The role of the neuropilins in tumour angiogenesis and tumour progression

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Summary

Neuropilins (NRPs) are multifunctional receptors for class 3 semaphorins, which are responsible for axon guidance during the development of the nervous system in vertebrates, and for vascular endothelial growth factors (VEGFs), essential for vascular development and angiogenesis in disease. There is now a large body of evidence that NRPs also mediate tumour angiogenesis and progression, and they have also emerged as novel therapeutic targets in cancer. Many neoplastic cell types express NRPs, and NRP1 and NRP2 upregulation is positively correlated with tumour progression and poor patient prognosis in several cancer types (Pellet-Many et al., 2008). Recently, NRPs have been shown to play novel roles in the tumour stem cell niche and in regulation of tumour immunity. This chapter focuses on the role of NRPs in tumour angiogenesis and tumour progression, focusing on the role of the NRPs as modulators of VEGF function, and highlighting approaches to therapeutic targeting of NRPs in cancer.

Neuropilin Structure

NRP1 and NRP2 are transmembrane glycoproteins that share a similar domain structure and have 44% amino acid sequence homology. The structures of NRP1 and NRP2 are divided into 5 main domains: large extracellular regions containing two CUB (a1/a2) domain, FV/FVIII (b1/b2) domain, and cMAM domains, a single transmembrane domain, and a short cytoplasmic region of 44 amino acid residues in NRP1 and 43 in NRP2 (Kolodkin et al., 1997; Pellet-Many et al., 2008). In the extracellular region, CUB (a1/a2) domains are important for binding to semaphorins. The b1/b2 domains are required to bind VEGFA and also contribute to semaphorin binding. The role of the NRP MAM domain is unclear, but it is thought to be important for protein stability and to play a

role in NRP1 oligomerization, largely based on function of other MAM domains present in diverse proteins (Nakamura and Goshima, 2002).

NRP1 is a glycoprotein but its glycosylation varies between different cell types. NRP1 glycosylation occurs by the addition of an O-linked heparin sulphate and/or chondroitin sulphate glycosaminoglycan (GAG) moiety preferentially to serine 612 in the linker region between the b2 and MAM domains. GAG modifications may enhance both VEGF binding to NRP, and cell survival, and downregulate VEGFR2 expression levels in vascular smooth muscle cells (Frankel et al., 2008; Shintani et al., 2006). In glioma cells, overexpression of a non-GAG form of NRP1 (NRP1 S612A) leads to enhanced cell invasion in a 3D matrix and increased levels of tyrosine phosphorylated p130Cas, indicating that GAG modified NRP1 plays a negative role in regulating invasion. It is suggested that the balance between GAG modified and unmodified NRP1 might be important for determining invasive potential (Frankel et al., 2008).

VEGF signalling

Vascular endothelial growth factor (VEGF) is a potent angiogenic factor, essential for the development of the vasculature. VEGF levels are up-regulated in many tumours and its contribution to pathogenic angiogenesis in cancer, eye diseases and other disorders is well established.

The mammalian VEGF (vascular endothelial growth factor) family consists of five homodimeric polypeptides of ≈40 kDa: VEGFA, -B, -C, -D and PlGF (placental growth factor) (Ferrara et al., 2003) (Zachary and Gliki, 2001). VEGFE is a virally-encoded isoform of VEGF. Since its discovery in 1989, VEGF has emerged has an important signalling protein involved in both vasculogenesis (the formation of the circulatory system) and angiogenesis (the growth of blood vessels from pre-existing vasculature) (Senger et al., 1983). VEGFA is alternatively spliced to generate VEGFA121, VEGFA145, VEGFA165, VEGFA189 and VEGFA206, which are endowed with different biological properties. (Neufeld et al., 1996) (Poltorak et al., 1997). VEGFA121 and VEGFA165 are the most abundant isoforms in mammals, which differ in their biological properties. VEGFA121 lacks exons 6 and 7, and VEGFA165 lacks exon 6.

The binding of VEGFs to NRP1 & NRP2 appears to be mediated by two distinct domains. In VEGFA165, these correspond to the basic heparin-binding domain encoded by exon 7 and the carboxy terminus of exon 8 (Jia et al., 2006) (Soker et al., 1998). Binding of VEGFA121, which lacks exon 7, to NRP1 has been more controversial. Gitay-Goren, H., et al. were unable to detect VEGFA121 binding. However, Pan, et al (2007b) have shown that VEGFA121 can directly bind to NRP1 using in vitro surface Plasmon resonance (SPR) analysis. However it should be noted that the K_D observed for both VEGFA121 (~.2uM) and VEGFA165 (~.1uM) in Pan et al (2007b) were significantly lower than previously published work yielding a K_D of ~.5nM for VEGFA165 binding to NRP1 using cell free ligand binding assays (Gitay-Goren et al., 1996) (Pan et al., 2007b) (Jia et al., 2006).

VEGF activity is mediated by high affinity tyrosine kinase receptors (VEGFR). There are three main subtypes of VEGFR (VEGFR-1 (Flt-1), VEGFR-2 (KDR/Flk-1) and VEGFR-3 (KDR/Flk-4). VEGFR1 is critical in the regulation of migration of endothelial precursors as well as mature monocyte/macrophages. VEGFR2 is the major transducer of VEGF function in vascular endothelial cells (ECs), whereas VEGFR3 is required for lymphatic endothelial function (Alitalo and Carmeliet, 2002)

VEGFs bind with different affinities to VEGFRs. VEGFA binds to VEGFR1 and VEGFR2; VEGFC and VEGFD bind VEGFR2 and VEGFR3; VEGFB and PIGF bind only to VEGFR1; and VEGFE binds only to VEGFR2. VEGFs binding to NRP1 and NRP2 modulates the biological outcome of VEGF/VEGFR signalling (Gluzman-Poltorak et al., 2000) (Koch et al., 2011; Pellet-Many et al., 2008; Soker et al., 1998). NRP1 is known to interact with some heparin-binding isoforms of VEGFA, B, E and PIGF, whereas NRP2 interacts with VEGFA, C and D (Hagberg et al., 2010) (Karpanen et al., 2006). NRP1 is a high-affinity receptor for VEGFA in both endothelial and tumour cells, and NRP1/VEGFR2 co-expression can enhance VEGF-induced chemotaxis in comparison with cells expressing only VEGFR2 (Soker et al., 1998). Co-expression of NRP1 with VEGFR2 also enhances VEGF binding to VEGFR2, VEGFR2 phosphorylation and VEGF-induced signalling and migration (Whitaker et al., 2001), though NRPs are not required neither for high affinity binding of VEGFA to VEGFR2, nor for VEGFA activation of VEGFR2, and downstream signalling pathways (Pellet-Many et al 2008).

Expression of Neuropilins in Cancer

Numerous studies have reported expression of NRPs in diverse human tumours (Table 1). NRP expression is detected both on tumour vessels and also on a large variety of cancer cells types. NRP2 is also expressed by a variety of neoplastic cell types (Table 1).

Tumour	NRP1	NRP2
Astrocytomas	x	ND
Neuroblastomas	x	x
Gliomas	x	ND
Glioblastomas	x	ND
Pituitary tumours	x	ND
Endocrine pancreatic Tumours	ND	x
Pancreatic adenocarcinomas	x	x
Gastric cancer	x	ND
Colon cancer	x	ND
Acute Myeloid Leukemia (AML)	x	ND
Chronic Lymphocytic Leukemia B	x	ND
Breast Cancer	x	ND
NSCLC	x	x
Lung Cancer	x	x
Melanomas	x	x
Prostate Cancer	x	x
Ovarian carcinomas	x	ND
Bladder cancers	ND	x
Osteasarcomas	ND	x

Table 1: Neuropilins (NRP)s expression in cancer cells. Cells from indicated tumours we probed for NRP1 & NRP2expression. (X) indicates detection of protein and / or mRNA expression, (ND) indicates no data or inconsistent data; Adapted from (Grandclement and Borg, 2011).

NRPs can influence tumour progression in multiple ways. NRPs form complexes with VEGF Receptors (VEGFR1 and VEGFR2) and thereby enhance VEGF signalling through VEGFR2 (Pellet-Many et al., 2008). Thus, NRPs can influence tumour vascularisation. NRPs also regulate other receptor signalling pathways important in stimulating growth of tumour cells, endothelial cells and/or tumour-associated stromal cells (eg PDGFR , c-Met, and TGFBR; covered in other chapters of this book), and therefore also have the potential to mediate growth and migration of tumour cells, VEGF-independent tumour vascularisation and expansion of fibroblasts and other stromal cells. Lastly, the NRPs are expressed in monocytic cells and on regulatory T-cells, and have been implicated in recruitment of immunomodulatory cells to cancers.

Neuropilin Function and Cancer

Several studies have highlighted the role of NRP in multiple aspects of cancer biology. In vitro studies have pointed to a role for NRP1 in mediating endothelial and tumour cell motility. Evans et al, have shown that tyrosine phosphorylation of p130Cas, a key molecule required for cell motility, is stimulated by HGF and PDGF in glioma cells and VEGF in endothelial cells via NRP1. Furthermore they showed that knockdown of NRP1 or p130Cas was able to inhibit growth factor-mediated migration of glioma and endothelial cells. This highlights the role of a NRP1 / p130Cas pathway in the regulation of endothelial and tumour cell motility, which has implications for the mechanisms involved in angiogenesis and tumour metastasis (Evans et al., 2011). Fantin et al have recently shown that NRP1 is important for actin remodelling and filopodia formation in endothelial tip cells via CDC42. This leads to proangiogenic signals to be converted into tip cell responses that are important for vessel sprouting and branching (Fantin et al., 2015). Other studies support a role for NRP1 in mediating cancer cell migration. For example, NRP1 knockdown using targeted siRNA inhibited breast carcinoma cell migration (Bachelder et al., 2003). However, the mechanisms mediating the role of NRP1 in cancer cell migration are presently unclear.

Several in vivo and clinical studies have pointed to important roles for NRPs in cancer growth in vivo (Graziani and Lacal, 2015). Miao et al reported that inducible overexpression of NRP1 in prostate carcinoma cells in vivo resulted in larger and highly vascular tumours, at least partly driven by VEGF, since NRP1 overexpressing tumours exhibited increased VEGF expression (Miao et al., 2000). Parikh et al showed that subcutaneous xenografts of stably transfected

KM12SM/LM2 human colon cancer cells overexpressing NRP-1 led to increased tumor growth and angiogenesis in nude mice. (Parikh et al., 2004). Expression of NRP1 is thought to have important implications for tumour metastasis and therapeutic intervention. Studies have shown that NRP1 is predominantly expressed in metastatic cells and its inactivation by the use of an anti–NRP1-binding peptide is sufficient to induce breast cancer tumour cell apoptosis (Bachelder et al., 2001) (Barr et al., 2005). Evidence that VEGFA binding to NRP1 is important for NRP1's role in tumour growth has come from a study showing that a NRP1 knockin mouse model containing a mutation in the b1 domain, which prevents VEGF binding ($Nrp1^{Y297A/Y297A}$), display reduced growth of syngeneic B16-F1 mouse melanomas (Fantin et al 2014).

NRP1 and NRP2 have also been linked with tumour growth and disease progression in human cancer (Guttmann-Raviv et al., 2006) (Ellis, 2006) (Latil et al., 2000). For example, NRP1 is up-regulated in gastrointestinal carcinomas, which appears to be correlated with increased invasive behaviour (Hansel et al., 2004). Co-expression of NRP1 and NRP2 is also associated with NSCLC tumour progression (Kawakami et al., 2002).

Despite the majority of NRP studies reporting a pro-tumourigenic role, others suggest that NRP expression in cancer may play different roles in different tumour types. In Panc-1 cells, overexpression of NRP1 reduced tumour incidence and volume in vivo, and NRP1-targeted siRNA was shown to increase tumour incidence in the same model (Gray et al., 2005). In addition to its VEGFR dependent actions, there is growing evidence that NRP1 has important functions in tumours independent of VEGFRs and possibly receptor tyrosine kinases for other cytokines . Studies in melanomas in which VEGFRs 1 and 2 are absent have shown that NRP1 is able to promote invasion through the activation of selected integrins, which can then recruit VEGFA and metalloproteinases and therefore modulate downstream signalling (Graziani and Lacal, 2015).

Cancer Biology Stem Cells

Cancer stem cells (CSCs) have been described in various cancers. Recently, studies have been done using a mouse model of skin tumourigenesis in order to understand the role of the vascular niche and VEGF signalling on controlling the stemness (the ability to self-renew and differentiate) of squamous skin tumours during the early stages of tumour progression. In this study, it was observed that VEGF signalling through VEGFR2 in endothelial cells is critical to sustain

angiogenesis and to create a vascular niche for CSCs. NRP1 also played an essential role in skin tumourigenesis in this work, as conditional genetic deletion of NRP1 in the epidermis reduced the number of induced squamous skin tumours. Furthermore, specific deletion of NRP1 from the tumour epithelial cell compartment abrogated the ability of VEGF to stimulate tumour cell proliferation. Taken together, these results highlighted the essential role of NRP1 in maintaining a VEGF autocrine loop which contributes to tumour initiation and CSC expansion in skin tumours, with important implications for the prevention and treatment of epithelial cancers (Beck et al., 2011) . In a further study in 2012, Hamerlik et al found that NRP1 is important for the proliferation of human glioma stem-like cells by maintaining autocrine VEGF production, allowing for sustained activation of downstream intracellular prosurvival pathways and promotion of Glioblastoma tumour growth, invasiveness, and enhanced resistance to bevacizumab (Hamerlik et al., 2012).

Tumour Angiogenesis

In cancer, angiogenesis plays a fundamental role during the transition of tumours from a benign to a malignant state. So far, several proteins have been identified as angiogenic factors. Among those are vascular endothelial growth factor (VEGF), basic fibroblast growth factor (bFGF), angiopoietins (Ang), transforming growth factor (TGF)-α, TGF-β, tumour necrosis factor (TNF)-α, platelet-derived endothelial growth factor, granulocyte colony-stimulating factor, placental growth factor, interleukin-8, hepatocyte growth factor, and epidermal growth factor (Hoeben et al., 2004).

The recognition of VEGF pathway as a key regulator of angiogenesis has led to the development of several VEGF-targeted agents, including agents that prevent VEGFA binding to its receptors (Sun et al., 2005), antibodies against VEGFA (Willett et al., 2004) and small molecules that inhibit the kinase activity of VEGFR-2 thereby block growth factor signalling (Ciardiello et al., 2003) (Drevs et al., 2000). In 2004, Bevacizumab (Avastin), a humanized monoclonal antibody against VEGFA, became the first anti-angiogenic drug approved by the FDA for the treatment for metastatic colorectal cancer in combination with chemotherapy. However, while in some cancer types, bevacizumab displayed a synergistic effect (Deng et al., 2013), in others, bevacizumab had an antagonist effect (Kabbinavar et al., 2003). A possible explanation for this relies on the fact that a treatment that aims to reduce the blood supply of a tumour is also likely to reduce the delivery of any other therapy such as chemotherapy (Niu and Chen, 2010). On the other hand, bevacizumab

can induce normalization of newly formed vessels, and thus allow enhanced delivery of chemotherapy to the tumours (Jain, 2005). Furthermore, Avastin has recently been removed as a breast cancer therapy due to adverse effects associated increased cardiovascular toxicity, although this decision is still controversial (Seddon et al., 2014). Thus there is an argument for additional anti-angiogenic therapies displaying reduced adverse effects to anti-VEGF therapy.

There is limited evidence that specifically supports a major role for NRP1 in tumour angiogenesis, though it is very likely that NRP1 contributes to VEGF-dependent tumour neovascularisation consistent with its role in VEGF-induced endothelial cell migration in cell culture studies and in post-natal angiogenesis in genetic models (Fantin et al 2014, Gelfand et al 2014). A peptide that inhibits VEGF binding to NRP1 has been reported to inhibit angiogenesis and growth of tumour xenografts (Starzec et al., 2006).

Tumour Lymphangiogenesis

The metastatic spread of cancer cells to distant organs is the major cause of morbidity in cancer patients. Recent studies have shown that the lymphatic vascular system is one of the main routes for the spread of tumorigenic cells leading to metastasis (Duong et al., 2012; Yoshimatsu et al., 2016). The distribution and structural features of the lymphatic system, as detailed in chapter 2, make it particularly suited to its emerging role as a major route of metastasis. The process by which the lymphatic system mediates metastasis is called lymph node metastasis and involves the migration of cancer cells from primary tumours to lymph nodes via the lymphatic vessels (figure 1). Once the cancer cells pass though the lymph nodes, they can then further metastasize to distant organs via the blood vascular system. Studies have shown that about 80% of metastasis occurs via the lymphatic system (Leong et al., 2006), starting from the site of the primary tumour, spreading through the lymphatic system via entry of invasive cancer cells into permissive lymphatic capillaries, then metastasizing at regional sentinel lymph nodes before disseminating systematically to distant organs (Duong et al., 2012).

Lymphangiogenesis (the growth of new lymphatic vessels from pre-existing ones (see chapter 2 for more details) has been shown to closely correlate with prognosis in various types of cancer. In 2001, a study using a breast carcinoma mouse model revealed that lymphangiogenesis in tumours promoted the metastasis of cancer cells to the sentinel lymph nodes (Skobe et al., 2001). Tumour

lymphangiogenesis has also been found to be a better predictor of cancer metastasis and survival rate in melanoma patients compared to tumour size (Dadras et al., 2003; Mandriota et al., 2001).

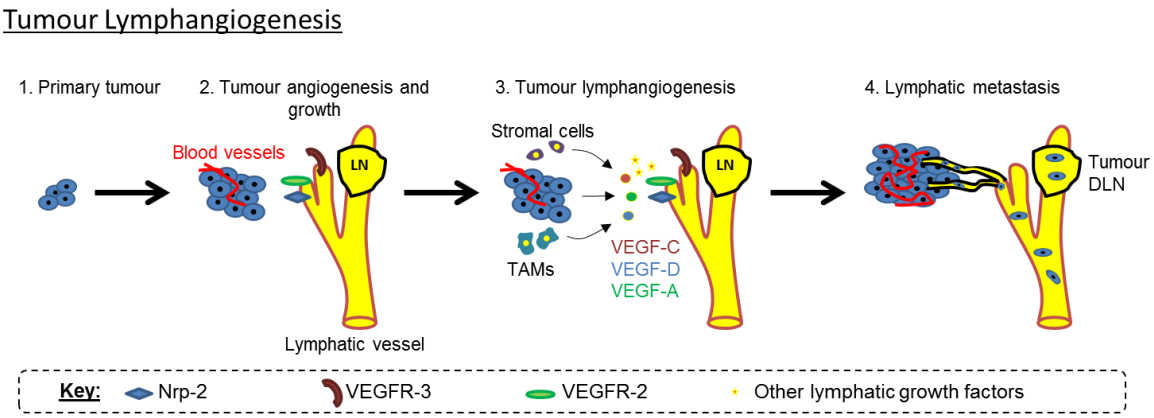

Figure 1. Stages leading to lymph node metastasis. As early stage tumours grow they develop their own blood supply via tumour angiogenesis. Tumour lymphangiogenesis arises mainly from the pre-existing lymphatic vasculature. Tumour lymphangiogenesis is regionally induced during tumourigenesis by lymphatic growth factors such as VEGFC/D/A which are secreted by tumour cells, stromal cells and inflammatory cells (e.g. tumour-associated macrophages (TAMs)). These growth factors bind to their specific receptors and mediate the formation of tumour neo-lymphatics, which facilitate the intravasation of tumour cells into the lymphatic vessels. Once tumour cells enter the lymphatic vessels they can reach the lymph nodes, which are a preferred site for lodgement of metastasizing tumour cells. LN, lymph node; DLN, draining lymph node; TAMs, tumour-associated macrophages. Figure adapted from review by Duong T, Koopman P, et al., 2012.

The most well established growth factors associated with tumour lymphangiogenesis are VEGF-C and VEGFD and, to a lesser extent, VEGFA. These structurally related growth factors are secreted by tumour cells as well as stromal cells, including cancer-associated fibroblasts and macrophages (Ji et al., 2014; Riabov et al., 2014; Schoppmann et al., 2002). The secretion of VEGFC/VEGFD correlates with lymphatic metastasis in various cancers such as breast and prostate cancer, which are prone to metastasis (Alitalo and Carmeliet, 2002). VEGFC overexpression induces the enlargement of tumour-associated lymphatic vessels and induces intercellular gaps which increase lymph flow and facilitate the intravasation of tumour cells into the lymphatics, respectively (Skobe et al., 2001; Tammela et al., 2007). Some studies have suggested that primary tumours can induce

neo-lymphangiogenesis in the sentinel lymph node even before the arrival of metastatic cells, thereby providing a 'pre-metastatic niche' that may support the survival of incoming metastatic cancer cells (Hirakawa et al., 2007; Hirakawa et al., 2005; Ishii et al., 2010; Liersch et al., 2012; Tobler and Detmar, 2006). This was shown in transgenic mice overexpressing VEGFA or VEGFC (Hirakawa et al., 2007; Hirakawa et al., 2005). VEGFD also contributes to the provision of a 'pre-metastatic niche' by inducing the dilation/enlargement of the collecting lymphatics, resulting in increasing lymph flow. This has been shown in a mouse model of VEGFD overexpressing tumours, in which the production of prostaglandins by the collecting lymphatic endothelium is altered leading to inhibition/blocking of smooth muscle cell contraction in these vessels (Karnezis et al., 2012).

VEGFC and VEGFD mediate their effects on both physiological and tumour lymphangiogenesis by binding to their cognate receptors VEGFR-3 and NRP2. VEGFC/VEGFR-3/NRP2 signalling is important for the proliferation, migration and survival of lymphatic endothelial cells (Tammela et al., 2005). Blocking VEGFR-3 signalling has been shown to inhibit tumour lymphangiogenesis as well as lymph node metastasis in animal models (25). VEGFC can also bind to VEGFR-2, which is expressed by both blood and lymphatic endothelial cells (Joukov et al., 1996). This could represent an alternative pathway that VEGFC can induce lymphangiogenesis and potentially also angiogenesis in tumours.

In the vascular system, NRP2 expression is restricted to the veins and lymphatic vessels (Yuan et al., 2002). NRP2 binding to ligand leads to the internalization of NRP2 along with VEGFR-3, resulting in increased affinity of LECs towards VEGFC gradients during lymphatic development (Karpanen et al., 2006). NRP2 has been reported to mediate VEGFC induced lymphatic sprouting alongside VEGFR-3 by modulating lymphatic endothelial tip cell extension and preventing tip cell stalling and retraction during vascular sprout formation (Xu et al., 2010). NRP2 expression is upregulated during tumour lymphangiogenesis, and an anti-NRP2 antibody was shown to reduce tumour lymphangiogenesis and metastasis to the sentinel lymph node and distant organs (Caunt et al., 2008).More recently, NRP2 was reported to mediate tumour lymphangiogenesis in colorectal carcinoma via activation of integrinα9β1/FAK/Erk signalling independent of the VEGFC/VEGFR-3 signalling pathway (Ou et al., 2015) NRP2 has also been shown to mediate anti-lymphangiogenic effects in tumours, thereby playing a protective role against tumour metastasis,

when mediating signalling by members of the semaphorin family of ligands. A recent study by Mumblat et al, reports that furin cleavage-resistant semaphorin-3C (sema3c) can induce the collapse of the cytoskeleton of LECs in a neuropilin-2-, plexin-D1-, and plexin-A1-dependent manner (Mumblat et al., 2015). This effect is not seen with cleaved sema3C (p65-Sema3C). Mumblat et al, generated an active point mutated furin cleavage-resistant sema3C and found that tumors derived from LM2-4 cells expressing this recombinant sema3c, implanted in mammary fat pads, grew at a slower rate, had reduced numbers of blood vessels and lymph vessels, and metastasized much less effectively to lymph nodes. Semaphorin-3F (sema3F) has also been shown to play a protective role against head and neck squamous cell carcinoma (HNSCC) (Doci et al., 2015). Sema3F re-expression in orthotopic HNSCC metastasis mouse models was shown to reduce lymphangiogenesis and lymph node metastasis in these mice, and Sema3F signalling in LECs predominantly required NRP2. NRP1 has not been directly implicated in tumour lymphangiogenesis but has been shown to play an important role in normal lymphatic development. A study by Bouvrée et al, which utilised a mouse model with a mutation in the Semaphorin 3A (Sema3A) binding domain of NRP1, a Semaphorin 3A global knockout and a PlexinA1 deficient mouse model, reported a direct role for Sema3A-Nrp1-PlexinA1 signalling in regulating lymphatic valve development (Bouvree et al., 2012). For a more detailed review on NRPs function in lymphatic development see Ochsenbein et al.(Ochsenbein et al., 2014) and chapter 2.

Role of NRPs in Tumour Immunomodulation

NRP1 plays roles in tumour progression beyond mediating tumour angiogenesis and tumour invasion. As described in chapter 5, NRP1 plays a role in the immune system and recently has been described to play a role in immune modulation of tumours. This chapter will focus on the role of NRPs in the emerging area of “cancer-immunity” (Figure 2). The interplay between cancer and the host immune system is a dynamic process, sometimes termed cancer immunoediting, which shapes the immunogenicity of developing tumours. Three sequential phases of cancer immunoediting have been proposed: elimination, equilibrium and escape and these phases represent a continuum of the interplay between tumour and immune system, shifting between elimination, equilibrium and escape depending on the state of the immune system and inherited or acquired properties of the tumour cells (Dunn et al., 2004). The development of clinical cancer

is in part the consequence of the tilted balance between host immunity and immune tolerance/suppression. Multiple pathways of suppression are at play in tumour microenvironments, including macrophages, regulatory T cells (Tregs), regulatory B cells (Bregs), myeloid derived suppressor cells (MDSCs), plasmacytoid dendritic cells (pDCs) and molecules such as checkpoint inhibitors. NRP1 is expressed on macrophages, Tregs and dendritic cells, thus it is a candidate molecule in eliciting immune tolerance leading to cancer development and progression.

Studies of CD4+CD25+FOXP3+ Tregs, previously called suppressor T cells, have yielded important insights into the role of NRP1 in tumour immunity. Tregs can either directly contact cytotoxic CD8+ T cells or indirectly do so by secretion of immune suppression cytokines such as TGFβ and Interleukin (IL)-10 to inhibit the activation and proliferation of T effector cells. Before we describe NRP1 function, it should be noted that NRP1 expression patterns between human and mice T-lymphocytes appear to be different. NRP1 is expressed on a majority of murine Tregs—up to 70 % of circulating Tregs, whereas it is largely downregulated in tTregs derived from the human thymus (Milpied et al., 2009) and in human pTregs derived from peripheral immune organs in healthy state, although NRP1+ pTregs have been identified in lymph nodes from patients with inflammation (E et al., 2012) (Yadav et al., 2013). However, several studies show that NRP1 is upregulated in Tregs in human cancer. Thus, it has been reported that NRP1 was significantly upregulated on Tregs isolated from the peripheral blood of chronic lymphocytic leukaemia (CLL) patients in comparison with healthy donors (Piechnik et al., 2013). Chaudhary's group showed that NRP1 was upregulated on Tregs isolated from the peripheral blood of patients with pancreatic adenocarcinoma and colorectal cancer with liver metastasis compared with healthy donors (Chaudhary et al., 2014). In metastatic melanoma patients, there was a significant increase in NRP1 expression in tumour infiltrating CD4+ T cells in comparison with peripheral blood CD4+ T cells (Jackson et al., 2014). In addition, NRP1 expression in Tregs isolated from metastatic tumour draining lymph nodes (TDLN) was significantly higher than in metastasis-free lymph nodes in cervical cancer (Battaglia et al., 2008). Interestingly, several lines of evidence indicate that NRP1+ Tregs were reduced after clinical treatment. Reduction of NRP1+ Treg levels was observed in TDLN of patients with cervical cancer following preoperative chemoradiotherapy, and this effect showed a good correlation with the reduction of tumour mass (Battaglia et al., 2008). Piechnik and colleagues found that a significant reduction of NRP1+ Tregs from the peripheral blood of CLL patients followed treatment with the anti-angiogenic drug thalidomide (Piechnik et al., 2013),

suggesting that the reduced level of the chemoattractant VEGF may result in less NRP1+ Treg migration towards the tumour. Collectively these findings suggest: (1) Treg elimination enhances the generation of T effector cells mediating the destruction of the cancer cells; (2) targeting NRP1 is one of therapeutic approaches to prevent Treg infiltration into tumours; (3) NRP1 may be a useful proof of concept pharmacodynamic (PD) biomarker to assess patient's response following immune, chemoradiation and targeted therapies.

Direct evidence of functions for NRP1 in Tregs came from Hansen and colleagues, who proposed that NRP1 mediates Treg migration towards VEGF cues secreted by tumour and stromal cells in the microenvironment. They generated CD4+ T cell-specific NRP1 knockout (KO) mice and demonstrated a significant inhibition of tumour immune escape in various transplantation models and in a spontaneous, endogenously driven melanoma model associated with a strong reduction of tumour growth and increased tumour-free survival. They found a significant reduction of tumour-infiltrating Tregs accompanied by increased activation of CD8+ T cells within these tumours. Importantly, the impaired tumour growth in NRP1 CD4+ T cell specific KO mice could be restored by adoptive transfer of NRP1+ Trges from wild-type mice. Furthermore, it was also reported from the same group that NRP1 is not essential for the immune suppression activity *in vitro*, since when sorted CD4+CD25+ T cells (Tregs) and CD4+ CD25- T cells (T effector) were cocultured, there was no difference in the suppression activity of cells isolated from NRP1 KO mice and wild-type mice. These results indicated that the VEGF/NRP1 axis is a key player of Treg infiltration into the tumour site resulting in a diminished CD8+ cell anti-tumour immune response and enhanced tumour progression (Hansen et al., 2012).

The function of CD8+ cells is to detect cellular abnormality and to protect the host from pathogenic invasion and malignancy. In addition to Treg-mediated tolerance, another mechanism of the control of adaptive immunity is peripheral T cell tolerance, which is critical in preventing pathological immune response mediated by excessive CD8+ T cell activity, and is especially important to limit the activation of self-reactive T cells harboured in the periphery of healthy individuals (Bouneaud et al., 2000). However, this tolerance also forms a strong barrier to inhibit anti-tumour immune activities since many cancer antigens are also expressed in healthy tissue (Rosenberg, 1999). Jackson and colleagues reported that tumour infiltrating NRP1+ CD8+ T cells were increased in metastatic melanoma patients in comparison with healthy donor peripheral

blood cells. Furthermore, they found that NRP1 expression was induced in tolerant self-reactive CD8+ cells in mouse, but was dispensable for the tolerant phenotype since NRP1 KO mice displayed the same functional defects as wild-type self-reactive T cells (Jackson et al., 2014). Several groups reported that CD8+ T cell tolerance was partially regulated by the co-inhibitory surface markers PD-1 and CTLA-4 (Berrien-Elliott et al., 2013; Curran et al., 2010). However it is not clear if NRP1 co-expresses with and/or acts like other immune checkpoint inhibitors such as CTLA-1, PD-1 and PD-L1, and contributes to the inhibitory function. It was also reported that NRP1 was one of the most upregulated genes in exhausted CD8+ cells after chronic infection (Wherry et al., 2007). However it is still unknown if NRP1 expressing CD8+ cells also represent exhausted CD8+ cells in cancer.

Natural killer T (NKT) cells are true T cells, which play a major role in regulating immune responses by bridging the innate and adaptive immune systems. Type I NKT cells, also called invariant NKT (iNKT) cells, express a semi-invariant T cell receptor (TCR) recognising lipid antigens presented by the non-classical MHC class molecule CD1d (Terabe and Berzofsky, 2008). iNKT cells have been shown to have a role in tumour immunosurveillance. In general iNKT cell numbers are decreased in solid tumours including melanoma, colon, lung, and breast cancers, as well as head and neck squamous cell carcinoma (Vivier et al., 2012). Increased iNKT cells in tumour are associated with a better prognosis and this may be because iNKT cells produce large amounts of pro-inflammatory cytokine IL-17 (Milpied et al., 2011). It was found that NRP1 was expressed on thymic recent emigrant iNKT cells, but not on long-lived mature NKT cells (Milpied et al., 2011). However, ligands and functions of NRP1 on iNKT cells remained to be explored. More details of NRP1 in NKT cells are discussed in chapter 5.

Tumour associated macrophages (TAMs) are tissue-resident cells that differentiate from circulating monocytes in peripheral blood and are a major cellular component of murine and human tumours. It has been reported that in most human cancers, macrophage infiltration is correlated with aggressive diseases and poor prognosis. However in colon, gastric and prostate cancers, macrophage infiltration resulted in a better outcome (Komohara et al., 2014). There are two opposing phenotypes in TMAs. The phenotype of TAMs is regulated by specific tumour-derived chemokines and cytokines that polarise macrophages to a proimmune ‘‘M1’’ phenotype via toll-like receptor (TLR) agonists and Th1 cytokines (e.g., interferon gamma (IFNγ) and tumour

necrosis factor alpha (TNFα), or to an immunosuppressive/proangiogenic ‘‘M2’’ phenotype mediated by Th2 cytokines, eg ILs-4, -13 and -10 (Romagnani, 2000; Roszer, 2015). M1 macrophages can act in a proimmune manner directly, by phagocytosis, and indirectly, by production of IL-1b, IL-12 and TNFα and reactive molecular species, and by presenting antigen via major histocompatibility complex (MHC) class II molecules to activate CD8+ cells to destroy cancer cells. In contrast, M2 macrophages can enhance production of the anti-inflammatory cytokine, IL-10, to reduce expression of proinflammatory cytokines; they amplify metabolic pathways that can suppress adaptive immune responses; and they upregulate cell-surface scavenger receptors, such as mannose receptor (MRC1/CD206); mechanisms that suppress immunity and promote angiogenesis in favour of tumour growth (Roszer, 2015).

Recently Casazza and colleagues found that there are different niches within a tumour, which can be categorised either normoxic or hypoxic regions. In the hypoxic region, hypoxia-induced semaphorin 3A (Sema3A) acts as a chemoattractant for M1 macrophages by triggering VEGFR 1 phosphorylation through a heterocomplex of NRP1 and the Sema3A receptor, PlexinA1 (pA1)/PlexinA4 (pA4). Once M1 macrophages arrived in the hypoxic region, NRP1 expression was repressed and M1 macrophages retained in the hypoxic region where they were educated to become M2 macrophages, allowing them to exert immunosuppression and induction of angiogenesis, thus promoting tumour growth. In NRP1-repressed macrophages, Sema3A continued to regulate M2 in an NRP1-independent manner by eliciting pA1/pA4-mediated stop signals, which retained M2 macrophages inside the hypoxic niche. In macrophage-specific NRP1 KO mice, M1 macrophages were trapped in normoxic regions, where they maintained their immune response and prevented the release of angiogenic factors, hence inhibiting tumour growth and metastasis. Thus the migration of macrophages from normoxic to hypoxic regions of the tumour microenvironment is precisely controlled by the Sema3A/NRP1/VEGFR1/pA1/pA4 signalling pathway. Casazza’s study also revealed that Sema3A, not VEGF, is the chemoattractant for macrophage migration, and that there was no additive, synergistic or antagonistic effects when both were added together in an *in vivo* subcutaneous macrophage chemotaxis assay (Casazza et al., 2013). Interestingly it has been reported that Sema3A binds to NRP1 and recruits VEGFR1 to induce neural progenitor cell repulsion, and prolonged interaction of Sema3A and NRP1 induces apoptosis in these cells. Furthermore, VEGF antagonised these effects by directly competing with

Sema3A binding to NRP1 (Bagnard et al., 2001). However, it is unclear how the Sema3A/NRP1 axis plays two opposite roles in these different cell types.

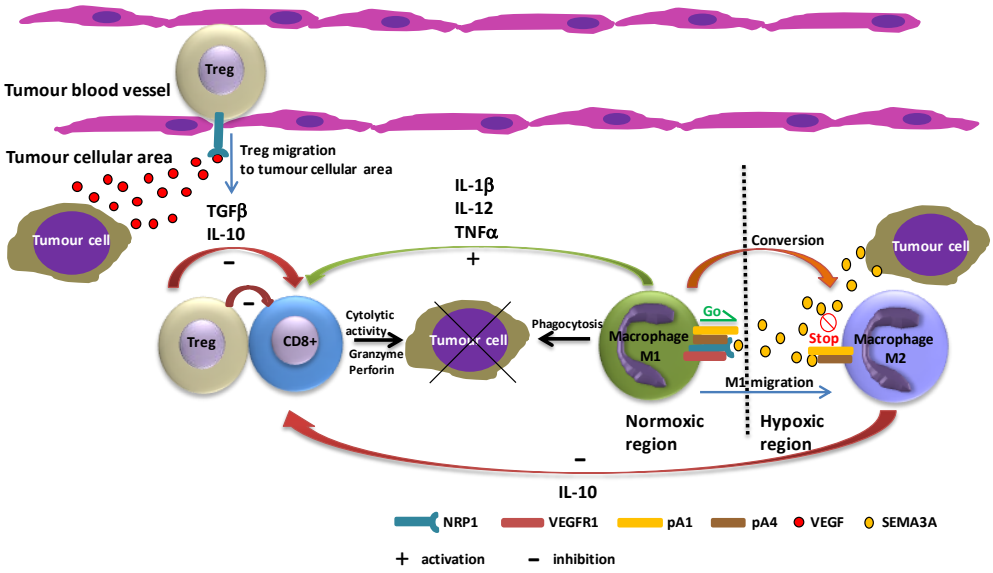

Figure 2. The role of NRP1 in cancer immununity

Tumour secreted VEGF mediates NRP1-expressing Treg migration into tumour, where Tregs either via direct contact or secretion of inhibitory factors such as TGFβ and IL-10, suppress CD8 T cell anti-tumour activity. NRP1 expressing M1 macrophages in normoix region migrate towards SEMA3A secreted by tumour cells in hypoxic region where they lose NRP1 and convert into M2 macrophges to suppress anti-cancer immune response. NRP1 also mediates M1 to M2 macrophages conversion.

Unlike NRP1 which acts predominantly as a co-receptor for Sema3A, NRP2 binds to Sema3B, C, D and F (Prud'homme and Glinka, 2012). It has been reported that the Sema3F /NRP2 axis mediates repulsive migration on human thymocytes and lymphoblastic leukemia/lymphoma tumour cells (Mendes-da-Cruz et al., 2014). Otherwise, there are limited reports on the role of NRP2 in the immune system in the literature, and details are described in chapter 5.

Taken together, the work considered above indicates that NRP1 guides Treg and macrophage migration towards the guidance cues, VEGF and Sema3A, similar to its roles in endothelial migration and neuronal migration/repulsion/outgrowth in development. NRP1 appears to play an immunosuppressive role in cancer and to promote tumour progression. Therefore, NRP1 is of growing interest as a target for anti-cancer therapy, potentially with a triadic mode of action: anti-angiogenesis, immunomodulation and inhibition of tumour metastasis.

Therapeutic Targeting of Neuropilins in Cancer.

Because of its role in mediating several aspects of tumour progression, NRPs are emerging as key targets for development of anti-cancer therapeutics. A key advantage of targeting NRPs is the expected reduced impact on vascular homeostasis, in contrast to VEGF-targeted therapies such as bevacizumab (Avastin), which are associated with a range of cardiovascular side effects. This chapter provides an overview on the current strategies being explored for development of anti-NRP therapeutics for cancer (Figure 3).

Therapeutic targeting of Neuropilin-2 in tumour lymphangiogenesis

The VEGFC/VEGFD-VEGFR3-NRP2 axis is the most well established pathway known to function specifically towards the induction of lymphangiogenesis in pathological conditions such as cancer. A number of studies have shown that targeting this signalling pathway by blocking either VEGFC/VEGFD, VEGFR3 or NRP2 function, using antibodies or soluble constructs, can reduce tumour lymphangiogenesis and inhibit tumour metastasis in experimental models (Wissmann and Detmar, 2006).

A monoclonal antibody against the Nrp-2 co-receptor was shown to bind exclusively to the b1-b2 domains of Nrp-2, thereby directly blocking VEGF binding without affecting semaphorin binding to the CUB (a1-a2) domains (Caunt et al., 2008). Caunt et al showed that by preventing VEGFC binding to Nrp-2, this anti-Nrp-2 antibody blocked lymphatic endothelial cell migration but not proliferation, and resulted in a reduction of VEGFC-driven lymphangiogenesis, but without affecting vascular permeability in vivo. More importantly, using two mouse models of breast adenocarcinoma (66C14) and rodent glioblastoma (C6), they showed that anti-Nrp-2 treatment led to a reduction in tumour lymphangiogenesis and inhibited the development of metastasis to sentinel lymph nodes and distant organs in these mice. These data imply that targeting VEGFC binding to Nrp-2 may be a promising tool to block tumour metastasis via targeted inhibition of tumour lymphangiogenesis.

Anti-NRP1 mAbs

Research has been done by Genentech on the development of antibodies that target the b1 domain of NRP1, thus blocking VEGFA binding to NRP1, and inhibiting VEGFR2-NRP1 complex

formation, VEGF-induced migration and sprouting of endothelial cells. Pan et al (2007) showed that an antibody targeted to the b1 domain of NRP1 caused a range of effects in endothelial cell cultures, including inhibition of VEGFR2 complex formation, VEGF-induced migration and vascular sprouting, reduced angiogenesis in a neonatal retinal neovascularization model, and inhibition of tumour growth and tumour vascularization in mouse xenograft models (Guttmann-Raviv et al., 2006; Pan et al., 2007a). NRP1 inhibition, on its own, had a small effect on tumour growth, but the combination of anti-NRP1 antibody with bevacizumab had an additive effect, leading to a stronger reduction of tumour growth. These findings suggested that the combination of anti-NRP-1 and anti-VEGF agents could improve the survival of patients with advanced malignancies (Guttmann-Raviv et al., 2006; Pellet-Many et al., 2008) (Pan et al., 2007a)

Genentech have generated several additional anti-NRP monoclonal antibodies that block NRP1 interactions with VEGFA and semaphorins (Appleton et al., 2007; Bumbaca et al., 2012; Pan et al., 2007a). In 2014, Genentech conducted a phase I clinical trial using an anti-NRP1 antibody, MNRP1685A, in patients with advanced solid tumours. Results showed that MNRP1685A was well-tolerated as a single agent, but had modest clinical activity. MNRP1685A was also used in combination with bevacizumab and paclitaxel in patients with advanced solid tumours (Weekes et al., 2014), and when co-administered with bevacizumab, caused a high rate of clinically significant proteinuria, resulting in the cessation of the phase I clinical trial (Patnaik et al., 2014).

Small Interfering RNAs or microRNAs

Small interfering RNAs are small pieces of double-stranded RNA that can be used to "interfere" with the translation of proteins by binding to and promoting the degradation of messenger RNA (mRNA) at specific sequences, thus inhibiting the production of specific proteins. siRNA have been used to target NRP1 resulting in reduction of human tumour growth, angiogenesis and metastasis formation in models of hepatocellular carcinoma (Berge et al., 2010; Raskopf et al., 2010), acute myeloid leukemia (Lu et al., 2008), and lung cancer (Hong et al., 2007).

NRP1 is also involved in targeting several microRNAs (miR) that are known to be involved in angiogenesis and invasion: miR-9, miR-181b, and miR-320 {Wu, 2014 #75}{Zhang, 2012 #74}. MicroRNAs (miRNAs) have been implicated in regulating diverse cellular pathways and there is

evidence that various miRNAs function as oncogenes or tumour suppressors. Zhang et at, in 2012 observed that miR-320a may suppress the invasion and metastasis of colorectal cancer (CRC) by directly binding to the 3'UTR of NRP-1. Thus, miR-320a might work as a novel potential marker to identify CRC patients that are at an elevated risk for developing liver metastasis (Zhang et al., 2012). These findings support the possible development of siRNA and microRNA-based agents as anti-angiogenic and/or anticancer drugs.

Cell-Penetrating Peptides

Another approach to NRP1-targeted therapy is cell-penetrating peptides (CPPs). CPPs express a C-terminal consensus sequence (R/KXXR/K), referred to as the C-end rule (CendR) motif that interacts with the b1/b2 domain of NRP1 (Sugahara et al., 2009; Teesalu et al., 2009). This interaction induces the internalisation of the CPP into NRP1-expressing cells via an endocytic mechanism. Once inside NRP1-expressing cells, CPPs are able to target NRP-1 expressing tissues (Teesalu et al., 2009). Importantly, CPPs can also be modified in order to create tumour penetrating peptides (TPPs) and be able to deliver drugs into tumours (Teesalu et al., 2013). As NRP1 is expressed in several cancer types, co-administration of TPPs with cancer drugs, is emerging as an attractive approach as it could allow cell internalization of high molecular weight drugs that cannot cross the plasma membrane and selective targeting of tumour tissues (Sugahara et al., 2010) (Graziani and Lacal, 2015).

Small molecule inhibitors

There has been growing interest in developing inhibitors of VEGFA interactions with NRP1 (Jia et al., 2006). Jia, et al. developed a peptide antagonist of VEGF binding to NRP1 (EG3287), capable of binding specifically to NRP1 through the b1 domain. Thus, EG3287 inhibited VEGFA165 binding to endothelial cells and to breast carcinoma cells endogenously expressing NRP1 but not KDR or Flt-1. This study also demonstrated that the C-terminus of VEGFA encoded by exon 8 plays a key role in EG3287 (and VEGFA165) binding to NRP1. In particular, a critical role is played by the C-terminal arginine encoded by exon 8 in VEGFA binding to the NRP-1 b1 domain (Jia et al., 2006). EG3287 also antagonised VEGFA165 binding to NRP1 in human tumour cells, and enhanced their sensistivity to cytotoxic chemothrapeutic agents (Jia et al., 2010) . Another peptide based on a modification of EG3287 (EG00086) reduced the viability of A549 lung cancer cells and, similar to EG3287, enhanced the cytotoxicity of the chemotherapeutic agents, 5-fluorouracil (5-FU) and

paclitaxel (Jia et al., 2014). Based on structure-function analysis of EG3287, Jarvis et al developed the first small molecule inhibitor for NRP1, called EG00229 (Jarvis et al., 2010). In this study, data generated by analysis of NRP1 b1 domain mutants, X-ray crystallography and NMR spectroscopy of NRP1 bound to EG00229, showed that EG00229 is able to bind the NRP1 b1 domain at a defined site, and thereby reduce VEGFR2 phosphorylation in endothelial cells and as cell migration *in vitro* (Jarvis et al., 2010). In addition, EG00229 enhanced the potency of the chemotherapeutic drugs paclitaxel and 5-fluorouracil in tumour cells (Jarvis et al., 2010).

Recently, two groups reported anti-tumour activity of EG00229 in vivo. Grun, et al performed a study using a subpopulation of epidermal cancer stem cells (ECS cells), that form rapidly growing, invasive and highly vascularized squamous cell carcinomas. These cells produce high levels of VEGFA, which is important for aggressive tumour growth, and accordingly, treatment with bevacizumab reduces tumour vascularity and growth. However, these cells lack VEGFR1 and VEGFR2 and therefore VEGF signalling appears to occur principally via NRP1. EG000229 treatment of tumour burdened mice reduced tumour spheroid size and tumour invasion, and attenuated tumour growth (Grun et al., 2016). These findings suggest that antagonism of VEGF binding to NRP1 may inhibit tumour growth via a mechanism independent of VEGFR activation.

Miyauchi and colleagues examined effects of NRP1 inhibitors in glioma models. NRP1 is expressed on glioma-associated microglia and macrophages (GAMs) from glioma patients of varying grades. Genetic ablation of NRP1 specifically in mouse CNS microglia and macrophages, delayed glioma progression accompanied by reduced tumour growth pace, longer survival period, less vascularity, and increased M1/M2 GAM ratio. Strikingly, the inhibitory effect of genetic loss of NRP1 in these cells was reproduced by dosing glioma bearing wild type mice with EG00229 (Miyauchi et al., 2016). These two studies demonstrate proof of concept in targeting NRP1 with small molecule inhibitors as a potential therapy for suppression of cancer progression in vivo.

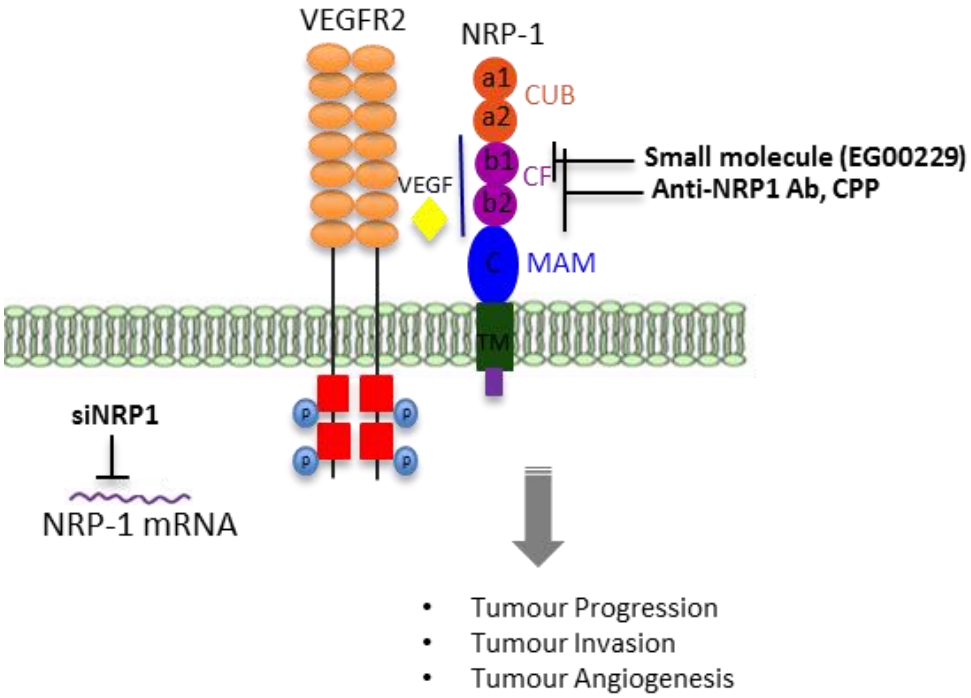

Figure 3. Neuropilin structure and strategies for therapeutic targeting in cancer.

Summary

Both NRP1 & NRP2 play important roles in the regulation of vascular development during tumour progression, but whereas NRP1 is primarily important for angiogenesis, NRP2 plays a key role in tumour Lymphangiogenesis. Recent studies implicate NRP1 as a mediator of cancer immunomodulation functioning as a regulator of tumour infiltrating lymphocytes, including T-regulatory cells and tumour associated macrophages. Due to its pleiotropic effects in tumour progression, NRP1 & NRP2 provide promising molecular targets for cancer therapies. Though at the time of writing this chapter we are not aware of any NRP-targeted therapeutic in ongoing clinical trials, several approaches to targeting NRP1 in cancer are currently being developed, and pre-clinical proof-of-concept studies suggest that some of these may offer future promise as anti-cancer therapeutics.

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