ both interact with the B domain of NRP-1. The b1 domain that forms part of the Semaphorin-3A binding domain of NRP-1, is also required for

the binding of $VEGF_{165}$, in addition to the b2 domain, and as a result $VEGF_{165}$ and Sema3A compete for binding to NRP-1 (Gu et al., 2002; Miao et al., 1999; Vander Kooi et al., 2007).

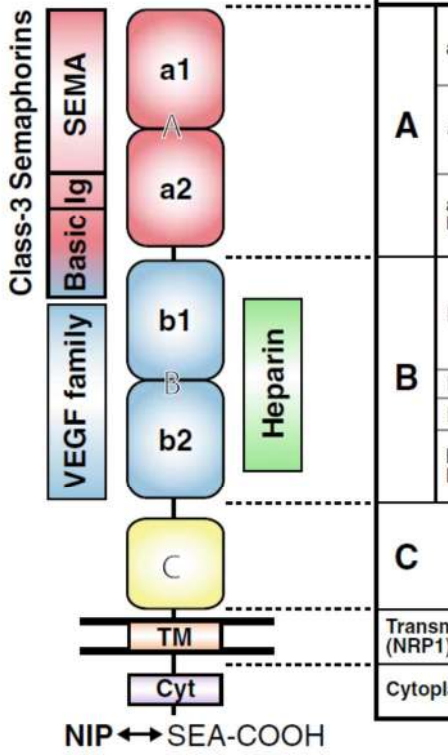
It has been shown that VEGF-A exon 7 is crucial for NRP binding (Soker et al., 1996). On the other hand, $VEGF_{121}$, as one of the splice forms of VEGF, differs from $VEGF_{165}$ by lacking 44 amino acids encoded by VEGF exon 7, which is responsible for binding to NRP-1. In this respect, no binding to NRP-1 was detected by $VEGF_{121}$, although recent studies has revealed that $VEGF_{121}$ can bind NRP-1 (Pan et al., 2007), but comparing with $VEGF_{165}$, $VEGF_{121}$ is not able to "bridge" NRP-1 and VEGFR-2 (Pan et al., 2007; Shraga-Heled et al., 2007). It is likely that the C-terminal residues encoded by exon 8 are responsible for the interaction of $VEGF_{121}$ with NRP-1.

As already mentioned, Neuropilin-1 (NRP-1) is a unique multifunctional transmembrane glycoprotein, a receptor for vascular endothelial growth factor A_{165} (VEGF-A_{165}) and the neuronal guidance molecule Semaphorin3A (SEMA3A) with key roles in vascular and neuronal development. It is worth mentioning that the primary structure of this gene is highly conserved within vertebrate species. NRP-1 was first identified in neurons of the developing nervous system and considered as a receptor for several class 3 Semaphorins as secreted proteins (i.e., Sema3A, Sema3B, Sema3C and Sema3F), which differ in their potential to interact with the two neuropilins and are essential for chemorepulsive and growth cone collapsing and repulsive signals in vitro (Chen et al., 1997; Feiner et al., 1997; Kolodkin et al., 1997). Among all members of Semaphorin family, Sema3A binds exclusively to neuropilin-1. In NRP-1 the a1 and a2 as well as the b1 domains are required for Sema3A binding and signal transduction (Gu et al., 2002; Vander Kooi et al., 2007) (Figure 4).

In addition to playing a critical role in the developing nervous systems, NRP-1 is expressed in a variety of non-neural cells and can mediate various intercellular signals to modulate diverse aspects of physiological and pathophysiologic functions (Banerjee et al., 2000; Gluzman-Poltorak et al., 2000; Pavelock et al., 2001; Reese et al., 2000; Robert et al., 2000; Soker et al., 1998). In endothelial cells, NRP-1 enhances the VEGF-A mediated biological signals via binding to VEGFR2. It was previously thought that NRPs are not able to transduce VEGF signals on their own, and may function only as enhancers of VEGFR2-mediated VEGF signalling, but recently it has been reported that NRP-1 can transduce VEGF signals in the absence of tyrosine kinase VEGF receptors (Wang et al., 2007).

NRP-1 has also been involved in tumour growth and angiogenesis. In tumour cells, which often lack VEGFR-2, it has been supposed that $VEGF_{165}$ acts as an autocrine survival

factor through an NRP-dependent process (Soker et al., 1998). In general, transmembrane proteins signal through their cytoplasmic domains, however, in NRPs no signalling motif has been detected in their cytoplasmic regions and, consequently, whether and how this domain signals is not clear so far. On the other hand, NRP-1 can promote signalling in endothelial cells by its intracellular domain (Wang et al., 2003). NRP-1 seems to mediate a $VEGF_{165}$/ NRP1/ VEGF-2 pathway resulting in tumour growth. Recent studies indicate that the range of growth factors with the ability of binding to neuropilins are not limited to members of Semaphorin and VEGF gene families. Neuropilin-1 functions as a receptor for platelet-derived growth factor BB (PDGFBB)(Banerjee et al., 2006), transforming growth factor β (TGFβ)(Glinka and Prud'homme, 2008), and fibroblast growth factor 2 (FGF2)(West et al., 2005). Another recently introduced ligand of Nrp-1 is galectin 1, a homodimeric lectin expressed in tumour-associated endothelial cells. Binding of galectin 1 to Nrp-1 stimulates VEGFR2 phosphorylation and triggers VEGFR2-mediated migration of endothelial cell (Hsieh et al., 2008). NRP-1 contains a single serine residue (S612), which can be altered by the attachment of a heparan sulphate or chondroitin sulphate glycosaminoglycan. This modification has been observed in smooth muscle cells as well as in endothelial cells and might modulate responses to VEGF (Shintani et al., 2006).



NRP domain		Amino acid positions	Related function
A	a1-NRP1	H46, S47, S51, 52, K53, D78, R80	SEMA3A binding
	a2-NRP1	–	SEMA3A binding
	a1a2 NRP1/2	–	SEMA3A/F binding
		–	Enhances $VEGF_{165}$ binding to B-NRP1
B	b1-NRP1	–	SEMA3A binding
		–	$VEGF_{165}$ binding
		S346, E348, T349	$VEGF_{165}$ binding
		R359, K373	Heparin binding
	b2-NRP1	R513, K514, K516	Heparin binding
	b1-NRP2	E284, E291	$VEGF_{165}$ binding
	b1b2-NRP1/2	–	SEMA3A/F binding
		–	$VEGF_{165}$, PlGF-2, VEGF-C binding
		–	Heparin binding
C		–	NRP dimerization
Transmembrane (NRP1)		G_{868}VLLGAVCG_{876}	NRP1 dimerization
Cytoplasmic		SEA	Interaction with NIP; essential for NRP1-dependent ECs migration

(Geretti et al., 2008)

Figure 4. Structural features and functions of Neuropilin-1 (Nrp-1). The a1a2 (pink), b1b2 (blue), c (yellow), transmembrane (orange), and cytoplasmic (purple) domains of Nrp-1, along with the ligand binding functions and the relevant targeted amino acid residues have been shown color-coded.

1.5 Semaphorin Family

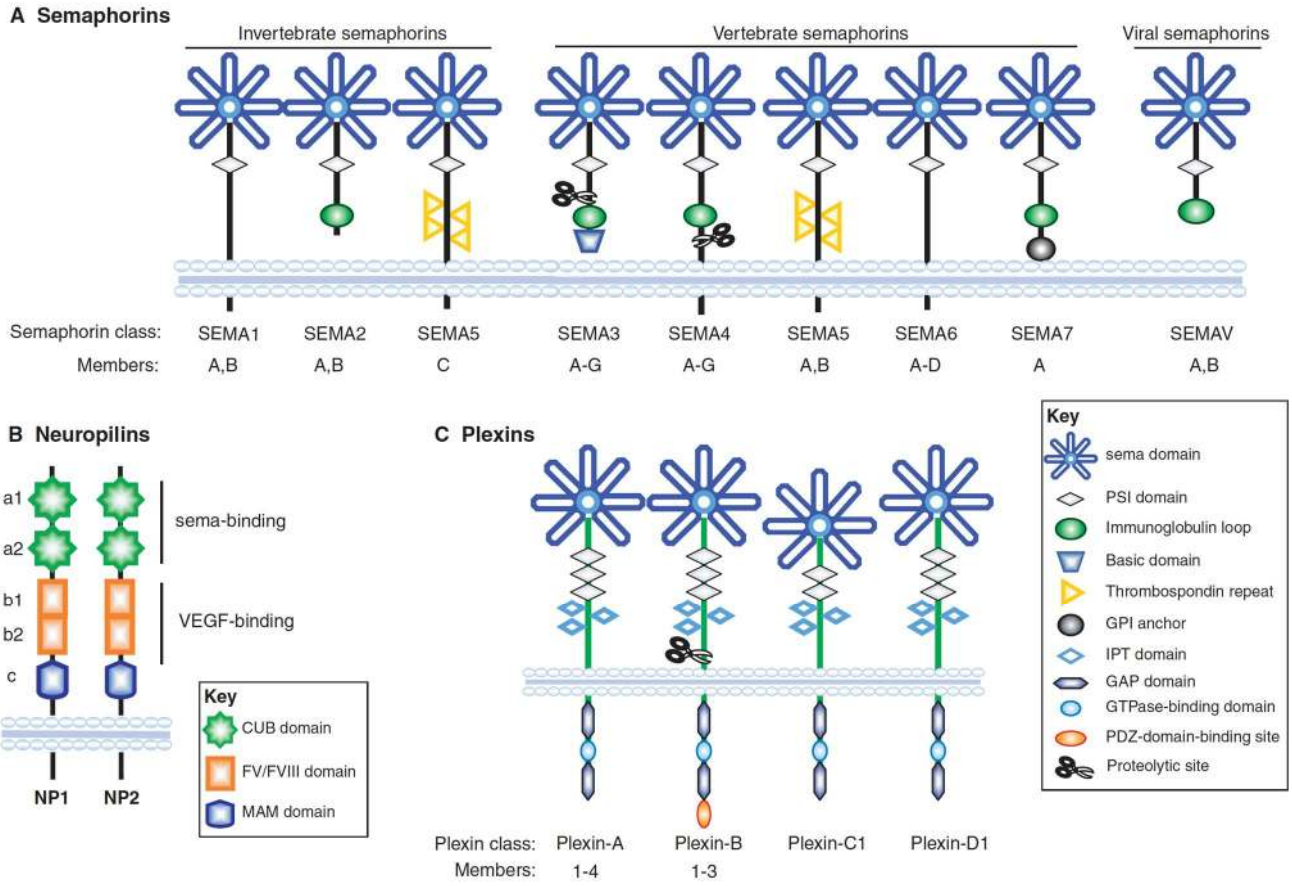
Semaphorins and their receptors were originally characterized as factors of the complex regulatory system responsible for the guidance of axons during the central nervous system development. Semaphorin receptors were subsequently found to be expressed by multiple types of cells, including endothelial cells and a variety of cancer cells (Neufeld and Kessler, 2008).

The Semaphorins are a large and phylogenetically conserved family of proteins including both secreted and transmembrane guidance cues (Yazdani and Terman, 2006). Semaphorins can either promote or inhibit tumour progression through the regulation of processes such as angiogenesis, metastasis and cell survival during cancer progression (Gaur et al., 2009). The family of Semaphorins contains 21 vertebrate genes and eight additional genes found in invertebrates. All Semaphorins are characterized by the presence of an amino-terminal domain essential for their signalling. *In vivo* studies in invertebrates and vertebrates

have definitively shown that Semaphorins serve as pivotal repulsive cues during neural development (Tran et al., 2007). Among all family members, class 3 Semaphorins are the only secreted ones in vertebrates. Sema3A, originally called "Collapsin-1", was the first vertebrate Semaphorin identified and was first characterized as a neruopilin-1 ligand. Mice in which the Sema3A gene has been disrupted show dramatic axon guidance deficiencies (Kitsukawa et al., 1997). Sema3A is basically expressed in tissues that encompass peripheral nerves and functions as a repellent. Transmembrane Semaphorins can also act as repellents. Among members of Semaphorin family, Class 3 Semaphorins and specifically Sema3A are competitive inhibitors of $VEGF_{165}$ binding to NRPs and have been recognized to negatively mediate tumour growth. Key members of class 3 Semaphorins, i. e. Sema3A and Sema3D are synthesized as homodimers linked by disulphide binds, and this dimerization is crucial for their bioactivity (Klostermann et al., 1998; Koppel and Raper, 1998). Specific members of class 3 Semaphorin family have been supposed previously to have both tumour suppressor (Sema3B and 3F) (Campioni et al., 2008) and anti-angiogenic (Sema3A and 3F) effects and to be down-regulated with tumour progression. Indeed, the expression of Sema3B and Sema3F is down-regulated with increasing lesion severity in ovarian cancer (Drenberg et al., 2009; Osada et al., 2006). Additionally, increased ratio of vascular endothelial growth factor to Semaphorin is a poor prognostic factor in ovarian carcinomas (Joseph et al., 2010) and also during lung cancer progression (Campioni et al., 2008; Tomizawa et al., 2001).

1.5.1 Semaphorin receptors

All semaphorins in higher vertebrates contain a signature Semaphorin domain of nearly 500 amino acids, which plays a crucial role in mediating the association of these lignads with signalling receptors of plexin family. Semaphorin receptor complexes are composed of plexins or plexins along with neuropilins (Figure 5). The main receptors for semaphorins are plexin family members including nine various proteins in higher vertebrates (Tamagnone and Comoglio, 2000) as high affinity receptors for Semaphorins. Several members of Semaphorin family bind plexins directly, whereas some secreted vertebrate semaphorins such as Sema3A, bind to the obligate co-receptors neuropilin-1 or neuropilin-2 to form neuropilin- plexin holoreceptor complex. The neuropilins form signalling complexes by associating with type A plexins. In these complexes, the neuropilins serve as the binding receptors and the plexins as the signal-transducing elements (Takahashi et al., 1999; Tamagnone et al., 1999).



(Capparuccia and Tamagnone, 2009)

Figure 5. Schematic representation of Semaphorin family members and their receptors (neuropilins and plexins). A) All members of Semaphorin family are recognized by the presence of a large Sema domain and one PSI domain. Among vertebrate Semaphorins, those belonging to classes 4, 5, and 6 are transmembrane proteins, while those in class 7 are membrane bound through glycophosphatidylinositol (GPI). Instead, class -3 Semaphorins as secreted members of this family, have C-terminal basic charged sequences required for binding to neuropilins. Several members of Semaphorin family contain immunoglobulin-like domains. **B)** Neuropilins are transmembrane non-tyrosin kinase co-receptors characterized by two CUB domains (also called a1 and a2 domains), two FV/ FVIII coagulation factor-like domains (also referred to as the b1 and b2 domains) and a meprin-like MAM domain or the c domain). **C)** Plexins consist of one Sema domain, two to three PSI domains and three IPT domains.

1.6 Tumour Microenvironment and Metastatic Disease

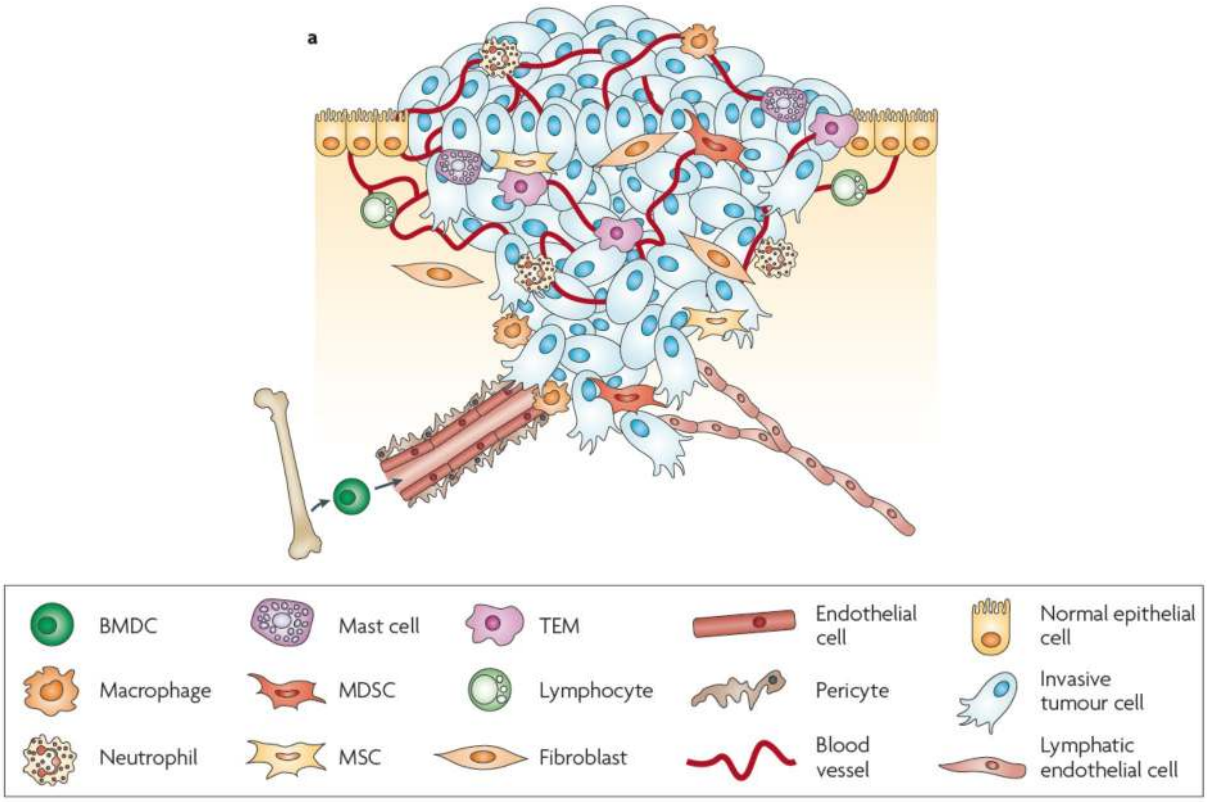
A tumour is not just a bunch of cancer cells with the ability to multiply infinitely, but is composed of cancer stem cells, mature cancer cells, metastatic cancer cells, stromal cells, endothelial cells, tumour- infiltrating macrophages and a broad variety of fibroblasts, all of which embedded in an extracellular matrix (ECM) and constitute what is usually called the "tumour microenvironment" (Joyce, 2005). The microenvironment of solid tumours is a

heterogeneous setting with specific features such as acidic pH, low nutrient levels, elevated interstitial fluid pressure (IFP) and variable levels of oxygenation characteristic of abnormal vascular network existing in tumours (Figure 6).

Interestingly, the tumour microenvironment, particularly in respect with the massive infiltration of dysregulated immune cells can promote tumour growth, angiogenesis, and metastasis (Joyce, 2005). It is believed that metastatic potential of tumour cells is regulated by interactions between the tumour cells and their extracellular environment (ECM). In fact, tumour microenvironment was once believed to be a simple bystander that accompanies the growth and the evolution of tumour masses, primarily and essentially composed of malignant cells, able to proliferate restlessly. Now, the other components of a tumour are known to actively sustain its growth, suppressing host immune response (Kusmartsev and Gabrilovich, 2006), secreting pro-tumourigenic growth factors (Mantovani et al., 2010), modelling the ECM and most prominently, fostering angiogenesis (Schmid and Varner, 2007; Shojaei et al., 2008) and promoting metastatic spread (De Palma et al., 2007).

Angiogenesis and metastatization are invariably linked, as blood vessels constitute the main route for cell dissemination. Subsequently, excessive angiogenesis leads to the formation of a poorly efficient vasculature and the consequent establishment of a chronic state of hypoxia (Wilson and Hay, 2011).

Clinical and experimental evidence suggests that altered gene expression in response to the hypoxic environment plays an important role in favour of metastasis (Keith et al., 2012), as will be discussed in Chapter 1.14.



(Joyce and Pollard, 2009)

Figure 6. Tumour microenvironment. Tumour cells in primary tumours are encompassed by a complex microenvironment consisting of various types of cells including endothelial cells, stromal fibroblasts and a broad range of bone marrow-derived cells (BMDCs) including macrophages, TIE-2 expressing monocytes (TEMs), myeloid- derived suppressor cells (MDSCs), and mesenchymal stem cells (MSCs).

1.7 Metastasis Mechanisms

Metastasis, the spread of malignant cells from a primary tumour to distant sites, creates the biggest problem to cancer treatment and is the main cause of death of cancer patients. It occurs in a series of distinct steps represented as a "metastatic cascade" including Epithelial-Mesenchymal Transition (EMT), invasion, anoikis (apoptosis in response to inappropriate interaction between cell and extracellular matrix), angiogenesis, transport through vessels and outgrowth of secondary tumours. Noteworthy, alterations that disturb normal control of anoikis, confer to the tumour cells the ability to live in completely inappropriate extracellular matrix environment.

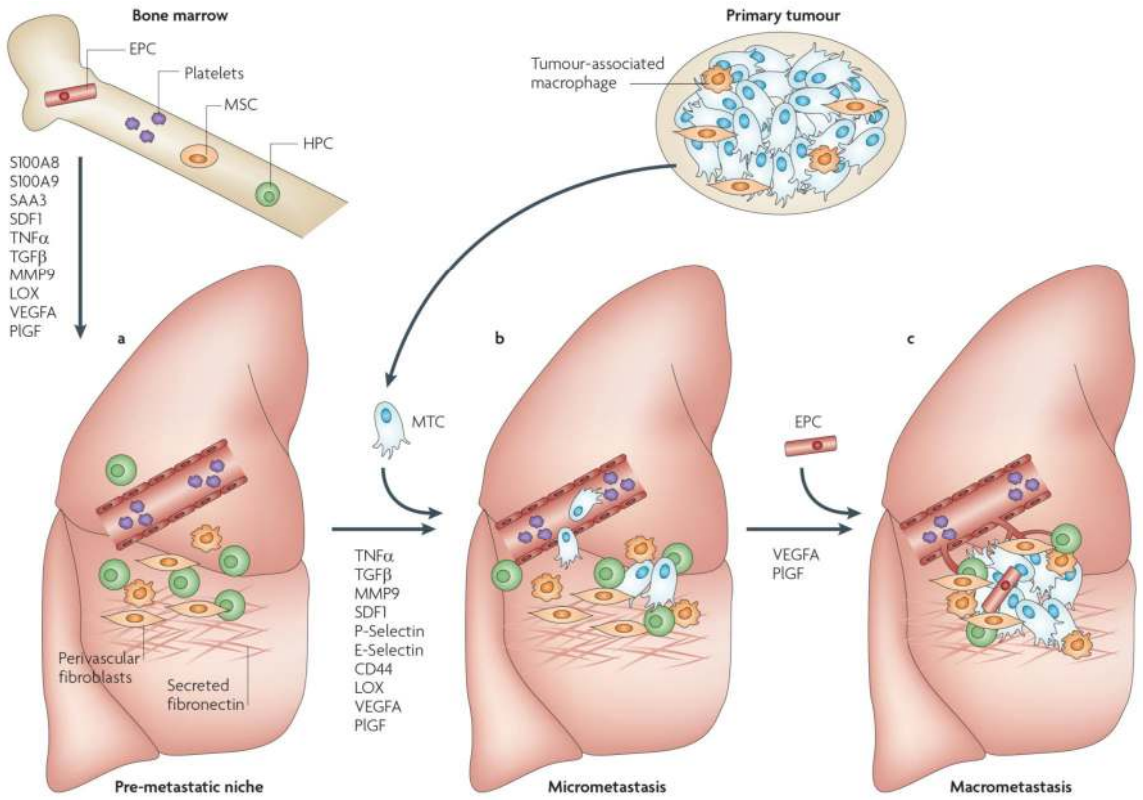
According to recent findings, beside the requirement of stem cell-like properties, the role of the tumour stroma and paracrine interactions of the tumour with cells in distant anatomical sites, or the existence of a "premetastatic niche" has well been established.

In fact, the dissemination of malignant cells from the primary tumour to secondary sites was consistently considered to be a late-stage phenomenon.

However, a large body of evidence demonstrated that metastasis initiation might begin earlier than what was previously believed. Using advanced molecular techniques, it has been shown that tumour cells are very often present in the blood and bone marrow of cancer patients before the incidence of clinical or histopathological metastasis (Alix-Panabieres et al., 2008). However, a novel hypothesis suggests that premalignant cells might disseminate during early stages of tumour progression and prime their own microenvironments or metastatic niches *in situ* (Chin, 2003). Instead, circulating cancer cells with no metastatic potential, might prepare distant sites to be engrafted by more invasive cell types (Bidard et al., 2008). Of the millions of tumour cells entering the circulation, only few of them will successfully lodge and proliferate at secondary sites (Weiss, 1980, 1990). The classical view on the metastatic cascade, starting from a primary epithelial neoplastic lesion includes: Epithelial-Mesenchymal Transition (EMT) and rupture of the basement membrane barrier; disaggregation of tumour cells from the tumour mass; Invasion of the adjacent tissue; intravasation into pre-existing and newly formed blood and lymph vessels; transport through vessels; extravasation from vessels; establishment of disseminated cells (which can stay dormant for a prolonged period of time) at a secondary anatomical site; and outgrowth of micrometastases and macrometastases (Figure 7). In order to successfully metastasize, tumour cells have to overcome all the physiological barriers caused by these steps. The necessity of a "premetastatic niche" at the target site, before the onset of first tumour cells to the distant site has been proved by different studies, although the exact time of the establishment of "premetastatic niche" is not known. Distant tumours induce increasing levels of the pro-inflammatory chemokines at the secondary target sites of tumour-bearing mice, which in turn are infiltrated by myeloid cells that crucially modify the local environment priming it for subsequent localization (Hiratsuka et al., 2002; Hiratsuka et al., 2006).

Epithelial tissues, representing the origins of most solid tumours are separated from the stroma by a basement membrane. During metastasis, epithelial tumour cells are released from adjacent cells and breach the basement membrane barrier. This process is suggested to involve specific cell alterations which are usually referred as EMT (Christofori, 2006; Thiery, 2002). EMT, a conserved critical process for embryonic development, concerns the release of epithelial cells from the surrounding tissue (Radisky, 2005). During EMT, epithelial cells acquire characteristics resembling those of mesenchymal cells, therefore inducing cellular

invasion into neighbouring tissues. Cells undergoing EMT usually acquire a spindle-shaped morphology. The major signalling pathways and molecules inducing EMT include Receptor Tyrosine Kinases (RTK), the transforming growth factor β (TGFB) superfamily, WNT, NOTCH, hedgehog pathway (Huber et al., 2005; Massague, 2008) and NF-kB (Huber et al., 2004). Instead, the reverse process, Mesenchymal-Epithelial Transition (MET), seems to be involved in establishing secondary tumours with an epithelial appearance, reminiscent to the primary one (Thiery, 2002). EMT can promote metastasis in different ways. First of all, the loss of cell-cell adhesion permits tumour cell invasion. Infiltration to tissues and vessels can be supported by a secondary property of cells that have undergone EMT, i.e. secretion of protein-degrading enzymes like matrix metalloproteinases (MMPs) (Jechlinger et al., 2003). MMPs are often overexpressed in tumours and are able to remodel the Extracellular Matrix (ECM) in the tumour microenvironment, eventually releasing and processing mitogenic and angiogenic factors sequestered by ECM. Cleavage of ECM components provides criptic sites that stimulate cell migration (Giannelli et al., 1997). In order to invade tissues and vessels, cells must reach the ability to migrate. In brief, cell migration starts with the extension of cell membrane projections that is driven by a continuous cycle of actin polymerization and depolymerization. Invasive tumour cells can migrate either as single cells or collectively in the form of files, clusters or sheets.



(Psaila and Lyden, 2009)

Figure 7. Schematic representation of the formation of a metastatic niche. The figure illustrates the pre-metastatic, micrometastatic to macrometastatic transition. **a)** Secretion of growth factors including vascular endothelial growth factor-A (VEGF-A), placental growth factor (PlGF), transforming growth factor-β (TGF-β)), inflammatory S100 chemokines and serum amyloid A3 (SAA3) by the primary tumour are upregulated in premetastatic areas. This can eventually result in clustering of bone marrow-derived haematopoietic progenitor cells (HPCs). HPCs secrete a wide range of pre-metastatic factors such as tumour necrosis factor-α (TNF-α), matrix metalloproteinase 9 (MMP9), and TGF-β. Stimulated fibroblasts, secrete fibronectin, a pivotal adhesion protein in the niche, and lysyl oxidase (LOX) expression is also elevated, with a role in modulating the local extracellular matrix. **b)** MTCs engraft the niche to populate micrometastases. **c)** Infiltration of endothelial progenitor cells (EPCs) to the early metastatic niche regulates angiogenic switch and facilitates the progression towards macrometastases.

1.8 Metastasis associated with Acute and Chronic Tumour Hypoxia

The mechanisms by which tumour hypoxia might increase metastatic potential may include the increased rate of mutagenesis, genetic instability and enhanced epigenetic regulation of gene expression (Erler et al., 2006; Graeber et al., 1996; Krishnamachary et al., 2003; Rofstad, 2000; Subarsky and Hill, 2003; Sullivan and Graham, 2007). The development of tumour hypoxia is essentially correlated with the formation of neovasculature. Vessels developed within tumour masses are immature, expanded, and tortuous (Vaupel, 2004). Because of an incomplete basement membrane, they are hyperpermeable and prone to

excessive branching, blind ends and neovascular shunts (Baluk et al., 2003; Kallinowski et al., 1989; Vaupel et al., 1989). For tumour cells, the consequence of being far from the vascular supply or so-called diffusion-limited hypoxia was proposed by Thomlinson and Gray in 1955 as the first concept of hypoxia in tumours (Thomlinson and Gray, 1955). It was proposed later that perfusion-limited hypoxia (also known as acute hypoxia), due to alterations in blood flow, might play an important role in solid tumours (Brown, 1979; Sutherland and Franko, 1980). Angiogenesis is a necessity of tumour growth.

A key pro-angiogenic gene in this regard, is hypoxia-responsive VEGF (Dvorak et al., 1999). Its expression is regulated by the hypoxia-inducible factor 1 (HIF-1) which is stabilized under hypoxic conditions (Semenza, 2001, 2007). HIF-1 has been considered as the master regulator of hypoxic response (Semenza, 2003) and drives expression of multiple genes that are involved in the metastatic process, although there are other transcriptional factors such as NF-kB, p53, AP-1, C/EBPb, Egr-1 and SP-1 that respond to an hypoxic environment (Hirota and Semenza, 2006; Mabjeesh and Amir, 2007; Subarsky and Hill, 2003; Zhou et al., 2006). HIFs are frequently up-regulated in cancer and metastasis since their downstream target genes can promote growth and survival (Maynard and Ohh, 2007). Nearly 1-1.5% of the genome is transcriptionally regulated by hypoxia and many pro-metastatic genes are known to be regulated by HIF-1α.

1.9 Tumour- Infiltrating Cells

Similar to other solid tumours, colon carcinomas are also infiltrated by various cell types including tumour-associated macrophages (TAMs), myeloid-derived suppressor cells (MDSCs), mast cells, cancer-associated fibroblasts (CAFs), monocytes, neutrophils, CD8 and CD4 T-cells, dendritic cells (DCs), natural killer (NK) cells, endothelial cells, endothelial progenitor cells (EPCs), platelets, and mesenchymal stem cells (MSCs) (Murdoch et al., 2008). It was originally proposed that the presence of these cells is the remnant of a truncated immune response mounted by the host against the tumour that is ultimately unable to eradicate the malignancy. Now, tumour-infiltrating immune cells are considered as potent tumour-promoting agents with multiple genetic and pharmacological evidence, although specific contribution of each cell type is difficult to dissect, largely due to their overlapping functions. It is now extensively approved that tumour vessels are composed not only of sprouting vessels from pre-existing ones, but also of heterogeneous precursor cells(Furuya and Yonemitsu, 2008). Bone marrow-derived EPCs (endothelial progenitor cells) (Asahara et

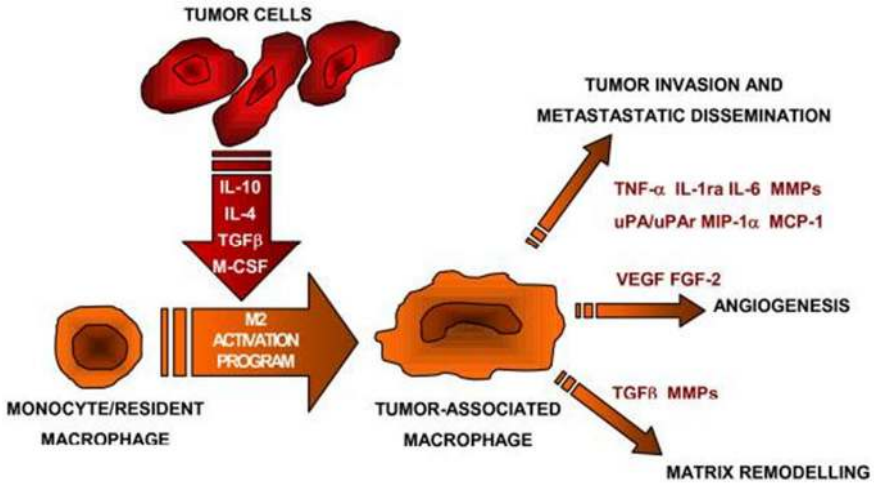
al., 1997), tissue-derived EPCs (Bruno et al., 2006; Zengin et al., 2006), and some hematopoietic stem cells (HSCs) (Hattori et al., 2002; Lyden et al., 2001) are believed to evolve into tumour-associated endothelial cells (TECs). Comparison of molecular profiles between normal ECs and TECs including cDNA microarray analysis and proteomic mapping, have detected several key molecules as TECs-specific markers (Seaman et al., 2007; Shih et al., 2002). Some highly expressed molecules such as VEGFR2 which are not specific to TECs seem to contribute to the modulation of permeability, proliferation, migration, anti-apoptosis, matrix remodelling, and many other aspects of tumour neovascularization (Bussolati et al., 2003; St Croix et al., 2000; Wang et al., 2002). Despite a general concept, not all of above mentioned cell populations differentiate into TECs. Some of these cells localize in close proximity of tumour vasculature as heterogeneous cells of uncertain differentiation capacities and contribute to tumour growth by mediating its proinflammatory microenvironment. In such a milieu, tumour vessels take advantage of specific signalling molecules (Furuya et al., 2004), this cocktail of infiltrating cells preserves tumour associated inflammation, angiogenesis, and immunosuppression, which sequentially promotes tumour progression and metastasis. Two important factors for this breach are inflammation and soluble chemoatractants secreted by both tumour and stromal cells (Jedinak et al., 2010). The initial role of these stromal cells is not tumour promotion but instead, these cells contribute to some antitumour properties (de Visser et al., 2006). But during tumourigenesis, the dynamic interaction between stromal and tumour cells changes, eventually being in favour of tumour progression (de Visser et al., 2006) possibly involving an “education” of immune cells *in loco* by malignant cells.

1.9.1 Tumour-Associated Macrophages

Tumour-Associated Macrophages (TAMs) derive from blood monocytes that are recruited to the tumour by growth factors, chemokines, and angiogenic factors such as colony-stimulating factor 1 (CSF-1), and VEGF (Lewis and Pollard, 2006; Pollard, 2009; Sica et al., 2008). The presence of low interleukin (IL)-12 and high IL-10 levels in the tumour microenvironment induce the differentiation of monocytes into TAMs (Fricke and Gabrilovich, 2006). Macrophages are phenotypically plastic and, depending on the microenvironment stimuli, can acquire different functions and morphology in a continuum of phenotypic conditions whose edges are represented by either M1 (anti-tumour) or M2 (pro-tumour) polarization states (Mantovani et al., 2007; Mantovani et al., 2004; Sica et al., 2006;

Van Ginderachter et al., 2008). TAMs with M2 polarization are a major tumour-infiltrating population of the cells (Coussens and Werb, 2002; Mantovani et al., 2008) and are an essential component of inflammation-associated carcinogenesis (Figure 8). The number of M2 macrophages is preferentially raised in the poorly vascularized areas of the tumour, which are marked by low oxygen concentration. Under such a condition, these cells cooperate with cancer cells in regulating HIF-1 dependent transcription of angiogenesis inducers such as VEGF-A, FGF-2, and CXCL8 (Mantovani et al., 2002). High TAM density in tumours is now considered as a poor prognostic indicator in various tumours, including CRC (Bacman et al., 2007; Bingle et al., 2002; Lewis and Pollard, 2006; Paik et al., 2004). TAMs promote tumour growth and metastasis through inducing angiogenesis and enhancing tumour cell migration/invasion and ECM degradation (Barbera-Guillem et al., 2002; Condeelis and Pollard, 2006; Leek et al., 1996; Shieh et al., 2009; Takanami et al., 1999). TAMs in the stroma express COX-2, and the relationship between COX-2 and colonic adenoma formation is well established (Adegboyega et al., 2004). Colon carcinoma cells induce TAMs to secrete VEGF, which promotes angiogenesis as well as metastasis (Barbera-Guillem et al., 2002; Pollard, 2004; Sickert et al., 2005). TAMs express many other pro-angiogenic factors, such as FGF2, TNF-α, IL-1β, IL-8 (CXCL8), COX-2, platelet derived growth factor-β (PDGF-β), hepatocyte growth factor (HGF), matrix metalloproteinase (MMP)-7, and MMP12 (Barbera-Guillem et al., 2002; Burke et al., 2003; Leek et al., 1996; Mizukami et al., 2007; Shieh et al., 2009; Takanami et al., 1999). Colon carcinoma cells produce CSF-1, which recruits macrophages to the tumour periphery where they secrete motility and angiogenic factors that facilitate tumour cell invasion and metastasis (Green et al., 2009). TAMs contribute to the epithelial- to- mesenchymal transition (EMT), which is a primary event for cancer metastasis (Bataille et al., 2008; Bates et al., 2007). TGF-β, which is produced by both colon cancers (Paduch and Kandefer-Szerszen, 2009) and macrophages (Mantovani et al., 1992), plays a significant role in the process of EMT. Moreover, cancer cells can induce stromal cells (including macrophages) to secrete MMPs (MMP2 and MM9), cysteine cathepsins and serine proteases that contribute to cell invasion and intravasation by cleaving cell- adhesion molecules such as E-cadherin and the ECM. On the other hand, suppressive mediators secreted by TAMs can suppress T-cell activation and proliferation (Mantovani et al., 1992). Two major series of evidence connect macrophages and cancer: first, the correlation of chronic inflammation leading to macrophage accumulation with the initiation of cancer and its progression (Coussens and Werb, 2002; Mantovani et al., 2008; Robinson and Coussens,

2005); second, a high density of TAM is associated with poor prognosis in more than 80% of studies (Lin et al., 2002; Murdoch et al., 2008). Experimentally, TAM depletion by means of bi-phosphonate compounds (i.e. zoledronic acid) severely impairs tumour growth in different models of tumour growth and progression (Giraudo et al., 2004). Indeed, TAMs accumulate in critical regions of tumours such as hypoxic areas, and hypoxia triggers a pro-angiogenic plan in these cells (Murdoch et al., 2004). Macrophages are specifically noticed as multifunctional cells due to their diverse and opposing activities in pro- *vs.* anti-inflammatory, immunogenic *vs.* tolerogenic, and tissue devastating *vs.* tissue restoration processes. Macrophages from healthy or inflamed tissues are able to lyse tumour cells, to present tumour-associated antigens to T-cells, and to express stimulatory cytokines for T- and NK-cells (Fidler and Schroit, 1988). The adaptability of macrophages might be taken as an advantage by tumour cells to acquire distinct abilities at different stages of tumour progression. Cancer cells can stimulate TAMs to produce matrix metalloproteinases (MMPs) in a paracrine manner through the secretion of different stimuli such as interleukins, growth factors and CD147 as an extracellular matrix metalloproteinase inducer. In addition, the invasiveness of the tumour cells might be supported by epidermal growth factor (EGF) secreted by TAMs in response to tumour-derived CSF-1, which consequently leads to the activation of several genes associated with the migration of tumour cells (Goswami et al., 2005). In fact, the invasion and chemotaxis of metastatic cancer cells as a subpopulation of tumour cells, relies on the co-migration of tumour-associated macrophages (Goswami et al., 2005; Wyckoff et al., 2004; Wyckoff et al., 2007).



(Calorini and Bianchini, 2010)

Figure 8. Origin and function of M2-polarized macrophages.

1.9.2 Myeloid-Derived Suppressor Cells

Myeloid-Derived Suppressor Cells (MDSCs) (Gabrilovich et al., 2007) are a heterogeneous population of immature myeloid cells that have suppressive effect on adaptive immune responses (Nagaraj and Gabrilovich, 2008). Numbers of MDSCs are increased in the blood of mice and patients with cancer, including CRC (Mandruzzato et al., 2009). When they are immature, they express endothelial markers such as CD31 and VEGFR2 and thus have the ability to join the tumour endothelium. It has been demonstrated that this heterogeneous population of immature myeloid progenitors have tumour angiogenesis-promoting activity in both mouse tumour models (Shojaei et al., 2007a) and human cancers (Almand et al., 2001; Diaz-Montero et al., 2009). These cells are composed of neutrophils, monocytes, and dendritic cells. In mice they are classified by the expression of Cd11b and Gr1 on their surface. Subsets of these cells have the potential to prevent antitumour activities of T cells and Natural Killer (NK) cells (Murdoch et al., 2008). Indeed, wide range of tumour-produced stimulators, including VEGF-A, colony stimulating factors, prostaglandins, SCF, S100A8, S100A9, CCL2, and a large number of interleukins can recruit $Cd11b^{+}Gr1^{+}$ cells to the site of tumour (Gabrilovich and Nagaraj, 2009). MDSCs may differentiate into mature TAMs (Movahedi et al., 2008; Umemura et al., 2008). Beside their immunosuppressive activities, when isolated from tumour bearing mice, these cells also express higher amounts of pro-angiogenic factors including matrix metalloproteinases (MMPs) (Yang et al., 2004; Yang et al., 2008), which consequently increases the bioavailability of VEGF-A. It has been demonstrated that $Cd11b^{+}Gr1^{+}$ myeloid cells also play a role in VEGF-A- independent angiogenesis and growth in mouse tumours (Shojaei et al., 2007a). This effect is associated to the secretion of the pro-angiogenic factor Bv8, which leads to the proliferation and migration of endothelial cells in the absence of VEGF-A (Shojaei et al., 2007b). Recent studies have also supported a role for $Cd11b^{+}Gr1^{+}$ cells in creating a pre-metastatic niche (Yan et al., 2010), where bone marrow-derived cells prepare the microenvironment sites prior to the settlement of tumour cells (Psaila and Lyden, 2009).

1.9.3 Mast Cells

In some human tumours, infiltration of mast cells is linked with increased vascularity and tumour growth, invasion and poor clinical consequences (Crivellato et al., 2008; Groot Kormelink et al., 2009). Recent CRC studies have shown that a lower number of mast cells in the site of tumour are associated with hypovascularity and better survival in CRC patients

(Gulubova and Vlaykova, 2009). Stem cell factor (SCF) secreted by tumour cells *in vivo* has been involved in the accumulation of mast cells in the border of growing tumours (Huang et al., 2008). Activated mast cells secrete many pro-angiogenic and growth stimulatory factors such as VEGF (Crivellato et al., 2008; Grutzkau et al., 1998), FGF-2, heparin, histamine, TNF-α, angiopoietin-1, and proteases, thus triggering neoangiogenesis. It has been also shown that mast cells are a substantial element for preneoplastic polyp development (Gounaris et al., 2007). Accordingly, the number of mast cells is significantly higher in primary CRCs, as well as in poorly differentiated tumours comparing with non-neoplastic tissues.

1.9.4 Cancer Associated Fibroblasts

Cancer Associated Fibroblasts (CAFs) are the major cellular component of reactive stroma in primary and metastatic cancer and play a key role in CRC progression (Kalluri and Zeisberg, 2006; Ostman and Augsten, 2009). CAFs are composed of heterogeneous components including tissue-resident fibroblasts, stromal smooth muscle cells and bone marrow (BM)-derived fibroblasts (Direkze et al., 2004). Residual tissue fibroblasts and fibroblast precursors stimulated by PDGF and TGF-β are also supposed to be the source of CAF (Peddareddigari et al., 2010).

Cancer-associated fibroblasts are mostly specified based on the expression of α-smooth muscle actin (α-SMA), fibroblast-activated protein (FAP), fibroblast-specific protein-1 (FSP1), neuron-glial antigen-2 (NG2), and PDGF β-receptor. Studies have shown that for colon cancer patients with high levels of stromal FAP, the risk of having aggressive disease progression and developing metastases or recurrence is higher (Henry et al., 2007). CAFs are a source of growth factors and chemokines that promote tumour growth and metastasis and tumour cell proliferation, survival and invasion respectively (Kalluri and Zeisberg, 2006). Furthermore, CAF-originated chemokines recruit bone marrow-derived cells, macrophages, and other immune cells to the site of tumour. The secretion of VEGF, FGF, TGF-β), and CXCL12 by CAF plays a crucial role in the promotion of tumour growth and angiogenesis (Dong et al., 2004; Hlatky et al., 1994).

1.9.5 Tie-2-Expressing Monocytes (TEMs)

Tie-2, an angiopoietin receptor, mainly expressed by endothelial cells and hematopoietic stem cells, is also found in a class of monocytes (Venneri et al., 2007). In cancer patients, Tie-2 expressing monocytes (TEMs) are detected in blood and the tumour microenvironment, where they correspond to the main monocyte population and are distinct from TAMs (Venneri et al., 2007). Interestingly, TEMs have been also detected in many tumours including colon carcinoma (Goede et al., 2012). They contribute to tumour angiogenesis and growth, and likely possess a superior angiogenic capacity. The Tie-2 ligand, angiopoietin-2, which is mainly expressed by hypoxic tumour cells and tumour endothelial cells is the major factor involved in their recruitment (Lewis et al., 2007; Murdoch et al., 2007).

1.9.6 Neutrophils

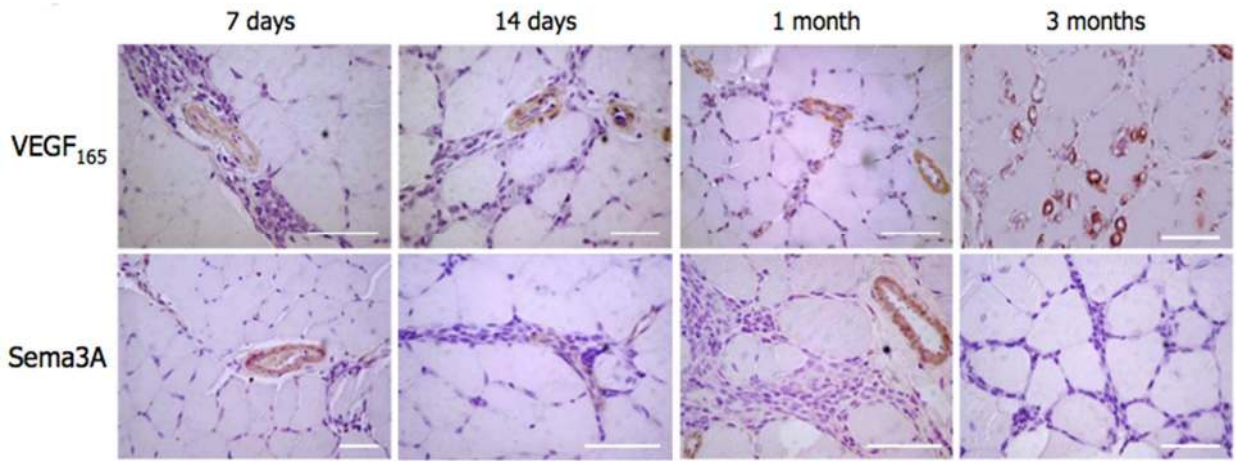
Infiltration of neutrophils has been detected in different stages of inflammation. In addition, increased number of neutrophils is evident in patients with various cancers including gastric and colon cancer (Roncucci et al., 2008). Remarkably, neutrophils are an essential element of oxidative stress-associated pathogenesis of chronic inflammatory bowel disease (IBD)-related RCR (Roessner et al., 2008). More recently, it has been shown that neutrophils are strongly involved in tumour angiogenesis. Neutrophils are recruited into the tumour site from the circulation. Once inside the tumour, neutrophils can secrete factors such as oncostatin M, which stimulates tumour cells to secrete more vascular endothelial growth factor (VEGF). Tumour-associated neutrophils can modulate tumour angiogenesis by releasing a variety of proteases contributing to degradation and remodelling of ECM. Of these proteases, MMP9 has been shown to have the most effective role in mediating tumour angiogenesis. Proteolysis of the ECM by this MMP releases angiogenic factors such as VEGF and FGF-2 that are usually attached in an inactivated form to the ECM. Cytokine-activated neutrophils also secrete VEGF and CXCL8 by de-granulation. These potent pro-angiogenic factors then act directly on the nearby vasculature to promote tumour angiogenesis. Moreover, the interaction of neutrophils with adhesion molecules (ICAM-1) on the endothelial cell surface may also stimulate angiogenesis (Tazzyman et al., 2009). However, neutrophils have been also shown to have antitumor activities since factors released by neutrophils such as ROS, proteases, and cytokines such as TNF-α can kill tumour cells directly (Di Carlo et al.,

2001a; di Carlo et al., 2001b). Thus, it was recently proposed that, depending on the tumour microenvironment, TANs (Tumour- associated Neutrophils) can have pro- or antitumour properties (Fridlender et al., 2009).

1.9.7 Neuropilin-1 Expressing Mononuclear cells (NEMs)

Neuropilin-1 expressing myeloid cells were originally discovered at the site of angiogenesis in mouse skeletal muscles injected with AAV-VEGF-A_{165} (Zacchigna et al., 2008). AAV-mediated overexpression of $VEGF_{165}$ was shown to recruit a large number of infiltrating mononuclear cells, interspersed among muscle fibres, persisted in the tissue for several months, and accompanied VEGF-induced angiogenesis (Zacchigna et al., 2008). These cells were further characterized as Nrp-1^+/$CD11b^+$/$Gr1^-$ cells involved in the process of blood vessel maturation and normalization and then designated as NEMs. Interestingly, a similar cellular infiltration was observed in adult tissues of inducible VEGF transgenic mice (Grunewald et al., 2006), pointing towards an invariable role of NEM recruitment in supporting VEGF-driven angiogenesis.

Remarkably, ectopic expression of Sema3A in the mouse skeletal muscle through AAV-mediated gene transfer elicited the attraction of NEMs, even in the absence of any angiogenic process (Zacchigna et al., 2008). Along with the observation that $VEGF_{121}$ did not recruit NEMs in the same model, these data strongly support a crucial role of Nrp-1 in mediating cell mobilization. A detailed representation of NEM infiltration upon AAV-mediated overexpression of either $VEGF_{165}$ or Sema3A is shown in Figure 9.



(Zacchigna et al., 2008)

Figure 9. Similar cell recruitment by $VEGF_{165}$ and Sema3A *in vivo*. Immunohistochemistry against α-SMA antigen in mouse skeletal muscles previously injected with either AAV-$VEGF_{165}$ or AAV-Sema3A demonstrates that both $VEGF_{165}$ and Sema3A, through binding their mutual co-receptor Nrp-1, can attract NEMs to the site of angiogenesis. Formation of arteries in muscles overexpressing $VEGF_{165}$ is due to the pro-angiogenic effect of this VEGF-A isoform, although such an effect is not detectable in mouse skeletal muscles injected with AAV-Sema3A.

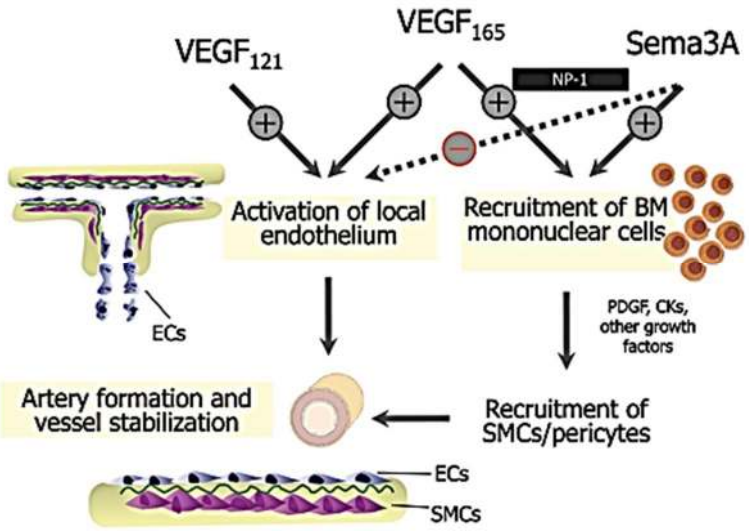
Indeed, NEMs promote vessel maturation and artery formation through the secretion of factors causing the recruitment of smooth muscle cells to the site of angiogenesis. Noteworthy, gene expression profiling showed that NEMs produce relatively low levels of canonical pro-angiogenic factors (Carrer et al., *submitted for publication*), remarkably distinguishing this cell type from other bone marrow-derived populations sustaining angiogenesis.

NEMs can be directly purified from the bone marrow as $CD11b^{+}/Nrp\text{-}1^{+}/Gr1^{-}$ cells and, when injected to the site of angiogenesis, they can actively sustain full vessel maturation (Zacchigna et al., 2008).

Prolonged expression of $VEGF_{165}$ in the skeletal muscles of adult rodents induces a strong angiogenic response, with an increase in the number of capillaries and newly formed arteries surrounded by α- smooth muscle actin (α-SMA) positive cells.

Consistently, $VEGF_{121}$ does not disply any arteriogenic capacity, as is not able to elicit the formation of α-SMA+ vessels at physiological doses. Importantly, $VEGF_{121}$ promote EC proliferation at an extent comparable to $VEGF_{165}$, as expected by its exquisite angiogenic potential. Accordingly, the Molecular Medicine Laboratory proposed a working model in which vessel maturation is elicited specifically by $VEGF_{165}$ in an indirect way, which implies the recruitment of NEMs as accessory cells, peculiarly able to attract mural cells thus allowing vessel coverage and stabilization (Figure 10). Indeed, NEMs contribute to arterial

formation through a paracrine effect resulting in the activation and proliferation of local smooth muscle cells present in the tissue.



(Zacchigna et al., 2008)

Figure 10. Role of NEMs in vessel stabilization. The recruitment of NEMs by both $VEGF_{165}$ and Sema3A as ligands of Nrp-1 plays a major role in vessel maturation. A wide range of growth factors secreted by this population attracts smooth muscle cells and pericytes to the site of ongoing angiogenesis, which in turn contribute to vessel coverage and stabilization.

NEMs do not infiltrate mouse experimental tumours, consistent with their ability to favour vessel stabilization, and do not have an equivalent human counterpart so far, as Gr1 is a marker present only in mice. Hence, their relevance in the context of human malignancy is still unexplored.

Importantly, Sema3A has been shown to exert anti-tumour activity in a variety of mouse models, including xenotransplantation (Kigel et al., 2008), orthotopic implantation (Carrer, *unpublished*) and spontaneous carcinogenesis (Maione et al., 2009). Whether this effect could be, at least in part, due to recruitment of NEM has been extensively investigated at the Molecular Medicine Laboratory. Indeed, administration of purified NEMs significantly inhibited tumour growth (Carrer, *unpublished*), an event concomitant with the normalization of tumour vasculature and improved oxygen perfusion. Previous data strongly point towards NEMs as a strong normalizing agent in the context of tumour angiogenesis. NEM-induced improvement in tumour perfusion contributes to the inhibition of tumour progression through amelioration of tumour-associated hypoxia.

1.9.8 Lymphocytes and Dendritic Cells

It has been shown that tumours without early metastatic invasion signs contain increased number of recruited immune cells and elevated amount of markers of T-cell migration, activation, and differentiation (Pages et al., 2005). In fact, the type, density, and location of T-cells in some colon cancer tissue samples, has been shown to be a better predictor of patient survival than histopathological results used to stage CRC (Galon et al., 2006; Morris et al., 2008). Moreover, the role of B-cells in human CRCs is not well described, but B-cells may inhibit antitumor T-cell responses by antigen-nonspecific mechanisms (Shah et al., 2005). Dendritic cells (DCs), both myeloid and plasmacytoid DCs are able to induce primary and secondary T- and B- cell responses, thanks to their antigen presenting capacity. Tumour-derived factors such as VEGF, β-defensin, CXCL12, HGF, CXCL8, and PGE_2 recruit immature DCs (iDCs) in the site of tumour but prevent their maturation (Gabrilovich et al., 1998), resulting in a cellular infiltrate composed of few mature DCs but abundant iDCs. These iDCs promote tumour angiogenesis by releasing pro-angiogenic cytokines and functioning as a source of endothelial progenitors (Curiel et al., 2004). On the other hand, mature DCs may induce tumor-specific cytotoxic T lymphocyte (CTL) activity against colon tumor growth in vitro and in vivo (Wu et al., 2009).

1.9.9 Platelets

Platelets are nucleus-less cells basically involved in hemostasis. On the other hand, they have been also observed to play a crucial role in tissue repair and the conservation of endothelial function (Mazzucco et al., 2010). Moreover, evidence suggests that increased number of platelets can contribute to tumour progression, possibly through enhanced production of growth factors normally required for tissue repair. Other evidence indicates a role of platelets in tumour metastasis and angiogenesis (Karpatkin, 2003; Nash et al., 2002). In cancer patients, platelets are activated by thrombin, ADP, or by direct contact with molecules on the surface of tumour cell membranes. Platelet activation outcome is the generation of thromboxane A2 and the release of the storage contents from both alpha granules and dense granules that contain pro-angiogenic factors such as VEGF, PDGF, and CXCL12 (Stellos et al., 2009). Platelets are also involved in colon cancer metastasis by being accumulated on embolic tumour cells, thus protecting them from elimination by the immune system (Kopp et al., 2009).

1.9.10 Mesenchymal Stem Cells

Colon tumours, as many other types of solid malignancies, contain various multipotent cells, including Mesenchymal Stem Cells (MSCs), Endothelial Progenitor Cells (EPCs), and pericyte progenitor cells beside cancer stem cells (Ricci-Vitiani et al., 2009). Remarkably important among these are the MSCs, which are multipotent nonhemopoietic cells that reside in the bone marrow and can differentiate into various types of mesenchymal cells. They are characterized by the expression of a large number of adhesion molecules and stromal cell markers including CD73, CD105, CD44, CD29, and CD90 and the absence of hematopoietic markers (CD34, CD45, and CD14) or endothelial markers (CD34, CD31, and vWF). MSCs give rise to a large number of cytokines and growth factors such as PDGF, FGF, and CXCL12. They also express growth factor receptors and ECM proteins.

In bone marrow, MSCs and MSC-derived stromal fibroblasts sustain hematopoiesis. However, in primary tumours, they are present in large numbers and contribute to the formation of tumour- associated stroma. They also promote tumour growth and metastasis (Karnoub et al., 2007) partly by their immunosuppressive effects and pro-angiogenic properties. MSCs can also be differentiated into endothelial and pericyte-like cells that support tumour growth. In addition, MSCs have been supposed to play a role in promoting the cancer stem cells' survival (Ning et al., 2008).

1.10 Lymphatic vasculature and its role in metastatic spread in cancer

Lymphatic vasculature is an important gate of metastasis in various cancer types. Spread of tumour cells to lymph nodes has been usually recognized as an early event in metastatic disease relevant to the staging of the cancer (Tuttle, 2004). In addition, lymphatic vasculature and lymph nodes are fundamental for immune function.

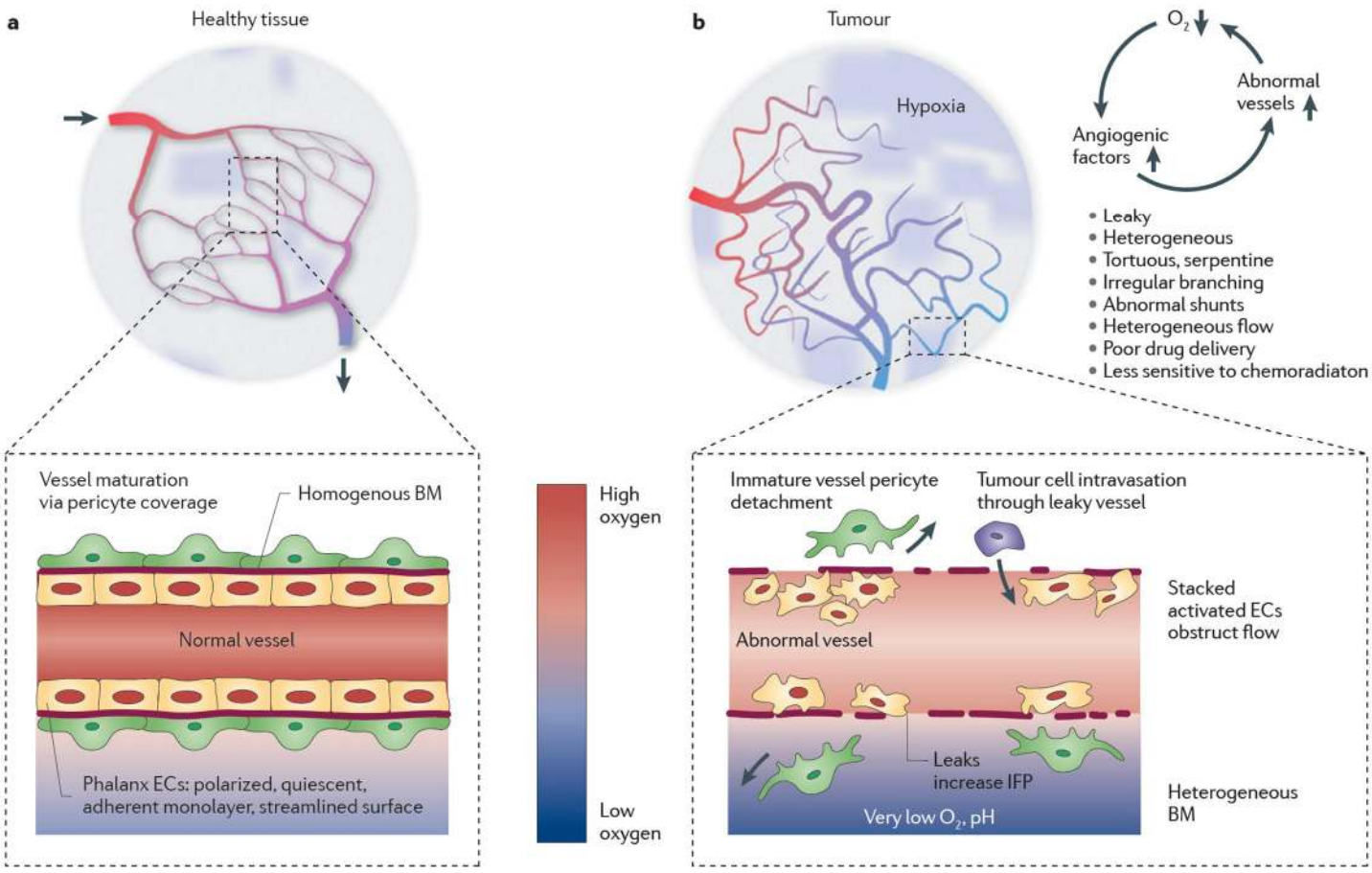
The lymphatic capillaries are composed of an irregular basement membrane and a single layer of endothelial cells deprived of tight junctions. These characteristics are crucial for the function of lymphatic capillaries, since they simplify the ingression of lymphocytes, proteins and fluids into the vessels (Oliver and Detmar, 2002; Skobe and Dana, 2009). Moreover, pericytes or smooth muscle cells are missing in lymphatic capillaries, although the larger lymphatics are also composed of smooth muscle cells involved in pushing lymph through the vessels (Saharinen et al., 2004). In fact, preventing regional lymph node metastasis in some animal models has been associated with lower metastasis rate at distant sites, proclaiming the existence of a pathway for organ metastasis through lymph nodes (Alitalo et al., 2004;

Krishnan et al., 2003; Stacker et al., 2002).

1.11 The cancer vasculature

As previously mentioned, tumour vessels branch in an irregular serpentine manner, with the tendency to connect to each other haphazardly (Baluk et al., 2005; Jain, 1988, 2005; Mazzone et al., 2009; Nagy et al., 2010). In addition, the tumour vasculature is highly heterogeneous and displays a wide range of vessel subtypes including capillaries, 'mother' vessels, which are characterized as large, leaky vessels encompassed by pericyte-depleted thin walls, and also glomeruloid vessel protuberances and vascular malformations(Nagy et al., 2010; Pettersson et al., 2000). Some vessels seem oversized whereas smaller vessels display some sort of immaturity (Fukumura et al., 2010; Jain and Stylianopoulos, 2010).

In fact, comparing to the healthy vasculature, as a result of a persistent, uncontrolled production of angiogenic factors, which tips the balance in favour of excessively active vessel growth, tumour vessels are structurally and functionally abnormal (Figure 11). Tumour perfusion is chaotic in such a way that blood may flow easily in certain areas while being stagnant in others (Fukumura et al., 2010; Jain, 1988). Irregularities in the flow patterns in tumour vasculature, subsequently causes an interruption in the process of constant delivery of nutrients as well as chemotherapeutics (Jain, 2005; Jain and Stylianopoulos, 2010). Indeed, in some areas, ECs with indiscriminate shape are piled upon each other and block blood flow by extending several outgrowths, while in other locations, ECs fade away or die behind intracellular gaps. These abnormalities are detectable in a wide variety of tumour types but not every tumour demonstrates the same degree of vessel abnormality (Rocha and Adams, 2009).



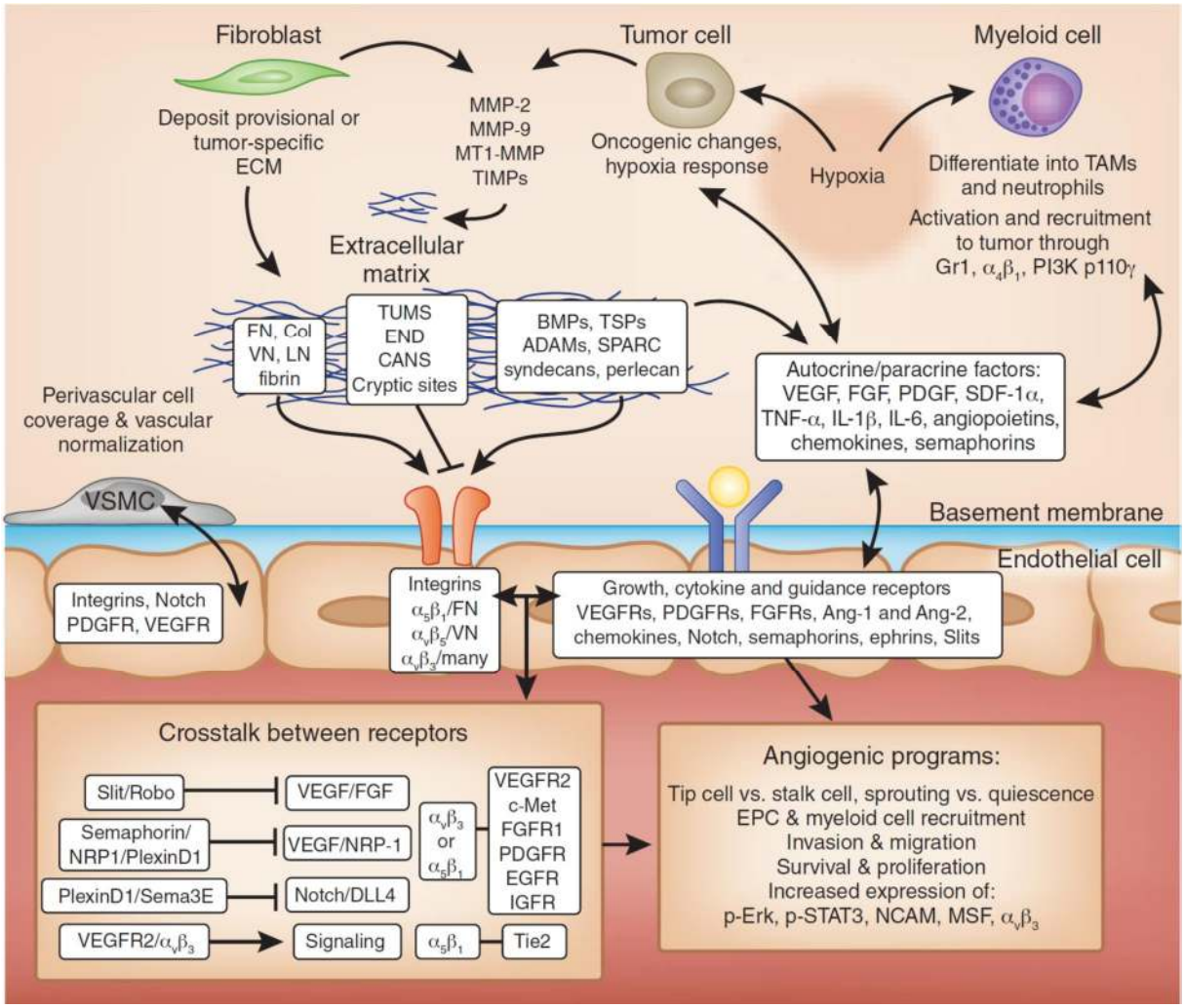
(Carmeliet and Jain, 2011)

Figure 11. Tumour vessels are abnormal in their function and structure. a) In a healthy normal tissue, a well-structured vasculature is formed, which is encompassed by normal pericyte coverage of the vessel wall.
b) Instead, in tumours, the vascular network displays structural and functional anomalies, mainly at the level of vessel wall, resulting in poor perfusion and severe hypoxia in some certain regions. BM, EC, and IFP stand for basement membrane, endothelial cell, and interstitial fluid pressure respectively.

Moreover, the vessel wall of tumour is also abnormal. Since their wall is compressed by tumour or stromal cells, diameters are asymmetrical. Tumour-associated endothelial cells have a unique activated phenotype compared to normal quiescent endothelial cells. Whereas normal vessels are lined with a tightly connected and impenetrable monolayer of endothelial cells aligned in the direction of blood flow, simulated tumour endothelial cells can easily detach from the basement membrane and pile upon each other (Baluk et al., 2005; Mazzone et al., 2009; Ozawa et al., 2005). Consequently, some tumour endothelial cells might die or dismount, resulting in the formation of some gateways to facilitate the access of escaped cancer cells to the blood flow through so-called ‘mosaic vessels’ (Jain, 1988) lined with both endothelial and tumour cells. In fact, tumour endothelial cells generate sprouts and form

abluminal extensions, with their tip cells pervading the tissue. On the other hand, tumour endothelial cells go through endothelial-to-mesenchymal transition leaving their default position.

Tumour blood vessels are exposed to a distinct combination of stimuli in their regional environment and are controlled by abnormal signalling pathways (Figure 12). In other words, cancer cells by their oncogenic transformation features or response to hypoxic conditions, release a collection of stimuli that trigger the transformation of their local environment. These factors stimulate the remodelling of the extracellular matrix to facilitate tumour growth and progression. Tumour cells as well as stimulated endothelial cells secrete a variety of soluble factors involved in the mobilization and homing of bone marrow-derived myeloid cells and their subsequent differentiation into tumour-associated macrophages and neutrophils. In such an environment, receptors on the surface of endothelial cells bind factors that trigger intracellular signalling pathways and thus promote a cascade of phenotypic changes leading to migration, invasion, survival and proliferation of these endothelial cells.



(Weis and Cheresh, 2011)

Figure 12. Regulators of endothelial activation and tumour angiogenic response. FN, fibronectin; Col, collagen; VN, vitronectin; LN, laminin; TUMs, tumstatin; END, endostatin; CANS, canstatin.

1.12 Abnormal vessel maturation in pathological angiogenesis

During physiological vessel maturation, alterations in blood flow, metabolic requirements, and growth factor secretion results in vessel remodelling. Some vessels follow the process of maturation through the secretion of extracellular matrix (ECM) by endothelial and encompassing stromal cells ensued by recruitment of pericytes and vascular smooth muscle cells to the growing vessels (Hellstrom et al., 2001). Pericytes and vascular smooth muscle cells on their surfaces express Angiopoietin-1 (Ang-1) that subsequently binds to Tie-2 receptors on the surface of endothelial cells. This close interaction between endothelial cells and mural cells (vascular smooth muscle cells and pericytes) results in the activation of TGF-

β, and consequent stimulation of basement membrane production (Holderfield and Hughes, 2008). The basement membrane is a specialized ECM construction made of sheet-like matrices that are closely attached to the cells and one of its major roles is to provide structural support for the nascent vessels (Gustafsson and Fassler, 2000). Whereas under normal physiological circumstances, interaction between Ang-1and its receptor stabilizes the integrity of vessel junctions, secretion of high levels of Ang-2 by sprouting endothelial cells, compete for binding to Tie-2, leading to destabilization of pre-existing vessels (Adams and Alitalo, 2007; Eklund and Olsen, 2006).

In a healthy tissue, mural pericytes with their stellate-like appearance and their unique ability to encompass the endothelium play a crucial role in sustaining a mechanical backbone for delicate endothelial channels. Pericytes are basically located around EC junctions and by forming umbrella-like constructions cover the gaps between ECs. Pericytes and their adjacent ECs are implanted within the same basement membrane and thus establish tight connections between each other. Additionally, among the most important functions of the basememt membrane, it is noteworthy to mention its crucial role in selective transport of components and cells and storage of angiogenic factors (Diaz-Flores et al., 2009; Eble and Niland, 2009). The basement membrane of the tumour vessel is deprived of tight connections with endothelial cells, is made of various irrelevant layers and has an abnormal thickness. As a result of constant tumour vessel remodelling, it is highly probable that under such a condition, endothelial vessels are no longer encompassed by a basement membrane. Pericytes as shields of endothelial cells secrete low levels of endothelial survival factors, such as VEGF and are eventually responsible for blood flow irregularities. Indeed, pericyte coverage of tumour vasculature is much less than normal tissues and is highly variable between tumours and even in the same tumour. In addition, since mural cells prevent breaching of the vessel wall by the intravasation of cancer cells, it seems reasonable that tumour cells spread through pericyte-deficient vessels is highly facilitated.

In a large number of human diseases and tumours, abnormal vessels are considered as one of the main characteristics of the malignancy. Indeed, an abnormal vasculature is now considered a hallmark of solid tumours (Jain, 2002; Jain et al., 2002). In other words, from the physiological point of view, the basic differences between the normal and tumour tissues originate from the tumour vasculature. Vascular network in tumours is composed of two categories of vessels: the pre-existing vessels in normal tissues into which the tumour has invaded; and tumour microvessels born through the process of neovascularization as a

consequence of increased expression of pro-angiogenic factors. In this respect, the two kinds of vessels form different structural and physiological abnormalities (Brown and Giaccia, 1998). These abnormalities in concert with the compression of blood vessels by cancer cells (Padera et al., 2004) eventually impair blood supply to the tumour. As a consequence, blood flow is often sluggish or delayed and highly irregular. In addition, the vessels are exceedingly permeable, if compared to those in healthy tissues. The oxygen diffusion distance in solid tumours can be nearly 100-150 µm (Dewhirst et al., 1994). Accordingly, tumour cells away from the effective oxygen diffusion distance may be exposed to low oxygen tensions, thus affected by hypoxia. Similarly, in such a hypoxic area, anticancer chemotherapeutics could not efficiently reach tumour masses at toxic concentrations (Durand, 1994).

The clinical and pathological relevance of vessel abnormalization is further underscored by the fact that through up-regulation of angiogenic factors by cancer and stromal cells, which in turn further supports vessel disorganization, hypoxia stimulates non-productive angiogenesis in a constant self-reinforcing circuit. Indeed, abnormal tumour vasculature might disrupt the function of immune cells in tumours. As a consequence, abnormal tumour vasculature can cause tumour resistance to radiation therapy and a wide range of chemotherapeutics.

In this respect, traditional anti-angiogenic-based therapies focus on inhibiting the formation of new blood vessels and/ or to demolish existing ones to starve the tumour (Folkman, 1971). Originally foreseen as a promising therapeutic approach to treat a broad variety of solid tumours, anti-angiogenic compounds have met a significant disappointment when translated to the clinical practice. Much of this setback is possibly due the aberrant organization of tumour vasculature and the intrinsic tumourigenic potential of tumour-associated hypoxia. In this respect, normalizing tumour vasculature improves tumour vessel perfusion and oxygenation, which consequently leads to reduced metastasis rate and more efficient chemo and immune therapy approaches (Hamzah et al., 2008; Mazzone et al., 2009; Stockmann et al., 2008). In fact, preclinical evidence has demonstrated that normalization of vascular abnormalities might be considered as a promising therapeutic paradigm for cancer and other vascular diseases affecting more than half a billion people worldwide.

1.13 Anti-angiogenic therapies

As mentioned before, VEGF is an essential mediator of tumour-associated angiogenesis. Concomitantly, in 1971, anti-angiogenic therapy was proposed as a tactic to

treat solid tumours. Fourteen years after the cloning of VEGF-A in 1989, the first VEGF-targeted agent, the anti-VEGF monoclonal antibody bevacizumab (Avastin, Genentech) as a VEGF-neutralizing antibody showed clinical benefits in metastatic colorectal cancer patients when combined with chemotherapy (Figure 13). Nevertheless, in certain cancers, the clinical use of VEGF blockers such as VEGF receptor tyrosine kinase inhibitors which block the signaling of pathways such as VEGF, are only effective when exploited as monotherapy, whereas they might be toxic in combination with chemotherapy (Jain et al., 2006; Jain et al., 2009).

VEGF-targeted therapy might limit the permeability of the vessels induced by VEGF, constrict dilated ones and as a result improve blood flow.

To date, two main obstacles currently impede non-surgical treatment of solid tumours. First of all, tumour physiological barriers hinder the delivery of therapeutics at effective concentration to all cancer cells (Jain, 1998). Moreover, acquired drug resistance originating from genetic and epigenetic mechanisms decreases the effectiveness of available drugs (Browder et al., 2000; Klement et al., 2000). Anti-angiogenic therapy has the ability to overcome these problems or at least decrease their impact. This specific sort of therapy targets the tumour vessel network by affecting endothelial cells. In fact, a majority of cancer cells for their growth and survival depend on a small number of residual or circulating endothelial cells. Blocking VEGF or its receptor VEGFR2 results in the apoptosis of endothelial cells and as a consequence, a reduction in vessel diameter, density and permeability (Kadambi et al., 2001; Tsuzuki et al., 2000; Yuan et al., 1996).

However, anti-angiogenic therapy seems more challenging than anticipated. Many patients with metastatic cancer are refractory or develop resistance to VEGF inhibitors. Additionally, whether anti-angiogenic therapy can cause tumour cells to become more malignant has yet to be further elucidated (Ebos et al., 2009; Paez-Ribes et al., 2009). Indeed, various anti-angiogenic techniques seem to be more effective in preclinical than clinical settings.

As mentioned before, a fraction of cancer patients are refractory to VEGF-inhibitor therapy (Bergers and Hanahan, 2008). The extent of resistance differs from one cancer to another, varies between micro and macrometastatic state, and also depends on the type of VEGF blocker exploited. Patients can be either intrinsically refractory or develop resistance during the process of treatment. In this regard, several mechanisms contributing to changes in tumour cells, endothelial cells or other stromal cells have been speculated to explain the

occurrence of resistance in both cases (Bergers and Hanahan, 2008; Carmeliet, 2005; Jain et al., 2006; Jain et al., 2009). At a more advanced stage, due to the production of other pro-angiogenic factors, tumour angiogenesis might become VEGF independent and consequently respond poorly to anti-VEGF treatment.

By normalizing tumour vasculature (before its elimination), low-dose anti-angiogenic therapy improves the delivery of drugs and oxygen to the tumour. Whereas anti-angiogenic therapy alone can prevent tumour growth, a large body of evidence has demonstrated that this sort of therapy along with radiation and chemotherapy results in long term cures in mice (Browder et al., 2000; Klement et al., 2000). Since both radiation therapy and chemotherapy strictly rely on sufficient blood flow to the tumour to transport oxygen and drugs respectively, normalization of the tumour vessels through anti-angiogenic therapy can be considered as a putative explanation why standard anti-angiogenic therapies give rise to a better outcome when combined with radio/chemotherapy. In recent years, some challenging questions have been raised in the field of anti-angiogenic cancer therapy: in order to achieve tumour shrinkage and starve primary tumours from oxygen, should tumour vessels disruption be the focus of therapy, or instead, should they be normalized to improve responses to routine anticancer therapies? Based on the fact that each individual approach has its own advantages, one novel possibility is to combine these two strategies to complement current anti-angiogenic therapeutic policies. Moreover, it has been previously demonstrated that in a mouse model of pancreatic islet tumourigenesis, inhibition of major VEGF-A receptors (VEGFR1 and VEGFR2) using monoclonal antibodies could initially block angiogenesis and tumour growth (Casanovas et al., 2005). However, due to the resistance to anti-VEGF receptor therapy, successive tumour progression accompanied by revascularization of the tumour was observed. This resistance was then characterized by the compensatory upregulation of VEGF-A, FGF-1, FGF-2, FGF-7, and FGF-8, ephrin-A1, and Ang-2.

Despite significant investments and several small molecule inhibitors of angiogenesis currently subjected to clinical trials (Ivy et al., 2009), bevacizumab, as an anti-VEGF anti-angiogenic antibody is now used as a first line treatment only along with conventional chemotherapy regimens for treatment of metastatic colorectal cancer. Many retrospective studies now suggest that antineoplastic effects of this drug might be due to normalization of abnormal tumour vasculature, reduced intratumoral hydrostatic pressure along with increased vessel permeability, which allows more effective drug delivery to the tumour(Ferrara et al., 2004; Jain, 2001; Willett et al., 2004). Interestingly, since radiotherapy and certain

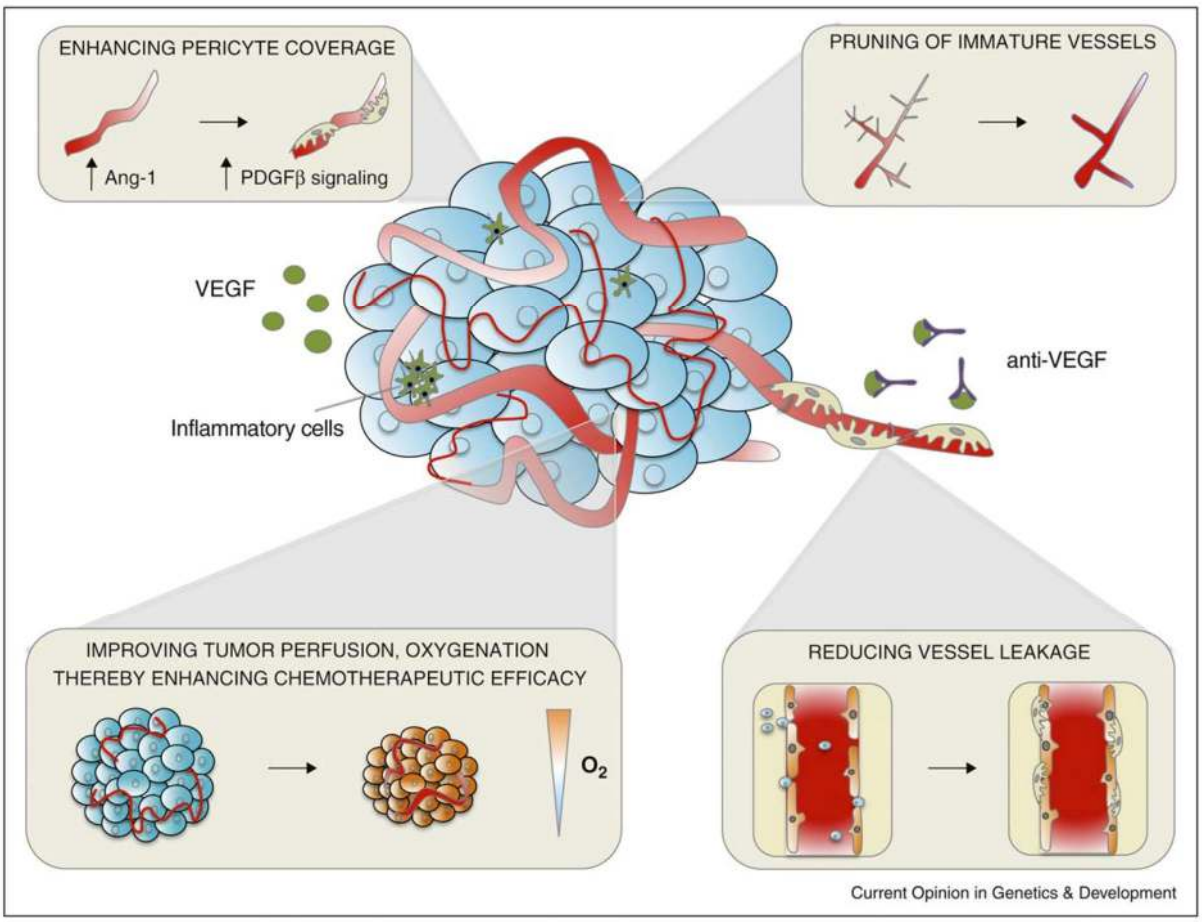
chemotherapy approaches strictly rely on the formation of reactive oxidative species to hit tumour cells, tumour hypoxia could reduce the efficacy of conventional anticancer treatments (Moeller et al., 2007).

On the other hand, induction of hypoxia through vessel relapse after VEGF blockade can trigger a more invasive and metastatic pattern, while in other cases, tumour stem cells by acquiring extra mutations can become hypoxia-tolerant. Although HIF-1 can be considered as an important therapeutic target, there are other multiple pathways responsive to hypoxia. Moreover, *in vivo* studies hypothesize that alternative angiogenic pathways might be activated when a single factor such as HIF-1 is inhibited. Another important influence of hypoxia is to induce radio/chemotherapy resistance in solid tumours. In fact, under hypoxic conditions, by activating HIF-1 mediated pathway, tumour cells have demonstrated an increased chemoresistance (Comerford et al., 2002) and radioresistance (Moeller and Dewhirst, 2006). On the other hand, it has been also demonstrated that chronic hypoxia can increase radiosensitivity due to a decreased homologous recombination capacity and DNA double-strand break repair in hypoxic tumour cells (Chan et al., 2008). In this regard, overexpression of the ATP-binding cassette (ABC) transporters family members in cancer tissues have been shown to pump anticancer drugs out of the cancer cells and thus to be responsible for chemoresistance to a wide range of these reagents. Indeed, HIF-1α has been described as an ABC transporter genes transcription regulator in tumour cells (Gottesman et al., 2002; Park et al., 2006). On the other side of the coin, hypoxic tumour cells secrete some amounts of VEGF, which is subsequently associated with progression of angiogenesis, increase in paracrine/autocrine growth factor secretion, tumour growth, and increase in the rate of metastasis. It has been also shown that $VEGF_{165}$ –transfected xenografts show highly vascularized tumours with an accelerated growth rate and enhanced chemoresistance (Zhang et al., 2006).

Altogether, these observations highlight the diversity and complexity of the tumour angiogenic response. As a consequence, better understanding of the full range of either HIF-dependent or HIF-independent angiogenic mechanisms seems a basic necessity to be able to design optimal combinations of anti-angiogenic agents. In other words, knowing how anti-angiogenic therapeutics act *in vivo* and what types of drugs can be combined to result in better outcome in certain patients are crucial steps in achieving successful anti-angiogenic strategies. Beside this, tumour vessel normalization can play a critical role in increasing the efficacy of anticancer therapies by ameliorating the oxygenation of the tumour and subsequent

modulation of the secretion of various cytokines and growth factors to the site of malignancy. Accumulative body of evidence shows that tumour vessels are normalized after anti-VEGF therapy, resulting in fewer but more efficient vessels with proper pericyte coverage, which supports better drug delivery to the tissue (Dickson et al., 2007; Inai et al., 2004; Mancuso et al., 2006). In other words, anti-angiogenic therapies affecting both endothelial proliferation and vessel stabilization have been shown to be more effective on tumour progression than only targeting the VEGF signalling (Bergers et al., 2003). Despite the fact that prolonged anti-VEGF therapy can eventually demolish most of the vessels, tumours can shift towards alternative pathways of angiogenesis and become resistant to VEGF blockade. Consequently, an interesting area of research at present can be to answer the question whether and how the normalization of vessels by anti-VEGF therapy can be sustained to achieve a prolonged benefit.

Therefore, anti-angiogenic therapy requires especial consideration of signalling components and receptor crosstalks among a wide range of cell types, considering the fact that tumour cells can persist growing in VEGF-independent manner (Shojaei et al., 2007b) by utilizing alternative pro-angiogenic signallings. These complicated interactions play a crucial role in angiogenic remodeling and can be involved in *de novo* or acquired resistance. To achieve a successful anti-angiogenic strategy, it is of high importance to consider these signaling effects in the context of various cell types involved in the continually altering tumour microenvironemt. These therapeutic responses can be extended to the suppression of endothelial progenitor cells infiltration and damages of cancer stem cells (CSCs) niche located in the proximity of tumour vasculature. It is also expected that anti-angiogenic therapies provide better prognosis by indirect disruption of cancer stem cells (Gilbertson and Rich, 2007).



(De Bock et al., 2011)

Figure 13. Anti-VEGF therapy acts as a normalizer for tumour vessels. Anti-VEGF treatment prunes the immature blood vessels and thus stabilizes the persisting vessels by enhancing their pericyte coverage through the induction of Ang-1 and PDGFRβ signalling. Accordingly, neutralization of VEGF reduces tumour vessel permeability and ameliorates tumour perfusion and oxygenation, which consequently results in a better penetration and higher efficacy of chemoterapeutics.

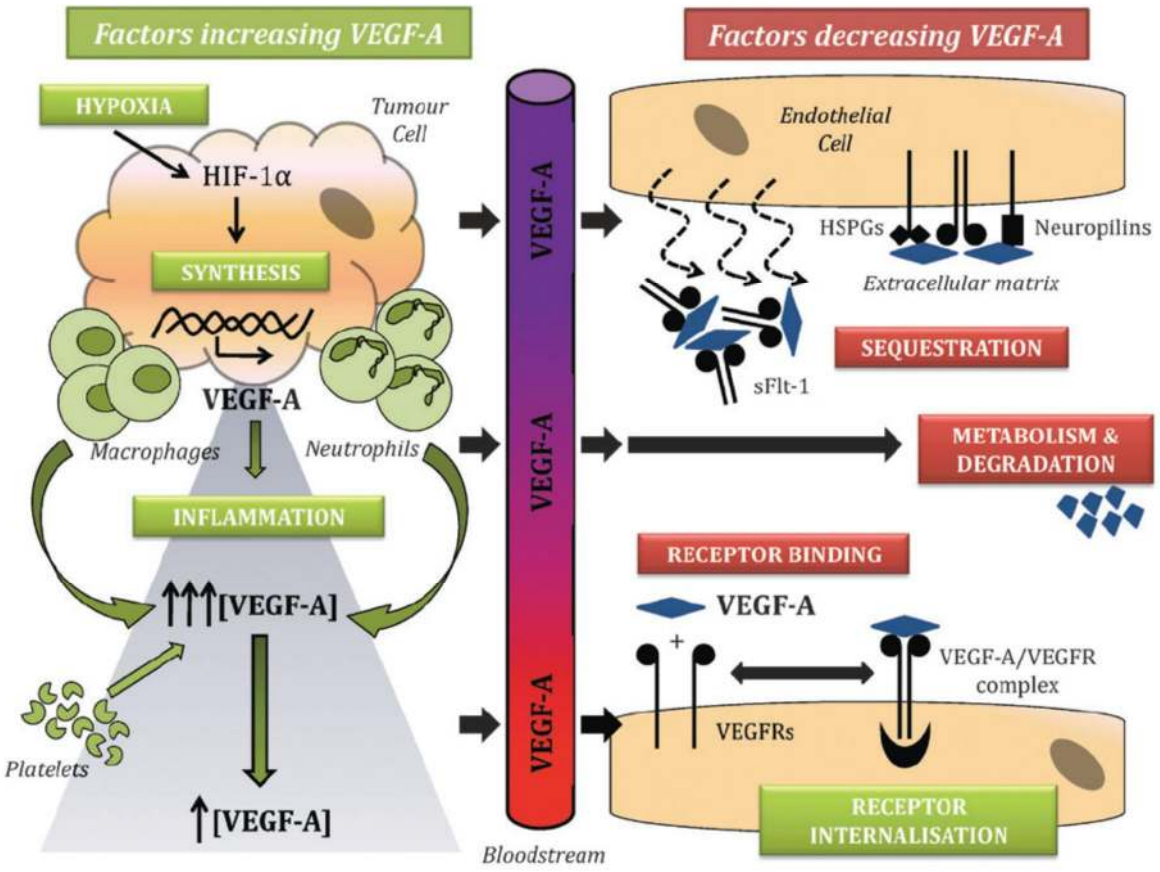
1.14 Role of hypoxia in physiological and pathological angiogenesis

Hypoxia is among the most important physiological stimuli to induce angiogenesis and, consistently, a substantial component of tumour progression. Hypoxia is generally defined as a reduction in the ambient oxygen concentration. During the process of hypoxia-induced tumour progression, cancer cells can establish an increased potential for invasive growth as well as regional and distant metastasis (Vaupel and Mayer, 2007; Vaupel et al., 2004). Indeed, hypoxia is established in the most interior regions of solid tumours due to abnormal vasculature and irregular intratumour blood flow (Yasuda, 2008).

In ischaemic conditions, tissue perfusion is reduced in such a way that oxygen availability is no longer sufficient to fulfil tissue metabolic requirements. In fact, hypoxia is a well-known regulator of many aspects of cell biology (Simon and Keith, 2008). Cells respond

to such environmental stress by activating several regulatory pathways. Expectedly, expression of almost all crucial angiogenic factors, including vascular endothelial growth factor (VEGF) is induced by hypoxia, as improving blood supply is the primary response to inefficient oxygen delivery (Figure14). Most of the effects of the hypoxia are associated with the transcriptional activity of hypoxia inducible factor-1 (HIF-1). HIF-1 mediates a transcriptional response in a cell type specific manner involving an organized expression of growth factors by diverse cell types within the hypoxic tissue. On the other hand, beside hypoxia, other pathways can also increase the expression of HIF-1α in tumours under normoxic conditions (so-called hypoxic mimicry conditions). Production of vascular endothelial growth factor (VEGF) in tumour cells is regulated by the activated HIF-1α regulated system. Increased levels of VEGF subsequently induce the accumulation of HIF-1α and promote tumour metastasis through angiogenesis. In fact, intratumoural hypoxia, as a frequent observation in metastatic cancers results in the activation of Hypoxia-inducible factors. HIFs are involved in different steps of metastatization, including metastatic niche formation through increasing the expression of various enzymes implicated in remodelling collagen at the metastatic site and recruiting bone marrow-derived cells (BMDCs) to the metastatic niche. In the case of breast cancer patients, overexpression of HIF-1α is associated with an increased risk of metastasis and mortality and may benefit from HIF-inhibitor therapy (Wong et al., 2012).

Additionally, it has been found that loss of HIF-1α in endothelial cells decreases nitric oxide (NO) synthesis, delays tumour cell migration through endothelial layers, and reduces tumour cell metastasis whereas loss of HIF-2α has opposite effects (Branco-Price et al., 2012). These contradictory effects stem from differential regulation of NO homeostasis that subsequently modulates VEGF expression in an NO-dependent response loop, but still highlight the pivotal role of HIF-mediated transcriptional program for the regulation of tumour angiogenesis.



(Latham et al., 2010)

Figure 14. Factors regulating the blood concentration of VEGF-A. VEGF-A is produced and secreted by inflammatory cells of the tumour microenvironment under hypoxic stress. This results in the establishment of a VEGF-A concentration gradient in the blood. Excess VEGF-A can be metabolized and eliminated as nitrogenous waste. However, VEGF-A is also removed from the bloodstream through binding to endothelial receptors (VEGFR1 and 2) and co-receptors (HSPGs, neuropilins) or sequestered within the bloodstream by the soluble form of VEGFR1, sFlt-1.

2. AIM OF THE STUDY

The target of this research was primarily to unravel the roles of the two major expressed isoforms of VEGF-A (termed as VEGF-A_{165} and VEGF-A_{121}) in human tumour angiogenesis, development and prognosis. Previous findings of the Molecular Medicine laboratory have demonstrated that these two VEGF-A splicing isoforms have divergent effects in mouse models of both physiological and pathological angiogenesis. In particular, despite a comparable capacity to activate the local endothelium, only VEGF-A_{165} successfully elicited full maturation of newly-formed blood vessels. Indeed, VEGF-A_{165} overexpression resulted in improved vessel architecture and function whereas VEGF-A_{121} primarily stimulated mere capillary sprouting. This difference is particularly relevant during tumour angiogenesis, as in such a context abnormal vascularization is favourable and leads to accelerated tumour growth and increased invasiveness. Thus, using tissue specimens of colorectal carcinoma (CRC) as a pathological model of angiogenesis, the initial idea was to assess whether human malignancies display a preferential expression of one of these isoform and to correlate it with the outcome of CRC in a predefined and statistically representative set of the patients. The expression levels of the two isoforms in the tumour mass were compared with levels in a matched, non-malignant tissue sample. The VEGF-A_{165} /VEGF-A_{121} ratio was then correlated with the clinical outcome and with patient prognosis.

Moreover, biological diversity of VEGF isoforms intriguingly is correlated with their differential ability to recruit a peculiar myeloid sub-population of bone marrow-derived myeloid cells (subsequently characterized as NEMs), which in fact exert a potent paracrine activity on pericytes and smooth muscle cells, by promoting the coverage and consequent stabilization of neoformed blood vessels. When mice skeletal muscles were pre-conditioned with $VEGF_{165}$ as one of the recruiters of NEMs, challenging the same location with malignant cells resulted in two chronological predominant outcomes: first, the significant infiltration of Nrp-1^+/$CD11b^+$ BM-derived cells to the site of tumour angiogenesis, and second, the indicative prevention of tumour growth through its vascular network normalization. In other words, when recruited to the tumour microenvironment, an outstanding role of this unique Nrp-1^+/$CD11b^+$ myeloid cells is to contribute to the maturation of tumour vasculature. Recruitment or ectopic administration of NEMs in mouse tumours results in decreased tumour growth, possibly as a final outcome of normalization of tumour vasculature. We thus also explored the possibility that human tumours expressing abundant levels of $VEGF_{165}$

indeed exhibited improved clinical outcome and NEM infiltration. In this respect, another novel aspect was to evaluate the existence of NEMs at the sites of human tumours expressing abundant levels of $VEGF_{165}$.

Subsequently, we investigated the expression of Semaphorin-3A (Sema3A) in human cancers, as it has been described as a potent NEM recruiter, similarly to $VEGF_{165}$, but differing for the absence of any angiogenic activity.

3. MATERIALS AND METHODS

3.1 Patients recruitment

Consecutive patients scheduled to undergo surgical resection of tumour mass at the Department of Surgery of the Azienda Ospedaliero-Universitaria 'Ospedali Riuniti di Trieste' of Trieste, Italy were recruited for this study, with no specific criteria for exclusion. All patients were enrolled according to protocols approved by the Ethical Committee of the Azienda Ospedali- ero-Universitaria 'Ospedali Riuniti di Trieste', after written informed consent was obtained. The tumour mass was entirely removed and samples were frozen at –80°C. Apparently healthy mucosa samples were harvested for each patient, at least 10 cm away from the tumour mass.

For each patient, tumour paraffin inclusions were sectioned and analysed by an independent group of pathologists. Tumour diagnosis and staging were determined according to commonly accepted criteria, including TNM and Dukes' classification, UICC staging, grade, vascular and lymphatic extra-parietal invasion, as routinely determined in all patients with colorectal cancer.

3.2 RNA Extraction Procedure

Total RNA from the CRC biopsy specimens was extracted using TRIzol reagent (Invitrogen) according to manufacturer's instructions. RNA extraction was then followed by DNase treatment using DNaseI (Roche) to remove DNA contamination. The isolated RNA was then subjected to the synthesis of first-strand cDNA using hexameric random primers (Invitrogen). Reverse transcription was carried out using Moloney murine leukaemia virus reverse transcriptase (MLV RT). The cDNA was then used as a template for PCR amplification to detect the expression level of $VEGF_{165}$ and $VEGF_{121}$.

3.3 Relative quantification of VEGF splicing isoforms

VEGF-A_{165} and VEGF-A_{121} relative abundance was quantified through semi-quantitative PCR. A set of primers matching exon 3 and exon 8 (thus able to amplify all VEGF isoforms) was employed. Primers sequences: CAG CAC AAC AAA TGT GAA TGC (Forward primer) and GAG GCT CCT TCC TCC TGC (Reverse primer). Thermal cycling conditions were 94°C for 5 min, followed by 40 cycles of 94°C for 15 sec, 65°C for 15 sec,

and 72°C for 15 sec. The obtained amplicons were then separated using polyacrylamide gel electrophoresis and visualized by ethidium-bromide staining. Band intensity was measured using VersaDoc imaging system (BioRad) as volume intensity/area (mm^2). Values were normalized against background signal. When either mucosa or tumour sample could not be visualized, corresponding patient was discarded from the study.

3.4 Quantification of total VEGF-A and Sema3A mRNA

To assess the abundance of VEGF-A and Sema3A transcripts in human tumours and matching mucosae, cDNA was amplified by Real-Time PCR (BioRad), using a human specific TaqMan assay (Applied Biosystems). Following assays were used: Hs00173626_m1 (for VEGF-A) and Hs00173810_m1 (for Sema3A). The reaction was performed according to manufacturer's instructions. GAPDH transcript abundance was similarly assessed using a human GAPDH-specific TaqMan assay (Applied Biosystems) and utilized as a house-keeping gene to normalize expression values.

3.5 Histology

Samples for histological evaluation were formalin-fixed and paraffin-embedded for routine post-surgical analysis at the Department of Anatomo-Pathology at Cattinara Hospital, in Trieste. A small subset of samples was snap-frozen in liquid nitrogen, as indicated in the text.

For immunohistochemistry, 5-µm sections were hydrated and different antigen retrieval methodologies were performed as listed in Table 2 in the chapter of Results. The following anti-Nrp-1 antibodies were tried at different dilutions and different incubation conditions (clone EPR3113, ABCAM; clone EPR3113, EPITOMICS; Immunogen NS0-derived rh Neuropilin-1, R&D). After treatment, slides were rinsed in PBS and signals were developed using 3, 3'-diaminobenzidine as a substrate for peroxidase chromogenic reaction [DAB Plus Substrate System (Thermo Scientific)].

For vessel immunofluorescence, 5-µm paraffin-embedded sections were stained with the following antibodies were used: anti-vWF (Polyclonal Rabbit Anti-Human Von Willebrand Factor, DakoCytomation), Cy3-conjugated anti-α-SMA (clone 1A4, Sigma), diluted 1:100 in PBS + 2% BSA. Antigen retrieval was performed using citrate buffer. Nuclei were counterstained with DAPI. Images were acquired using a Leica MLB upright fluorescence microscope (Leica Microsystems, Wetzler, Germany) equipped with a Coolsnap

CF CCD camera (Roper Scientific, Evry, France). Signals were quantified by the use of ImageJ software.

For NEM detection, 4-µm frozen sections were air dried and post-fixed with Zinc solution (BD Pharmingen). The following antibodies were diluted 1:100 in PBS supplemented with 2% BSA: anti-Nrp-1 (clone EPR3113, ABCAM) and anti-CD11b (clone M1/70, BD Pharmingen). Nuclei were counterstained with DAPI. Images were acquired using a Leica MLB upright fluorescence microscope (Leica Microsystems, Wetzler, Germany) equipped with a Coolsnap CF CCD camera (Roper Scientific, Evry, France).

3.6 Cell culture and treatments

HUVEC (human endothelial cell line) were purchased from Clonetics (San Diego, CA) and cultured in their own medium, provided by the manufacturer. 4T1 (mouse breast cancer cell line), T241 (mouse fibrosarcoma cell line) and B16.F10 (mouse melanoma cell line) were all purchased from ATCC (Rockville, MD) and cultured in RPMI medium 1640 (4T1) or Dulbecco's modified Eagle's medium (T241 and B16.F10), both supplemented with 10% Foetal Bovine Serum.

$5x10^5$ cells were seeded onto 60-mm tissue culture dish and, upon reaching 70-80% confluency, were submitted to environmental stress.

Hypoxic conditioning was achieved through incubation in a dedicated hypoxic chamber for the indicated periods of time. An anoxic chamber and an O_2-controlled hypoxic incubator (Ruskinn INVIVO2 200) were employed.

3.7 HIF-1α quantification

RNA was extracted from cell cultures using Trizol Reagent (Invitrogen) according to manufacturer instructions. cDNA was obtained through retro-transcription as described above and utilized as template for Real-Time PCR. HIF-1a expression was quantified using SYBR Green technology employing the following primer set: GAA TGC TCA GAG GAA GCG AAA (Forward primer) and ACA GTC ACC TGG TTG CTG CA (Reverse primer). Data were normalized against β-actin gene expression.

3.8. Statistical analysis

One-way ANOVA and Benferroni/Dunn's post-hoc test was used to compare multiple groups. Pair-wise comparison between groups was performed using the Student's t test. $P<0.05$ was considered statistically significant. Graphs report ± SEM in each column.

4. RESULTS

4.1 Sample collection from colorectal cancer patients

In this study, colorectal carcinoma (CRC) was considered as a human model of pathological angiogenesis. Biopsies of tumour and adjacent healthy mucosa were harvested from 78 colorectal cancer patients scheduled to undergo surgical resection of the primary tumour mass. This cohort of patients was homogeneous for sex and tumour location, and included mostly patients with advanced malignancy (Stage III and IV). For a detailed description of the cohort of enrolled patients, see Table 1 (Appendix).

Collected samples were then stored in TRIzol at -80°C until RNA extraction was performed. Notably, among these analysed specimens, for a small set of CRC patients (n=19), an additional biopsy corresponding to the peripheral zone of the primary tumour was collected. To avoid any contamination at the moment of surgical resection, mucosa was harvested at least 10 cm far from the core lesion, reasonably allowing to consider it as a normal, "healthy" mucosa (Figure 1).

Next, by employing the competitive PCR method, all known VEGF-A isoforms were amplified, using primers designed to amplify all splicing variants (annealing at the level of two exons, 4 and 8, always included in the final transcript, as shown in Figure 2). By measuring the intensity of the corresponding bands (volume intensity/mm^2) on ethidium bromide-stained polyacrylamide gel, the relative abundance of the two mainly expressed splicing isoforms of VEGF-A, i.e. VEGF-A_{165} and VEGF-A_{121}, was then assessed. In practice, the intensity of the bands corresponding to these two isoforms was separately measured, subtracted from the background intensity, and then used for further calculations. In this way, the ratio between VEGF-A_{165} and VEGF-A_{121} was calculated in both mucosa and tumour of 78 CRC patients. Such a balance is hereafter indicated as the VEGF-A_{165}/VEGF-A_{121} ratio. Importantly, this technique permits to specifically investigate the relative abundance of different VEGF-A isoforms in the same sample. Absolute quantification of mRNA levels was not considered in these settings, since the objective of this study was to specifically correlate the ratio between the two isoforms, and not their absolute levels, with a series of clinical and histological parameters. In this respect, the VEGF-A_{165}/VEGF-A_{121} ratio might represent a very informative parameter to understand if the expression of one isoform or another is favourable in certain clinical settings. Indeed, as extensively discussed in the "Introduction", cell transformation and microenvironmental stimuli can alter isoform usage to

sustain tumour progression.

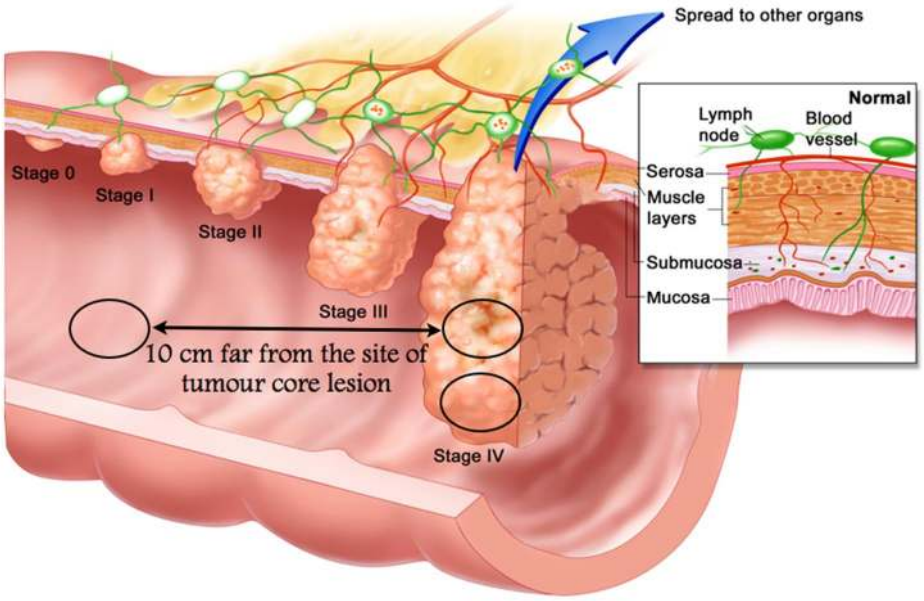


Fig. 1. Sample collection. In a set of colorectal cancer patients, biopsy specimens from the core of the lesion and adjacent healthy mucosa were collected at the moment of surgical resection and processed for analyses. In a small category of the CRC patients, additional biopsies from the periphery of the lesions were harvested to assess the difference between the expression level of $VEGF_{165}$ and $VEGF_{121}$ isoforms corresponding to the location of the malignancy.

Splice variant	Amplicon length
VEGF206	352 bp
VEGF189	301 bp
VEGF 183	283 bp
VEGF165	229 bp
VEGF148	194 bp
VEGF121	97 bp

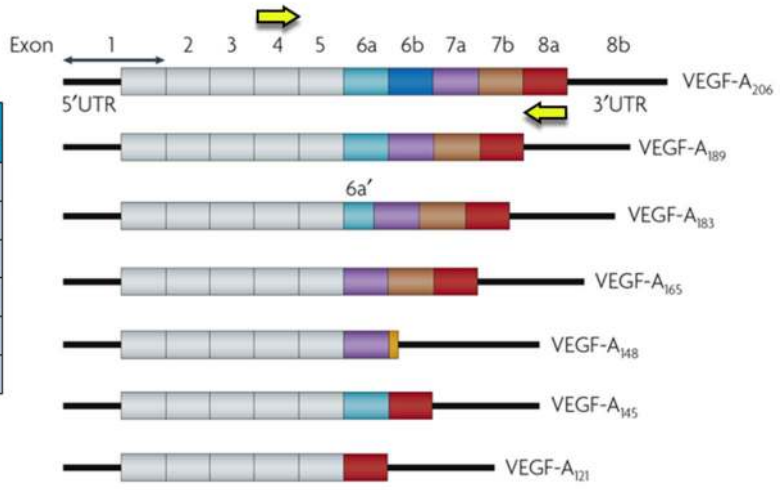


Fig. 2. Primer design. A primer pair was designed on exon 4 (Forward primer) and exon 8 (Reverse primer) in order to amplify all splicing isoforms of VEGF-A with pro-angiogenic effects. Localization in respect to VEGF gene structure is schematically shown in the Figure. The length of the resulting PCR amplicons has been reported in the adjacent table.

4.2 Evaluation of primer efficiency

Competitive PCR is a well-established and widely used technique for semi-quantitative analysis (Zentilin and Giacca, 2007). It allows precise and reproducible measurements, but its accuracy is strongly affected by primers specificity: in this respect, it is crucial that primers amplify both competitors with the same efficiency (Zentilin and Giacca, 2007). In the specific case of VEGF isoform amplification, the two primers perfectly match to all isoforms, and thus these act as internal competitors for the reaction.

The efficacy of PCR primers was assessed by amplifying PCR serial dilutions of a mixture containing equimolar concentrations of two plasmids carrying either $VEGF_{165}$ or $VEGF\text{-}A_{121}$ cDNA (pAAV- $VEGF_{165}$ and pAAV- $VEGF\text{-}A_{121}$, respectively – previously utilized in the Molecular Medicine Laboratory). Gel analysis confirmed that our primer set was indeed able to amplify either isoform with the same efficiency, as clearly shown in Figure 3, where both bands are equally intense for any of the tested reactions.

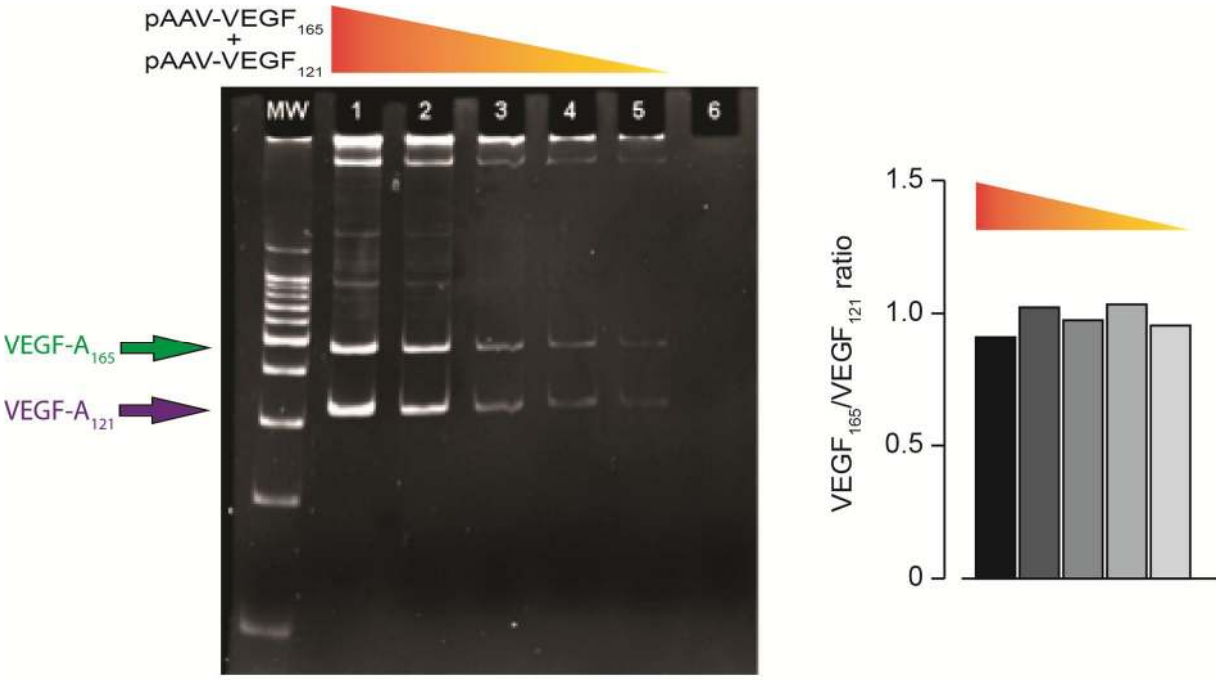


Fig. 3. Primers efficiency assessment. Electrophoresis on polyacrylamide gel demonstrates the equivalent efficacy of the designed primers to amplify two different isoforms of VEGF-A by PCR. Serial dilutions of a mixture, containing equimolar concentrations of two plasmids carrying either $VEGF_{165}$ or $VEGF_{121}$ cDNA were used as templates for a series of PCR reactions. The relevant PCR products were then loaded in adjacent wells (1-5). Bands corresponding to $VEGF_{165}$ and $VEGF_{121}$ display equal intensity in all tested PCR conditions. As a negative control, dH_2O was used as a template for this reaction (line 6). 100 bp molecular weight was loaded in the first well. Quantifications are shown in the graph on the right.

4.3 Robustness of the band intensity quantification

In order to evaluate the accuracy and reproducibility of technique exploited for quantification of VEGF-A isoforms, in a randomly selected subset of samples, VEGF-A_{165}/VEGF-A_{121} ratio was repetitively measured 3 to 4 times per each and the mean of these values was calculated. As shown in the Figure 4, the vast majority of the measurements displayed only minimal variations, almost entirely encompassed in a 25% confidence interval (C.I.).

In keeping with these data, the above described methodology was considered as a robust and reliable procedure for VEGF-A isoform quantification and further applied to the whole sample set to assess the VEGF-A_{165}/VEGF-A_{121} ratio.

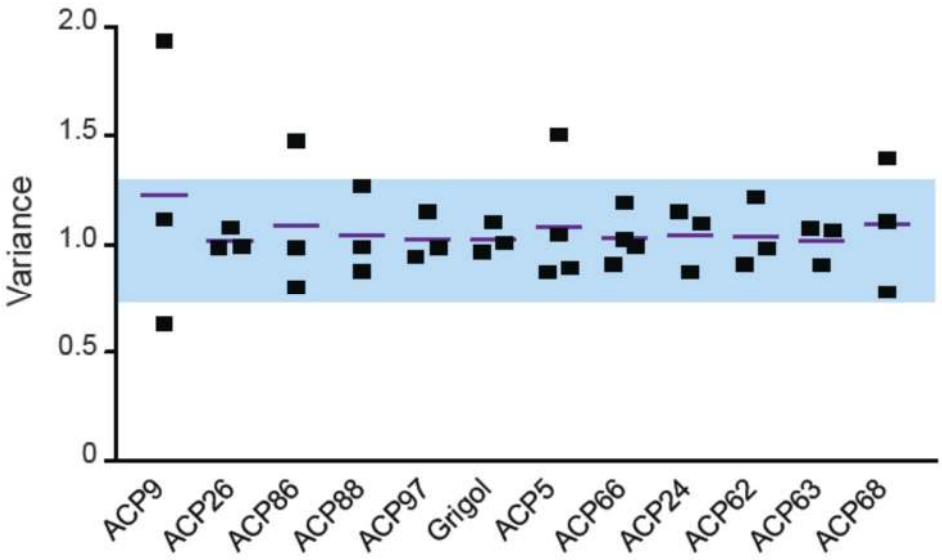


Fig. 4. Robustness of VEGF splicing analysis. Twelve randomly selected tumour samples were subjected to repeated quantification of $VEGF_{165}$/ $VEGF_{121}$ ratio. After RNA extraction and consequent PCR amplification of the two isoforms, $VEGF_{165}$/ $VEGF_{121}$ ratio was measured, and this methodology was repeated up to three to four times for each individual sample and loaded on different polyacrylamide gels. The mean value of all the measurements for each sample was calculated. Variance of $VEGF_{165}$/ $VEGF_{121}$ ratio from mean value was evaluated for each measurement and plotted as squared dots in the graph above.

4.4 $VEGF_{121}$ is the most abundant VEGF-A isoform in both healthy mucosa and tumour samples

As the first novel observation, and in disagreement with published data (Ferrara, 2010), it was unveiled that $VEGF_{121}$ was the most abundant isoform expressed in CRC specimens. In fact, after PCR amplification and gel-electrophoresis, the $VEGF_{121}$-related amplicon was constantly found to be more abundant than that corresponding to $VEGF_{165}$ (Figure 5).

Graphical representation of PCR band quantification is shown in Figure 6, where average intensity of either $VEGF_{121}$- or $VEGF_{165}$-related bands are plotted. The graph clearly shows that $VEGF_{121}$ is significantly more expressed than $VEGF_{165}$, both in mucosa (mean= 33037 vs. 14945 Int/ mm^2) and tumour (mean= 35628 vs. 17904 Int/ mm^2).

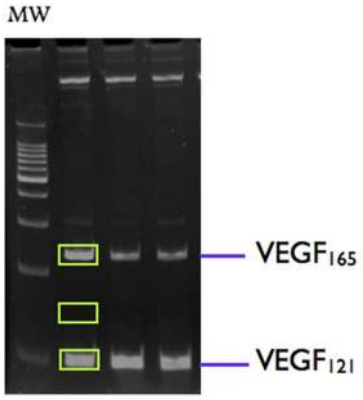


Fig. 5. Representative image of PCR-amplified VEGF isoforms. Band intensity (coloured rectangles) was evaluated and normalized for background value. $VEGF_{121}$ (lower bands) is invariably the most abundant isoform expressed in colorectal cancer specimens.

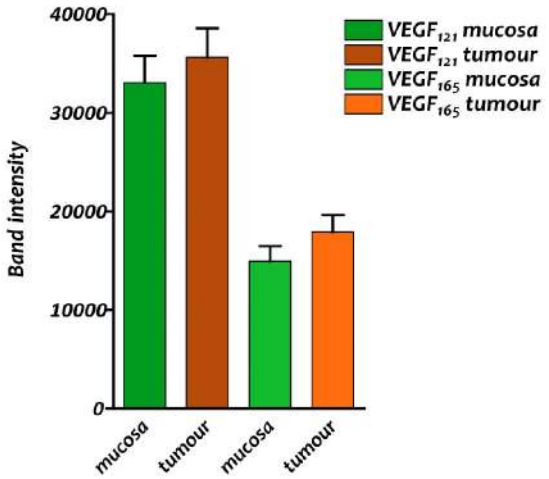


Fig. 6. Isoform quantification. Quantification of $VEGF_{121}$ and $VEGF_{165}$ expression in both healthy mucosae and tumour mass specimens. Values are expressed as arbitrary units of band intensity. Bars show mean ± s.e.m.

4.5 Lack of significant difference between VEGF-A splicing isoforms in CRC and paired mucosa samples

The $VEGF_{165}/VEGF_{121}$ ratio in tumour and in mucosa was assessed for 78 patients. The mean of these values for mucosal and tumour samples were 0.442 ± 0.207 and 0.483 ± 0.226 respectively (no statistically difference was observed) (Figure 7). Further, the ratio between $VEGF_{165}/VEGF_{121}$ in the tumour and $VEGF_{165}/VEGF_{121}$ in its paired mucosa ({$VEGF_{165}/VEGF_{121}$ Tumour}/{$VEGF_{165}/VEGF_{121}$ Mucosa}) was evaluated for each patient (hereinafter referred to as T/M distribution), and plotted in Figure 8 (each dot represents a single patient).

VEGF-A isoforms balance did not appear obviously regulated in CRC patients. Indeed, the $VEGF_{165}/VEGF_{121}$ ratio average was unaffected upon malignant transformation ($\{VEGF_{165}/VEGF_{121}$ Tumour$\}/\{VEGF_{165}/VEGF_{121}$ Mucosa$\} \simeq 1$). As a consequence, T/M distribution was clearly centred on 1 (Figure 8). However, the aforementioned patient distribution was broad, reflecting the high heterogeneity of VEGF isoforms proportion in CRC patients. This was more evident in Figure 9, where changes in $VEGF_{165}/VEGF_{121}$ ratio were shown for each of the analysed patient. The vast majority of the samples showed a marked variation in VEGF-A isoforms content between normal and malignant tissue specimens, thus clearly suggesting that carcinogenesis has a strong impact on VEGF-A splicing, even though this phenomenon does not follow an obvious trend.

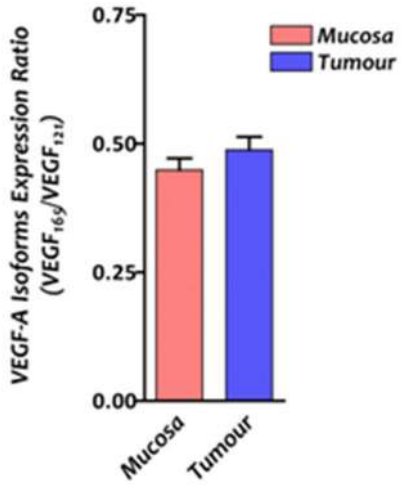


Fig. 7. Isoform quantification in mucosae and tumours. VEGF-A splicing balance was expressed as the ratio between $VEGF_{165}$ and $VEGF_{121}$. $VEGF_{165}$/ $VEGF_{121}$ ratio in mucosae and tumours was calculated for the entire sample set. Bars show mean ± s.e.m. No statistical difference was observed between mucosae and tumours.

Band intensity quantification in either normal mucosa or tumour mass specimens revealed that there was no significant difference in the total expression level of $VEGF_{165}/VEGF_{121}$ between tumour and its paired mucosa in a large set of colorectal cancer patients. In other words, despite a massive inter-personal variability in isoform ratio, no obvious trend in the modulation of VEGF-A splicing was observed. However, considering the mucosa and tumour sample of each patient, a large variability could be observed within the ratio of $VEGF_{165}/VEGF_{121}$ in mucosa vs. its matched tumour sample; this ratio has been either elevated in some tumour samples comparing their paired mucosae or dramatically decreased in the rest of the specimens.

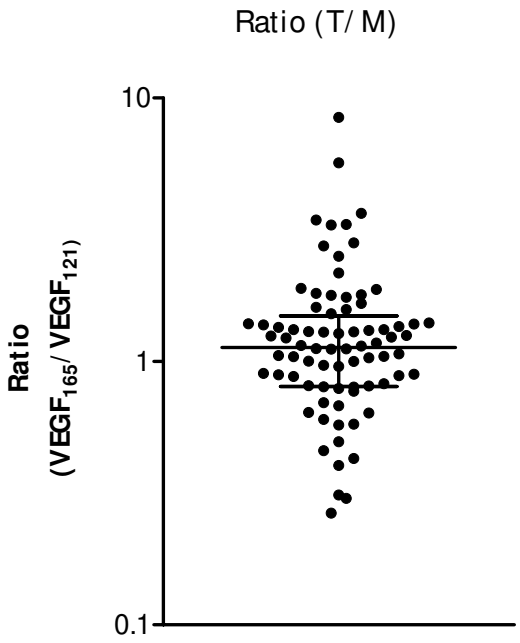


Fig. 8. Patient distribution according to T/M ratio. Using band quantification approach, $VEGF_{165}$/ $VEGF_{121}$ ratio was separately measured in healthy mucosa and tumour sample of each patient. The obtained value from tumour was then divided to the corresponding value in the mucosa. As shown here, the final ratio values, ($VEGF_{165}$/ $VEGF_{121}$ in tumour divided to $VEGF_{165}$/ $VEGF_{121}$ in mucosa of each patient) was plotted in a scatter graph. Each dot corresponds to an analysed patient. Values were distributed around value 1, indicating the fact that in the majority of the patients, $VEGF_{165}$/ $VEGF_{121}$ ratio had been remained unchanged between mucosae and their tumour counterparts.

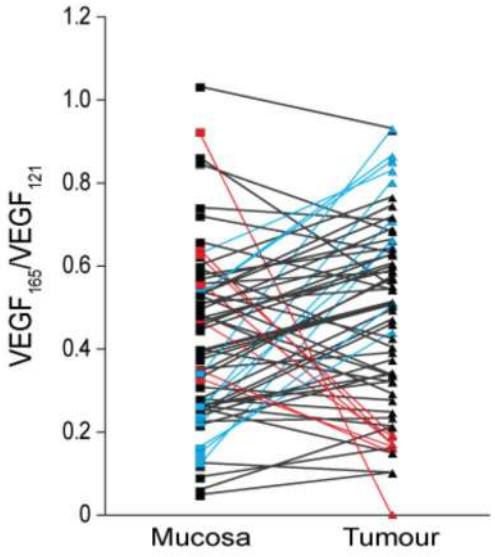


Fig. 9. Variability in $VEGF_{165}$/ $VEGF_{121}$ ratio between mucosa and tumour mass of each patient. Large heterogeneity was observed in the data set. Black lines denote individual patients whose ratio has been found substantially unchanged between normal and malignant tissue. Conversely, red and blue lines denote decreased and increased ratios, respectively.

4.6 The correlation between $VEGF_{165}/VEGF_{121}$ ratio and tumour grading in colorectal cancer patients

Primary aim of this project was to correlate the VEGF isoforms balance with clinical tumour outcome in the cohort of the CRC patients, seeking for a parallelism between the observed molecular and clinical heterogeneity, although clinical data was not available for some of the patients for whom experimental analysis was performed (see Table 1 in Appendix for complete clinical evaluations of the recruited patients).

Tumour grading and other major clinical parameters had been routinely assessed at the moment of surgical resection by independent pathologists. Based on above described quantifications, the mean $VEGF_{165}/VEGF_{121}$ ratio was calculated in two groups of CRC patients with low grading and high grading tumours (G=0&1 and G=2&3, respectively). In both cases, $VEGF_{165}/VEGF_{121}$ ratio in tumour mass was higher than in mucosa, even if this trend did not reach statistical significance. Comparing the average $VEGF_{165}/VEGF_{121}$ ratio in tumours from the two subgroups of patients, it was noticeably lower in high grading tumours, although this difference did not reach statistical significance (Figure 10).

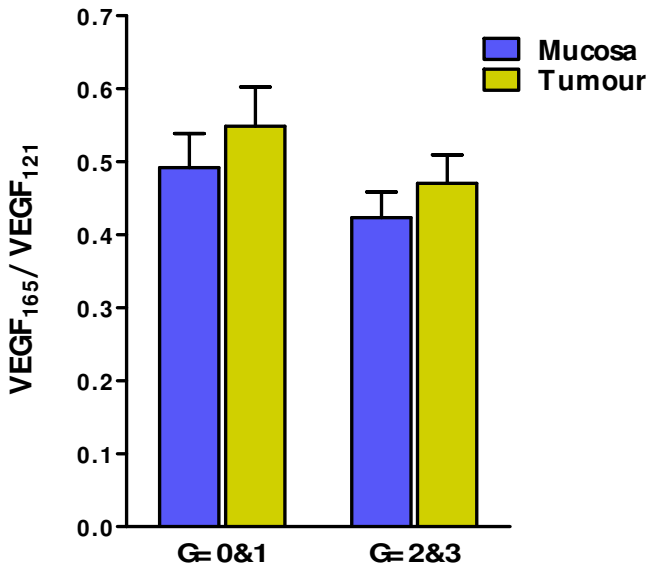


Fig. 10. VEGF splicing and tumour grading. $VEGF_{165}/VEGF_{121}$ ratio in low grading and high grading CRC patients. $VEGF_{165}/VEGF_{121}$ ratio was calculated in mucosae and tumours of patients showing differentiated tumours (G=0 and G=1, assessed by independent pathologists). The same was assessed in patients with grading level of 2 and 3 (G=2&3). Coloured bars show mean ± s.e.m. Interestingly, in patients with higher grading level, this ratio had been decreased in both mucosae and tumour specimens compared to G=0&1.

Lymph node infiltration (N) is another important hallmark of poor clinical outcome. Interestingly, T/M ratio was lower in patients with infiltrated lymph nodes (N≥1), as shown in Figure 11, even if this decrease did not reach statistical significance.

Both observations, even though statistically not relevant, might imply that higher $VEGF_{165}$ (or lower $VEGF_{121}$) expression to some extent hurdles tumour progression towards poorly differentiated stages.

It is worth mentioning the fact that this correlation is not maintained for other clinical parameters, most notably tumour stage (S), as shown in Figure 12.

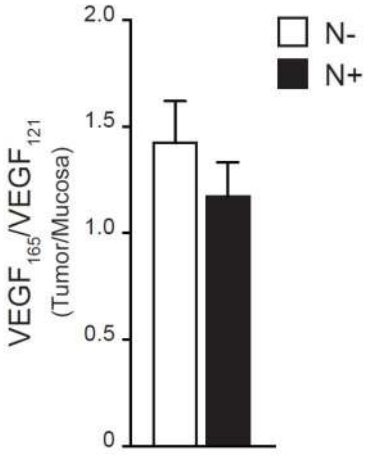


Fig. 11. VEGF splicing and lymph node infiltration. T/M ratio ($VEGF_{165}$/ $VEGF_{121}$ in tumour vs. mucosa) in patients showing lymph node infiltration (N^+) is lower if compared to patients who were diagnosed with no lymph node infiltration (N-). Bars show mean ± s.e.m.

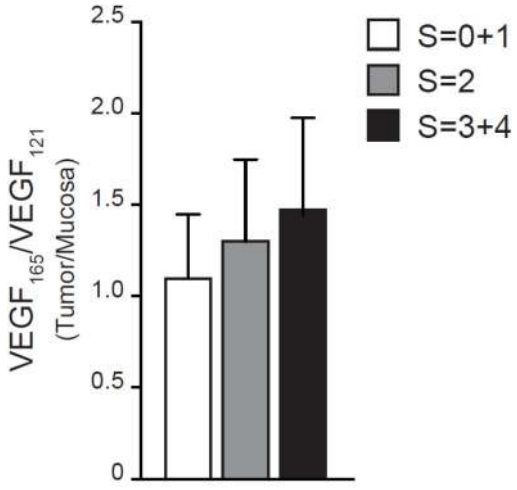


Fig. 12. VEGF splicing and tumour staging. T/M ratio in patients accrued according to cancer staging at the moment of surgery.

4.7 Patients with higher $VEGF_{165}/VEGF_{121}$ expression ratio in their tumour mass demonstrate better outcome

Exploiting AAV-mediated gene transfer, it was demonstrated that $VEGF_{165}$ or $VEGF_{121}$ conditioning clearly exerts distinct effects on tumour growth in mouse animal models, as detailed in the Introduction section. In particular, $VEGF_{165}$ acts in a peculiar and rather surprisingly tumour suppressive manner, hampering tumour growth through the recruitment of NEMs and eventual vessel normalization (Carrer et al., *submitted*). Although in the CRC samples we did not detect any obvious correlation between higher $VEGF_{165}$ expression and better prognosis, possibly due to the vast dispersal of data, we nevertheless selected a restricted subset of CRC patients (n=6), in whom the $VEGF_{165}/VEGF_{121}$ ratio was strongly higher in the tumour mass, compared to its paired normal mucosa. In particular, these patients were selected because their T/M ratio exceeded a 90% C.I., shown as green dots in Figure 13.

Strikingly, this small subset of patients (hereinafter referred to as $VEGF_{165}^{hi}$) displayed a significantly reduced lymph node infiltration, compared to the rest of the patients in the selected cohort (Figure 14). In contrast, the patients who conversely expressed higher levels of $VEGF_{121}$ (T/M ratio exceeding 90% C.I., violet dots in Figure 13, hereafter $VEGF_{121}^{hi}$) did not exhibit any difference compared to the whole residual dataset (hereafter $VEGF_{165}^{int}$, black dots in Figure 13). This is in agreement with experimental evidence, supporting a differential impact of VEGF isoforms on tumour progression.

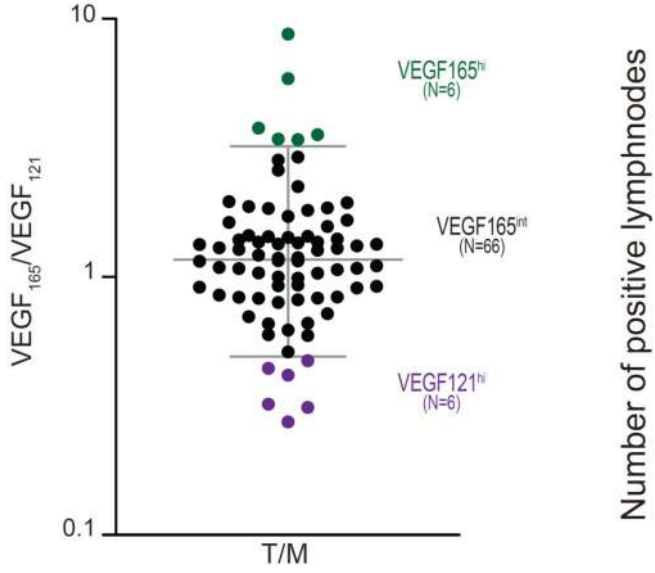


Fig. 13. Patient distribution according to VEGF splicing. Patients were divided into 3 groups. Individuals showing a T/M ratio that exceeds a 90% CI were denoted as $VEGF_{165}^{hi}$ (green dots) and $VEGF_{121}^{hi}$ (purple dots), for highest and lowest ratios, respectively. Remaining patients were denoted as $VEGF_{165}^{int}$ (black dots). Lines denote median ± 90% CI.

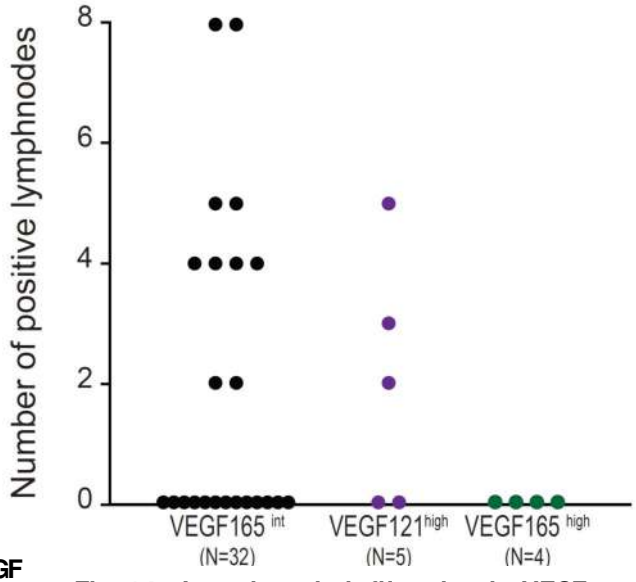


Fig. 14. Lymph node infiltration in $VEGF_{165}^{hi}$ patients. Number of positive lymph nodes in $VEGF_{165}^{int}$ (black dots), $VEGF_{121}^{hi}$ (purple dots) and $VEGF_{165}^{hi}$ (green dots) patients is shown in this graph. $VEGF_{165}^{hi}$ patients show no lymph node infiltration.

4.8 $VEGF_{165}^{hi}$ patients exhibited a more mature tumour vasculature

Preliminary evidence in experimental animal models clearly demonstrates that $VEGF_{165}$-driven tumour inhibition is correlated with an improvement in the tumour vasculature maturation, as mentioned before. In order to assess whether the clinical amelioration observed in $VEGF_{165}^{hi}$ patients was linked to improved tumour vascularization, immunofluorescence (IF) analysis was performed on paraffin-embedded tumour specimens, routinely harvested at the time of surgical resection for standard histopathological analysis. The morphology of the vascular network in patients with high expression level of $VEGF_{165}$ was compared with the cohort of the patients with high expression level of $VEGF_{121}$. Double immunostaining against von Willebrand factor (vWF) and alpha-smooth muscle actin (α-SMA) was employed; the former as a marker of endothelial cells and the latter as a marker of mural cells (specifically smooth muscle cells). As it is appreciated in the following images (Figure 15), vessels of $VEGF_{165}^{hi}$ tumours appeared more normalized and mature in respect to those of $VEGF_{121}^{hi}$ patients. In this regard, α-SMA was considered as an indicator of vessel maturation, numerically expressed as a ratio between α-SMA and vWF fluorescence (both measured as pixels over a fixed threshold) displaying higher value for $VEGF_{165}^{hi}$ sample (Figure 16). Moreover, the intensity of vWF was also measured in tumour vessels of all subsets of the patients showing higher intensity in the sample belonging to $VEGF_{121}^{hi}$ patient (Figure 17). Importantly, the number of vessels was not affected by isoform selection (data not shown), and CD31 positivity assessed independently by routine histopathological analysis did not show any variation (data not shown). This again points toward a specific role of $VEGF_{165}$ in promoting full vessel maturation, despite the fact that it retains the strong angiogenic capacity common to all VEGF-A isoforms.

Unfortunately, given the paucity of $VEGF_{165}^{hi}$ samples it was not possible to establish any significant correlation with effectiveness of neo-adjuvant therapy, a clear and definitive indicator of vessel normalization (Carmeliet and Jain, 2011).

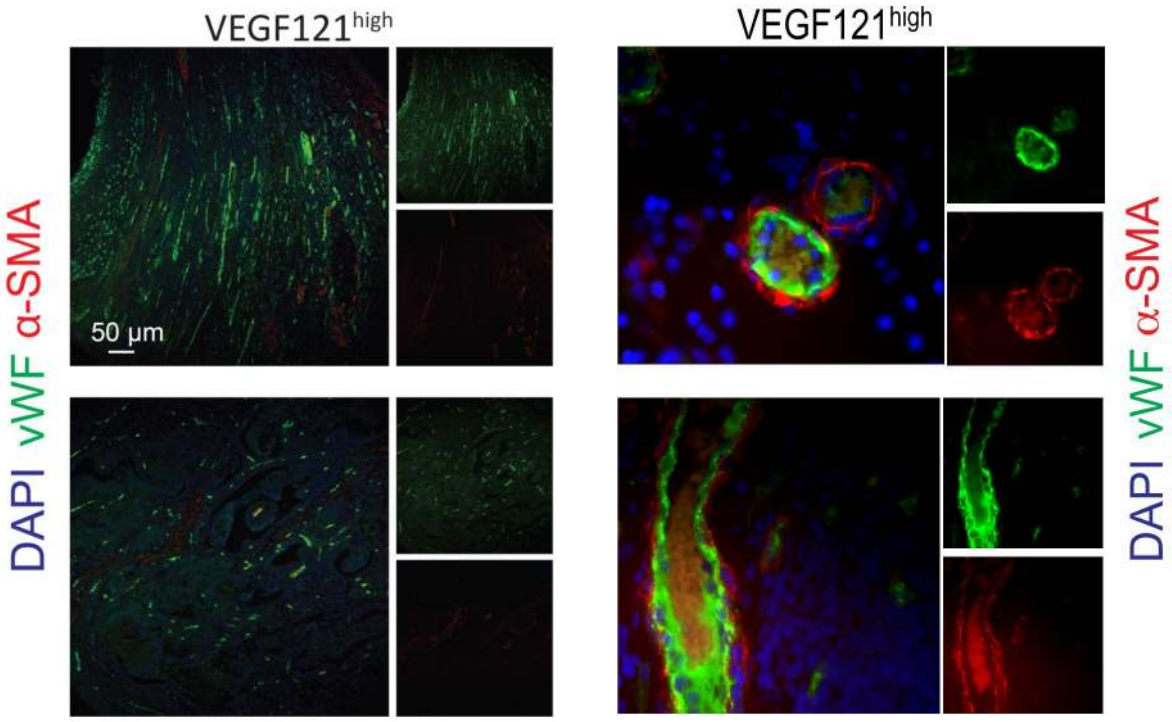
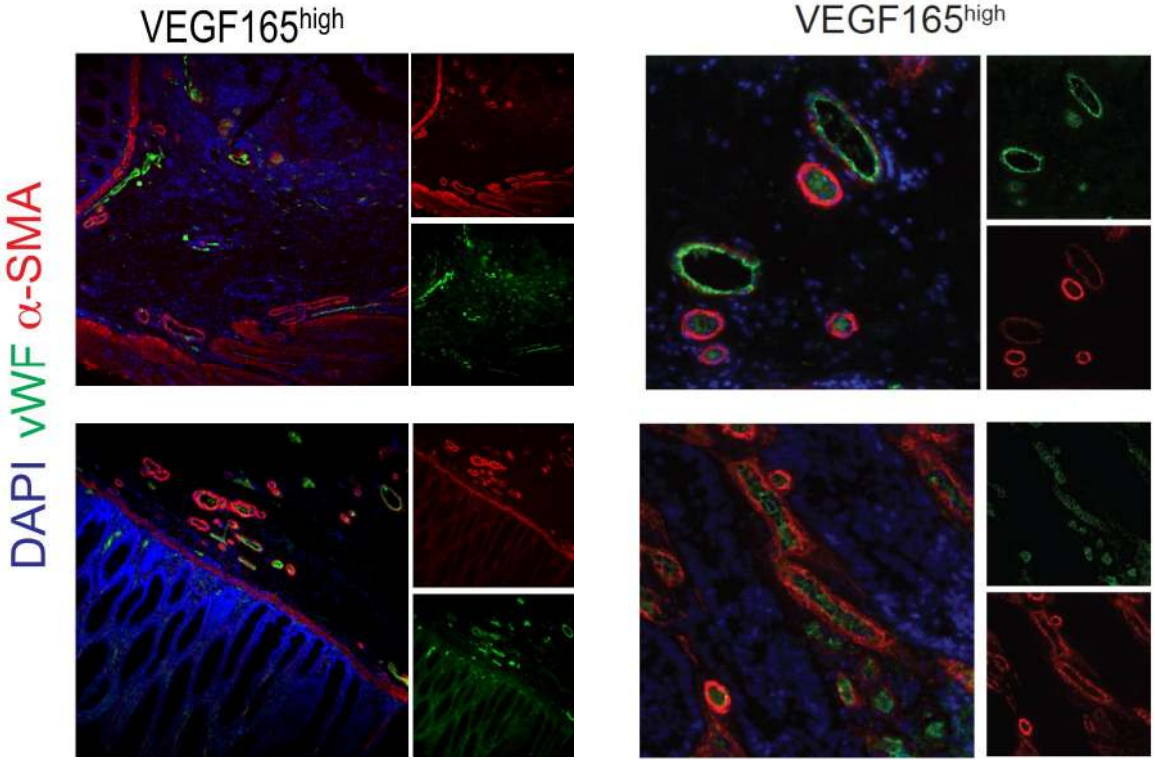


Fig. 15. Tumour vessel analysis. Double immunostaining against vWF and α-SMA in paraffin-embedded tumour sections harvested from $VEGF_{121}^{hi}$ (panels above) and $VEGF_{165}^{hi}$ (panels below). Anti-vWF antibody labels endothelial cells (EC) and is shown in green, whereas antibody against α-SMA labels mural cells and is shown in red. As displayed in right-hand panels above, in $VEGF_{121}^{hi}$ patients, vessels are scantly covered with smooth muscle cells indicating poorly mature vascular network. In left-hand panels, this observation has been shown with lower magnification. In contrast, in the panels below, immunofluorescence experiments for $VEGF_{165}^{hi}$ patients are shown. Most of the vessels show a high coverage of smooth muscle cells and pericytes (α-SMA$^+$) both at lower (left-sided panels) and higher (right-sided panels) magnifications. Split anti-vWF (green) and anti-α-SMA (red) signals were also shown besides merged images. Nuclei are counterstained in blue with DAPI.



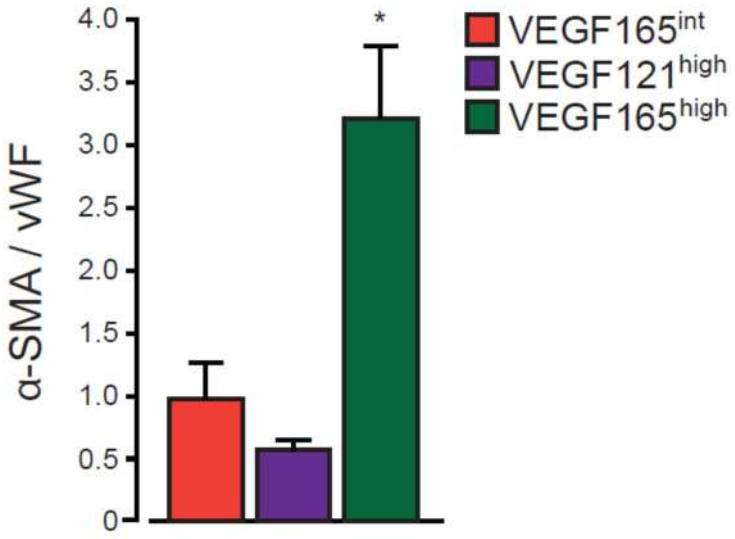


Fig. 16. Quantification of IF analysis. Quantitative evaluation of IF analyses in $VEGF_{165}^{int}$, $VEGF_{121}^{hi}$ and $VEGF_{165}^{hi}$ patients according to the ratio of α-SMA/ vWF. Bars show mean ± s.e.m. Asterisk denotes statistical significance ($p<0.05$). $VEGF_{165}^{hi}$ patients clearly show enhanced vessel maturation.

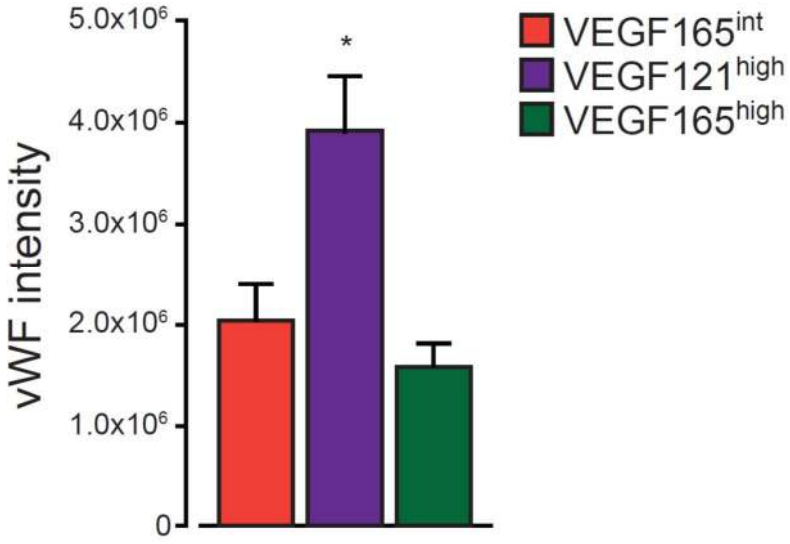


Fig. 17. Quantification of vascular area. vWF intensity in $VEGF_{165}^{int}$, $VEGF_{121}^{hi}$ and $VEGF_{165}^{hi}$ tumours. Bars show mean ± s.e.m. Asterisk denotes statistical significance ($p<0.05$).

4.9 The effect of higher $VEGF_{165}/VEGF_{121}$ ratio on the occurrence of metastasis in colorectal cancer patients

There is an ample consensus that hypoxia consequent to impaired vessel functionality results in reduced tumour cell growth and subsequent reduced tumour mass, however whether normalization is beneficial in impeding tumour metastasis or, on the contrary, favours the metastatic process is still unclear (De Bock et al., 2011). Hence, in keeping with the observation that $VEGF_{165}$ elicits vessel stabilization, the correlation between the presence of distant metastasis and the ratio of the expression of $VEGF_{165}/VEGF_{121}$ was investigated. We found that, in metastasis-free patients (M=0), the $VEGF_{165}/VEGF_{121}$ ratio was significantly lower when compared to metastasis-bearing patients (M=1), as shown in Figure 18 A. These data appear consistent with recently published data supporting the concept that vascular normalization induced by anti-angiogenic therapies might elicit tumour cell spreading and metastasis in some settings (Ebos et al., 2009; Paez-Ribes et al., 2009). In this respect, it is worth mentioning the role of hypoxia in the up-regulation of $VEGF_{165}$ and in hypoxia-induced tumour metastasis, which will be discussed in subsequent sections.

Further exploring the issue, it was evident that in patients with no sign of metastasis, the mean value of $VEGF_{165}/VEGF_{121}$ expression in both mucosa and tumour did not show any significant difference (see Figure 18A). However, in M=1 patients this value was decreased in mucosae when compared to the tumour samples, and, notably, equally reduced if compared with mucosae from M=0 patients (Figure 18B). This was completely unexpected, as mucosa samples should represent “normal” tissue specimens, unaffected by either tumour presence or its outcome. It might be conceptualized that the down-regulation of $VEGF_{165}$ or, instead, the up-regulation of $VEGF_{121}$ in the mucosa sample of metastatic patients sets some sort of predisposition in CRC patients to develop very aggressive tumours. Since there are no clues to better understand the abundance of which one of these isoforms is more affected during the process of metastasis, this topic needs to be better elucidated.

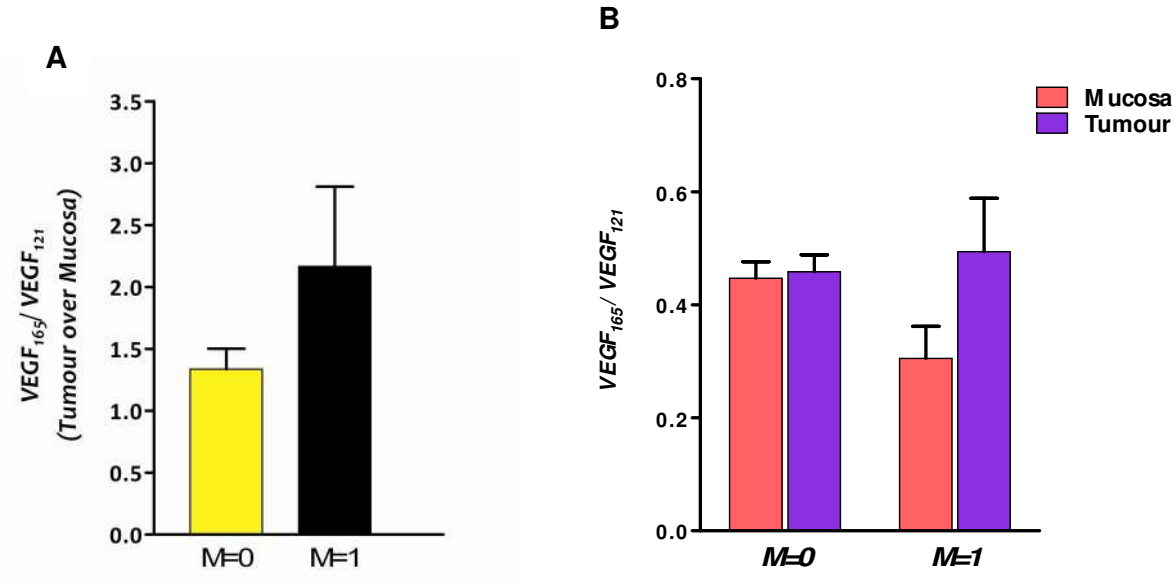


Fig. 18. VEGF splicing and metastasis A) T/M ratio in metastasis positive (M=1) and negative (M=0) patients. Ratio was elevated in M=1 patients. **B)** $VEGF_{165}/VEGF_{121}$ ratio in mucosae and tumours of M=0 and M=1 patients. Bars show mean ± s.e.m.

4.10 Detection of NEMs in tissue sections of CRC patients

In our laboratory, using a mouse model of tumour xenotransplantation, we unveiled a dissimilar and somewhat opposite effect of $VEGF_{165}$ and $VEGF_{121}$ on tumour growth, by which the former decreased tumour growth while the latter increased it, compared to untreated animals. This difference was ascribed to the diverse ability of these two isoforms to recruit NEMs to the site of angiogenesis (Carrer et al., *submitted*). As previously mentioned in the "Introduction", Neuropilin-1 Expressing bone marrow-derived Myeloid cells (NEMs) have been recently considered as a crucial population of BM-originated cells recruited to the site of physiological and pathological angiogenesis. NEMs have been recognized as $CD11b^{+}Nrp1^{+}Gr1^{-}$ cells in diverse animal models (Carrer et al., *unpublished*; Maione et al., *unpublished*), but their detection and characterization in humans have been hampered by several obvious difficulties, above all, the lack of Gr-1 homologs in humans and the impossibility to overexpress $VEGF_{165}$ or Sema3A in human tissues.

In respect of the above issue, partial but critical focus of this project was to detect the presence of NEMs in human tumours, taking advantage of the fact that a handful of tumours did express above-the-average levels of $VEGF_{165}$ (see Figure 13), a well-known attractor of NEMs (Zacchigna et al., 2008). For this purpose, 5 µM-thick sections of paraffin-embedded CRC tissues from $VEGF_{165}^{hi}$ patients were analysed. Immunohistochemistry (IH) experiments were performed using an anti-Nrp-1 antibody. Unfortunately, despite using various antigen

retrieval procedures in order to unmask Nrp-1 antigen and trying different protocols, results of IH experiments were not satisfying. Some representative images are shown in Figure 19 and a complete list of unmasking solutions used for antigen retrieval is detailed in Table 2.

The feasibility of such an approach was reasonably hindered by original sample processing. As these samples were initially collected for routine histopathological analysis, they underwent strong, automatized fixation and processing, not compatible with poorly sensitive immunostaining. In this respect, it is important to note that Nrp-1 is a poor immunogen, and good staining antibodies have not been developed yet.

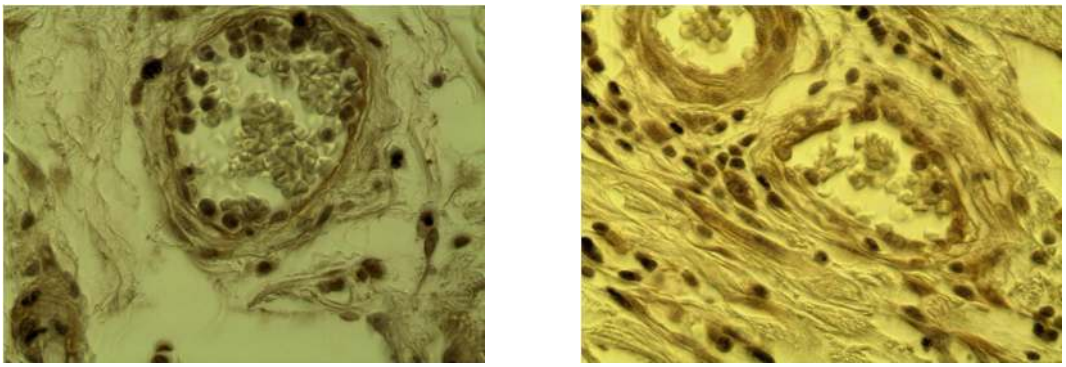


Fig. 19. IHC analysis. Immunohistochemistry experiments on paraffin-embedded tissue sections of CRC patients: anti-Nrp-1 immunostaining was tentatively performed on paraffin-embedded tumour sections. Representative images unveiling negative results are shown.

IHC	Paraffin-embedded tissue sections	L.A.B solution
IHC	Paraffin-embedded tissue sections	Citrate buffer PH6
IHC	Paraffin-embedded tissue sections	EDTA PH8 & Tris-HCl PH8
IHC	Paraffin-embedded tissue sections	Trypsin working solution PH11
IHC	Paraffin-embedded tissue sections	Proteinase K
IHC	Paraffin-embedded tissue sections	Proteinase K and Trypsin

Table 2. Reagents used for Nrp-1 antigen retrieval on CRC paraffin-embedded tissue sections.

To overcome these problems, we took advantage of the commonly recognized fact that commercially available Nrp-1 antibodies work almost exclusively on freshly-frozen tissues. For this reason, an additional set of CRC patients (n=19) were recruited for the above described VEGF isoforms analysis. For each of these patients, a frozen biopsy was harvested at the time of surgical resection, and kept at -80°C for further immunofluorescence (IF) analysis. Five-µM cryosections were randomly obtained from some of these samples and then subjected to IF staining to check whether the freezing procedure of the specimens in the hospital was reliable enough to maintain the morphological features of the tissue. IF experiments were performed using anti-CD11b and anti-Nrp-1 antibodies, in order to unveil

eventual double positive cells, which might be considered as putative human NEMs.
Unfortunately, quantification of VEGF isoforms revealed that none of these recently collected samples showed strong expression level of $VEGF_{165}$. Instead, all of them showed a balanced $VEGF_{165}/VEGF_{121}$ ratio between tumour and mucosa (as shown in Figure 20, where all the dots fall within a 90% C.I. calculated on the entire sample set). In keeping with this observation, and despite the fact that a significant number of infiltrating $CD11b^+$ cells could be appreciated around the tumour vessels, no double positive cells ($CD11b^+/Nrp\text{-}1^+$) could be found in the tested cryo-sections (Figure 21).

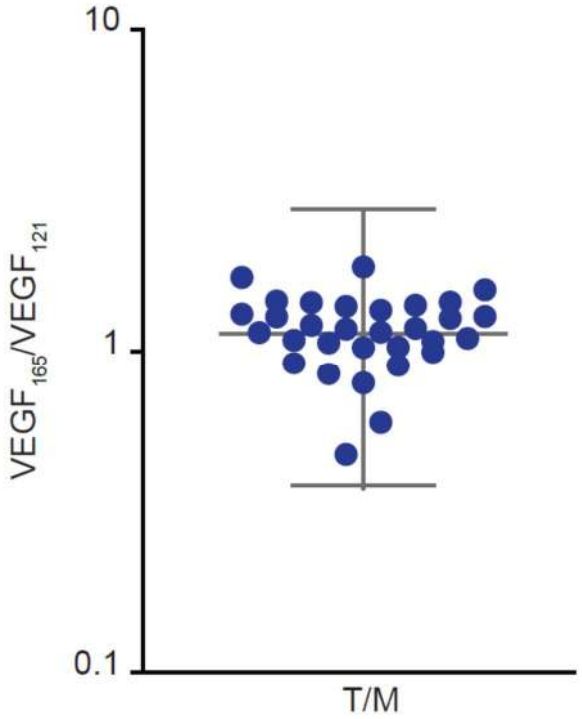


Fig. 20. T/M distribution according to $VEGF_{165}/VEGF_{121}$ ratio in a small subset of newly collected CRC samples. None of these samples showed high $VEGF_{165}/VEGF_{121}$ ratio.

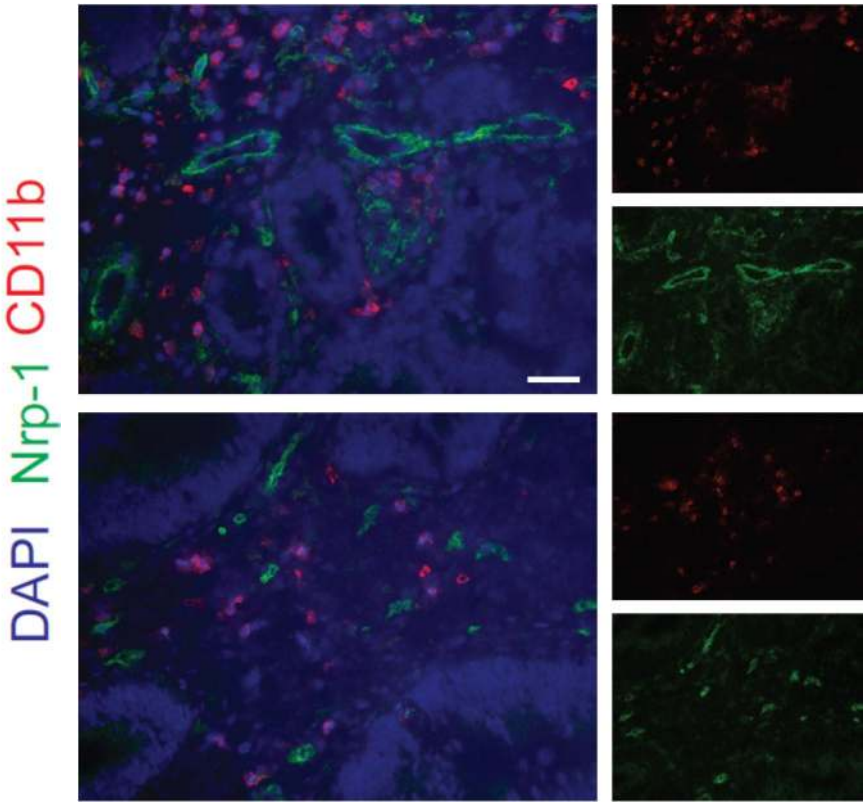


Fig. 21. NEMs are not found in tumours that do not up-regulate $VEGF_{165}$. Double immunostaining against Nrp-1 and CD11b was performed in newly collected CRC frozen samples. No evidence of the presence of Neuropilin-1 expressing bone marrow-derived mononuclear cells was found, as expected standing the low expression levels of $VEGF_{165}$ isoform.

4.11 Quantification of Sema3A abundance in human CRC

To further explore the hypothesis that NEM recruitment contributes to better cancer outcome, and based on the fact that Nrp-1 has basically been recognized as a co-receptor for Semaphorin3A, in parallel, we evaluated Sema3A expression in the same cohort of patients recruited for the VEGF isoform analysis. Noteworthy, Sema3A, similar to $VEGF_{165}$, has been demonstrated to be a potent NEM recruiter (Zacchigna et al., 2008). The obtained cDNAs were thus used as templates for real-time PCR amplification to detect the expression levels of the Sema3A gene, both in tumours and normal mucosae. For each patient, expression of Sema3A was calculated as a ratio between tumour- and mucosa-expressed Sema3A. In the whole data set, Sema3A expression did not show any obvious shift towards up- or down-regulation in the tumour mass, with only a modest tendency to down-modulation (Figure 22). Nonetheless, in a restricted group of patients (n=8), Sema3A appeared far up- regulated in the tumour mass (above 90% CI). Strikingly, these patients (hereinafter referred to as $Sema3A^{hi}$) had more benign tumours as compared to the remaining patients (hereinafter referred to as

Sema3A^{low}). In particular, as shown in Figures 23 and 24, Sema3A^{hi} patients showed a reduced lymph node infiltration, decreased metastatic spread and longer survival (all patients are alive 36 months after surgery). Remarkably, these Sema3A^{hi} tumours showed enhanced vessel maturation, as unveiled by vWF/α-SMA double immunostaining (data not shown). These data are in perfect concordance with the anti-tumorigenic effect of Sema3A, previously observed in tumour xenograft animal models and even more remarkably, with the possibility that it might recruit tumour-hampering NEMs also in human malignancies.

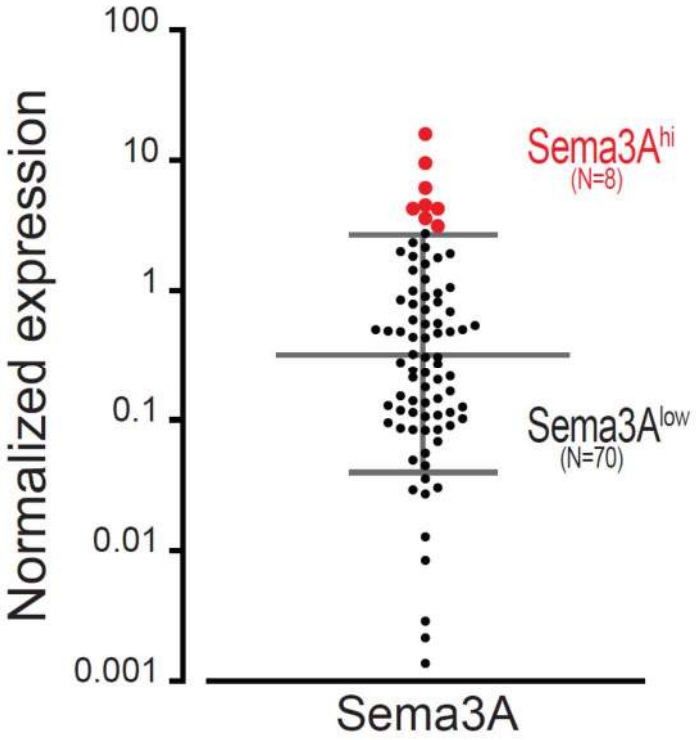


Fig. 22. Distribution of accrued patients according to T/M ratio of Sema3A expression. Tumour expression did not show any tendency towards up- or down-regulation. Patients exceeding a 90% CI were denoted as Sema3A^{hi} and labelled as red dots. Remaining patients were identified as Sema3A^{low} (black dots). Lines denote median ± 90% CI.

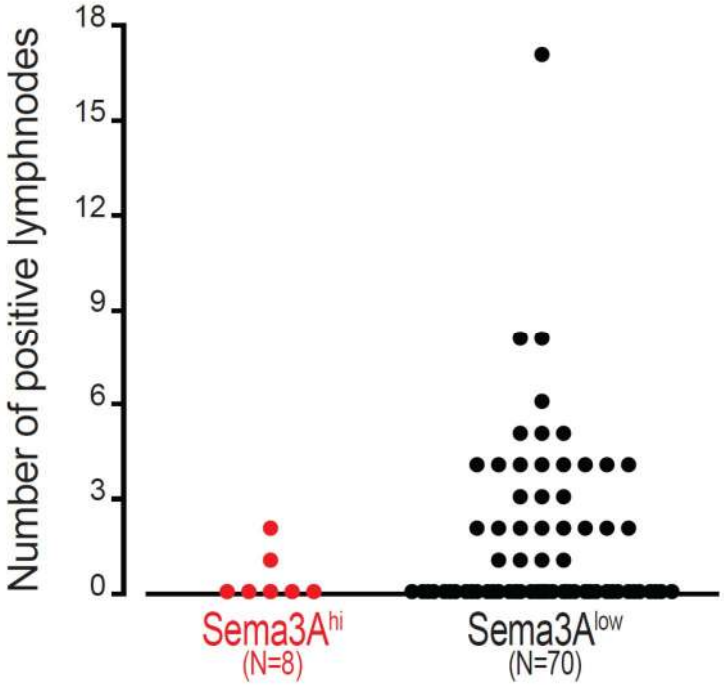


Fig. 23. Lymph node infiltration in Sema3A^{hi} patients. Number of positive lymph nodes in Sema3A^{hi} and Sema3A^{low} patients. Infiltration was markedly reduced in the former group.

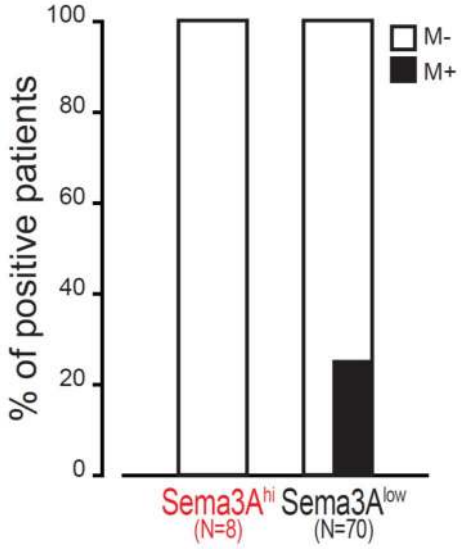


Fig. 24. Occurrence of distant metastases in Sema3A^{hi} and Sema3A^{low} patients. Metastasis positive patients were represented as proportion of the entire subset of patients (in black within white column in the Figure).

Next we determined the levels of Sema3A expression in the additional set of CRC tumours and mucosa pairs (n=30, including the 19 samples described above) for which cryopreserved samples were available. Distribution of the results obtained is shown in Figure 25. Only one of these patients showed a level of expression falling above the 90% CI that was considered in the previous experiments, and was thus chosen for further examination. Double immunostaining against Nrp-1 and CD11b was performed on 5 µM cryosections of this sample. As shown in Figure 26, in this single sample, putative NEMs, defined as double positive cells for CD11b and Nrp-1, could be recognized. Their distribution was sparse and mostly concentrated in perivascular locations. Of note, double positive cells were evident only in this single sample out of 10 tested, out of which the other 9 displayed neither $VEGF_{165}$ nor Sema3A overexpression.

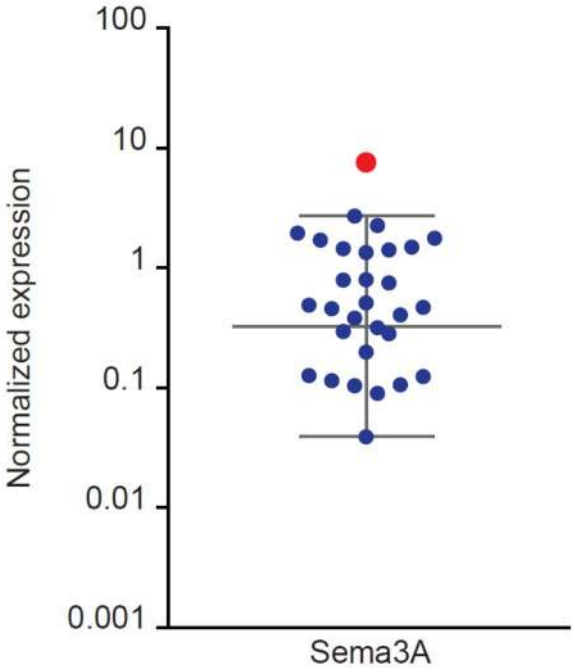


Fig. 25. T/M distribution according to Sema3A expression in newly collected CRC patients. One sample displayed features equivalent to Sema3A^{hi} patients described in Figure 22, and is labelled as a red dot in the Figure.

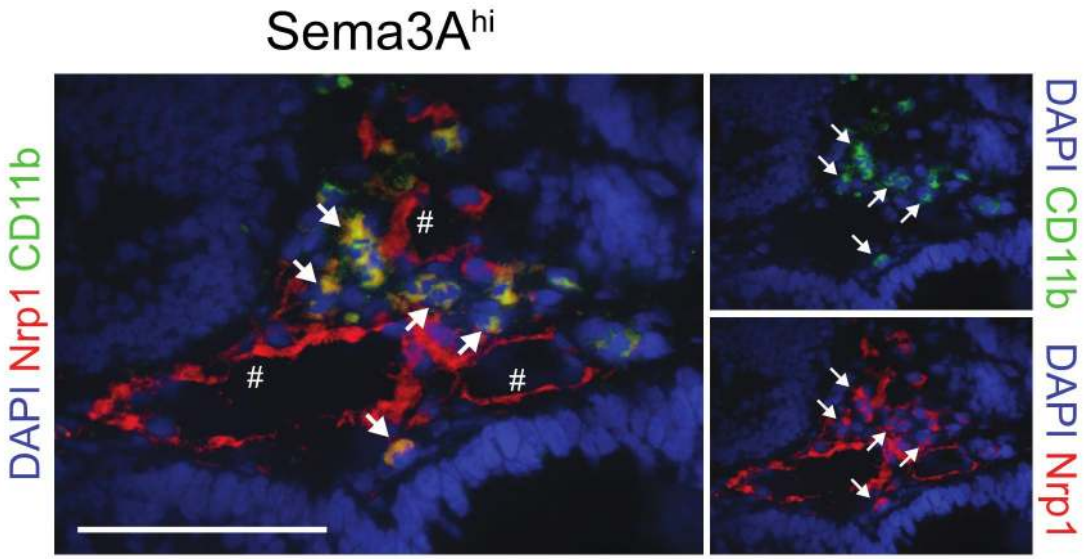
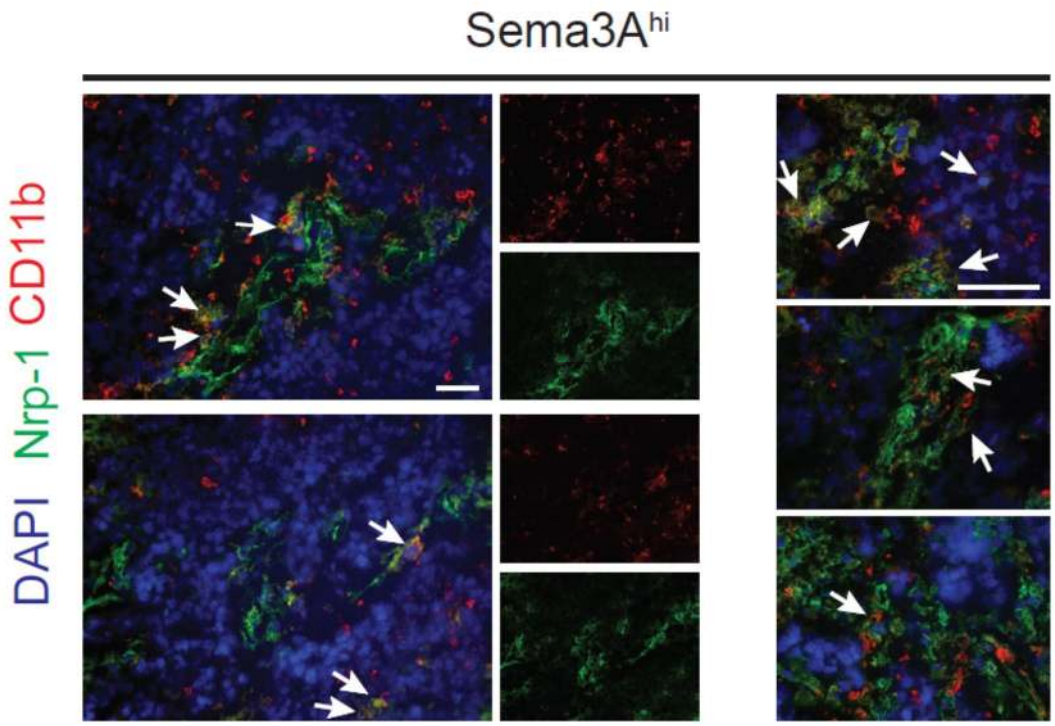


Fig. 26. NEMs infiltrate tumours expressing high levels of Sema3A. Detection of NEMs as double positive cells for Nrp-1 and CD11b antigens. Immunofluorescence analysis showed the presence of NEMs (highlighted by white arrows) in a patient overexpressing Sema3A. Split signals were shown besides merged images. Gates (#) denote vessels. Nuclei were counterstained in blue with DAPI.



4.12 $VEGF_{165}/VEGF_{121}$ ratio in lung cancer patients

Next we became interested in assessing whether our observations in colorectal carcinoma had any relevant implication in other human malignancies. For this purpose, thanks to a collaboration with the Institute of Genetics and Molecular Oncology (Istituto Oncologico Veneto-IRCCS) in Padova, a number of frozen lung cancer specimens were collected (n=42). The expression levels of $VEGF_{165}$ and $VEGF_{121}$ isoforms were measured employing the approaches described in Chapter 3. As shown in Figure 27, the $VEGF_{165}/VEGF_{121}$ ratio showed a wide distribution also in this sample set (mean value=0.27±0.16). In the case of lung carcinoma, the expression level of $VEGF_{121}$ was consistently higher than that of $VEGF_{165}$. Furthermore, a restricted subset of patients displayed a significantly increased $VEGF_{165}/VEGF_{121}$ ratio (above 90% C.I.). Similar to the case of colorectal carcinoma, in lung cancer patients with the higher expression level of $VEGF_{165}$ ($VEGF_{165}^{hi}$), no lymph node infiltration was detected (Figure 28).

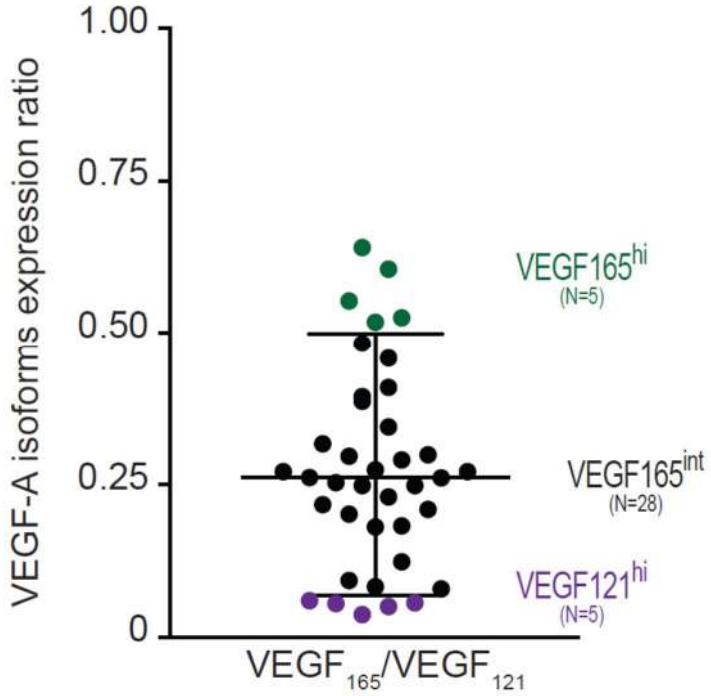


Fig. 27. VEGF isoforms in lung cancer. In a cohort of lung cancer patients, $VEGF_{165}$/ $VEGF_{121}$ ratio was measured. This ratio demonstrates a broad distribution among the patients and similar to the cohort of colorectal cancer patients, again a small subset of the patients shows higher level of expression of $VEGF_{165}$.

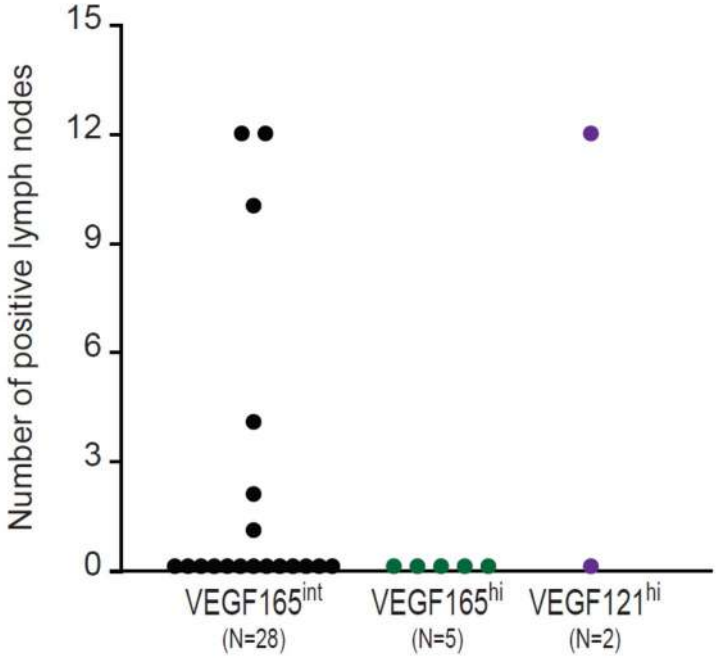


Fig. 28. Number of positive lymph nodes in $VEGF_{165}^{int}$, $VEGF_{165}^{hi}$ and $VEGF_{121}^{hi}$ lung cancer patients.

4.13 Analysis of Sema3A expression in the same set of lung cancer patients

Normalized Sema3A expression (over GAPDH) in the same cohort of lung cancer patients was also assessed by quantitative PCR approach. Sema3A expression was then plotted as in Figure 29, again unveiling a restricted number of the patients (4 out of 40) who expressed tumour levels of Sema3A significantly above average (exceeding 90% C.I.). Amongst these samples, one (ACP29) showed up-regulation in both $VEGF_{165}$ content and Sema3A expression.

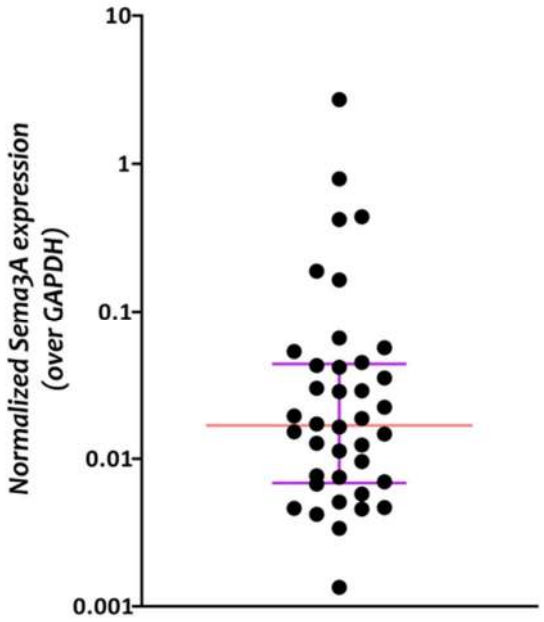


Fig. 29. T/M distribution of lung cancer patients according to Sema3A expression.

4.14 Hypoxia induces specific $VEGF_{165}$ up-regulation

As previously mentioned, three distinct biopsies (from the healthy mucosa, periphery and core of the lesion) was collected in a small subset of the CRC patients (n=19). It is widely recognized that the marginal ("periphery") and the central ("core") areas of a tumour mass possess clearly distinct features, at both structural and molecular level. To assess whether these differences affect $VEGF_{165}/VEGF_{121}$ ratio, standard isoform quantification was applied to this restricted set of samples.

The $VEGF_{165}/VEGF_{121}$ ratio was consistently up-regulated in the core of the lesion compared to the periphery and mucosa samples of the same patients. As shown in Figure 30, $VEGF_{165}/VEGF_{121}$ ratio in mucosa displayed a significant degree of homogeneity, being most samples gathered close to an average value of 0.53±0.12 (23% Coefficient of Variation). The average value of $VEGF_{165}/VEGF_{121}$ ratio was increasingly higher in the periphery and the core of the tumour (0.61±0.19 and 0.66±0.2, respectively). Heterogeneity was also increased in tumour datasets (both in periphery and core), displaying 32% and 37% as Coefficient of Variation, respectively.

Once more, these data demonstrate that malignant transformation has a profound impact on VEGF-A alternative splicing regulation. Progression towards a less structured, less vascularised and more chaotic area of tumour mass consistently entails an up-regulation of $VEGF_{165}$.

It is generally conceived that the most interior part of the tumour mass is more hypoxic. Due to poor and inefficient vascularization in the core of the tumour, it was originally envisaged that hypoxia might induce a specific preference for $VEGF_{165}$, an isoform specifically involved in vessel maturation as widely discussed throughout this thesis.

This issue was further investigated to unveil the molecular events leading to VEGF isoform selection in a highly hypoxic environment.

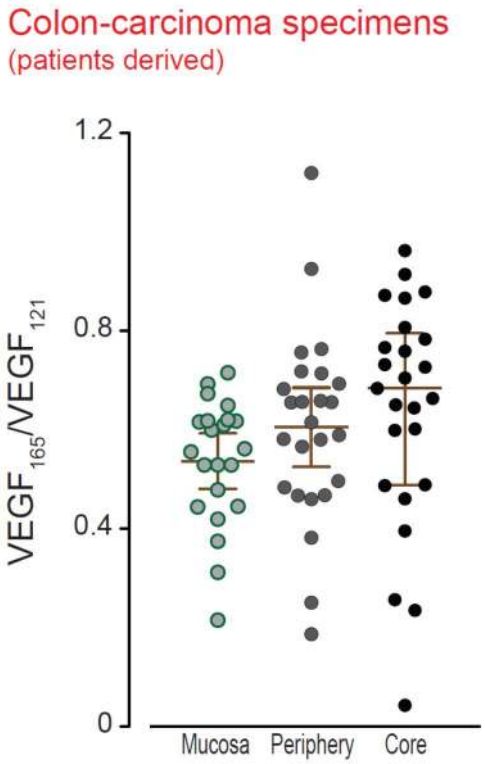


Fig. 30. VEGF splicing depends on local environment. $VEGF_{165}/VEGF_{121}$ ratio was evaluated in normal epithelial tissue (mucosa, light grey dots), border of tumour mass (periphery, dark grey dots) and interior of tumour mass (core, black dots). Horizontal lines denote mean ± s.e.m. Progressive increase in $VEGF_{165}/VEGF_{121}$ ratio was observed.

4.15 Effect of hypoxia on the expression levels of VEGF-A isoforms in different tumour cell lines

In order to assess the putative impact of low oxygen tensions on VEGF-A alternative splicing, two sets of experiments were performed. In a preliminary experiment, in order to create a hypoxic environment, a classic hypoxic chamber was employed. Two different cell lines (4T1 breast carcinoma and HUVEC endothelial cells) were subjected to a strong hypoxic conditioning for 2 or 24 hours. Wild-type cells were maintained in normoxia for the duration of the experiment. Then, using TRIzol, the RNA was extracted and, after cDNA preparation, the $VEGF_{165}/VEGF_{121}$ ratio was quantified. In fact, prolonged hypoxia conditioning induced the up-regulation of $VEGF_{165}$ in a time dependent manner in both cases, whereas $VEGF_{121}$ expression was not significantly affected, as shown in Figure 31.

Noteworthy, the above described experiment was performed exploiting an old hypoxic chamber that essentially creates an anoxic environment, using N_2 to replace O_2 into the chamber. This represents a largely non physiological condition, thus a further experiment was performed employing an advanced hypoxia incubator chamber, in which the concentration of CO_2 and N_2 was finely tuned during the experiment's duration. In this respect, three various tumour cell lines were employed; T241 (fibrosarcoma), 4T1 (breast carcinoma), and B16-F10 (melanoma). Cells were exposed to hypoxia conditioning (1% O_2 tension), for 2 and 18 hours. Isoform quantification analysis revealed that, in all three conditions, $VEGF_{165}$ was the preferred isoform compared to the shorter isoform, and that hypoxia significantly increased the isoform balance towards $VEGF_{165}$ (Figure 32).

Both experiments clearly demonstrated that malignant cells respond to hypoxia by altering the balance between VEGF isoforms, preferentially producing $VEGF_{165}$. This effect is consistent in all tumour cell lines tested so far, but its kinetic and magnitude vary significantly. The reason for this variability is currently under investigation; upon hypoxic conditioning, the HIF pathway was equally activated in all three cell lines, as revealed by negative feedback regulation of HIF-1α mRNA and shown in Figure 33. Similarly, VEGF-A total mRNA was equally up-regulated in all cases (data not shown).

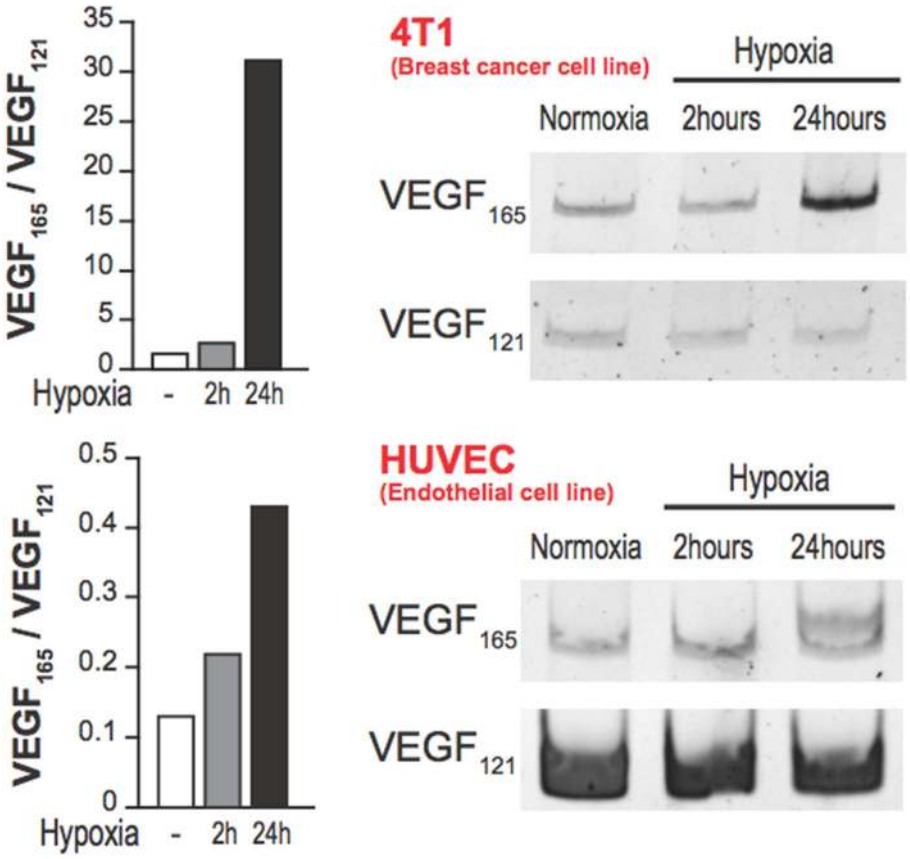


Fig. 31. Hypoxia and VEGF splicing. 4T1 (breast cancer) and HUVEC (endothelial) cell lines were exposed to hypoxic conditioning for 2 and 24 hours using a routine hypoxic chamber. $VEGF_{165}$/ $VEGF_{121}$ ratio was assessed by PCR approach: gel electrophoresis results are shown on the right. Quantifications are shown on the left as column graphs. Noticeable increase was observed upon severe hypoxic conditioning.

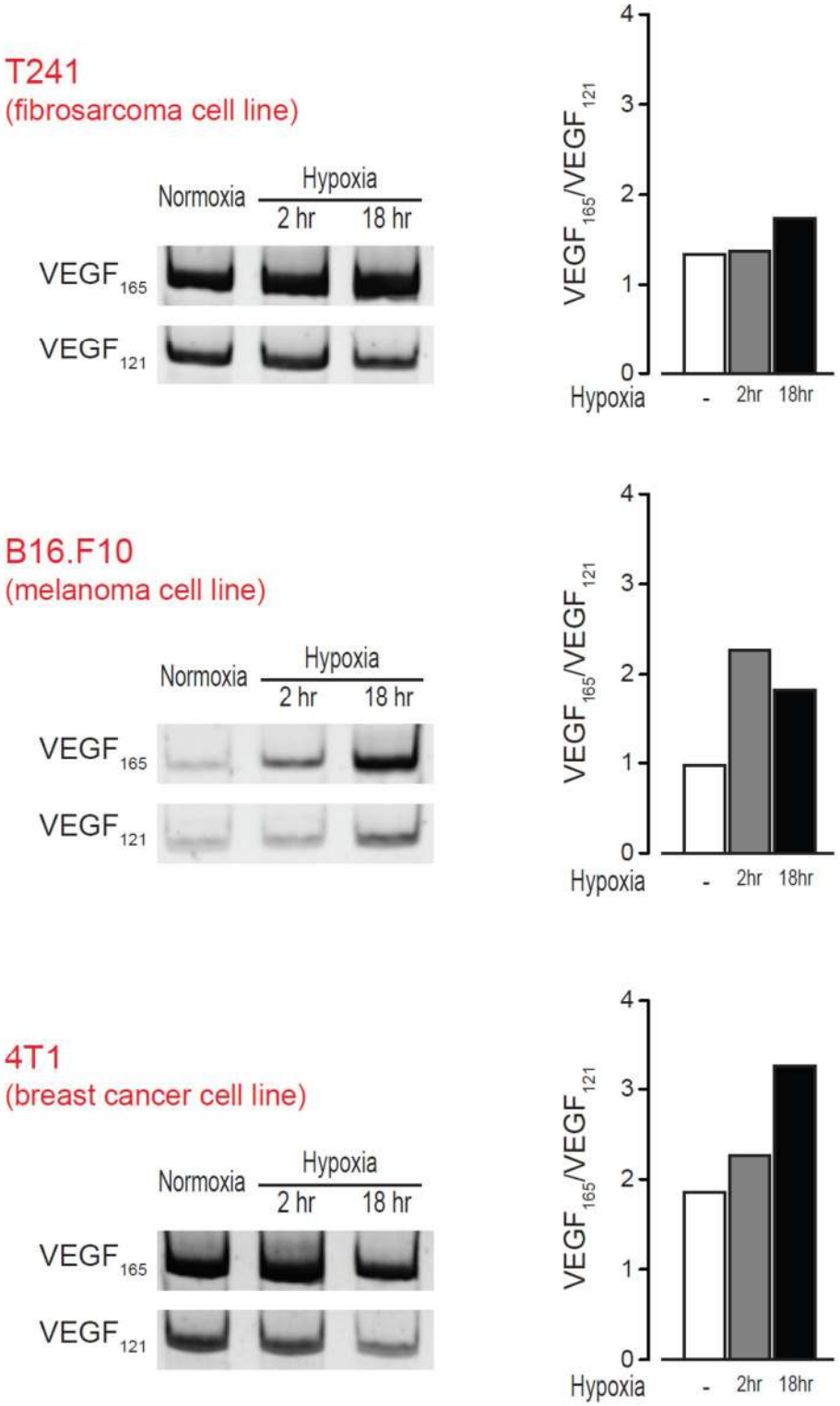


Fig. 32. Hypoxia affects VEGF splicing. Using a hypoxia incubator chamber, various cancer cell lines were exposed to hypoxia to assess the effect of low oxygen levels on the regulation of alternative splicing of VEGF isoforms in a time-dependent manner. As a control, cells were cultured under normoxia condition. In the case of T241 cell line, the expression level of $VEGF_{165}$ was clearly increased after 18 hours of hypoxia conditioning. B16-F10 cell line shows up-regulation of $VEGF_{165}$ but in a slightly different manner. As evident in the gel image, $VEGF_{165}$ has been overexpressed during hypoxia exposure time, however, based on band quantification data, visible switching towards $VEGF_{165}$ up-regulation might need longer than 18 hours of exposure to hypoxia. Instead, in 4T1 cell line, based on the band quantification analysis, this continuous modulation towards the overexpression of $VEGF_{165}$ was evident.

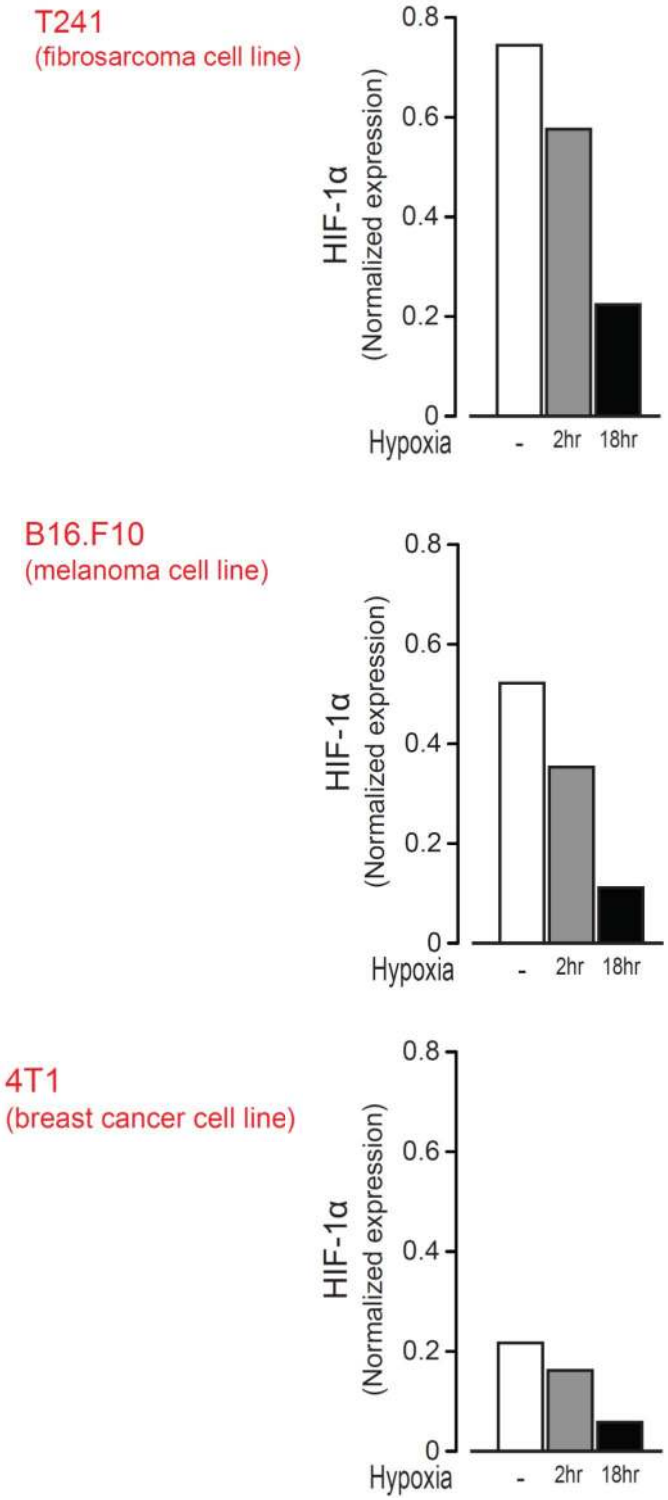


Fig. 33. HIF-1 is negatively regulated upon hypoxic conditioning. Using Real- Time PCR approach, the effect of hypoxia on the expression of HIF-1α was assessed in three aforementioned cancer cell lines. In fact, under prolonged hypoxia, all three cell lines showed a HIF-1α negative feedback in response to hypoxia in a time-dependent manner.

5. DISCUSSION

The project described in this thesis primarily examines the balance of the two most expressed splicing isoforms of VEGF-A, specifically $VEGF_{165}$ and $VEGF_{121}$, on the pathology of CRC. In our study, CRC was considered as an example of pathological angiogenesis and used to assess the specific roles of two isoforms of VEGF-A, certainly the best characterized among the several of pro-angiogenic factors known.

Indeed, $VEGF_{165}$ and $VEGF_{121}$ have well-recognized different biological properties. Most notably, $VEGF_{121}$ is freely diffusible in the extracellular matrix, whereas the longer isoform is more strongly bound to the extracellular matrix, and released upon enzymatic digestion of ECM components by metalloproteinases. In addition, $VEGF_{165}$ and $VEGF_{121}$ differ in their capacity to bind Neuropilin-1 (Nrp-1), an important co-receptor able to enhance VEGF-A binding to canonical receptors. Consistently, compelling evidence now suggests that $VEGF_{165}$ and $VEGF_{121}$ in fact elicit significantly different angiogenic response *in vivo*, in terms of magnitude, vessel structure, kinetic and functional outcome. We thus wondered whether tumours might preferential express one specific isoform to sustain the highly atypical vascularization that invariably distinguishes all solid tumours. To tackle this issue, 78 CRC patients were recruited in this study and, at the moment of therapeutic surgical resection, a biopsy of tumour mass was dedicated to RNA analysis. Similarly, for each patient, a paired sample of normal, healthy mucosa was collected. $VEGF_{165}$ and $VEGF_{121}$ relative abundance was evaluated in tumour and matched mucosa samples. No clear tendency toward up-regulation of either isoforms could be detected. A striking variability in isoform balance was observed: $VEGF_{165}/VEGF_{121}$ ratio was decreased impaired in the malignant tissue in the vast majority of the samples. Alteration in VEGF splicing balance was appreciable in a significant number of patients, although the relevance of this phenomenon for cancer biology and progression remains somehow elusive.

Others have described increased $VEGF_{121}$ expression in bladder cancer (Catena et al., 2007), an event associated with enhanced angiogenesis and worse outcome. Previous experiments performed at the Molecular Medicine Laboratory on xenotransplanted tumour models, suggest that conditioning the microenvironment with $VEGF_{121}$ results in accelerated tumour growth. More remarkably, $VEGF_{165}$-conditioning significantly inhibits tumour growth, consistent with a specific anti-tumour activity of $VEGF_{165}$. Importantly, such inhibitory activity is due to a modification of the local microenvironment, notwithstanding the

potent pro-angiogenic activity of all VEGF-A isoforms. This in fact, unravels a level of complexity in VEGF biology.

In CRC samples, $VEGF_{121}$ was invariably found to be more expressed compared to $VEGF_{165}$, both in tumours and mucosa samples, contrary to the general assumption that considers the latter the most abundant VEGF-A isoform in vertebrates. Whether this feature reflects a tissue-specific characteristic or underpins a sort of predisposition in individuals who will develop CRC, is probably worth further investigations.

We assessed the correlation between tumour grading (G) and the relevant $VEGF_{165}/VEGF_{121}$ ratio, both in the mucosa and tumour samples of the CRC specimens studied during this project. Although not statistically significant, for the patients for whom clinical data were available, we observed that the $VEGF_{165}/VEGF_{121}$ ratio was higher in both mucosae and tumour counterparts of the samples belonging to the patients categorized with the low grading level (a distinction that joins in the same category G= 0 and G= 1 patients). Conversely, in the samples with grading levels evaluated as 2 and 3 (intermediate and high), $VEGF_{165}/VEGF_{121}$ ratio was reduced. This finding might suggest an inverse correlation between tumour grading and $VEGF_{165}$ expression. In addition, in patients displaying a lower tumour grade, the expression level of $VEGF_{165}$ was higher in both tumour and its corresponding mucosa. In higher levels of grading, cells are morphologically very abnormal and poorly differentiated; they grow more quickly, and they may metastasize easily (www.cancer.gov). In this case, the $VEGF_{165}$/ $VEGF_{121}$ ratio resulted decreased in both tumour and matched mucosa, consistent with a possible role of $VEGF_{121}$ in promoting tumour progression. Due to the high variability of the results, it is unlikely that the determination of $VEGF_{121}$ levels might possess prognostic or predictive value, however it appears important to unravel the molecular mechanisms of tumour growth and local invasion.

Considering metastasis as another important clinical feature in the context of CRC, we compared $VEGF_{165}/VEGF_{121}$ ratio in mucosa and tumour samples of each CRC patient with or without metastasis (M) occurrence. In this regard, total $VEGF_{165}/VEGF_{121}$ ratio in tumour *vs.* mucosa of metastasis negative (M^-) patients was evidently lower than in the patients with the occurrence of metastasis. Since $VEGF_{165}$ promotes vessel normalization, this observation appears to be in line with recent findings indicating that the presence of a more mature vascular network, while decreasing tumour growth, on the contrary increases the propensity to metastatic spread. In particular, this was highlighted by recent observations in patients who received anti-angiogenic treatment (Loges et al., 2009). As a result of vascular normalization,

it is plausible that cancer stem cells or metastatic cancer cells have the opportunity to enter the blood circulation through large, mature blood vessels, carrying a regular flow, rather than exploiting small, narrow, stagnant branches of a disorganized vascular network. Noteworthy, extravasation of cancer cells invariably requires physical endorsement at the level of extracellular matrix, which works as tracks for spreading cells. In this respect, mature vessels, sheathed with more structured ECM components, might represent a more suitable route for invasion (Naito et al., 2012).

Not taking into account the concept of vessel normalization, it might more simply be speculated that the overexpression of $VEGF_{165}$ might favour metastasis through different mechanisms. Accordingly, it has been recently demonstrated that VEGF acts as a negative regulator of pericytes (Greenberg et al., 2008). We have also previously reported the formation of aberrant vascular structures upon AAV-mediated overexpression of $VEGF_{165}$ (Zacchigna et al., 2007).

At a closer look, examining the VEGF isoform balance, uniquely in mucosa samples, it was clearly evident that the $VEGF_{165}/VEGF_{121}$ ratio in the normal tissue of metastatic (M^+) CRC patients was markedly lower when compared to the equally "normal" tissue of non-metastatic (M^-) patients. Indeed, the $VEGF_{165}/VEGF_{121}$ ratio was substantially comparable when comparing the two datasets relative to M+ and M- tumours. This finding raises the intriguing possibility that high $VEGF_{121}$ expression sets a predisposition in normal epithelial tissues towards the development of highly metastatic malignant diseases. This hypothesis is consistent with the superior tumorigenic potential of $VEGF_{121}$ revealed by tumour xenotransplantation experiments in mouse models (Carrer et al., *submitted*).

One of the most recent, novel findings of our laboratory was the detection of a unique population of bone marrow-derived myeloid cells mobilized to the site of human tumour malignancies through $VEGF_{165}$ expression. These cells (Neuropilin-1 Expressing Monocytes, NEMs) are characterized as a subset of $CD11b^+Nrp1^+Gr1^-$ myeloid cells. By attracting NEMs, $VEGF_{165}$ induces the pericyte coverage of the newly-formed blood vessels (Zacchigna et al., 2008). NEMs secrete a broad range of cytokines and growth factors involved in the recruitment of mural cells (pericytes and vascular smooth muscle cells) to nascent blood vessels. This phenomenon plays a crucial part in the stabilization and maturation of new vascular network.

To understand whether $VEGF_{165}$ retains the above described properties also in the context of human malignancies, we identified a small subset of the patients (n=6) who

expressed particularly high levels of $VEGF_{165}$. We compared vascular morphology in such tumours with the one present in tumours showing a singularly reduced $VEGF_{165}/VEGF_{121}$ ratio. Interestingly, no sign of lymph node infiltration was detectable in the $VEGF_{165}^{hi}$ patients, consistent with a protective role of this longer VEGF isoform. Moreover, immunofluorescent analyses of the tumour samples belonging to this subset of the patients revealed that their vessels were covered with an increased number of α-SMA^+ cells; unveiling the more mature structure of the vascular network in tumours specifically expressing high levels of $VEGF_{165}$.

It was not possible to assess the presence of Nrp-1^+ myeloid cells in $VEGF_{165}^{hi}$ tumours, due to technical reasons. Hence, the question whether NEM recruitment in fact accounts for blood vessel maturation in those tumours still remains unresolved. More interestingly, with respect of metastasis, our data might imply that NEM recruitment indeed favour invasiveness, as many other tumour-infiltrating myeloid cells.

Very similar to the case of AAV-mediated delivery of $VEGF_{165}$, massive cellular infiltration of NEMs was also promoted by AAV-Sema3A injection in the mouse skeletal muscle (Zacchigna et al., 2008). Hence, we wondered whether the same effects on the maturation of tumour vessels could be observed as a consequence of the spontaneous Sema3A overexpression in tumour masses. A small group of patients bore tumours that overexpressed Sema3A. Remarkably, these $Sema3A^{hi}$ patients displayed improved clinical outcome and longer survival and their tumours exhibited a more mature vascular network, as unveiled by immunofluorescence analysis. In a set of recently collected frozen samples of CRC patients (none of which up-regulating $VEGF_{165}$), the presence of NEMs as $CD11b^+$/Nrp-1^+ myeloid cells could also be assessed in patients overexpressing Sema3A. This represents the first evidence of the presence of NEMs at the site of angiogenesis in human malignancies.

Another section of this study was dedicated to evaluate the effect of hypoxia on the splicing regulation of VEGF-A *in vitro*. Since the cores of the CRC lesions were reasonably more hypoxic, we wondered whether this condition might affect the regulation of $VEGF_{165}$ isoform production. In particular, arising from the observation that the $VEGF_{165}/VEGF_{121}$ ratio was increased in the core of the tumour samples, we hypothesized that environmental stresses might favour the selection of the longer isoform. Thus, a set of hypoxic conditioning experiments using different cell lines (including endothelial and tumour cell lines) was performed. Collectively, the results of these experiments revealed that hypoxic conditioning up-regulated the levels of $VEGF_{165}$ in a time-dependent manner. $VEGF_{165}$ up-regulation could

be conceived as the result of massive HIF-1α activity likely established in hypoxic, poorly vascularised tumours. There might be a strict link between this finding and the observation that the tumours with higher levels of $VEGF_{165}$ are more prone to metastasize, since HIF-1α loss-of-function results in decreased tumour growth, vascularization and invasiveness (Du et al., 2008; Lee et al., 2009a; Lee et al., 2009b; Liao and Johnson, 2007; Maxwell et al., 1997; Ryan et al., 1998; Stoeltzing et al., 2004). Further experiments are clearly needed to further address this issue and define the molecular mechanisms linking hypoxia, HIF-1 up-regulation and the regulation of VEGF precursor RNA splicing.

Taken together, the results of this thesis demonstrate that the modulation of VEGF splicing is a very complex process, which ultimately affects several aspects of tumour cell biology. The different VEGF isoforms modulate the tumour microenvironment by both directly influencing tumour vessel formation and by stimulating the tumour infiltration by specific circulating cell populations. A better understanding of the factors affecting VEGF pre-mRNA processing is required to shed light into this intricate network of interactions.

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8. APPENDIX

Patient ID	T				N		M	G			Lymphatic Reaction			Vascular invasion		Lymphatic invasion		Dukes				Staging			VEGF-A isoform ratio		
	1	2	3	4	0	≥1		0+1	2	3	Absent	Scant	All the rest	Present	Absent	Present	Absent	A	B	C	D	0+1	2	3+4	Mucosa (165/121)	Tumour (165/121)	T/M
10GO				X		X	No			X	-	-	NA		X	X				X				X	0,85886	0,54932	0,63959
62			X		X		Yes		X		-	-	NA		X		X				X			X	0,11625	0,65932	5,6714
73	X					x	No			X	-	-	X		X		X	X						X	0,63915	0,1696	0,26535
10A				X		x	No		X		-	-	X	NA	NA	NA	NA			X				X	0,37035	0,50984	1,37664
3A	no clinical data																								0,84391	0,68039	0,80624
59	X				X		No		X		-	-	NA		X		X		X			X			0,04621	0,10017	2,16775
15A			X		X		No		X		-	-	X	NA	NA	NA	NA		X				X		0,21342	0,70698	3,31269
64	no clinical data																								0,47810	0,33368	0,69793
2GO Rip					X		No	X			-	-	NA		X	NA	NA	X				X			0,73862	0,70721	0,95747
7A	no clinical data																								0,24959	0,46858	1,87737
7GO			X		X		No	X			-	-	X		X		X		X				X		0,27764	0,24524	0,88333
57	no clinical data																								0,12382	0,09927	0,80176
74				X	X		No	X			-	X			X		X		X				X		0,24238	0,79935	3,29793
8A			X		X		No		X		-	-	X	NA	NA	NA	NA		X				X		0,38098	0,50329	1,32103
69				X		X	No	X			-	-	X		X		X			X				X	0,39336	0,51104	1,29918
70			X			X	Yes	X			X	-		X		X					X			X	0,46663	0,18794	0,40276
76			X		X		No		X		-	-	X		X		X	X					X		0,22214	0,23164	1,04275
15GO	no clinical data																								0,27570	0,49110	1,78127
16GO				X		X	Yes		X		-	-	NA	X		X					X			X	0,32809	0,92379	2,81568
86			X		X		No		X		-	-	X		X		X	X				X			0,2355	0,37046	1,57304
11A	no clinical data																								0,08788	0,74203	8,44335
5A			X		X		No			X	-	-	X	NA	NA	NA	NA		X				X		0,52684	0,33531	0,63646
8GO	no clinical data																								0,62043	0,19320	0,31139
84			X			X	No		X		-	-	X		X		X			X				X	0,26095	0,21126	0,80961
85				X		X	No		X		-	-	X		X		X			X				X	0,48185	0,29026	0,60239
18GO		X			X		No	X			-	-	X		X		X		X			X			0,34812	0,38864	1,11639
68			X			X	Yes		X		X	-	-		X		X				X			X	0,15935	0,43746	2,74525
78	no clinical data																								0,26050	0,31996	1,22827
1GO			X		X		No			X			NA		X	NA	NA		X				X		0,32748	0,16218	0,49524
11GO			X			X	No			X			X		X		X			X				X	0,39021	0,50475	1,29353
40			X		X		No		X				X		X		X		X				X		0,53591	0,42302	0,78936
56				X		X	Yes		X		X			X		X					X			X	0,2556	0,14678	0,57428
63				X	X		No		X				X		X		X		X				X		0,21313	0,34161	1,60283
72			X		X		No		X				X		X		X		X				X		0,59234	0,40247	0,67945
80			X		X		No			X			X		X	X			X						0,1454	0,50037	3,44123
19GO			X		X		Suspicious	X					NA	X		X					X			X	0,25731	0,64528	2,50781
75				X		X	Yes		X		X			X			X				X			X	0,26071	0,33642	1,29039
13GO				X		X	No		X				NA		X		X			X				X	0,62548	0,82628	1,32105
42			X		X		No		X				NA		X		X	X					X		0,34702	0,14868	0,42844
66	no clinical data																								0,56125	0,16964	0,30225
1A-A	no clinical data																								0,05716	0,20875	3,65195
16A	no clinical data																								0,24126	0,45750	1,89633
2A				X		X	No		X		X			X		NA	NA			X				X	1,02993	0,92826	0,90128
65	no clinical data																								0,92149	0,00000	0,00000
67	no clinical data																								0,32595	0,57123	1,75249
79			X		X		No		X		X			X			X	X					X		0,39755	0,31789	0,79963

Patient ID	T				N		M	G			Lymphatic Reaction			Vascular invasion		Lymphatic invasion		Dukes				Staging			VEGF-A isoform ratio		
	1	2	3	4	0	≥1		0+1	2	3	Absent	Scant	All the rest	Present	Absent	Present	Absent	A	B	C	D	0+1	2	3+4	Mucosa (165/121)	Tumour (165/121)	T/M
MC1			X			X	NA		X				X	X		NA				X				X	0,61533	0,85517	1,38978
MC2		X			X		NA		X			X			X	NA			X			X			0,47656	0,21867	0,45885
MC3			X		X		NA	X				X		NA	NA	NA			X				X		0,64691	0,64677	0,99979
MC4			X		X		NA	X					X	NA	NA	NA	NA		X				X		0,52675	0,71508	1,35754
MC5			X		X		NA	X					X		X		X		X				X		0,5531	0,58271	1,05353
MC6		X			X		NA		X			X			X		X		X			X			0,59745	0,7497	1,25483
MC7				X		X	Yes		X			X		X		NA	NA				X			X	0,60731	0,62749	1,03322
MC8	X				X		NA	X			NA	NA	NA	NA	NA	NA	NA	X				X			0,618	0,7104	1,14951
MC9				X	X		NA		X				X	X		NA	NA		X					X	0,44191	0,79054	1,78892
MC10		X			X		NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA		X			X			0,69034	0,66746	0,96685
MC11		X			X		NA	X					X	NA	NA		X		X			X			0,71272	0,63409	0,88967
MC12	X				X		NA	NA	NA	NA	NA	NA	NA		X	NA	NA	X				X			0,41679	0,24055	0,57715
MC13			X		X		NA		X				X		X	NA	NA		X				X		0,61566	0,68807	1,11761
MC14			X			X	NA		X				X		X	NA	NA			X				X	0,55945	0,58519	1,04601
MC15			X		X		NA		X				X		X	NA	NA		X				X		0,443	0,44401	1,00227
MC16			X			X	NA		X				X	focale		X				X				X	0,31002	0,47087	1,51883
MC17			X		X		NA		X		NA	NA	NA		X	NA	NA		X				X		0,61338	0,47255	0,7704
MC18	no clinical data																								0,67049	0,76632	1,14292
MC19	no clinical data																								0,37248	0,02660	0,07142
S12	no clinical data																								0,34055	0,56593	1,66184
S41	no clinical data																								0,65602	0,53987	0,82295
S24	no clinical data																								0,46037	0,60286	1,30950
S10	no clinical data																								0,56337	0,66131	1,17385
S31	no clinical data																								0,50423	0,62333	1,23621
S21	no clinical data																								0,45194	0,62594	1,38503
S25	no clinical data																								0,49038	0,68664	1,40021
S38	no clinical data																								0,47043	0,58718	1,24820
S42	no clinical data																								0,44305	0,59683	1,34711
S43	no clinical data																								0,71835	0,62929	0,87603
S11	no clinical data																								0,59773	0,76225	1,27525
S19	no clinical data																								0,57478	0,64013	1,11371
S22	no clinical data																								0,50811	0,54250	1,06768

Table 1. Clinical evaluation. Full clinical data available for a cohort of CRC patients analysed in this study. T: tumour staging according to TNM classification; N: number of positive lymph nodes; M: the occurence of metastasis; G: tumour grading according to tumour invasiveness; Dukes' : tumour staging according to Dukes' classification; S: overall tumour staging according to ASCO guidelines; NA: not assessed. All data were collected by an independent group of pathologists.