

The Role of the Co-receptor Neuropilin-1 in Human Vascular Smooth Muscle Cells.

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List of Abbreviations

7AAD: 7-aminoactinomycin D.

α -SMA: α -smooth muscle actin.

Ang: Angiopoietin.

BMP1: Bone Morphogenetic Protein 1.

BSA: Bovine serum albumine.

CAM: Cell adhesion molecule.

Cas: Crk-associated substrate.

CD: Cluster of differentiation.

CDC: Cell division cycle protein.

CMV: Cytomegalovirus.

CNS: Central nervous system.

CPM: Counts per minutes.

CS: Chondroitin sulphate.

CSPG: Chondroitin sulphate proteoglycan.

CUB: Domain with homology with complement binding factors C1s/C1r, sea urchin fibropellins (called Uegf) and Bone Morphogenetic Protein 1 (BMP1).

Da: Dalton.

DAB: Diaminobenzidine.

DAG: Diacylglycerol.

DAPI: 4',6-diamidino-2-phenylindole.

ΔC: C-terminus deleted.

DMEM: Dulbecco's Modified Eagle Medium.

DNA: Deoxyribonucleic acid.

DRG: Dorsal root ganglia.

ds: Double stranded.

DTT: Dithiothreitol.

EC: Endothelial cell.

ECM: Extracellular matrix.

Efs/Sin: Embryonal fyn substrate/ Src interacting.

e.g.: exempli gratia.

eNOS: Endothelial nitric oxide synthetase (NOS3).

Eph: Ephrin receptor.

ERK: Extracellular signal-regulated kinase.

ET: Endothelin.

FV/VIII: Blood coagulation factors V and VIII homology domain.

FAK: Focal adhesion kinase.

FBS: Foetal bovine serum.

FcR: Crystallisable fragment receptor.

FGF: Fibroblast growth factor.

FKHR1: Forkhead transcription factor 1.

Flt: Fms-like tyrosine kinase.

Fms: Fibroblast motility stimulating factor.

Flk-1: Foetal liver kinase.

GAG: Glycosaminoglycan.

GAIP: G α -interacting protein.

GalNAc: N-acetyl-D-galactosamine.

GAP: GTPase activating protein.

GAPH: Glyceraldehyde 3-phosphate dehydrogenase.

GDP:: Guanosine diphosphate.

GEF: Rho guanine exchange factor.

GFP: Green fluorescent protein.

GIPC: RGS-GAIP interacting protein C terminus.

GlcA: D-glucuronic acid.

Grb2: Growth factor receptor bound protein 2.

GTP:: Guanosine triphosphate.

HAoSMC: Human Aortic smooth muscle cells.

HBMEC: Human brain microvascular endothelial cells.

HCAEC: Human coronary artery endothelial cells.

HCASMC: Human coronary artery smooth muscle cells.

HEF1: Human enhancer of filamentation.

HEK: Human embryonic kidney.

HELP: HEF1-Efs-p130Cas-like.

HEPES: 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

HGF: Hepatocyte growth factor.

HIF-1: Hypoxia inducible factor-1.

HRE: Hypoxia response element.

HRP: Horseradish peroxidase.

Hsp 27: Heat shock protein 27.

HSPG: Heparan sulphate proteoglycan.

HUVEC: Human umbilical vein endothelial cells.

IFN γ : Interferon γ .

Ig: Immunoglobulin.

IP₃: Inositol 1, 4, 5-triphosphate.

IPT: Immunoglobulin-like domains shared by plexins and transcription factors.

IQGAP: IQ (isoleucine and glutamine) motif containing GTPase activating protein.

ITR: Inverted terminal repeat.

kbp: Kilo base pairs.

kDa: KiloDalton.

KDR: Kinase insert domain-containing receptor.

LB: Lysogeny broth.

LDL: Low density lipoprotein.

LMCI: Lateral motor column.

LMCm: Medial motor column.

MAM: Meprin, A5 antigen, and receptor tyrosine protein phosphatase μ homology domain.

MAPK: Mitogen-activated protein kinase.

MASA syndrome: Mental retardation, Aphasia, Shuffling gait, Adducted thumbs syndrome.

MEK: MAPK/ERK kinase.

miRNA: microRNA.

MMP: Matrix degrading metalloproteinase.

MOI: Multiple of infection, number of viral particles per cell.

mRNA: Messenger ribonucleic acid.

MRS: Met-related sequence.

Ndst1: N-acetylglucosamine N-deacetylase/N-sulfotransferase 1.

NIP: Neuropilin interacting protein.

NO: Nitric oxide.

NOS: Nitric oxide synthase.

NRP: Neuropilin.

nt: Nucleotide.

ODD: Oxygen degradation domain.

ORF: Open reading frame.

PAEC: Porcine aortic endothelial cell.

PAF: Platelet activating factor.

PBS: Phosphate buffer saline.

PBST: Phosphate buffer saline tween.

PDGF: Platelet derived growth factor.

PDGFR: Platelet derived growth factor receptor.

PDZ: structural domain first found in post-synaptic density protein (PSD95), *Drosophila* disc large tumor suppressor (DlgA), and zonula occludens-1 protein (zo-1).

PE: phycoerythrin

PECAM-1: Platelet endothelial cell adhesion molecule-1.

PFA: Paraformaldehyde.

PHD: Prolyl hydroxylase domain-containing protein.

PI3K: Phosphatidylinositide 3 kinase.

PIP₃ Phosphatidylinositol (3,4,5)-trisphosphate

PKC: Protein kinase C.

PKD: Protein kinase D.

PLC: Phospholipase C.

PIGF: Placental growth factor.

PMSF: phenylmethanesulphonylfluoride.

PNS: Peripheral nervous system.

PVDF: Polyvinylidene fluoride.

Pyk2: Proline-rich tyrosine kinase 2.

Rac: Ras-related C3 botulinum toxin substrate.

Ras: Rat sarcoma.

Rho: Ras homolog gene family.

RGS: Regulator of G protein signalling.

RISC: RNA induced silencing complex.

RNA: Ribonucleic acid.

S612A: Serine 612 Alanine.

SDS: Sodium dodecyl sulphate (or sodium lauryl sulphate).

SDS-PAGE: Sodium dodecyl sulfate polyacrylamide gel electrophoresis.

SEM: Standard error mean.

Sema: Semaphorin.

SH2: Src homology domain 2.

SH3: Src homology domain 3.

SHP2: Src homology domain 2 containing tyrosine phosphatase.

siRNA: Small interfering RNA.

SMC: Smooth muscle cells.

SM-MHC: Smooth muscle-myosin heavy chain.

Sos: Son of sevenless protein.

Src: Sarcoma.

SV5: Simian virus 5.

TBS: Tris buffer saline.

TBST: Tris buffer saline tween.

TCEP: Tris(2-carboxyethyl)phosphine.

TFPI: Tissue factor pathway inhibitor.

TGF: Transforming growth factor.

Tris: Tris(hydroxymethyl)aminomethane.

Tyr: Tyrosine.

UDP: Uridine diphosphate.

VE-cadherin: vascular endothelial cadherin.

VEGF: Vascular endothelial cell growth factor. Unless specified, when VEGF is cited, it refers to the specific isoform VEGF-A₁₆₅.

VEGFR: Vascular endothelial growth factor receptor.

VEGFR1: Vascular endothelial growth factor receptor 1, also known as Flt-1 (Fms-related tyrosine kinase 1).

VEGFR2: Vascular endothelial growth factor receptor 2, also known as KDR (Kinase insert domain receptor), mice equivalent to Flk-1.

VEGFR3: Vascular endothelial growth factor receptor 3, also known as Flt-4 (Fms-related tyrosine kinase 4).

VPF: Vascular permeability factor.

VRAP: VEGF receptor associated protein.

VSM: Vascular smooth muscle.

VSMC: Vascular smooth muscle cells.

WT: Wild-type.

XIAP: X-linked inhibitor of apoptosis.

Declaration

'I, Caroline Pellet-Many, confirm that the work presented in this thesis is my own. Where information has been derived from other sources, I confirm that this has been indicated in the thesis.'

Abstract

Neuropilin-1 (NRP1) is a co-receptor required for neuronal and vascular development, which binds to class 3 semaphorins and VEGFs. NRP1 has been strongly implicated in VEGF-induced endothelial cell migration. VEGF has been shown to regulate vascular smooth muscle cell (VSMC) function *in vitro*. Evidence from mutant mice also suggests that NRP1 disruption *in vivo* can affect VSMC as well as endothelial function. I therefore investigated the role of NRPs in VSMC biological functions and more particularly in their migration. Western blotting showed that NRP1 and the related molecule, NRP2, were strongly expressed in human coronary artery SMC (HCASMC), whereas the major VEGF signalling receptor VEGFR2/KDR was not detectable. A high molecular weight NRP1-immunoreactive band (>250 kDa) was also strongly expressed in HCASMC, but was not detected either in cognate Human coronary artery endothelial cells (HCAEC) or in Human umbilical vein EC. The high molecular weight species was decreased significantly by treating the SMCs with chondroitinase, an enzyme that specifically chondroitin sulphate (CS) residues found in CS proteoglycan. Treatment with heparitinase, an enzyme that specifically heparan sulphate (HS) residues also resulted in a decrease of the high molecular weight band but to a lesser extent than chondroitinase. Finally, treatment of SMC with both enzymes caused the complete disappearance of the high molecular weight species. Hence, in SMCs, in addition to the known NRP1 species at 130 kDa, NRP1 exists as a glycosaminoglycan containing either chondroitin sulphate or heparan sulphate polysaccharide chains.

Mutational analysis of candidate O-linked glycosylation sites in the NRP1 extracellular domain showed that glycosylation occurred at serine 612. The importance of this glycosaminoglycan (GAG) modification was assessed by generating a construct of NRP1 lacking this GAG modification, called S612A. This was done by generation of an adenovirus NRP1 mutant with an alanine residue instead of the serine found in the wild-type

species, however, the over-expression of the S612A NRP1 mutant in VSMC caused no significant difference in PDGF-induced HCASMC migration.

VEGF was able to bind significantly to ECs and SMCs but did not induce a significant migratory response of SMCs in contrast to PDGF-AA and PDGF-BB. PDGF-BB-induced HCASMC migration in transwell assays was inhibited by EG3287, a NRP1-specific antagonist, which blocks the ability of VEGF-A₁₆₅ to bind to NRP1. Furthermore, the migratory response to PDGF-BB was significantly decreased by siRNA-mediated knockdown of NRP1, NRP2 or a neuropilin interacting protein (NIP1 or synectin), and by pre-treatment with soluble NRP1 or NRP1 b1 domain (NRP1 VEGF binding domain). NRP1 knockdown also inhibited the migratory response to PDGF-AA.

NRP1 was found to physically interact with PDGFR α , but not with PDGFR β , as determined by co-immunoprecipitation. PDGFR α , but not PDGFR β , phosphorylation was decreased in response to PDGF-AA and PDGF-BB when NRP1 was knocked down in HCASMC.

Intracellular signalling in response to PDGF-BB stimulation was investigated in HCASMCs with NRP1 knockdown. PDGF-BB stimulated tyrosine phosphorylation of the adapter protein p130Cas, which has been strongly implicated in cellular and molecular processes involved in cell migration. NRP1 knockdown reduced p130Cas phosphorylation, but had little effect on signalling pathways, such as ERK1/2, Akt, cofilin, Hsp27 and FAK. To investigate the contribution of the NRP1 intracellular domain in PDGF-induced migration and signalling, I generated a NRP1 construct lacking the intracellular domain by introducing a stop codon after the transmembrane domain. Overexpression of NRP1 lacking its C-terminus in HCASMC resulted in a decrease of PDGF-induced migration and activation of phospho-p130Cas. Furthermore, p130Cas knockdown also inhibited PDGF-induced HCASMC migration, thus reinforcing the importance of p130Cas phosphorylation in NRP1-dependent cell migration.

The findings that NRP1 is strongly expressed in HCASMC in a CS-GAG and a HS-GAG modified form and plays a role in the chemotactic response to PDGF-BB, highlight the possible involvement of NRPs in neointima formation in vasculoproliferative diseases.

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Chapter 1

Introduction

The formation of the vascular system and the function of the adult vasculature are complex processes that involve many cell types and are regulated by many factors, their receptors and effectors. During early embryonic vascular development, the differentiation of angioblasts from the mesoderm and the formation of primitive blood vessels from angioblasts are the two distinct steps which occur at the onset of vascularisation and define vasculogenesis (Risau and Flamme, 1995). Later, the primitive vascular system undergoes a complex process of remodelling of the primary vascular plexus with formation of new vessels from pre-existing ones: this process is known as angiogenesis (Risau, 1997; ten Dijke and Arthur, 2007) (Figure 1.1).

1.1 Vasculogenesis and development of the primary vascular plexus

In both avian and mammalian embryos, blood vessels are first observed in the yolk sac and it was initially thought that they arose from extraembryonic tissues. However, histological analysis later indicated that endothelial cells can also be observed in the embryo, suggesting that blood vessels come from an intraembryonic source. It is now commonly accepted, that endothelial cell progenitors exclusively arise from the mesoderm (Ferguson et al., 2005). The pluripotency of vascular precursors has also proved a complex issue to clarify. Indeed, colocalisation of endothelial and hematopoietic precursors (in the so-called blood islands) within the yolk-sac has been recognised since the beginning of the 20th century (Sabin, 2002) and is induced by signals from the endoderm (Ferguson et al., 2005). This raised the possibility that both cell types originate from a common precursor, the hemangioblast. The existence of this common precursor was suggested by defects in both cell lineages in embryos lacking the vascular endothelial growth factor receptor 2 (VEGFR2) (Shalaby et al., 1995). Also recent evidence indicates that cells with hemangioblast properties are present transiently in the posterior segment of the primi-

tive streak during gastrulation and are defined by the co-expression of VEGFR2 and the mesodermal T-box gene brachyury (Huber et al., 2004). Nevertheless, in some cases, hemangioblasts do not seem to be an essential step for all endothelial development, and direct differentiation of angioblasts from mesoderm is also thought to occur (Drake and Fleming, 2000; Pardanaud and Dieterlen-Livre, 1993). Moreover, the definitive isolation and localisation of the hemangioblasts within the embryo is still awaited. Drake (2003) described the following as essential steps in vasculogenesis: 1) the birth of angioblasts; 2) angioblast aggregation; 3) elongation of angioblasts into cord-like structures; 4) the organisation of isolated vascular segments into capillary-like-networks and, concomitant with step four; 5) endothelialisation and lumenisation. Targeted gene deletion established the vascular endothelial growth factor A₁₆₅ (VEGF-A₁₆₅) gene and its related receptor Flk-1/KDR/VEGFR2 as major regulators of the vasculogenesis process (Carmeliet et al., 1996; Ferrara et al., 1996; Fong et al., 1995). These regulatory systems are conserved amongst species especially in mammals and birds, reptiles and amphibians (Figg and Folkman, 2008).

1.2 Angiogenesis

1.2.1 Sprouting and non-sprouting angiogenesis

After the embryo has formed the primary vascular plexus, further blood vessels are formed through a process called angiogenesis. Angiogenesis can be of two types, sprouting and intussusceptive. Intussusception involves the splitting of pre-existing vessels *in situ* by transcapillary pillars or extracellular matrix (Patan et al., 1996) and is commonly found in the yolk sac and the developing lungs (Short, 1950) and during tumour vascularisation. By contrast, in sprouting angiogenesis, endothelial cells proliferate behind the tip cell of a growing branch in response to cytokines such as vascular endothelial growth factor (VEGF) and lumens form by vacuole fusion. Tip cells are specialised endothelial cells situated at the leading edge of vascular sprouts. They are lumenless and characterised by filipodia extensions that integrate directional cues from their environment (Gerhardt et al., 2003). Sprouting angiogenesis involves proteolytic degradation of the extracellular matrix followed by chemotactic migration towards an unvascularised region and proliferation of endothelial cells, formation of a lumen and finally functional maturation of the endothelium (Risau, 1997). In both forms of angiogenesis, the final step of the angiogenic process is to stabilise the newly-formed vessels through the recruitment of mural cells such as smooth muscle cells (SMCs) and pericytes (Gerhardt and Betsholtz, 2003; Hellstrom et al., 1999), and the integration of the vessel into the surrounding matrix. The endpoint of angiogenesis is a network that meets the nutritional and functional requirement of an organ *i.e.* transportation of oxygen and nutrients to, and removal of waste products from, the tissues.

1.2.2 Regulation of angiogenesis

Physiological angiogenesis occurs mainly during development. In the adult, this process is infrequent and occurs mostly in repair processes such as wound, fracture healing, and in the female reproductive system during the menstrual cycle and pregnancy. In these contexts, angiogenesis is transient and tightly regulated. By contrast, pathological and often uncontrolled or poorly regulated angiogenesis occurs in many diseases such as cancer, diabetic retinopathy, retinal vein occlusion, rheumatoid arthritis, psoriasis and cardiovascular diseases. In the majority of these diseases, the common characteristics of pathological vascularisation include abnormal vascular permeability, defective vascular remodelling and

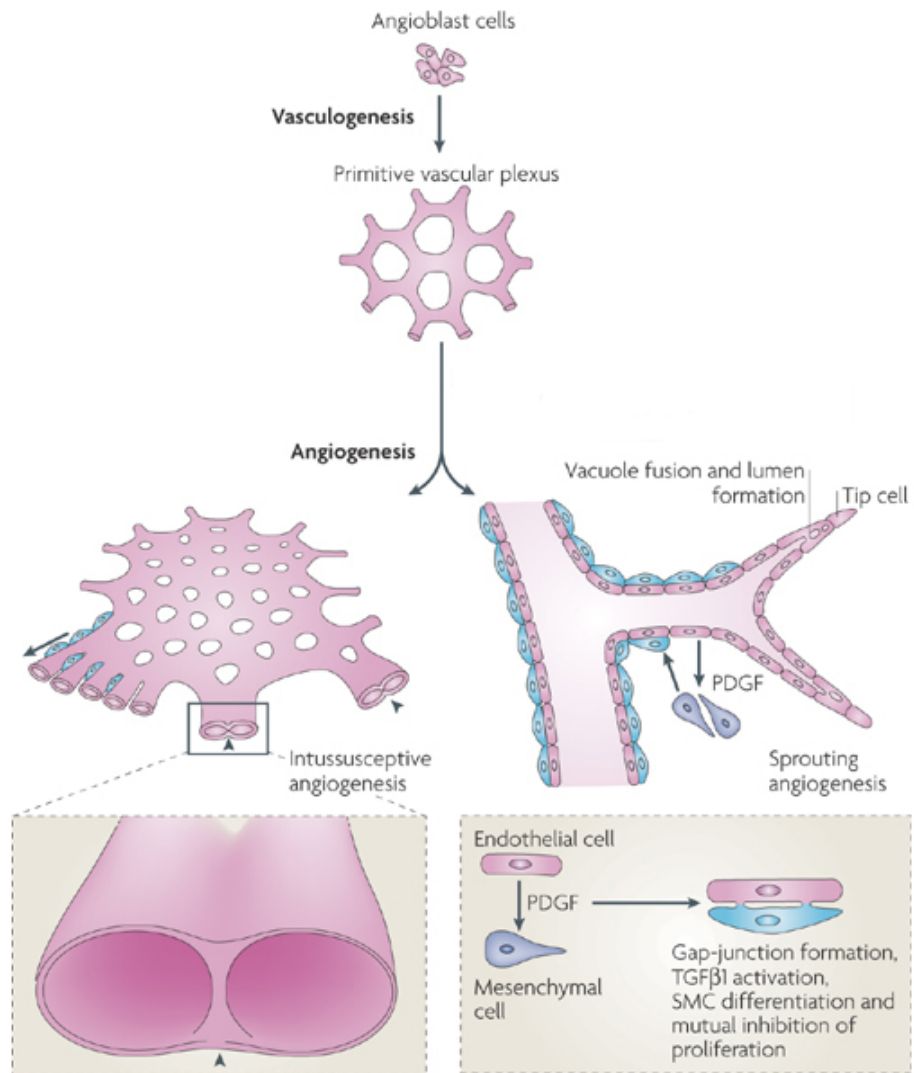


Figure 1.1: Vasculogenesis and angiogenesis

This illustration shows the processes of vasculogenesis corresponding to the *de novo* formation of blood vessels from endothelial cell precursors, the angioblasts and angiogenesis corresponding to the formation of blood vessels from pre-existing one, Figure from ten Dijke and Arthur (2007).

maturation leading to leakage, haemorrhaging and inflammation (Gerhardt and Betsholtz, 2003).

The first characterised endothelial specific growth factor, VEGF, is the most potent and critical vascular regulator, as it is required for vasculogenesis and angiogenesis during development as well as in the adult. Its expression must be tightly regulated in a spatial, temporal and quantitative manner to avoid profound vascular defects (Yancopoulos et al., 2000). This is illustrated by the disruption of both VEGF alleles in mice which mimics knockout of VEGFR2, resulting in the almost complete absence of a vasculature and death *in utero* between days 8.5 and 9.5. Even loss of a single VEGF allele leads to embryonic lethality due to severe vascular abnormalities between day 11 and 12, which constitute probably the most severe autosomal phenotype due to heterozygous deficiency known to date (Carmeliet et al., 1996; Ferrara et al., 1996).

Other growth factors such as angiopoietin-1 (Ang1) and ephrin-B2, are required for the maturation of the primary plexus, with ephrin-B2 and its receptor, ephrin receptor B4 (EphB4), having an important role in segregating developing blood vessels. Ang1 plays a role in the maintenance of a quiescent and stable mature vasculature. Disruption of this stabilising signal or its receptor Tie2 leads to embryonic lethality at E9.5 to E12.5 but does not impair the formation of the primary vascular plexus. However, vascular remodelling is compromised as the endothelial cells fail to form appropriate contact with their supporting cells, suggesting a role for mediating reciprocal interactions between the endothelium (expressing Tie2) and surrounding matrix and mesenchyme (expressing Ang1) (Suri et al., 1996). Deletion of ephrin-B2 and EphB4 genes result in fatal defects in early angiogenic remodelling that resemble those seen in mice lacking Ang1 or Tie2 (Wang et al., 1998; Adams et al., 1999). Ephrin-B2 is expressed by the endothelium of primordial arterial vessels while EphB4 marks the endothelium of primordial venous vessels (Gerety et al., 1999), indicating a role for both in the establishment of arterial-venous identity.

1.3 Biology of endothelial cells

All blood and lymphatic vessels are lined by endothelial cells and form a layer called the endothelium. First thought to be only a static physical barrier, the endothelium is now regarded as a dynamic and heterogeneous organ that possesses secretory, synthetic, metabolic, and immunologic functions (Aird, 2004).

1.3.1 Structure

Being composed of a single layer of flat scale-like cells that are all in contact with a basement membrane, the endothelium is described as a squamous epithelium. Endothelial cells are very flat, between $135\ \mu\text{m}$ in diameter and up to $5\ \mu\text{m}$ in depth (Limaye and Vadas, 2007). The cytoplasm is relatively simple with few organelles, mostly concentrated in the perinuclear zone. Another prominent feature of ECs is the presence of many pinocytotic vesicles which are involved in the process of transport of substances from one side of the cell to the other.

1.3.2 Function

Endothelial cells are involved in many aspects of vascular biology, including:

- Vasoconstriction and vasodilation, and hence the control of blood pressure,
- Coagulation (thrombosis & fibrinolysis),
- Atherosclerosis,
- Formation of new blood vessels (angiogenesis),
- Inflammation and oedema,
- Filtering and trafficking functions, particularly in the renal glomerulus and in the blood-brain barrier to allow transit of white blood cells in and out of the bloodstream.

Several of these essential functions will be briefly reviewed below.

Control of blood pressure

ECs regulate vascular flow and basal vasomotor tone, and therefore blood pressure, by the tightly controlled release of vasodilators, *eg.* nitric oxide (NO) and prostacyclin (PGI₂) and vasoconstrictors *eg.* endothelins and platelet activating factor (PAF), NO and endothelins, being the main regulators of basal vascular tone (Limaye and Vadas, 2007).

NO is synthesised by the constitutively active endothelial NO synthase that can be further induced by receptor dependent agonists such as thrombin or shear stress (Topper et al., 1996; Venema et al., 1994). NO has multiple effects in the vasculature, causing not only SMC relaxation by binding to guanylyl cyclase and hence maintaining basal vasomotor tone, but also inhibiting platelet aggregation (Mendelsohn et al., 1990), and inhibiting SMC proliferation (Zuckerbraun et al., 2007).

The endothelins (ETs) are a family of 21 amino acid peptides, of which ECs and SMCs produce mainly the ET-1 isoform. Production of ET-1 is induced by hypoxia, ischaemia and shear stress, which induce the transcription of ET-1 mRNA, with rapid secretion of ET-1 within minutes. The majority of ET-1 secretion is towards the abluminal side of the EC and thus it acts in a paracrine manner by binding to ET_A receptors on smooth muscle cells, to cause vasoconstriction. It also has some autocrine effects through the ET_B receptor on endothelial cells, activation of which causes NO and prostacyclin release (Rosendorff, 1997).

Coagulation

The quiescent endothelium possesses anticoagulant activity by providing an anti-thrombotic surface which inhibits the coagulation cascade. One of the major strategies used by ECs to maintain anticoagulant activity is to prevent activation of thrombin which, if activated, converts soluble fibrinogen into insoluble strands of fibrin, stimulates coagulation by causing platelet activation and the activation of several coagulation factors (Limaye and Vadas, 2007). ECs express heparan sulphate proteoglycan (HSPG), which along with glycosaminoglycans in the ECM, is able to stimulate antithrombin-III (Rosenberg, 1985). They also express tissue factor pathway inhibitor (TFPI) that prevents thrombin formation, and express thrombomodulin (Dielis et al., 2008). Therefore in the healthy endothelium, the balance is towards the expression of anticoagulant factors to ensure smooth uninterrupted blood flow in the vessels.

Angiogenesis

Angiogenesis corresponds to the formation of new blood vessels from pre-existing ones. This process involves the degradation of ECM by ECs, as well as their migration, proliferation and the formation of tubes in association with mural cells. To ensure the successful outcome of vessel formation, it is essential for ECs to carry out efficient cell-matrix attachments and cell-cell contacts.

Attachments between the EC and surrounding ECM are mediated by the integrin group of cell surface adhesion receptors. Integrins provide adhesive and signalling functions between ECs and the ECM, and this interaction is critical in maintaining EC polarity and alignment along the vasculature. The integrins link the cell with the ECM at focal adhesion points and interact with the actin cytoskeleton. This interaction stimulates cell contraction, thus allowing cell movement on adhesive contacts. This is reviewed later in this introduction (see 1.5.3.6).

In addition to interaction with the ECM, ECs must form cell-cell contacts in order to produce viable capillary like networks. This cell-cell adhesion is mediated by cell adhesion molecules, including vascular endothelial cadherin (VE-cadherin) and platelet endothelial cell adhesion molecule-1 (PECAM-1).

PECAM-1 (cluster of differentiation 31, CD31) is a 130 kDa member of the immunoglobulin superfamily and is highly expressed on the vasculature (Newman et al., 1990). It is able to form homophilic interactions on adjacent cells *via* its domains 1 and 2 (Newton et al., 1997). Moreover, heterophilic ligands for PECAM-1 include $\alpha_V\beta_3$ (Piali et al., 1995), glycosaminoglycans (Muller et al., 1992) and CD38 (Horenstein et al., 1998). Hence, PECAM-1 is thought to act as a docking molecule, which allows other proteins (including integrins) to form additional cell-cell and cell-matrix interactions.

The transmembrane protein, VE cadherin, forms homophilic interactions in a calcium dependent manner (Dejana, 1996). VE cadherins further interact with catenins, intracellular proteins linking the cadherins to the actin cytoskeleton. VE-cadherin is abundantly expressed at *adherens*-type cell junctions where it plays roles in the control of endothelium permeability and angiogenesis.

Inflammation and oedema

Endothelial cells are responsive to local agents such as histamine, which is released when local tissues are damaged. Consequently, the endothelial cells open up their intercellular junctions and allow the passage of large amounts of fluid from blood plasma so that the surrounding tissues become engorged with fluid and swollen, a condition called oedema.

The endothelium also plays a critical function in regulating the trafficking of leucocytes from the intravascular space to extravascular sites of inflammation. The first step in leucocyte transmigration is the arrest of leucocytes and contact with the ECs. This is mediated by the selectins, which allow the rolling and docking of the leucocyte on the endothelium (Vestweber, 2007). During the second step of the transmigration process, the leucocytes flatten and migrate along the endothelium, a process known as diapedesis. Finally, extravasation occurs by leucocyte migration through EC junctions and their subsequent attachment/migration on ECM components (collagen and fibronectin) (Vestweber, 2007). The main families of molecules involved in this trafficking process are the selectins, integrins, immunoglobulin supergene family and variants of the CD44 family (Limaye and Vadas, 2007).

1.4 Biology of vascular smooth muscle cells

1.4.1 Structure

Smooth muscle is a type of non-striated muscle, found within the *tunica media* layer of large and small arteries and veins (Figure 1.2), and around hollow organs such as the bladder, the uterus, the reproductive, gastrointestinal and respiratory tracts, the ciliary muscle, and the iris of the eye. Smooth muscle is fundamentally different from skeletal muscle and cardiac muscle in terms of structure, function and mechanism of contraction. Smooth muscle fibres are smaller (2-10 μm in diameter) than skeletal muscle fibres (10-100 μm) and are assembled in a different manner. Smooth muscle fibres making up the single-unit muscle are gathered into dense sheets or bands. The fibres run approximately parallel to each other, packed together, most often so that the narrower portion of one fibre lies against the wider portion of its neighbour. These fibres are connected by gap junctions between their cytoplasmic membrane (sarcolemma), which act as a low resistance pathway for the rapid spread of electrical signals throughout the tissue. In contrast, multi-unit smooth muscle fibres have no interconnecting bridges and are combined with surrounding connective tissue.

Smooth muscle cells are spindle-shaped, and, like striated muscle, can contract and relax. Their actin and myosin chains are not arranged into distinct sarcomeres that form orderly bands throughout the muscle cell; hence, the most striking feature of smooth muscle is the lack of visible cross striations. Nonetheless, there is an organised cytoskeleton consisting of the intermediate filament proteins vimentin and desmin, along with actin filaments. Moreover, differences can be observed between SMC types, for example vascular SMCs (VSMCs) differ from other smooth muscle cells with a predominance of vimentin filaments and a specific α -type actin.

1.4.2 Function

Smooth muscle controls slow, involuntary movements such as the contraction of the smooth muscle tissue in the walls of the intestines, of the uterus during labour and of the arteries to regulate blood flow. SMCs are coupled to one another by *adherens* junctions, and therefore, cells are mechanically linked to one another such that contraction of one cell triggers the contraction of its neighbour (Eddinger et al., 2005). Gap junctions couple adjacent cells chemically and electrically, facilitating the spread of molecules such as Ca^{2+}

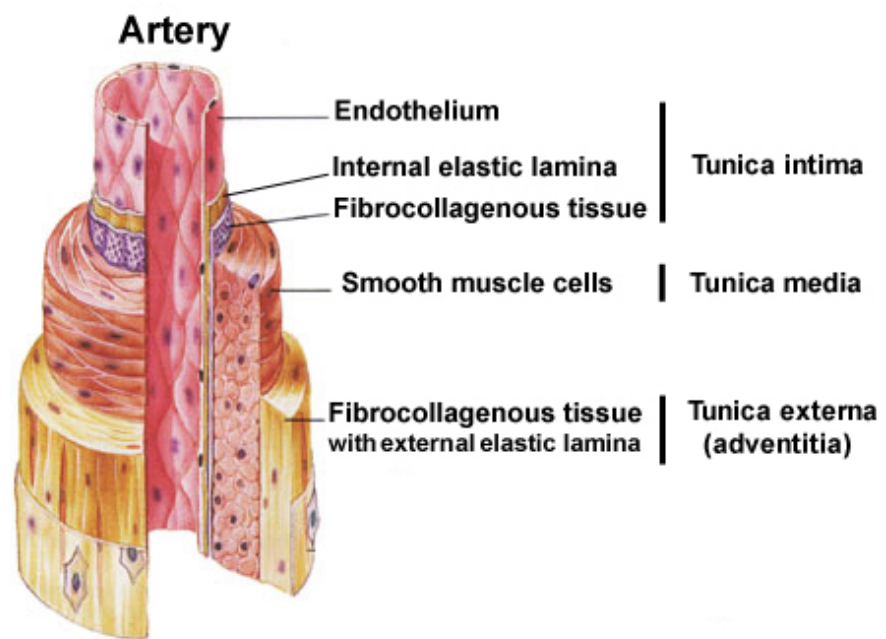


Figure 1.2: **Anatomy of a muscular artery**

This illustration shows the anatomy of a muscular artery with, from the lumen to the outside of the vessel: the endothelium, the tunica intima, tunica media and tunica adventitia, Figure adapted from Fox (2007).

or action potentials between SMCs. Moreover, smooth muscle may contract spontaneously or be induced by a number of physiochemical agents (e.g. oxytocin triggers contraction of the uterus, histamine contracts the SMC of airways in asthma), and also mechanical stimulation (such as stretch).

As mentioned before, SMC are essential for function of the vasculature as their contraction/relaxation state is responsible for alterations of the luminal diameter and allows the maintenance of an adequate blood pressure. However, in addition to their contractile abilities, SMCs can also perform synthetic functions and the modulation between the two phenotypes are characterised by changes in morphology, proliferation, migration rates and expression of different marker proteins (Rensen et al., 2007). Contractile SMCs are elongated, spindle-shaped cells and contain contractile filaments. They exhibit a very low rate of synthetic activity and express a unique repertoire of contractile proteins, ion channels and signalling molecules which are required to fulfill its contractile function (Owens et al., 2004). In contrast, synthetic SMCs are usually associated with vasculo-proliferative disorders involving abnormal neointimal SMC accumulation (see below) and with cell culture of SMCs. Synthetic SMCs are less elongated and have a morphology which is referred to as epithelioid or rhomboid (see Figure 1.3) (Hao et al., 2002). Synthetic SMCs contain a large number of organelles involved in protein synthesis and exhibit higher proliferative and migratory activities than contractile SMCs (Hao et al., 2002). Nevertheless, it is not really accurate to reduce the number of SMCs functions to either contractile or synthetic as a whole range of phenotypes between these two end states can be observed *in vivo* (Owens et al., 2004). Moreover, even in adult organisms, SMCs are not terminally differentiated and are capable of major changes in their phenotypes in response to their local environment such as injury and inflammation (Owens et al., 2004).

The protein markers most commonly used to differentiate the contractile and synthetic phenotypes are α -smooth muscle actin (α -SMA), smooth muscle-myosin heavy chain (SM-MHC) and smoothelin. The relevance of these markers is illustrated by the fact that, in the case of SM-MHC, its expression has never been detected in non-SMCs *in vivo*, and is the only marker protein that is also SMC specific during embryogenesis (Miano et al., 1994). Another relevant marker, smoothelin has a more uniform expression and is rapidly downregulated in cultured vascular SMCs switching towards a synthetic phenotype (Christen et al., 1999). Moreover, both SM-MHC and smoothelin are absent in myofibroblasts in models of arterial injury which are known to be highly synthetic (Christen et al., 2001).

As stated before, SMCs are able to alter their phenotype in response to local cues in their environment, such as the growth factor PDGF. PDGF-BB is able to downregulate α -SMA expression in rat aortic SMCs and stimulate proliferation and migration of SMCs of pig coronary SMCs (Hao et al., 2002; Li et al., 1997). Moreover, PDGFs are strongly implicated in mediating neointima formation *in vivo* as indicated by the fact that inhibition of both PDGF isoforms, PDGF-AA and PDGF-BB, decreased neointima formation in the rat model of arterial injury (Deguchi et al., 1999; Kotani et al., 2003). On the contrary, TGF- β seem to play an essential role in the induction of the contractile phenotype. Treatment of cultured SMCs with TGF- β increases the expression level of protein markers of the contractile phenotype such as α -SMA, SM-SMH and SM-calponin (Hautmann et al., 1997). Other environmental cues that dictate the behaviour of SMCs are the components of the ECM, mainly collagen, elastin and proteoglycans. One example is the effect of the proteoglycan heparin which has been shown to be important for the maintenance of the contractile phenotype and is also able to decrease SMC proliferation. Treatment of porcine SMCs with heparin reduced proliferation of both contractile and synthetic SMCs and increased the expression of the contractile markers desmin and smoothelin (Hao et al., 2002). Other examples are hyaluronan and versican, ECM proteoglycans that are present in low amounts in normal blood vessels, but increase dramatically in vasculo-proliferative disease. They are particularly upregulated in neointimal hyperplasia following balloon angioplasty and provide a permissive environment for arterial smooth muscle cell (ASMC) proliferation, migration, and macrophage adhesion (Wight, 2008). Moreover, physical factors also influence the phenotype of SMCs: the level of mechanical stress can either drive SMCs towards a contractile or a synthetic phenotype.

1.4.3 Role in atherosclerosis

1.4.3.1 Definition of atherosclerosis

Atherosclerosis is a disease affecting arterial blood vessels. It is generally regarded as a chronic, excessive, inflammatory and fibroproliferative response to various forms of damage to the endothelium and also the smooth muscle cell layer of the artery wall (Ross, 1993). Atherosclerosis, the principal underlying cause of myocardial and cerebral infarction or stroke and gangrene of the extremities, is responsible for the majority of deaths in western Europe, the USA and Japan (Ross, 1993). Briefly, atherosclerosis is thought to be initiated by injury which is likely to involve some form of oxidative stress, including free radical ox-

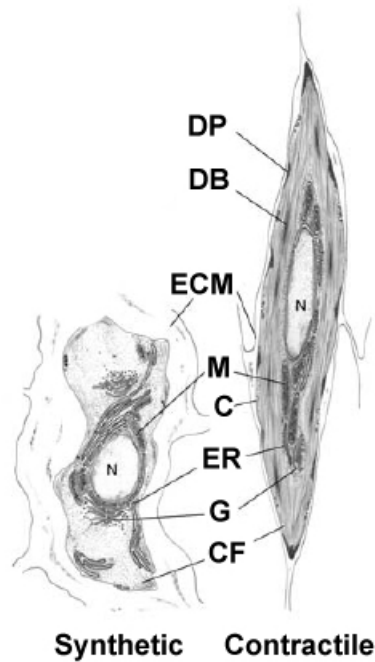


Figure 1.3: **Structural characteristics of synthetic and contractile SMCs**

This illustration shows the structural differences between the contractile and the synthetic SMCs. G: Golgi complex, M: Mitochondria, ER: Endoplasmic reticulum, ECM: Extracellular matrix, N: Nucleus, DB: Dense body, DP: Dense plaque, C: Caveolae, CF: Contractile filaments. Figure adapted from Rensen et al. (2007).

idation of low-density lipoprotein molecules (LDL) carrying cholesterol. In veins, the lack of oxygen probably limits the development of atherosclerosis. When oxidized LDL comes into contact with an artery wall, a series of reactions occur to repair the damage to the artery wall caused by oxidized LDL. The body's immune system responds to the damage to the artery wall by sending macrophages and T-lymphocytes (Faggiotto et al., 1984) and the first lesion type of atherosclerosis appears by accumulation of lipid-filled macrophages (or foam cells): "the fatty streak". These can be found randomly through the arterial tree but are commonly found at sites where the flow is decreased (bifurcations, curves) (Wissler and Vesselinovitch, 1983). The second category of lesions are the intermediate or "fibrofatty lesions" (Ross, 1993), consisting of layers of lipid-filled macrophages and T cells that alternate with layers of smooth muscle cells surrounded by a relatively poorly developed connective tissue matrix with collagen, elastic fibres and proteoglycans. As the lesions progress, the cells undergo a rearrangement that may lead to the final, advanced lesion or "fibrous plaque". The fibrous plaque is characteristically covered by a cap of connective tissue that contains smooth muscle cells surrounded by more connective tissue, consisting of collagen with some elastic fibres. The fibrous cap containing macrophages and T lymphocytes (Faggiotto and Ross, 1984) may provide stability to the lesion. More recently, the importance of SMC apoptosis was identified as a critical process determining plaque stability and thus the final and dramatic step of the atherosclerotic process, plaque rupture and the triggering of the acute thrombotic events causing myocardial or cerebral infarction (Clarke and Bennett, 2006).

1.4.3.2 Role of vascular smooth muscle cells in atherosclerosis

SMCs are known to have an important role in the initiation and early progression of atherosclerosis (Doran et al., 2008) and also in plaque stability (Clarke and Bennett, 2006). Very early, intimal thickening containing SMCs and the proteoglycans they produce, are found in regions of the vascular tree with turbulent blood flow such as bifurcations and branch points and are present in all humans at 1 year of age (Stary et al., 1992; Velican and Velican, 1976, 1985). This focal-type of intimal thickening correlates with sites of advanced atherosclerotic lesions observed later in life (Stary et al., 1992). Moreover, SMCs present in these intimal thickenings differ greatly from those found in the media. They switch from a "contractile" state to a "synthetic" state, express lower levels of proteins involved in contraction (such as α -SMA) and synthesise more extracellular matrix as well as proteases and cytokines (Owens et al., 2004; Worth et al., 2001). The SMCs found in the atherosclerotic lesions are also able to take up lipids (Stary et al., 1994) and to interact with monocytes and macrophages (Stary et al., 1994).

As stated previously, SMC apoptosis is now thought to have an important role in plaque destabilisation (Clarke and Bennett, 2006). The advanced atherosclerotic plaque consists of an SMC-rich fibrous cap overlying a lipid- and macrophage-rich necrotic core and those which are more prone to rupture contain a higher proportion of lipids and a preponderance of monocyte/macrophages with fewer SMCs in the cap (Davies et al., 1993).

1.5 Cytokines regulating angiogenesis and intra-cellular pathways involved

In the past several years, genetic manipulation of the mouse genome and identification of several molecules have provided insights into the molecular mechanisms that govern vessel formation. This complex process is tightly controlled by several pro-angiogenic cytokines in balance with other inhibitory factors, and their identification is important not only for understanding normal development but also for the generation of novel therapies for cancer and neovascular diseases generally. Clinically, the promotion of new blood vessel formation is desirable in situations of severe tissue ischemia such as ischemic heart or peripheral disease, often resulting from underlying atherosclerosis. In contrast, there is an increasing interest in the development of inhibitory molecules to block the initiation of angiogenesis in cancer and ocular diseases. Factors that have been implicated in the regulation of angiogenesis include vascular endothelial growth factor (VEGF), platelet-derived growth factor (PDGF), basic fibroblast growth factor (bFGF), transforming growth factor beta-1 (TGF- β 1), the Tie-1 and Tie-2 receptors, the angiopoietins, integrins and several coagulation factors (Beck and D'Amore, 1997).

1.5.1 VEGF

1.5.1.1 VEGF structure

The first VEGF member to be identified was VEGF-A, initially called vascular permeability factor (VPF) because of its ability to render microvessels hyperpermeable to plasma proteins at sites where it was over-expressed along with its receptors (Leung et al., 1989; Dvorak et al., 1991). Other early research established a clear role for VEGF-A as a potent mitogen for endothelial cells and was also able to induce their survival and migration (Connolly et al., 1989). The VEGF family of growth factors comprises a group of homodimeric glycoproteins most of which are implicated in the formation and modelling of the vascular system, and belong to a super-family of growth factors also including the PDGFs. Both growth factors consist of antiparallel homodimers covalently linked by two interchain disulphide bonds and possessing a characteristic cysteine knot structure that confers important binding properties (Keyt et al., 1996).

The VEGF family includes VEGF-A, -B, -C, -D, the related placental growth factor gene PlGF, the *orf* virus-encoded factor, VEGF-E, and VEGF-F found in snake venom. In

humans, six isoforms of VEGF-A are generated by alternate splicing: 121, 145, 165, 189 and 206 amino acids long, with VEGF-A₁₂₁ and VEGF-A₁₆₅ being the most abundant isoforms (Neufeld et al., 1996; Tischer et al., 1991) (Figure 1.4). VEGF-A₁₂₁ is readily diffusible and does not bind to heparin, while the larger isoforms, such as VEGF-A₂₀₆, bind heparin and are sequestered by the extracellular matrix. Heparin binding activity is due to the presence or absence of exons 6 and/or 7 that encode for heparin binding domains (Robinson and Stringer, 2001). The heparin binding, but also diffusible isoform, VEGF-A₁₆₅, is regarded as the most abundant and biologically active isoform and is able to induce proliferation and migration of endothelial cells *in vitro* and blood vessel permeabilisation *in vivo* (Neufeld et al., 1999). Moreover, VEGF-A₁₆₅ is regulated by oxygen availability (Shweiki et al., 1992), and VEGF-A₁₆₅ production in response to hypoxia is an important regulator of abnormal angiogenesis in several diseases such as diabetic retinopathy (Adamis et al., 1994), psoriasis (Detmar et al., 1994) and cancer (Shweiki et al., 1995). Gene targeting in mice further confirmed a pivotal role for VEGF-A in angiogenic processes, with the absence of only one VEGF-A allele causing severe disruption of the developing vasculature (Carmeliet et al., 1996; Ferrara et al., 1996).

VEGF-B is expressed in a wide range of tissue and particularly in the heart and skeletal muscle. Studies from two separate groups shown different results in VEGF-B knockout mice. The first group observed that knockout mice did not display any abnormal phenotype but had smaller hearts with conduction abnormalities suggesting that VEGF-B is required for normal heart function in adult animals but is not required for proper development of the cardiovascular system during development (Aase et al., 2001). The other group found that, despite appearing normal, hearts of VEGF-B^{-/-} mice were reduced in size and displayed vascular dysfunction after coronary occlusion and impaired recovery from experimentally induced myocardial ischemia (Bellomo et al., 2000).

VEGF-C plays an essential role in the development of the lymphatic system as VEGF-C^{-/-} mice die during late embryogenesis due to a defect in lymph vessel formation (Karkkainen et al., 2001). On the contrary, VEGF-D^{-/-} mutant mice are viable and do not display any abnormal phenotype, but both high levels of VEGF-C and VEGF-D are a prognostic factor for cancer patients as lymphatic growth around tumors may facilitate metastatic spread of malignant cells (Alitalo and Carmeliet, 2002).

VEGF-E is a VEGF-related molecule found to be encoded in the Orf virus, a parapox virus infecting sheep, goats and sometimes human and inducing a transient angiogenesis

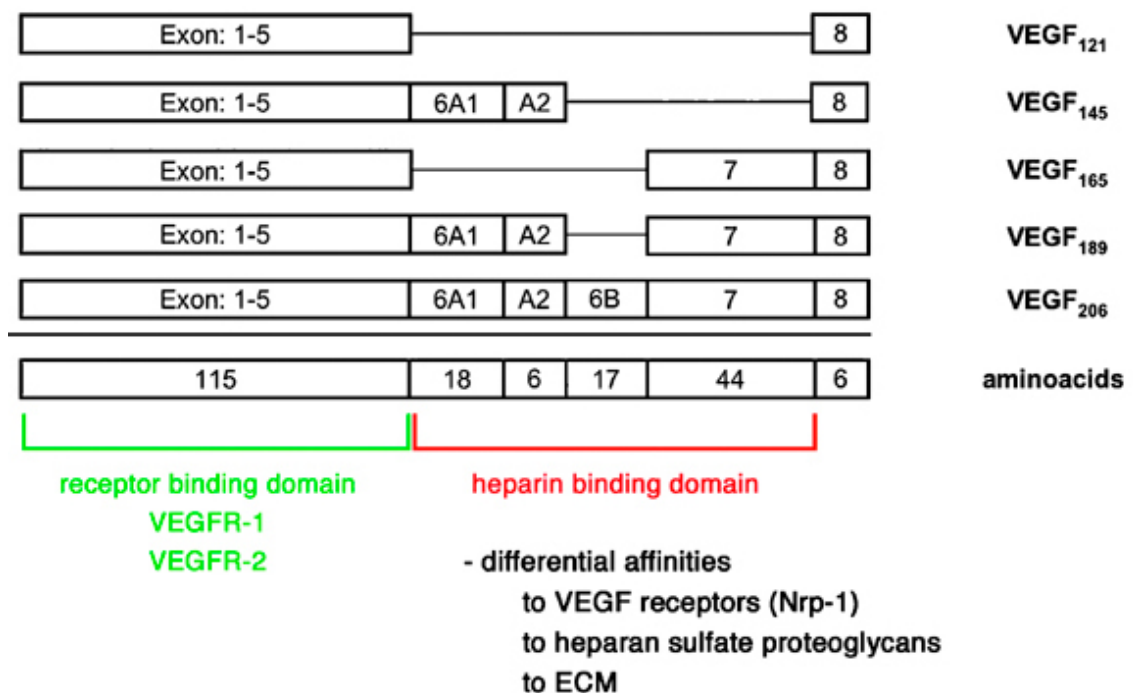


Figure 1.4: **VEGF-A isoforms result from alternative splicing**

VEGF-A isoforms are generated by alternative splicing and are 121, 145, 165, 189 and 206 amino acids long. The larger isoforms contain an heparin binding domain granting affinity to the ECM, Neuropilin 1 and HSPG. Domains 1 to 5 are conserved between all VEGF-A isoforms and are the VEGFR1 and VEGFR2 binding domains, Figure modified from Eming and Krieg (2006).

in the skin (Lyttle et al., 1994).

1.5.1.2 VEGF regulation

Up-regulation of VEGF-A by hypoxia is mediated *via* the binding of hypoxia inducible factor-1 (HIF-1) to the hypoxia response element (HRE) in the VEGF promoter. HIF-1 is composed of a 120 kDa HIF-1 α subunit complexed with a HIF-1 β subunit. Under physiological conditions, HIF-1 α undergoes rapid degradation. Two key prolines within its oxygen degradation domain (ODD) are hydroxylated by one of a three member family of prolyl-hydroxylase domain-containing proteins (PHDs 1-3), the activity of these enzymes being oxygen dependent (Semenza, 2001). Their action allows HIF-1 α interaction with ubiquitin ligase thus promoting the formation of a larger complex which is rapidly destroyed by proteasome activity. Under hypoxia, the hydroxylation of HIF-1 α is reduced, allowing HIF-1 α and HIF-1 β to combine at nuclear HREs of target genes such as VEGF, thereby allowing gene transcription. Not only hypoxia is able to increase the production of VEGF but it also allows stabilisation of its mRNA (Ikeda et al., 1995), and these two mechanisms provide a system by which ischemic tissues can increase VEGF production and secretion, so attracting the growth of new blood vessels (Shweiki et al., 1992).

1.5.1.3 VEGF receptors

VEGFs bind to three major receptor tyrosine kinases (RTK): VEGFR1/Flt-1 (fms-like tyrosine kinase-1), VEGFR2/KDR/Flk-1 (fetal liver kinase-1) and VEGFR3/Flt-4 (Neufeld et al., 1999) and also to accessory or co-receptors, the neuropilins (Soker et al., 1996). The three VEGFRs comprise an extracellular domain of seven immunoglobulin-like (Ig-like) domains containing ligand binding activity, a single hydrophobic transmembrane domain and a cytoplasmic region containing a bipartite tyrosine kinase domain divided into two parts by an insert sequence. Analysis of mutant and chimeric forms of VEGFR1 and VEGFR2 have shown that ligand binding occurs primarily to the second and third immunoglobulin-like domains (Barleon et al., 1997; Davis-Smyth et al., 1996). Figure 1.5 summarises the interactions between the different VEGF isoforms and their receptors. Briefly, VEGF-A₁₆₅ binds to VEGFR1 and VEGFR2 (Neufeld et al., 1999). VEGF-C and -D also recognise VEGFR2 with lower affinity and bind to a third receptor, VEGFR3. PlGF and VEGF-B bind with high affinity only to VEGFR1, and VEGF-E only to VEGFR2. VEGFR1 has a 10-fold higher affinity for VEGF-A₁₆₅, than VEGFR2 (Petrova et al., 1999). Nevertheless, VEGFR1 undergoes little detectable phosphorylation and weak signalling when bound

to VEGF-A₁₆₅, whereas, VEGF-A₁₆₅ binding to VEGFR2 results in autophosphorylation on several major sites and is followed by the activation of multiple downstream signalling pathways. All three VEGF receptors possess a similar overall structure, the tyrosine kinase domains being 80% identical at the amino acid level in the three receptors.

VEGFR1 is a 180 kDa protein which binds VEGF and can be alternatively spliced to generate a soluble, secreted form, sFlt1 (Kendall et al., 1996). VEGFR1^{-/-} mice die *in utero* from vascular defects at E8.5 (Fong et al., 1999). It was found that, increased hemangioblast commitment, is the primary defect in VEGFR1 knock-out mice. Mice expressing only the extracellular region of VEGFR1 develop normally (Hiratsuka et al., 1998); it is therefore thought that, at least during embryonic angiogenesis, VEGFR1 act primarily as a decoy that negatively regulates VEGFR2-mediated actions of VEGF. Nevertheless, the mechanisms by which VEGFR1 mediates its biological functions are only partially elucidated, especially in the adult. Although VEGFR1 may not be essential for mediating effects of VEGF in ECs, it is able to bind other members of the VEGF family and also may play a more significant role in other cell types, such as monocytes (Clauss et al., 1990). Moreover, some experiments suggested a role for VEGFR1 phosphorylation by VEGF in increasing the potency of VEGFR2 signalling (Autiero et al., 2003).

VEGFR2 is a 230kDa protein and is responsible for the transduction of most biological effects of VEGFs including EC migration, proliferation, survival and vascular permeability (Quinn et al., 1993; Waltenberger et al., 1994). Mice deficient for VEGFR2 die *in utero* at embryonic day 8.5 to 9.5. Their phenotype resembles that observed in mice lacking VEGF and results from an early defect in the development of haematopoietic and endothelial cells (Shalaby et al., 1995) and is characterised by a lack of EC development. Originally thought to be exclusive to the vascular endothelium, VEGFR2 has since been found to be expressed on neuronal precursor cells in the retina (Yang and Cepko, 1996). Moreover, VEGF was shown to have direct effects on neurons and glial cells, and stimulate their growth, survival and axonal outgrowth (Carmeliet and Storkebaum, 2002), indicating important roles for VEGF and its receptor in the nervous system.

Finally, VEGFR3 is expressed only in venous endothelial cells in the embryo, and is confined to the lymphatic system in the adult (Iljin et al., 2001). It has roles in cardiovascular development in the embryo, particularly lymphangiogenesis, and binds to VEGF-C and VEGF-D. In VEGFR3 deficient mice, large vessels become abnormally organised with defective lumens, leading to fluid accumulation in the pericardial cavity and cardiovascular

failure at embryonic day 9.5 (Dumont et al., 1998).

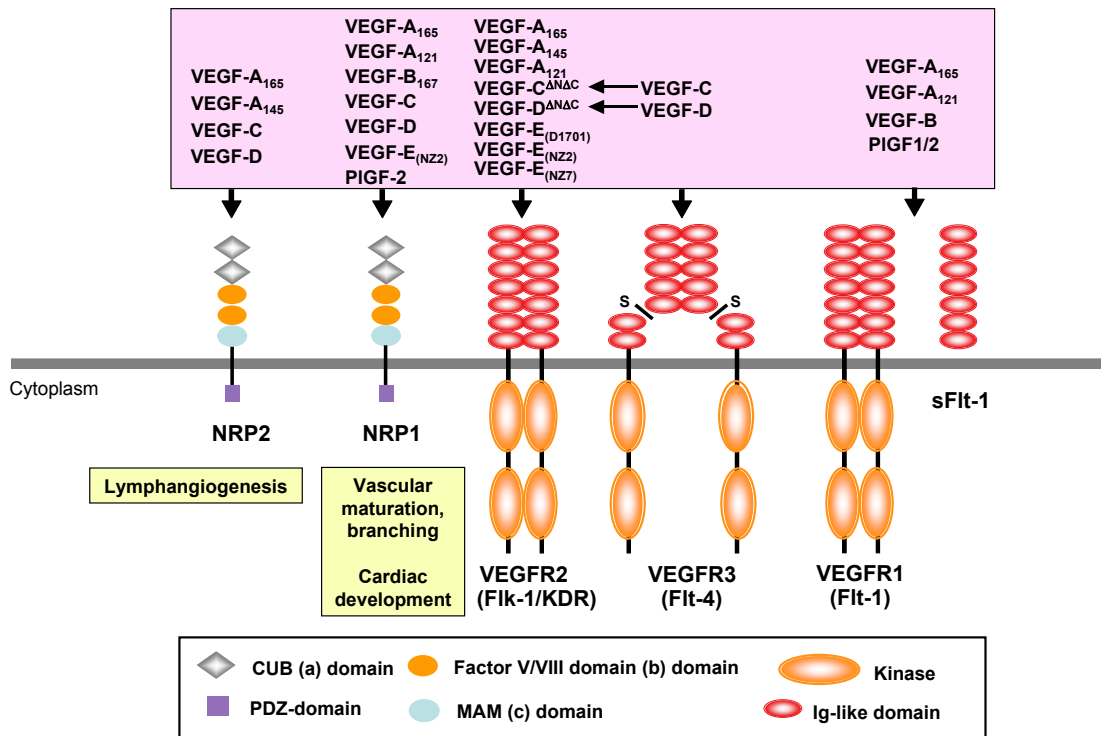


Figure 1.5: **VEGF family ligands and their receptors**

The RTKs that specifically recognise VEGF-A, Flt-1 (VEGFR1) and KDR (VEGFR2), possess an extracellular domain containing seven Ig-like loops (red ovals), a single hydrophobic membrane-spanning domain, and a cytoplasmic domain comprising a single kinase domain (yellow ovals) that is interrupted by a non-catalytic region, called the kinase insert. The extracellular domain of Flt-1 is also independently expressed as a soluble protein. VEGFs C, D and E also bind to KDR, while PIGF and VEGF-B bind to Flt-1 only. Flt-4 (VEGFR3) is a related receptor for VEGFs C and D that undergoes proteolytic processing to yield 120 and 75 kDa polypeptides with a disulphide bridge in the fifth Ig domain. Neuropilin 1 is a non-RTK receptor for VEGF-A₁₆₅, the PIGF-2 isoform, VEGF-B and VEGF-E. NRP1 comprises an extracellular region with two complement binding domains (CUB), two coagulation factor domains, one MAM (meprin, A5, μ tyrosine phosphatase) domain, a transmembrane region and a short cytoplasmic domain, Figure adapted from Pellet-Many et al. (2008).

1.5.1.4 VEGF signal transduction

Most biologically relevant VEGF signalling occurs through VEGFR2 which is also thought to be primarily responsible for endothelial functions of VEGF, vasculogenesis and angiogenesis (Zachary, 2005). Indeed, in contrast to VEGFR1, autophosphorylation of VEGFR2 is easily detected in endothelial cells and several phosphorylated tyrosine residues have been identified and found important for binding to effector molecules and propagation of intracellular signalling. Tyr 1054 and Tyr1059 are required for maximal kinase activity (Dougher and Terman, 1999).

Survival signalling

Phosphorylation of Tyr1175 by VEGF has been shown to activate phosphatidylinositol 3'-kinase (PI3K) (Dayanir et al., 2001) which in turn activates anti-apoptotic Serine/Threonine kinase Akt/PKB to mediate survival of endothelial cells (Gerber et al., 1998b). Akt then phosphorylates Bad and caspase-9 which are pro-apoptotic proteins (Brunet et al., 1999; Cardone et al., 1998), while long-term survival effects of VEGF may be mediated through the upregulation of anti-apoptotic proteins that inhibit caspase function, such as Bcl-2, $\alpha 1$ (Gerber et al., 1998a), survivin and XIAP (X-linked inhibitor of apoptosis) (Tran et al., 1999)(see Figure 1.6).

The integrity of cell junctions is also important in survival as it regulates the balance between the proliferative and confluent/quiescent phenotypes of the endothelial cells (Dejana, 2004). Deficiency or truncation of VE-cadherin, the calcium-dependent cell-cell adhesion glycoprotein, major component of the *adherens* junction, induces endothelial apoptosis and abolishes transmission of the endothelial survival signal by VEGF-A to Akt kinase and Bcl2 *via* reduced complex formation with VEGF receptor-2, beta-catenin, and phosphoinositide 3 (PI3)-kinase (Carmeliet et al., 1999).

Mitogenic signalling

VEGF strongly activates the extracellular signal-regulated kinases (ERKs) 1 and 2 through VEGFR2. Activation of the raf-1/MEK/ERK 1/2 cascade, rather than involving growth factor receptor-bound protein 2 (GRB2) phosphorylation pathway, and subsequent Sos (a guanylnucleotide exchange factor) and ras activation, occurs *via* a ras-independent pathway mediated through phospholipase C (PLC)- γ and protein kinase C (PKC) (Takahashi et al., 2001; Glikli et al., 2001). PLC- γ binds to phosphorylated Tyr1175 and mediates

the generation of diacylglycerol (DAG) and inositol 1,4,5-triphosphate (IP₃) leading to PKC activation and an increase in intracellular Ca²⁺ (Takahashi et al., 2001; Cunningham et al., 1999). Activated PKC then activates ERKs 1/2 via Raf-1 and MEK which is a major pathway leading to cell mitogenesis and proliferation (see Figure 1.6).

Chemotactic signalling

Chemotaxis corresponds to the directional response of a cell towards a gradient of chemoattractant in a fluid phase. This process is called haptotaxis when the chemoattractant molecules are attached to a support, like the surface of a dish or the extracellular matrix. Necrotaxis is a special type of chemotaxis which occurs when the chemoattractant molecules are released from necrotic or apoptotic cells. Finally, chemokinesis is the response of a cell to a chemical that causes the cell to make some kind of change in its movement by speeding it up, slowing it down or changing its direction (see Figure 1.7).

VEGF is strongly involved in endothelial cell migration by activating many proteins involved in chemotaxis. As the extracellular matrix (ECM) plays an essential role in stabilising blood vessels through interactions with endothelial and SMC sheathed in a basement membrane, breakdown of the later is a prerequisite for endothelial cell migration and formation of new vessels during development and disease (Zachary, 2005). This is triggered by the induction of matrix-degrading metalloproteinases (MMPs). MMP-2 and MMP-9 seem to play an important role in promoting angiogenesis and are upregulated along with VEGF and VEGFR2 in angiogenic lesions in a model of tumour angiogenesis (Bergers et al., 2000). Little is known of the link between VEGFR2 activation by VEGF and induction of MMPs.

The VEGF receptor-associated protein (VRAP) also called Tsad (T cell-specific adapter protein), binds to the phosphorylated Tyr951 of VEGFR2 and has been shown to mediate endothelial cell migration, as indicated by the reduced vascularisation and tumour growth seen in VRAP^{-/-} mice (Matsumoto et al., 2005). VRAP forms a complex with Src upon VEGF activation, indicating that it might regulate Src activation and permeability downstream of VEGFR2.

Focal adhesion kinase (FAK) is a key transducer of signals converging from RTKs and integrins important for migration (Zachary and Glikli, 2001). Both FAK and its substrate paxillin are phosphorylated in response to VEGF, and they are both known to be involved in focal adhesion turnover during cellular migration (Abedi and Zachary, 1997).

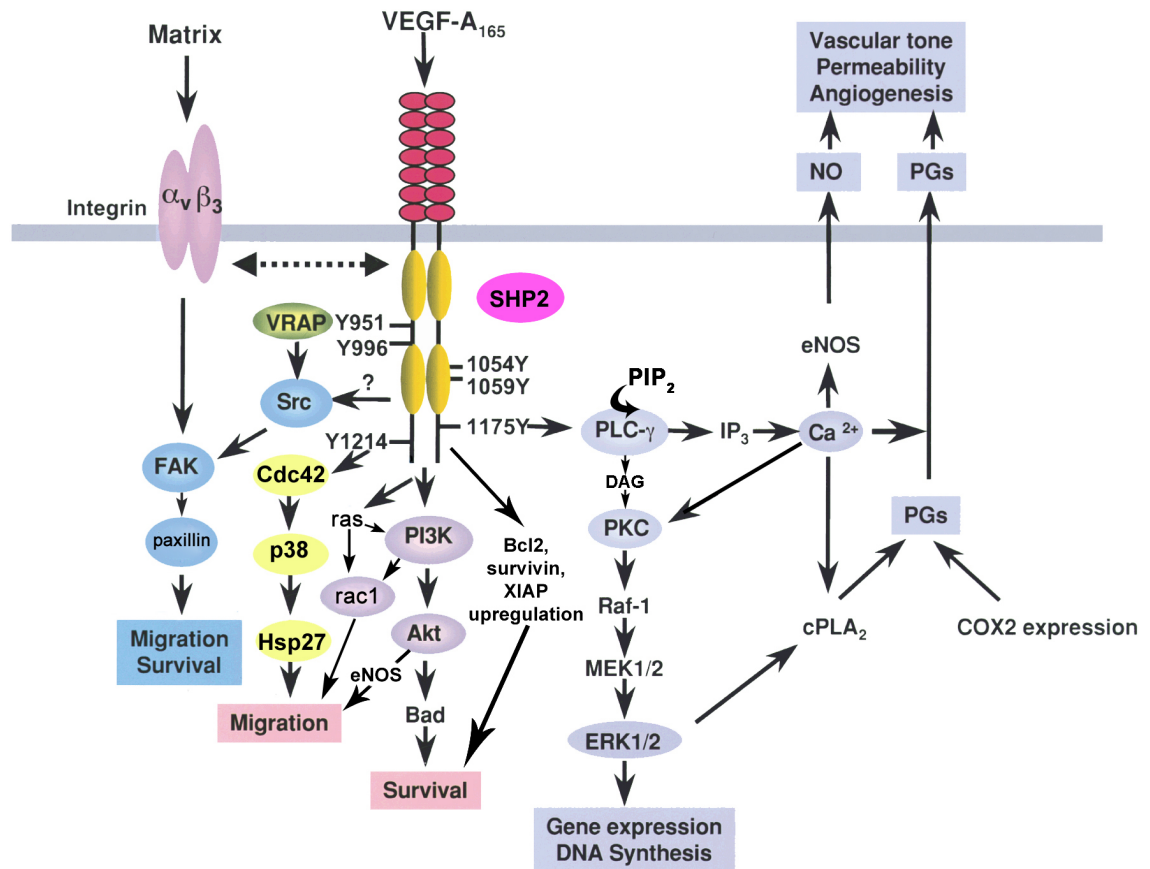


Figure 1.6: **Functional VEGF signalling in endothelial cells**

Activation of VEGFR2 occurs through ligand-induced dimerisation and autophosphorylation at several tyrosine residues in the intracellular domain. VEGF-induced cell survival is mediated in part *via* PI3K activation of Akt, which in turn inhibits the pro-apoptotic proteins Bad and caspase activity. Caspases are also inhibited by the anti-apoptotic protein Bcl2, survivin and XIAP. Increased tyrosine phosphorylation of FAK is a converging point for diverse endothelial cell survival stimuli, including VEGF, matrix-integrin interactions, and fluid shear stress. FAK and its substrate, paxillin, may also be important for endothelial cell migration and, hence, angiogenesis. Interactions between the integrin $\alpha_v\beta_3$ and VEGFR2 may also play a role in survival functions of VEGF. Short-term NO production induced by VEGF is mediated via increased cytosolic Ca^{2+} , resulting from activation of PLC- γ and subsequent generation of IP₃. Activation of Akt leads to phosphorylation and activation of endothelial NO synthase, providing a mechanism for sustained Ca^{2+} -independent NO synthesis which might be a pathway important for migration. The activity of the RTK also triggers the activation of the small GTPases ras and rac1 important for migration. PLC- γ -mediated production of DAG leads to activation of PKC, and this pathway plays an important role in mediating VEGF-induced activation of ERKs and EC proliferation. In turn, ERK activation mediates cPLA₂-mediated PG synthesis. Increased cytosolic Ca^{2+} also stimulates the cellular release of PGI₂. Figure modified from Zachary (2005).

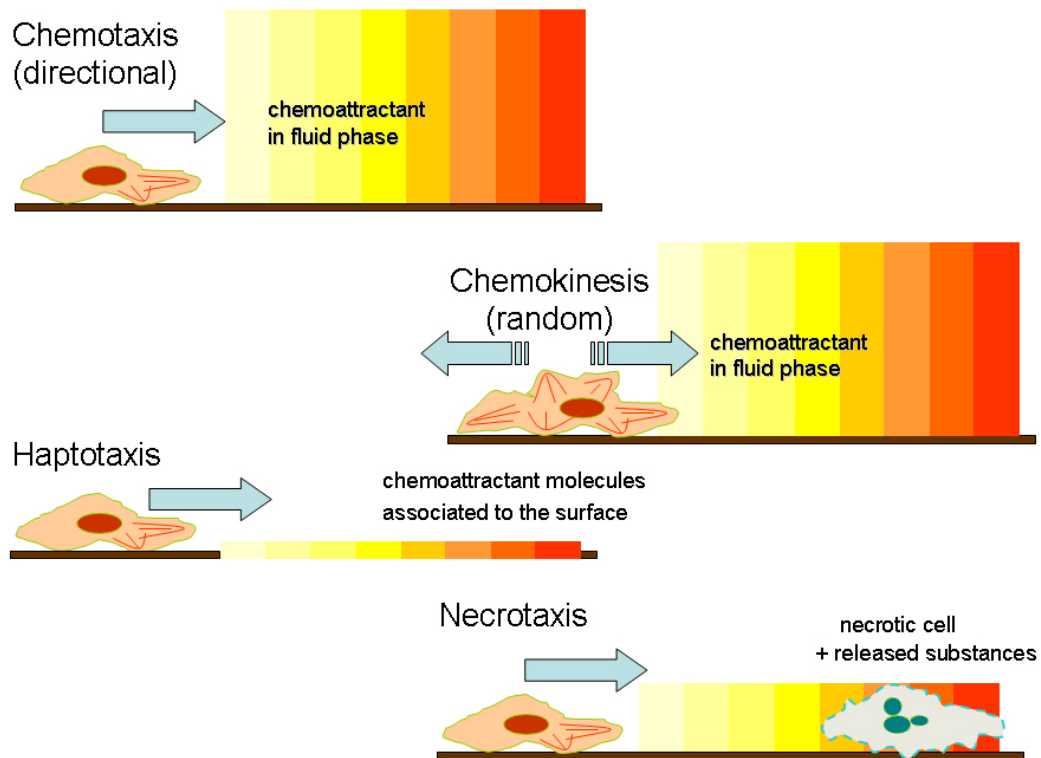


Figure 1.7: Representation of the different types of chemotaxis and chemokinesis
 This picture represents several chemotaxis processes and chemokinesis. Chemotaxis as such is the attraction of a cell towards a gradient of chemokine present in a fluid phase; necrotaxis a type of chemotaxis when the chemokine is released by a dying cell; and haptotaxis when the chemokine instead of being in a fluid phase is attached to a surface or in the ECM. Finally chemokinesis is the locomotory response of a cell in a non-directional manner. Figure from Khidai (1999).

Phosphorylation of FAK in response to VEGF occurs at Tyr397 and Tyr861, the latter being phosphorylated *via* a Src-dependent pathway (Abu-Ghazaleh et al., 2001).

The importance of Src in the migration of endothelial cells was demonstrated using a specific Src inhibitor that led to a decrease in migration of ECs (Abu-Ghazaleh et al., 2001). Furthermore, a dominant negative Src construct selectively inhibited VEGF-induced angiogenesis and reduced VEGF anti-apoptotic effects in the chick CAM model (Eliceiri et al., 2002). Src also participates in the formation of a complex between FAK and integrin $\alpha_V\beta_5$ stimulated by VEGF (Eliceiri et al., 2002). Although there is no evidence for vascular defects in mice lacking individual Src kinases, the previous results indicate an important role for Src in VEGF induced migration of EC for FAK signalling.

Phosphorylation of Tyr1214 on VEGFR2 is required for the VEGF-induced actin reorganisation and EC migration through the sequential activation of cell division cycle protein 42 (CDC42) and p38 MAP kinase (Lamallice et al., 2004). The major p38 substrate, MAPK-activated protein kinase-2 (MAPKAPK-2), is able to phosphorylate heat-shock-protein 27 (Hsp27) at serines 15, 78 and 82 (Landry et al., 1992; Stokoe et al., 1992). Hsp27 is a molecular chaperone known to positively regulate actin reorganisation and VEGF-induced migration of endothelial cells. It was recently found that serine 82 could also be activated *via* protein kinase D (PKD), independently of p38 kinase to stimulate EC migration and tubulogenesis (Evans et al., 2008). Indeed, while p38 kinase may contribute to signalling pathways involved in EC migration, and cell migration more generally, p38 kinase alone may not play a key role in the endothelial migratory response to VEGF, and a PKC/PKD pathway acting at least in part *via* Hsp27 phosphorylation is a more important contributor to VEGF chemotactic signalling (Evans et al., 2008).

IQ motif containing GTPase activating protein (IQGAP1) is a scaffolding protein that controls cellular motility by interacting directly with cytoskeletal, cell adhesion, and small G proteins, including Rac1, which it activates by inhibiting its intrinsic GTPase activity (Hart et al., 1996). It is strongly expressed in ECs and is able to bind directly to VEGFR2. IQGAP co-localises with phosphorylated VEGFR2 at the leading edges of migrating endothelial cells and its knock down by siRNA decreased VEGF-induced EC migration (Yamaoka-Tojo et al., 2004).

Finally, small GTPases of the Rho family play an important role in cell migration as well as other cellular functions such as the regulation of endothelial phenotype and permeability (Wojciak-Stothard and Ridley, 2002). RhoA and Rac1 are activated by VEGFR2

phosphorylation at Tyr951 in response to VEGF and are required for KDR-mediated HUVEC migration (Zeng et al., 2002). Rac1 is required for the assembly and maturation of endothelial junctions and its activity increases during junction formation (Lampugnani et al., 2002), whereas RhoA destabilises endothelial junctions (van Nieuw Amerongen and van Hinsbergh, 2002).

Vascular tone/permeability

Nitric oxide (NO) is a multifunctional gaseous molecule and a highly reactive free radical. It is synthesized from L-arginine, NADPH and oxygen by NO synthase (NOS). In the cardiovascular system, NO signalling has several important physiological functions. NO produced by the constitutive endothelial nitric oxide synthetase (eNOS also called NOS3) diffuses into the vessel wall and relaxes VSMCs in arteries, thereby increasing vessel diameter, lowering resistance and enhancing blood flow to tissues (Isenberg et al., 2009). Endothelial cell-derived NO mediates acute local self-regulation of arterial tone in response to changes in mechanical shear sensed by the endothelium and to circulating factors such as VEGF, VEGFR2 being the major mediator for the hypotensive effect of VEGF (Li et al., 2002). NO also diffuses into the lumen of vessels, where it regulates haemostasis by limiting platelet aggregation. Finally, NO acts in an autocrine manner to regulate endothelial permeability and angiogenesis. These vascular responses to NO occur over different timescales: haemostasis and vessel tone are regulated acutely, whereas angiogenesis requires a sustained increase of NO levels, see Figure 1.8.

Prostacyclin (prostaglandin I₂, PGI₂) is another well-known key regulator of vascular tone that is also produced upon VEGF stimulation (Glick et al., 2001; Wheeler-Jones et al., 1997). VEGF-induced PGI₂ production results from PKC-mediated ERK1/2 activation, ERK-mediated phosphorylation and activation of cytosolic phospholipase A₂ (cPLA₂) (Wheeler-Jones et al., 1997). Cyclooxygenase 2 (Cox2) catalyses the biosynthesis of prostaglandins from arachidonic acids. Prostaglandin H₂ is transformed into the primary prostanoids PGE₂, PGF_{2 α} , PGD₂, PGI₂ and thromboxane A₂ (TXA₂) that directly stimulate endothelial cell migration and angiogenesis *in vivo* (Vane et al., 1998).

VEGF is therefore a major regulator of endothelial cell function and is able to promote EC proliferation, migration, survival as well as regulating vascular tone: the major pathways involved are summarised in Figure 1.6.

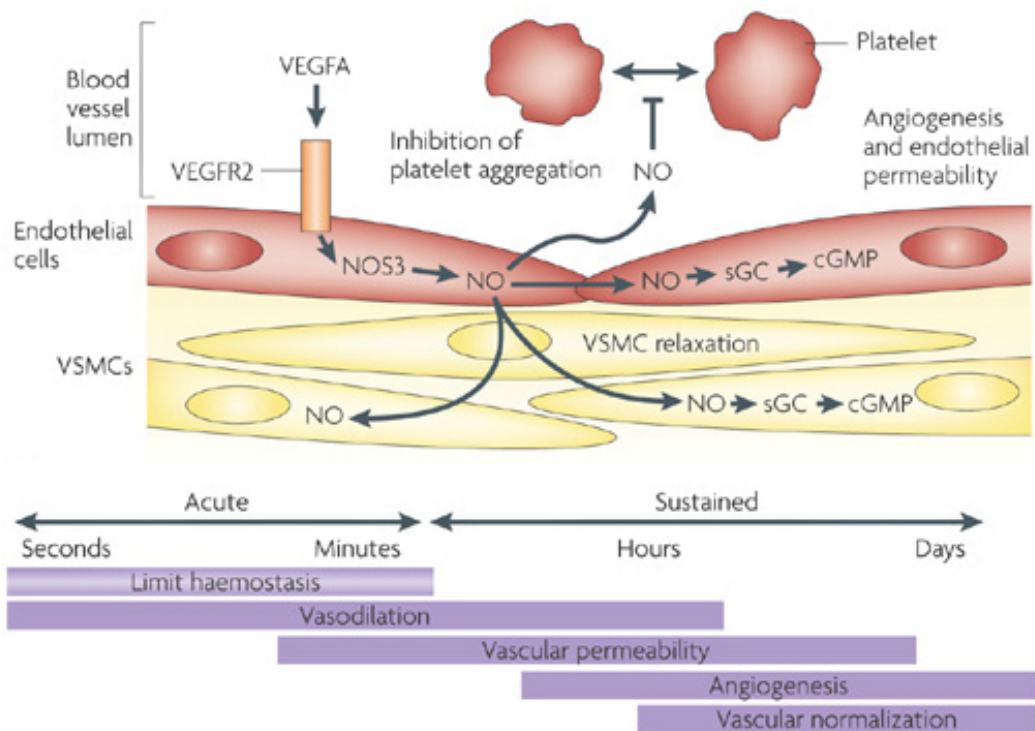


Figure 1.8: **Role of nitric oxide (NO) signalling in angiogenesis, vascular tone and haemostasis**

VEGF binding to its receptor VEGFR2 on endothelial cells activates endothelial nitric oxide synthase (NOS3, eNOS) to produce the diffusible signalling molecule NO. NO acts in an autocrine manner to stimulate endothelial cell growth and motility leading to angiogenesis. VEGF signalling through NO also contributes to increasing vascular permeability. NO diffuses into vessel walls, causing arterial vessels to relax and increase blood flow. NO also acts in a paracrine manner to prevent thrombosis by inhibiting platelet adhesion and aggregation. These different vascular activities of NO occur on different timescales. Figure modified from Isenberg et al. (2009).

1.5.2 PDGF

1.5.2.1 PDGF structure

Platelet-derived growth factor (PDGF) was first described as a platelet-dependent serum factor that stimulates the proliferation of arterial smooth muscle cells *in vitro* (Ross et al., 1974). Further studies revealed that PDGF is also produced by many other cell types such as neurons, endothelial and epithelial cells (Heldin and Westermark, 1999), which are in close contact with mesenchymal cells expressing PDGF receptors (PDGFR) suggesting an involvement in paracrine signalling for PDGF (Ataliotis and Mercola, 1997; DiCorleto and Bowen-Pope, 1983). Moreover, knock-out experiments showed that paracrine signalling appears to be the common route of signalling by PDGFs in embryogenesis, allowing cross-talk between different cell types to form a viable vascular system, brain, kidney and lungs (reviewed in Betsholtz et al., 2001). It is also clear now that in addition to its mitogenic role, PDGF also plays an important role in cell migration and survival (Claesson-Welsh, 1994).

Structurally, PDGFs and VEGFs belong to the same family of growth factors, characterised by eight conserved cysteine residues with similar spacing between the two cysteines. The A- and B-chains of PDGF are 126 and 110 amino acids long respectively and show 60% sequence homology. Two of the cysteine residues are involved in cysteine bonds between the two subunits in the PDGF dimer, and the other six are engaged in intrachain disulphide bonds (Haniu et al., 1994, 1993). More recently, two new members were added to the PDGF family: PDGF-C and PDGF-D which form a separate subfamily (Bergsten et al., 2001; Li et al., 2000). The four homodimers PDGF-AA, PDGF-BB, PDGF-CC and PDGF-DD and the heterodimer PDGF-AB have all been demonstrated to exist as endogenous cell products. PDGF-C and PDGF-D do not appear to heterodimerise with PDGF-A or PDGF-B as their core domains seem to be rather distantly related. Although PDGF-C and PDGF-D are closely structurally related, it remains to be established if they can form heterodimers with each other. Both the A-chain and the B-chain of PDGF are synthesised as precursor molecules that undergo proteolytic activity in their NH₂ termini and, in the case of the B-chain, also in its COOH terminus (Ostman et al., 1991). PDGF-C and PDGF-D possess a novel N-terminal motif referred to as a CUB domain (with homologies to the complement binding factors C1s/C1r, sea urchin fibropellins, called Uegf and Bone Morphogenetic Protein 1, BMP1) (Bergsten et al., 2001; Li et al., 2000). In contrast to the N-terminal pro-peptides of PDGF-A and PDGF-B, the CUB domains of PDGF-C

and PDGF-D do not appear to be obligatorily removed by proteolytic processing prior to secretion, and remain on the secreted PDGF-CC and PDGF-DD molecules (Bergsten et al., 2001; Li et al., 2000). Nevertheless, proteolytic removal of the CUB domain is necessary for binding of PDGF-CC and PDGF-DD to PDGF receptors (Figure 1.9).

1.5.2.2 PDGF receptors

PDGF isoforms trigger their effects on target cells by activating two structurally related protein kinase receptors. Because all isoforms are dimeric molecules, they bind two receptor molecules simultaneously and thus trigger their dimerisation upon binding (Bishayee et al., 1989; Seifert et al., 1989). While the α receptor binds the A-, -B and -C chains of PDGF with high affinity, the β -receptor binds only the -B chain and -D chain with high affinity. Thus, PDGF-AA and PDGF-CC induce the formation of $\alpha\alpha$ receptor homodimers, PDGF-DD induces $\beta\beta$ receptor homodimer formation, PDGF-AB, $\alpha\alpha$ -receptor homodimers or $\alpha\beta$ -receptor heterodimers, and PDGF-BB induces all three dimeric combinations of α - and β -receptors (Figure 1.9).

The PDGF α -receptor contains 1063 amino acid residues and the β -receptor 1067. The receptor precursors are converted in the endoplasmic reticulum to mature forms of 170,000 and 190,000 Da, respectively. Both receptor types are glycosylated through N-linked and O-linked sugar groups (Daniel et al., 1987) and their level of glycosylation varies among different cell types (Heldin et al., 1988). A broad spectrum of cells express the PDGF receptors, their common target, the fibroblasts and SMC of several species express both PDGF receptor isoforms, with the β isoform being the most abundantly expressed, though there are differences in expression between SMC of different species and arterial types. Other cell types exclusively express the α receptors, such as platelets (Vassbotn et al., 1994), or exclusively the β isoform, such as pericytes or myoblasts (Jin et al., 1990; Sundberg et al., 1993; Heldin and Westermark, 1999). More recently, Greenberg et al. (2008) found that there is an interaction between PDGFR β and VEGFR2 (KDR) and that this complex formation through heterodimerisation is implicated in the suppression of pericyte function and therefore negatively regulates tumour angiogenesis.

1.5.2.3 PDGF signal transduction

PDGF receptors

The different PDGFRs trigger overlapping, but not identical intracellular signals. Both α and β receptors are able to transduce a potent mitogenic signal (Heldin et al., 1985) and induce intracellular Ca^{2+} (Diliberto et al., 1992) mobilisation, whereas there is stimulation of migration through activation of the β receptor and a cell-type dependent migration through activation of the α receptor (Heldin and Westermark, 1999). Like other tyrosine kinase receptors, ligand binding induces receptor dimerisation, which allows autophosphorylation in *trans* of tyrosine residues in the intracellular domain (Heldin and Ostman, 1996). The PDGFR extracellular domains contains five Ig-like domains, the three most N-terminal ones joined through a disulphide bond and involved in ligand binding (Heidaran et al., 1990). Similarly to VEGFRs, PDGFRs possess a split tyrosine kinase domain, the two parts separated by a 100-amino acid residue hydrophilic stretch that does not possess any catalytic properties.

Autophosphorylation induced by ligand binding and receptor dimerisation has two important functions. Firstly, phosphorylation on tyrosine residues within the kinase domain (Tyr849 for the α receptor and Tyr857 for the β receptor) leads to an increase in the kinase efficiency and a suppression of phosphatase activity (Kazlauskas and Kovalenko, 2004). Secondly, tyrosine phosphorylation outside the kinase domain creates docking sites for transduction molecules containing Src homology domain 2 (SH2) domains, such as PI3K, PLC- γ , the Src family of tyrosine kinases, the tyrosine phosphatase SHP2 and a GTPase activating protein (GAP) for Ras.

Downstream effector molecules

PI3K has a central role in intracellular signalling transduction downstream of PDGFRs. It is activated by association of its SH2 domains with the tyrosine phosphorylated PDGFR, and mediates several cellular responses. It phosphorylates phosphatidylinositol 4,5-bisphosphate (PIP₂) to form phosphatidylinositol 3,4,5-triphosphate (PIP₃). PIP₃ is an upstream regulator of Rac/Rho activity, either by stimulation of GTP/GDP exchange activity or by inhibition of GAP activity (Hawkins, 1995), and Rac activation will in turn encourage lamellipodia extension and ruffle formation, hence playing a role in migration (Hooshmand-Rad et al., 1997). Moreover, PI3K will have anti-apoptotic effects through the serine/threonine kinase Akt/PKB (Dudek et al., 1997).

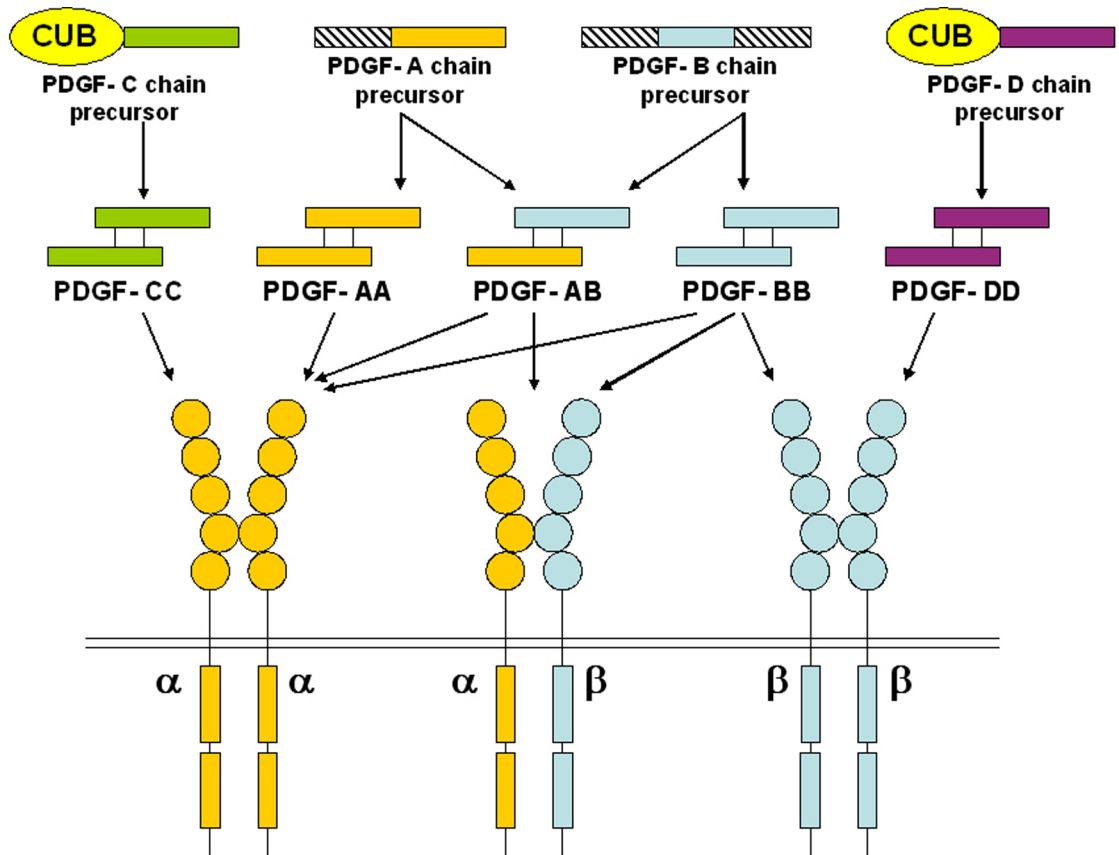


Figure 1.9: **Processing and receptor specificity of PDGF isoforms**

A, B, C and D chains of PDGF are synthesised as precursor molecules that form disulphide-bonded dimers and undergo proteolytic processing. While the A and B chain are secreted in their active forms, the C and D chain are still linked to their CUB domain. Different PDGF isoforms bind to and dimerise α - and β -receptors with different specificities. PDGF-CC and PDGF-DD must be released of their CUB domain to be able to bind to the PDGF receptors. Receptors are drawn to illustrate that extracellular parts consist of 5 Ig-like domains; ligands bind to 3 outermost Ig-like domains, and domain 4 is involved in direct receptor-receptor interactions. Intracellular parts of receptors contain two tyrosine kinase domains. Adapted from Betsholtz et al. (2001) and Heldin and Westermark (1999).

PLC- γ catalyses the hydrolysis of membrane PIP₂ to form IP₃ and diacylglycerol (DAG). The first increases intracellular Ca²⁺ and the second activates certain members of the PKC family. Interestingly, full activation of PLC- γ is dependent upon PI3K as the PIP₃ formed by the latter binds to PLC- γ and may help to anchor the enzyme to the membrane (Falasca et al., 1998). The importance of PLC- γ in cell mitogenesis seems to be dependent upon cell type (Kamat and Carpenter, 1997).

Phosphorylation of tyrosine residues situated in the juxtamembrane domain of PDGFR (Kypka et al., 1990), leads to the dephosphorylation of the Src Tyrosine in position 530, in the COOH-terminus which, in turn, leads to a change in conformation allowing substrate interaction, translocation of Src to the membrane and phosphorylation of other tyrosines within the molecule. Although Src is thought to be important for the cell mitogenic response, it was discovered that binding of Src to PDGFR α is not necessary for mitogenic signalling *via* this receptor (Hooshmand-Rad et al., 1998). Indeed, a mutant PDGFR α missing the two tyrosine residues responsible for Src binding was unable to associate with or activate Src family tyrosine kinases but was able to trigger mitogenicity, actin reorganisation and chemotaxis similar to the wild-type receptor (Hooshmand-Rad et al., 1998).

SH2 domain-containing tyrosine phosphatase 2 (SHP2) is a ubiquitous phosphatase that is activated by the binding of both its SH2 domains to phosphorylated tyrosine residues (Pluskey et al., 1995). Firstly, it is known to inhibit PDGFR signalling as it is able to dephosphorylate autophosphorylated receptors and its substrates (Klinghoffer and Kazlauskas, 1995). Alternatively, it may be involved in positive downstream signalling by acting as an adaptor that binds to Grb2/Sos and therefore activate Ras (Li et al., 1994), and also through its ability to dephosphorylate, thus activate, Src (Stokoe and McCormick, 1997).

GTPase activating proteins (GAP) bind to PDGFR β and initiate the conversion of Ras-GTP to Ras-GDP (Heidaran et al., 1990), and therefore have a modulatory role in Ras activation by PDGF receptors (van der Geer et al., 1997).

Grb2 is an adaptor protein that possess one SH2 and two SH3 domains. The SH2 domain is able to bind directly to the phosphorylated PDGFR or indirectly with the help of other molecules such as SHP2. The SH3 domains are involved in binding with Sos, a nucleotide exchange factor for Ras that converts inactive Ras-GDP to active Ras-GTP. Consequently, Ras activates Raf-1, which in turn, initiates the activation of the extracellular-signal reg-

ulated kinases (ERK) cascade to stimulate cell growth and migration (reviewed in Schlessinger, 1993 and Klinghoffer and Kazlauskas, 1995).

Regulation of PDGFR signalling

As mentioned earlier, several proteins, such as SHP2 and GAP, are involved in negative feedback which may in turn inhibit signal transduction from PDGF receptors (van der Geer et al., 1997). Another example is ERK that phosphorylates and thus inactivate Sos. Sos inactivation will in turn result in a decrease of Ras activation (Porfiri and McCormick, 1996).

PDGFR α versus PDGFR β

In vitro data show that there are some differences in signal transduction between PDGFR α and β and these can be explained by differential interaction with SH2 domain effector proteins (Rosenkranz and Kazlauskas, 1999). For example, GAP binds only to the β receptor (Heidaran et al., 1990). Another example is the importance of PI3K in PDGFR α signalling, whereas it does not seem to be an important effector for PDGFR β (Rosenkranz and Kazlauskas, 1999). In vascular smooth muscle cells, the signalling mechanisms of the α and β receptors are different and the α receptor promotes cellular hypertrophy whereas the β receptor mediates mitogenesis (Inui et al., 1994). This was discovered by selectively stimulating rat vascular SMC that express both α and β receptors with PDGF-AA or PDGF-BB (Inui et al., 1994). In this cell type, PDGF-BB elicited a mitogenic response; however, PDGF-AA increased only protein synthesis without activating DNA synthesis.

There is a clear difference in the phenotypes of knockout mice that lack either the α (Soriano, 1997) or the β receptor (Soriano, 1994). Both mutants die *in utero*, but whereas PDGFR α deficient mice exhibit incomplete cephalic closure and alterations in mutant vertebrae, ribs and sternum (Soriano, 1997), PDGFR β deficient mice are hemorrhagic, thrombocytopenic, and severely anemic and exhibit a defect in kidney glomeruli because of a lack of mesangial cells (Soriano, 1994). The defective development of blood vessels, with characteristic bleeding, has been correlated to the lack of microvessel pericyte recruitment, subsequent aneurysm formation and capillary rupture at late gestation (Lindahl et al., 1998). Nevertheless, the major vessels and the heart appear normal in the absence of PDGFR β ; this indicates that whereas it is essential in certain cell types during embryonic development, its complete role may be underestimated because of compensation by PDGFR α (Soriano, 1994).

1.5.2.4 Role of PDGF in development

Targeted disruption of the genes encoding the PDGF B-chain or the PDGFR β gave similar phenotypes in mice. Both mutants show an impaired kidney and blood vessels development with leaking microvessels that failed to attract supporting cells. However, in contrast to the β -receptor knock-out, the B-chain knock-out mice showed heart defects with an increased size and trabeculation of the myocardium. The fact that this defect is not seen in the β -receptor knock-out animals suggests that during normal development it is compensated by PDGF-BB acting *via* α -receptors (Leveen et al., 1994; Soriano, 1994).

Knock-out of the A-chain gene led to defective development of the lung alveoli, leading to death of the mice at around three weeks of age (Boström et al., 1996). PDGF-A^{-/-} mice also lack lung alveolar SMC, exhibit reduced deposition of elastin fibres in the lung parenchyma, and develop lung emphysema due to complete failure of alveogenesis (Lindahl et al., 1997).

The patterns of PDGF A- and B-chains and PDGF receptors expression during embryogenesis are complex and dynamic and suggest that signalling can be autocrine or paracrine, depending on the particular tissue and the stage of development (reviewed in Ataliotis and Mercola, 1997).

1.5.3 Other molecules involved in angiogenesis

1.5.3.1 Fibroblast growth factor (FGF)

The FGF family comprises at least 22 members in humans, of which FGF-1 (acidic FGF), FGF-2 (basic FGF) and FGF-4 play a role in angiogenesis *in vivo* (Fernig and Gallagher, 1994). Basic fibroblast growth factor (bFGF) has been shown to stimulate vessel growth (Cross and Claesson-Welsh, 2001) and bFGF deficient mice, although viable, present neuronal defects and also impaired regulation of their blood pressure (Dono et al., 1998). The redundancy among the various FGF family members may explain the absence of a stronger phenotype and makes it difficult to define a clear role for FGF-2 (bFGF). There are four structurally related FGF receptors (FGFR), all possess an extracellular binding region with three Ig-like domains, a single transmembrane domain and an intracellular domain with a split kinase domain (Jaye et al., 1992). FGF-2 binds as a monomer to FGFR1 and FGFR2 with high affinity and requires heparin or heparan sulphate proteoglycan (HSPG) to promote receptor dimerisation and signalling (Plotnikov et al., 1999).

FGF-2 is a strong activator of the ERK cascade, but it is unclear if it is able to stimulate the PI3K/Akt pathway. A recent report has shown that FGF-2 can stimulate the activation of Akt through FGFR1 but this is also dependent on the presence of the non-receptor tyrosine kinase Src (Sandilands et al., 2007).

1.5.3.2 Transforming growth factor-Beta (TGF- β)

In humans, the TGF- β s are a large family of 35 homodimeric peptides implicated in the regulation of proliferation, migration, survival, differentiation, and extracellular matrix synthesis in EC and VSMC (reviewed in Bertolino et al., 2005). In mammals, seven type I receptors, also termed activin receptor-like kinases (ALKs) 1 to 7 and five type II receptors have been identified for TGFs. Gene-targeting studies revealed the crucial role of TGF- β and its signaling components in angiogenesis. Targeted inactivation of TGF- β 1 caused embryonic lethality (E10.5) due to defects in the yolk sac vasculature and in the hematopoietic system (Dickson et al., 1995). TGF- β 1 mutant embryos have decreased wall integrity, with defective differentiation resulting in an inadequate capillary tube formation and a lack of SMC recruitment at the periphery of the newly formed vessel. A similar phenotype is exhibited by mice lacking the receptor T β RII (Bertolino et al., 2005). Moreover, mutations in two TGF- β receptors, endoglin (a co-receptor for TGF- β type II receptor, lacking intracellular enzymatic function) and activin receptor-like kinase 1 have been linked to a vascular dysplasia named hereditary hemorrhagic telangiectasia (Johnson et al., 1996; McAllister et al., 1994).

1.5.3.3 Angiopoietins and Tie receptors

Four members of the angiopoietin family of growth factors have been isolated: Ang1 to Ang4 (Jones et al., 2001). All of them seem to bind exclusively to the Tie2 (also known as Tek) receptor tyrosine kinase, whereas the ligand for the related Tie1 receptor remains unknown to date. Interestingly, Angiopoietins have different effects on endothelial cells. While Ang1 and Ang4 both activate Tie2, Ang2 and Ang3 behave as competitive antagonists for this receptor (Davis et al., 1996; Maisonpierre et al., 1997; Valenzuela et al., 1999). The Tie receptors consist of one extracellular domain with one complete and one incomplete immunoglobulin (Ig)-like domain that are separated by three epidermal growth factor (EGF)-like cysteine repeats. These are followed by three fibronectin type III homology domains, a single transmembrane domain and an intracellular region with a split kinase domain. Embryos lacking Tie2 die between embryonic day E9.5 and E12.5 as

a consequence of insufficient remodeling and maintenance of the primary vascular plexus and resemble to the phenotype observed in $Ang1^{-/-}$ mice (Maisonpierre et al., 1997; Sato et al., 1995; Suri et al., 1996). Both embryos present severe malformations in the heart, and also display vascular haemorrhage. The vasculature appears simplified with few supporting cells which suggests a role for Ang1 in mediating interactions between the endothelium (expressing Tie2) and surrounding matrix and mesenchyme (expressing Ang1) (Suri et al., 1996). Embryos lacking Tie1 also show defects in vascular maintenance due to impaired endothelial cell integrity and die later in development, between E13.5 and birth (Puri et al., 1995; Sato et al., 1995).

1.5.3.4 FAK

Focal adhesion kinase (FAK) is a non-receptor tyrosine kinase localised at the cell membrane where it mediates signals between the ECM and the cytoskeleton. FAK plays a prominent role in the mediation of signalling by integrins or growth factor receptors, hence controlling several crucial cell functions such as growth, survival, migration and differentiation (Parsons, 2003). Upon activation, FAK is phosphorylated at Tyrosine 397, creating a high-affinity binding site for signalling molecules carrying SH2 domains, which in turn phosphorylate FAK within its kinase and C-terminal domain resulting in its complete activation (Parsons, 2003). FAK has been found to play an important role in angiogenesis and has been implicated in the migration of endothelial cells in response to VEGF-A₁₆₅ (Abu-Ghazaleh et al., 2001) through its association with p130Cas, Pyk2, and Src (Cary et al., 1998).

1.5.3.5 p130Cas

The Cas family of adaptor protein comprises of p130Cas (Crk-associated substrate), HEF1 (Human enhancer of filamentation), Efs/Sin (embryonal fyn substrate/ Src interacting) and recently identified HELP (HEF1-Efs-p130Cas-like) (Singh et al., 2008), which have structural homology with several domains for protein-protein interactions and share similar effector proteins (Defilippi et al., 2006), but with different functional role and tissue distribution. The 130-kDa Crk-associated substrate, p130Cas, was originally identified as a protein highly tyrosine-phosphorylated in cells transformed by v-Src and v-Crk oncogenes (Sakai et al., 1994a). It was found to be ubiquitously distributed and particularly highly expressed in smooth muscle cells (Tang, 2009). p130Cas is composed of an SH3 domain that interacts with proteins containing a proline-rich motif such as FAK, a proline-rich

domain, a cluster of SH2-binding motifs and a bipartite C-terminus domain for interaction with both SH2 and SH3 motifs (Sakai et al., 1994a). p130Cas plays an important role in the formation of signalling complexes through its association with focal adhesion proteins, such as FAK, paxillin, and SH2 domain-containing signalling molecules, such as Crk (Sakai et al., 1994a; Polte and Hanks, 1995). Its activation is initiated after stimulation with various growth factors, such as endothelin-1 (ET) and PDGF, and also during cell adhesion (Casamassima and Rozengurt, 1997; Kodama et al., 2003).

1.5.3.6 Integrins

Vessel development requires the migration of EC, a process that involves cell adhesion molecules such as integrins to interact with the extracellular matrix (ECM). Integrins are heterodimeric transmembrane proteins that comprise a large family of over 15 α and 8 β subunits that can heterodimerise in over 20 combinations.

β_1 subunits form dimers with at least 12 distinct α subunits. They are found on almost all vertebrate cells: $\alpha_5\beta_1$, for example, is a fibronectin receptor, $\alpha_6\beta_1$ a laminin receptor and $\alpha_1\beta_1$ a collagen receptor on many types of cells (Shimaoka et al., 2002).

The β_2 subunits form dimers with at least four types of α subunit. They are expressed on the surface of white blood cells (leucocytes and macrophages) and have an essential role in enabling these cells to fight infection. The β_2 integrins present on white blood cells mediate cell-cell rather than cell-matrix interactions, binding to specific ligands on another cell, such as an endothelial cell, to attach firmly to ECs at sites of infection and migrate out of the bloodstream into the infected site (Shimaoka et al., 2002).

β_3 subunits are expressed by several different cells, including platelets, and bind several matrix proteins, including fibrinogen. Another integrin, named $\alpha_V\beta_3$, has been identified as having an interesting expression pattern among vascular cells during angiogenesis and vascular remodelling. Integrin-mediated adhesion triggers a number of downstream signals, including activation of the Ras/MAPK pathway (Eliceiri et al., 1998), thus inducing proliferation and cell survival by activating NF- κ B (Scatena et al., 1998). A major pathway stimulated by integrin-dependent cell adhesion is activation of the FAK pathway (Sieg et al., 1999), implicated in cell survival and migration. Furthermore, $\alpha_V\beta_3$ is able to stimulate migration *via* matrix metalloproteinases (MMPs)-induced degradation of the ECM (Leavesley et al., 1993). In ECs, $\alpha_V\beta_3$ not only stimulates MMP-2 production, but interacts with it, to further activate the newly synthesised enzyme (Brooks et al., 1996).

This interaction is critical in EC migration, and indeed, disruption of MMP- 2 binding to integrin $\alpha_V\beta_3$ inhibits angiogenesis and tumor growth *in vivo* (Silletti et al., 2001). Integrin $\alpha_V\beta_3$ is a receptor for a wide variety of ECM ligands including vitronectin, fibronectin, fibrinogen, von Willebrand factor, osteopontin and peptides with an exposed RGD (Arginine-Glycine-Asparagine) sequence. Finally, $\alpha_V\beta_3$ activation promotes its association with VEGFR2 and augments its signalling by doing so (Soldi et al., 1999).

1.6 Proteoglycans

Proteoglycans are glycoproteins that are heavily glycosylated. They have a core protein with one or more covalently attached glycosaminoglycan (GAG) chain(s). The chains are long, linear carbohydrate polymers that are negatively charged under physiological conditions, due to the addition of sulphate and uronic acid groups. Proteoglycans can be categorised depending upon the nature of their glycosaminoglycan chains. These chains may be: chondroitin sulphate and dermatan sulphate, heparin and heparan sulphate, or keratan sulphate (Varki et al., 2008).

Chondroitin sulfate (CS) and heparan sulphate (HS) chains are linked to hydroxyl groups on serine residues of certain proteins. Exactly how proteins are selected for attachment of glycosaminoglycans is not understood. Glycosylated serines are often followed by a glycine and have neighbouring acidic residues, but this motif does not always predict glycosylation. Both CS and HS synthesis initiates with the transfer of xylose by xylosyltransferase to specific serine residues within the protein core. Attachment of two galactose residues by galactosyltransferases I and II and glucuronic acid by glucuronosyltransferase I completes the formation of a core protein linkage tetrasaccharide. Each sugar is attached by a specific enzyme, allowing for multiple levels of control over GAG synthesis. Xylose is attached to proteins in the endoplasmic reticulum, while the rest of the sugars are attached in the Golgi apparatus. The pathways for HS/heparin or CS and dermatan sulphate (DS) biosynthesis diverge after the formation of this common linkage structure (Varki et al., 2008).

1.6.1 Chondroitin sulphate

CS chains are unbranched polysaccharides of variable length containing two alternating monosaccharides: D-glucuronic acid (GlcA) and N-acetyl-D-galactosamine (GalNAc) (Varki et al., 2008). Some examples of proteoglycans containing CS are aggrecan, versican, brevican, and neurocan, collectively termed the lecticans. Another example are syndecans, single transmembrane domain proteins that are thought to act as coreceptors, especially for G protein-coupled receptors. These core proteins carry three to five heparan sulfate and chondroitin sulfate chains which allow for interaction with a large variety of ligands including FGF, VEGF, TGF β , fibronectin and antithrombin-1.

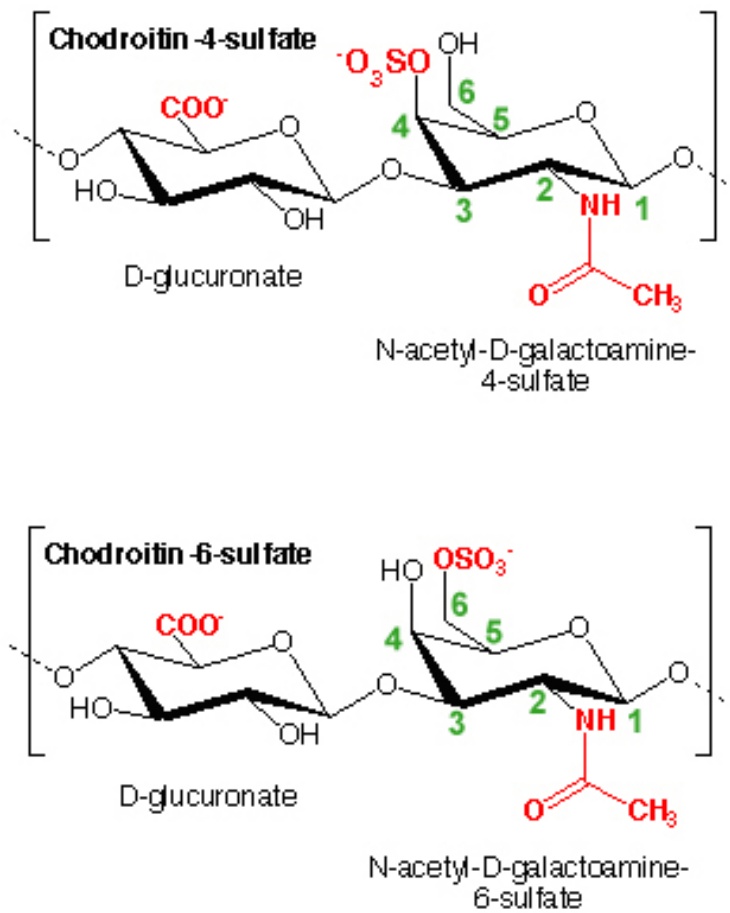


Figure 1.10: **Repeating disaccharide units composing CS**

Chondroitin sulphate (CS) chains are unbranched polysaccharides of variable length containing two alternating monosaccharides: D-glucuronic acid (GlcA) and N-acetyl-D-galactosamine (GalNAc). The most common for of CS are sulphated on the GalNAc residue in position 4 or 6.

1.6.2 Heparan sulphate

Heparan sulphate (HS) chains are unbranched polysaccharides of variable length containing two alternating monosaccharides: GlcA and N-acetyl-glucosamine (GlcNAc). HS binds to a variety of protein ligands and regulates a wide variety of biological activities, including developmental processes, angiogenesis, blood coagulation and tumour metastasis (Tumova et al., 2000). Attachment of the first N-acetylglucosamine (GlcNAc) residue to the tetrasaccharide linker is continued by the stepwise addition of GlcA and GlcNAc residues. These are transferred from their respective UDP-sugar nucleotides. This is carried out by one or more related enzymes whose genes are members of the exostosins (EXT) gene family of tumour suppressors.

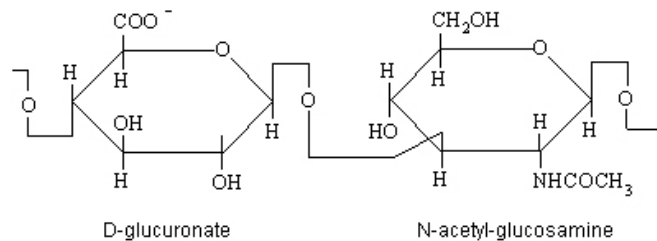


Figure 1.11: **Repeating disaccharide units composing HS**

Chondroitin sulphate (HS) chains are unbranched polysaccharides of variable length containing two alternating monosaccharides: D-glucuronic acid (GlcA) and N-acetyl-glucosamine (GlcNAc).

1.6.3 Role for heparan sulphate proteoglycan in angiogenesis

Endothelial-targeted deletion in N-acetylglucosamine N-deacetylase/N-sulfotransferase 1 (Ndst1), a biosynthetic enzyme catalysing the N-sulfation of specific glucosamine residues, results in decreased tumor angiogenesis, and angiogenesis *in vitro* in response to FGF-2 and VEGF-A₁₆₅, altered growth factor binding to isolated endothelial cells and heparan sulfate, and attenuation of ERK1/2 signalling (Fuster et al., 2007). Ndst1-deficient embryos die perinatally in a condition resembling respiratory distress syndrome (Fan et al., 2000). Both these findings suggest an important role for heparan sulphate proteoglycan (HSPG) in development and particularly in angiogenesis.

Several angiogenic growth factors, such as vascular endothelial growth factor (VEGF-A), fibroblast growth factor (FGF-2), and platelet-derived growth factor (PDGF-BB), depend on HS/heparin for full biological effect (Gitay-Goren et al., 1992; Rolny et al., 2002; Yajon

et al., 1991). The binding of the growth factors is regulated by differential sulfation of the polysaccharide backbone, creating a multitude of protein binding domains (Kreuger et al., 2005). In addition to their role in binding growth factors and presenting them to their receptors in a *cis* confirmation, a study by Jakobsson et al. (2006) show that VEGF-A₁₆₅-induced signalling is potentiated by HSPGs presented in *trans* to the receptor. Transactivation of VEGFR2 by HSPGs presented by perivascular cell types, or alternatively by the extracellular matrix/basement membrane, traps the receptor complexes at the surface of the cell, and hence, internalisation and degradation of activated receptor complexes are delayed, sustaining activation and downstream signalling (Jakobsson et al., 2006).

1.7 Neuropilins

1.7.1 Introduction

Neuropilin-1 (NRP1) was originally identified as the antigen of a specific monoclonal antibody called A5, raised against neuronal cell surface proteins presumed to be involved in neuronal recognition between the visual centres and the optic nerve fibres of *Xenopus laevis* (Takagi et al., 1991, 1987). It was subsequently named neuropilin because A5 bound to the superficial neuropile of the tadpole optic tectum (Fujisawa et al., 1995). Further work demonstrated an essential role of NRP1 in development of the embryonic nervous and cardiovascular systems (Gu et al., 2003; Kawasaki et al., 1999; Kitsukawa et al., 1997; Lee et al., 2002). NRP1 and the structurally-related molecule, NRP2, are receptors both for class 3 semaphorins, a family of secreted polypeptides with key roles in axonal guidance, and for various members of the VEGF family of angiogenic cytokines, but are thought to transduce functional responses only when co-expressed with other receptors: plexins in the case of semaphorins and VEGFR2 for VEGFs. NRPs are also highly expressed in diverse tumour cell lines and human neoplasms and have been implicated in tumour growth and vascularisation in vivo (Gagnon et al., 2000; Klagsbrun et al., 2002; Liang et al., 2007; Pan et al., 2007b). More recently, NRP1 was also found to be a novel mediator of the primary immune response (Bruder et al., 2004; Tordjman et al., 2002; Wulfig and Rupp, 2002).

These findings suggest that NRPs are multi-functional co-receptors essential for neuronal and cardiovascular development, and potentially with additional roles in other physiological and disease-related settings. However, despite the wealth of information regarding the likely biological functions of these molecules, many aspects of the regulation of cellular function *via* NRPs remain uncertain, and little is known concerning the molecular mechanisms through which NRPs mediate the functions of their various ligands in different cell types.

1.7.2 NRP structure

NRP1 and NRP2a are transmembrane glycoproteins of up to 923 and 926 amino acids, respectively, sharing a similar domain structure and an overall amino acid homology of 44% (Chen et al., 1997). NRPs comprise large extracellular regions with a multi-domain structure, a single transmembrane domain and small cytoplasmic domains (44 amino acids for NRP1 and 43 for NRP2). The carboxy-terminal three amino acids, SEA, present in

NRP1 and NRP2a comprise a consensus PDZ domain binding motif, which mediates association with a PDZ domain protein called NIP-1 (neuropilin interacting protein-1), also known as GIPC (RGS-GAIP-interacting protein) or synectin (Cai and Reed, 1999). The NRP extracellular region comprises two CUB (a1/a2) domains, two Factor V/VIII homology (b1/b2) domains (Takagi et al., 1991), and a single MAM (c) domain (Figure 1.12).

The CUB (a1/a2) domains share homology with the complement binding factors C1s/C1r, sea urchin fibropellins (called Uegf) and Bone Morphogenetic Protein 1 (BMP1). CUB domains comprising approximately 110 amino acid residues are found in functionally diverse, often developmentally regulated proteins, including the dorso-ventral patterning protein tolloid, BMP1, a family of spermadhesins (Bork and Beckmann, 1993). Most CUB domains contain four conserved cysteines which probably form two disulphide bridges and are predicted to form a beta-barrel structure similar to that of immunoglobulins. In NRPs, the CUB domains contain the major ligand-binding site for semaphorins.

The b1/b2 domains consist of two tandem regions each of approximately 150 amino acids, sharing homology with the C-terminal (C1/C2) domains of blood coagulation factors V and VIII. In factors V and VIII, C1/C2 domains are part of a larger functional domain, which promotes binding of membrane phospholipids on the surface of platelets and endothelial cells. The NRP1 b1 domain is essential for VEGF-A₁₆₅ binding, though the b2 domain is also required for optimal binding. In addition, the b1/b2 domains contribute to semaphorin binding.

The c or MAM domain is a 170 amino acid region, found in the extracellular domains of diverse proteins, including meprin (a cell surface glycoprotein), A5 antigen, and receptor tyrosine protein phosphatase μ (hence MAM). MAM domains are thought to mediate homophilic protein-protein associations important for homodimerisation or oligomerisation (Chen et al., 1998; Nakamura et al., 1998), and have also been implicated in regulating protein stability.

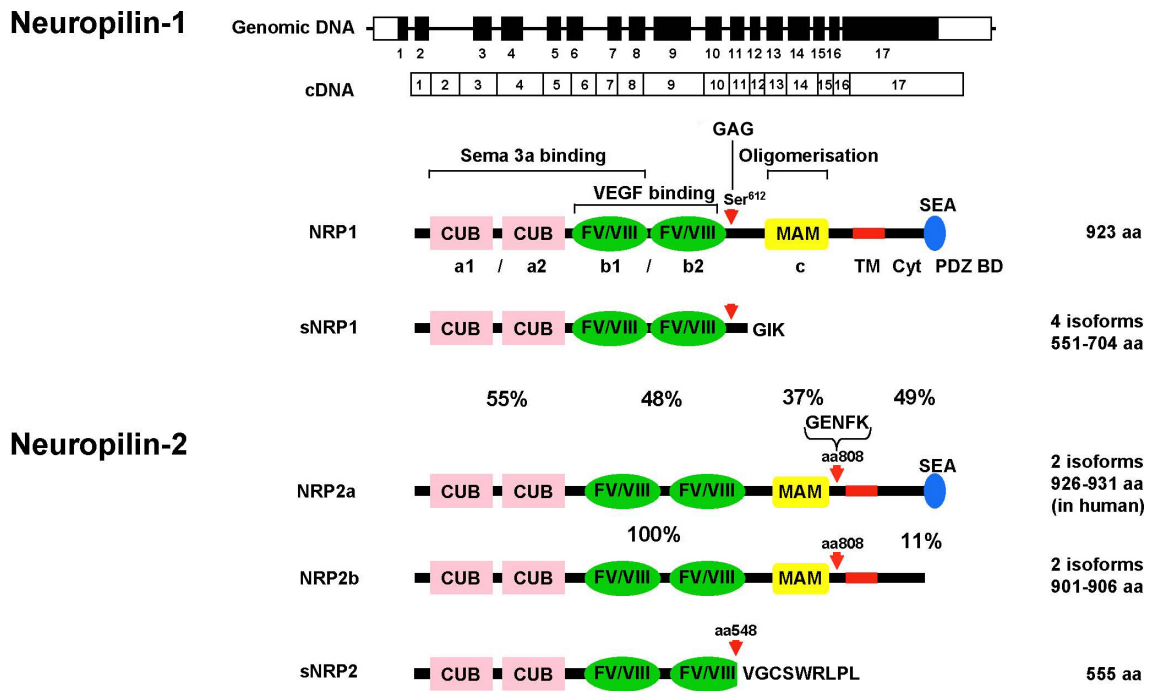


Figure 1.12: **Neuropilins domain structure homology**

This figure shows the protein structure of full-length and soluble neuropilins. Full-length NRPs are composed of two complement binding domains (CUB), two blood coagulation factors V and VIII homology domains (FV/VIII), one meprin-A5-phosphatase μ domain (MAM), a transmembrane domain and a cytoplasmic domain containing a PDZ binding domain (for NRP1 and NRP2a). Soluble forms do not include the MAM, transmembrane and cytoplasmic domains. Percentage homology between NRP1 and NRP2a and between NRP2a and NRP2b are displayed on the figure. On the top part are represented the genomic DNA structure of NRP1 as well as the cDNA composed of 17 exons. Figure adapted from Pellet-Many et al. (2008).

1.7.3 Genomic organisation and expression

NRPs are present in all vertebrates so far examined, including mammals, chicken *Gallus gallus* and the zebrafish *Danio rerio* and are highly conserved between species. Though homologues of the VEGFs and semaphorins have been found in *Drosophila*, *Caenorhabditis elegans* and other invertebrates, NRPs have not so far been identified in lower (non-vertebrate) organisms. Human NRP1 and NRP2 genes are located on chromosomes 10p12 and 2q34, respectively (Rossignol et al., 1999, 2000), and both encode full-length proteins with apparent molecular weights of 130-140 KDa (923 aa for NRP1 and 926 aa for NRP2a). The NRP1 gene spans over 120 Kb and is composed of 17 exons (Rossignol et al., 2000) while the NRP2 gene spans over 112 Kb and also comprises 17 exons (Rossignol et al., 2000). The strong similarities in the exon-intron organisation of the NRP1 and NRP2 genes, their exon and intron sizes, and positions of many of the splices sites suggests that they may have originated from a gene duplication event.

1.7.3.1 NRP1 isoforms

Alternative splicing results in the generation of several soluble isoforms forms of NRP1 and NRP2. One membrane-associated NRP1 isoform has been identified, called NRP1 (Δ exon16), which lacks the 51 nucleotides corresponding to exon 16 (Rossignol et al., 2000; Tao et al., 2003). NRP1 (Δ exon16) does not differ from the common full-length NRP1 in its binding to VEGF₁₆₅, dimerisation with VEGFR2 or regulation of VEGF₁₆₅ signalling (Tao et al., 2003). Four NRP1 mRNA isoforms have so far been reported (see Figure 1.12), all predicted to encode soluble proteins containing the CUB (a1/a2) and b1/b2 domains but lacking the MAM (c), transmembrane and cytoplasmic domains. These isoforms vary in size from 551 to 704 amino acid residues due to alternative splicing. However, only two soluble NRP1 isoforms, s₁₂NRP1 (NRP1 isoform b) and s_{IV}NRP1 (NRP1 isoform c), have so far unambiguously been shown to be expressed in protein form and are found in protein databases. s_{III}NRP1 contains a1/a2 and b1/b2 domains missing 48 aa at the C terminus of the b2 domain, but contains 13 extra amino acids resulting from a shift in the reading frame of exon 12 (Cackowski et al., 2004). Soluble isoforms s₁₂NRP1 (644 aa) and s₁₁NRP1 (704aa) are secreted by a variety of tissues, including kidney tubules and hepatocytes, where their role is currently unknown, but not by endothelial cells. Both bind to VEGF₁₆₅ and Sema3a (Gagnon et al., 2000). s_{III}NRP1 (551 aa) and s_{IV}NRP1 (609 aa) occur in both normal and cancerous human tissues.

Soluble NRPs may act as decoys, competitively binding and sequestering ligands such as VEGF₁₆₅ and Sema3a, and therefore negatively regulating functions mediated by these cytokines. For example, sNRP1 triggers tumour cells apoptosis mimicking the effect of VEGF₁₆₅ withdrawal (Benjamin and Keshet, 1997; Gagnon et al., 2000). However, the effects of sNRPs may be more complex than suggested by a simple decoy role. Thus, whereas an sNRP1 monomer sequesters VEGF₁₆₅ and inhibits its activity, sNRP1 dimers appear to deliver VEGF₁₆₅ to endothelial cell VEGFR2, thereby promoting angiogenesis (Yamada et al., 2001).

1.7.3.2 *NRP2 isoforms*

Membrane-bound NRP2 exists in two major isoforms, NRP2a which shares 44% overall homology at the amino acid level with NRP1, and NRP2b which is identical to NRP2a in its extra-cytoplasmic domain, but exhibits only 11% homology with NRP2a in its transmembrane and cytoplasmic regions (see Figure 1.12). In the mouse, a total of four NRP2a isoforms are generated by alternative splicing resulting in insertion of 0, 5, 17 and 22 (17+5) amino acids after residue 808, situated between the MAM and the transmembrane domain (Chen et al., 1997). In the human, only two forms of NRP2a have been cloned, NRP2a₍₁₇₎ and NRP2a₍₂₂₎, homologous to the corresponding mouse isoforms. The NRP2a₍₂₂₎ isoform (931 amino acids) results from the insertion of the five amino acids GENFK within the 17 amino acid insertion of NRP2a₍₁₇₎ (926 amino acids). So far, NRP2a₍₀₎ and NRP2a₍₅₎ isoforms have not been found in human tissues. These isoforms do not appear to differ in their ligand binding properties, but insertion of residues between the MAM and transmembrane domains might potentially alter its ability to form complexes with VEGFR2 or homodimerise. NRP2b displays little homology with NRP2a from residue 808 (Rossignol et al., 2000) with a distinct cytoplasmic domain lacking the carboxy-terminal PDZ domain recognition sequence, SEA, required for interaction with synectin (Cai and Reed, 1999). Similar to NRP2a, NRP2b₍₀₎ and NRP2b₍₅₎ isoforms result from alternative splicing and the insertion of none or five amino acids after amino acid 808. The marked differences in their cytoplasmic domains suggest that NRP2a and NRP2b isoforms may have divergent functions, a possibility supported by their differential tissue expression. NRP2a and NRP2b are both highly expressed in the brain, but NRP2a is preferentially expressed in the liver, lung, small intestine, kidney and heart, while NRP2b is present in heart and skeletal muscle (Rossignol et al., 2000). A soluble NRP2 isoform is also generated by alternative splicing (Figure 1.12), s₍₉₎NRP2 (1785 bp,

555 aa, 62.5 kDa) consisting of the two a1/a2 domains the b1 domain and a truncated b2 domain followed by the 8 amino acids VGCSWRLPL encoded by intron 9 (Rossignol et al., 2000).

1.7.4 Ligands for neuropilins

NRPs have the ability to bind with high affinity two structurally unrelated classes of ligands with distinct biological functions, the class 3 semaphorins and VEGFs (see Figure 1.13).

1.7.4.1 *Semaphorins*

Semaphorins constitute a large protein family including both transmembrane and secreted species expressed in diverse Metazoans (worms, insects, crustaceans, fishes, vertebrates) and also in viruses but not in plants or protozoans (Raper, 2000; Yazdani and Terman, 2006). The class 3 semaphorins are secreted proteins, most of which require NRP1 or 2 as obligate co-receptors. The major ligand for NRP1 is Sema3a (also named collapsin-1), a factor that induces collapse of the growth cone in selected sensory and sympathetic neurons, e.g. dorsal root ganglia, also repels DRG axons, and is essential for neurogenesis in development (Luo et al., 1993). NRP1 also binds Sema 3F with lower affinity and several other secreted class 3 semaphorins (see Figure 1.13), though the functional roles of these interactions are not clear in each case. The binding of Sema3a to the CUB domains of NRP1 is mediated by the sema domain, a conserved extracellular domain of 500 amino acids forming a β -sheet propeller structure held together by four disulphide bonds (Gherardi et al., 2004), while a carboxy-terminal region of Sema3a rich in basic residues interacts with the b1b2 domains (Gu et al., 2002; He and Tessier-Lavigne, 1997; Taniguchi et al., 2005). The most well-characterised ligands for NRP2 are Sema3c and 3f, and NRP2 also recognises Semas3b and 3g, but does not bind Sema3a. There is evidence that several NRP2 ligands (Sema3b, c and f) may act as antagonists of Sema3a (Chen et al., 1997; Tamagnone et al., 1999). The members of the class 3 semaphorins have different affinities for NRPs 1 and 2 and require distinct plexins to transduce their signal. Therefore different complexes combinations can be formed and allow a fine tuning of the downstream signal of plexins.

1.7.4.2 VEGFs

The most active and abundant isoform, VEGF-A₁₆₅ contains exon 7 but lacks exon 6, and is able to bind to both NRP1 and 2, indicating a key role for the domain encoded by exon 7 in NRP binding (Soker et al., 1996). However, VEGF-A₁₄₅ lacks exon 7 and binds only to neuropilin 2 (Gluzman-Poltorak et al., 2000). Though VEGF-A₁₆₅ was originally believed uniquely able to bind to NRPs with high affinity through its exon 7-encoded carboxy-terminal domain, it now appears that the residues encoded by exon 8 are crucial for NRP recognition. Peptides corresponding to exon 8 inhibit VEGF binding to NRP1 (Jia et al., 2006), and a naturally-occurring peptide, Tuftsin, with homology to exon 8 also binds NRP1 (von Wronski et al., 2006). Antibodies specifically blocking VEGF-A₁₆₅ binding to NRP1 also inhibited endothelial cell migration induced by VEGF-A₁₂₁ (Pan et al., 2007b). VEGF-A₁₂₁, lacking exon 7 but containing exon 8, was found to bind NRP1 *in vitro* using surface plasmon resonance, but was unable to promote complex formation between NRP1 and VEGFR2 (Pan et al., 2007b). These findings are consistent with a model in which initial VEGF binding to NRP1 is mediated via the extreme carboxy-terminal residues encoded by exon 8. NRP1 is either stabilised by further interactions between NRP1 and exon 7-encoded residue and/or the exon 7 domain functions as a bridge between NRP1 and VEGFR2. The larger isoforms, VEGF-A₁₈₉ and VEGF-A₂₀₆, bind heparin, are not readily diffusible, and are thought to remain sequestered by the extracellular matrix. A novel isoform, VEGF-A_{165b}, has a distinct carboxy-terminus, RSLTRKD, encoded by an alternative exon 8 and is unable to stimulate endothelial cell proliferation, angiogenesis or other biological activities (Bates et al., 2002). Possibly, the weaker biological activity of VEGF-A_{165b} is due to its inability to bind NRP1 and thereby to promote productive signalling complexes with VEGFR2. VEGF-B and VEGF-E have been shown to bind to NRP1 (Makinen et al., 1999; Wise et al., 1999), while VEGF-C and VEGF-D interact with NRP1 and NRP2 (Karkkainen et al., 2001; Karpanen et al., 2006). PlGF-2 also binds to NRP1 (Migdal et al., 1998). Whether binding of other VEGFs to NRPs results in complex formation with the respective receptor tyrosine kinases, VEGFR1/Flt-1, VEGFR2/KDR, or VEGFR3/Flt-4, and the biological relevance of these interactions, remains unclear. Also, the role of VEGF-A binding to NRP2 is not clear.

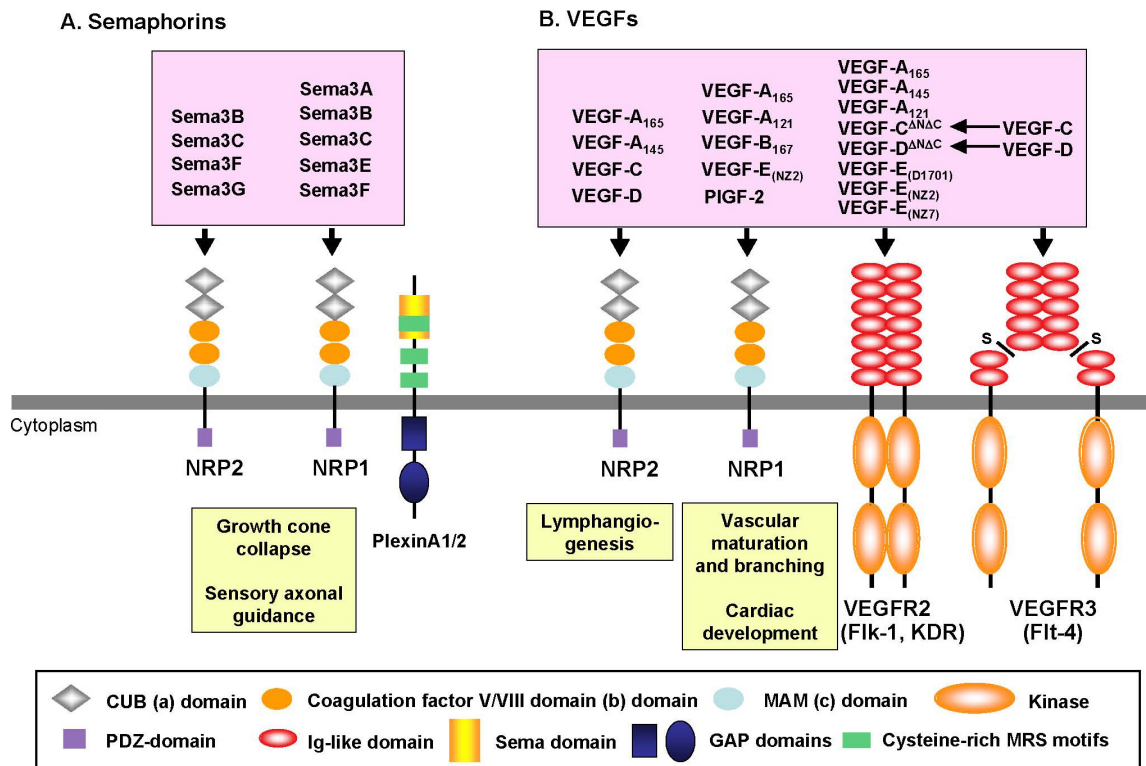


Figure 1.13: **Neuropilins, their ligands and co-receptors**

This figure shows the two different families of neuropilin ligands: Class 3 Semaphorins and VEGFs. Both comprise different isoforms and are classified in function of their binding properties to NRP1 and/or NRP2. Also, the neuropilin co-receptors are represented: Plexin A1/2 which participates to the binding module for Semaphorins and VEGFR2 (Vascular endothelial growth factor receptor 2 also called KDR for kinase insert domain-containing receptor) and VEGFR3 (Vascular endothelial growth factor receptor 3 also called Flt-4 for fms-related tyrosine kinase receptor 4) which participate to the formation of binding modules with NRP1 or NRP2 for VEGFS. Under the NRP1 and NRP2 symbols are indicated their biological functions in relation to the family of ligands (Semaphorins or VEGFs). Figure from Pellet-Many et al. (2008).

1.7.5 Neuropilin function in development

Targeted disruption of NRP genes has demonstrated an essential dual role of these molecules in neurogenesis and cardiovascular development (Table 1.1). NRP1 null mice die between E12 and E13.5 with a spectrum of cardiovascular and neuronal defects. Both the central nervous system (CNS) and peripheral nervous system (PNS) are severely affected with severe abnormalities in the trajectory and connection of efferent fibres of the PNS (Kitsukawa et al., 1997). Vessels of the yolk sacs are disorganised and the capillary network sparse. Aberrant embryonic macrovascular development is characterised by lack of development of the branchial arch, related great vessels and dorsal aorta, by a transposition of the aortic arch, and insufficient septation of the *Truncus arteriosus* (Kawasaki et al., 1999). NRP1 over-expression also results in embryonic lethality with excess capillary growth, haemorrhage in the head and neck and a malformed heart, in addition to anarchic sprouting and defasciculation of nerve fibres (Kitsukawa et al., 1995). These transgenic embryos also appear redder than normal, suggesting that the blood vessels are leaky perhaps due to an enhanced vascular permeability activity of VEGF-A₁₆₅. In contrast to NRP1 mutant mice, NRP2 null mice survive to adulthood with no obvious cardiovascular abnormalities, but exhibit a severe reduction of small lymphatic vessels (Yuan et al., 2002) and capillaries, as well as abnormal guidance and fasciculation of cranial and spinal nerves (Chen et al., 2000; Giger et al., 2000). Doubly deficient NRP1^{-/-}/NRP2^{-/-} mice exhibit earlier embryonic mortality than the single NRP1 knock out (E8 versus E12-13.5) and have a more severe vascular phenotype resembling the VEGF-A₁₆₅ and VEGFR2 knock-outs (Takashima et al., 2002), marked by large avascular areas in the yolk sacs, and head and trunk regions, and a lack of connections between blood vessel sprouts.

An essential role of NRP1 in vascular development has also been demonstrated in other vertebrate species. Morpholino NRP1 knock down in the developing zebrafish (Bovenkamp et al., 2004; Lee et al., 2002; Yu et al., 2004) produced a severe vascular phenotype characterised by a loss, or anarchic sprouting, of new capillaries from pre-existing intersomitic vessels, but had no effect on the formation of the major axial vessel, suggesting that, in zebrafish, NRP1 is not implicated in vasculogenesis (Lee et al., 2002). Defects were also observed in the developing zebrafish nervous system after NRP1 knockdown, characterised by aberrant migration and branching of motor neurons (Feldner et al., 2005).

Important insights into the relationship between the vascular and neural functions of NRPs have been yielded by analysis of tissue-specific knock-outs and mutant knock-in mice. An

essential role in cardiovascular development for vascular NRP1 has been demonstrated by the generation of endothelial-specific NRP1 knock out mice. These mice exhibit mid-to-late embryonic lethality, a poorly-branched vasculature and multiple defects in the major arteries and failure of septation of the major cardiac outflow tract (Gu et al., 2003). In contrast, knock-in mice expressing a mutant NRP1 with a 7 amino acid deletion in the CUB (a1) domain required for Sema 3A binding but retaining the ability to bind VEGF-A₁₆₅, survive to birth, possess no obvious cardiovascular defects, but exhibit aberrant pathfinding of sensory afferent nerves to synapses in the CNS and defasciculation of spinal and cranial nerves, most of the mice dying by P7. Specifically, the Semaphorin/NRP1 signalling axis is essential for formation of cranial and spinal nerve projections, guidance of peripheral projections of bipolar neurons of the vestibular ganglion and the central projections of a subset of axons of cutaneous sensory neurons, and for basal cortical neuron dendrite development (Gu et al., 2003). These findings indicate a critical role for Semaphorin binding to NRP1 in axonal pathfinding, but also demonstrate the compartmentalisation of the neuronal and vascular roles of NRP1. The importance of Semaphorin-Neuropilin interactions in axonal homing is further emphasised by the close similarities of the neuronal pathfinding defects in NRP1 and Sema 3A mutant mice (Gu et al., 2003; Kitsukawa et al., 1997; Taniguchi et al., 1997), and in mice lacking NRP2 (Chen et al., 2000; Giger et al., 2000; Huber et al., 2005; Sahay et al., 2005; Tran et al., 2007). The guidance of motor axons from the spinal cord during vertebrate limb bud development serves as a striking illustration of how the expression pattern of NRPs and their specific semaphorin ligands exquisitely choreographs the homing of specific subsets of axons. These findings indicate that in key respects the cardiovascular and neuronal guidance functions of NRP1 are not reliant on mutual dependence or cross-talk between the development of these networks, mediated by ligands for a shared receptor, but result from spatially distinct and divergent functions of NRP1 expressed in the vasculature or neurons. However, there is some cooperation between Sema 3A and VEGF binding in cardiovascular development because knock-in NRP1 mice deficient in Sema 3A binding and also null for NRP2 (Table 1.1), mice in which VEGF is only able to bind to NRP1, exhibit cardiovascular defects similar to those in endothelial-specific NRP1 null mice (Gu et al., 2003).

NRP expression may also play an important role in the early specification of arterial and venous fate during vascular development. In one day old chick embryos, NRP1 and NRP2 are co-expressed in the early extra-embryonic blood islands, but by the 13 somite stage expression of NRP1 and NRP2 has become restricted to, respectively, the arterial and

venous regions of the primary vascular plexus before blood has started to flow (Herzog et al., 2005). In 26-somite embryos, which have a functioning vasculature, NRP1 and NRP2 are differentially expressed in arteries and veins, while the NRP2 ligand, Sema 3F, was bound to NRP2-expressing cells (Coultas et al., 2005; Herzog et al., 2005). Similar to the expression patterns in the chicken embryo, NRP1 is preferentially expressed in the dorsal aorta of zebrafish embryos, and NRP2 transcripts are localised to the posterior cardinal vein (Coultas et al., 2005). It is unclear at present whether differential NRP expression is crucial for the early embryonic segregation of arterial and venous cells in mammalian embryos, though the mild phenotype in NRP2-deficient mice (Table 1.1), suggests that NRP2 expression may be less important in this respect.

1.7.6 Cellular functions

The most characteristic biological function mediated by NRPs in neuronal cells is chemorepulsion. NRP1 is essential for mediating Sema 3A stimulation of growth cone collapse and axon repulsion in DRG neuronal cultures (He and Tessier-Lavigne, 1997; Kolodkin et al., 1997), while NRP2 is responsible for Sema 3F-induced repulsion of superior cervical ganglia (Chen et al., 1997; Giger et al., 1998). Sema 3A and 3F have also been reported to promote chemorepulsion in porcine aortic endothelial (PAE) cells expressing, respectively, NRP1 and NRP2 (Bielenberg et al., 2004; Miao et al., 1999), though other investigators failed to find an inhibitory effect of Sema 3A on EC migration (Pan et al., 2007a). Sema 3A also appears to be required for the correct orientation and chemoattraction of cortical apical dendrites (Polleux et al., 2000). This chemoattractant role of Sema 3A is mediated at least in part via NRP1, as judged by the effects of function-blocking NRP1 antibody. Remarkably, the conversion of the Sema 3A/NRP1 axis from chemorepulsion to chemoattraction is determined by the distribution of soluble guanylate cyclase: guanylate cyclase is localised to the dendrite, and inhibition of either guanylate cyclase or protein kinase G disrupted dendrite outgrowth in response to Sema 3A (Polleux et al., 2000; Song et al., 1998).

Most evidence points to a role of NRP1 in endothelial cell migration and adhesion. Co-expression of NRP1 and VEGFR2 in PAE cells enhances VEGF binding and chemotaxis (Soker et al., 1998), and NRP1 mediates endothelial cell attachment to extracellular matrix (Murga et al., 2005). VEGF-A₁₆₅ also stimulates morphogenetic responses in renal epithelial cells, including sheet migration and tubulogenesis, through a mechanism dependent on VEGFR2 and which is also blocked by either neutralising antibody to NRP1 or by the

Genotype	Phenotype	References
NRP1^{-/-}	Die between E10 and E13.5 and present extended cardiovascular and neuronal defects. Embryos have impaired endothelial tip cell guidance in the developing CNS. Small and large vessels of the yolk sac are disorganised and its capillary network is sparse. Mice mutants show an agenesis of the brachial arch related great vessels and dorsal aorta. Zebrafish mutants displayed a loss or anarchic angiogenesis, without loss of vasculogenesis function. Disorganization of the nervous system was also observed.	Kitsukawa <i>et al.</i> , 1997 Gerhardt <i>et al.</i> , 2004 Kawasaki <i>et al.</i> , 1999 Lee <i>et al.</i> , 2002
NRP1^{Endo}^{-/-}	Mid to late embryonic lethality, abnormally poorly-branched vasculature, multiple cardiac defects including <i>Truncus arteriosus</i> , failure of septation of cardiac outflow tract.	Gu <i>et al.</i> , 2003
NRP2^{-/-}	Embryo survive to adulthood, but abnormal guidance and fasciculation of some cranial nerves and fewer small lymphatic vessels and capillaries.	Giger <i>et al.</i> , 2000 Chen <i>et al.</i> , 2000 Yuan <i>et al.</i> , 2002
NRP1^{-/-}/NRP2^{-/-}	Earlier embryonic mortality than NRP1 knockout alone (E8) with a phenotype that resembled those of VEGF-A165 and VEGFR2 (KDR) knockouts.	Takashima <i>et al.</i> , 2002
NRP1^{tg}	Embryonic lethality with excess capillary growth, haemorrhage in the head and neck. Embryos have a malformed heart with anarchic sprouting and defasciculation of nerve fibres.	Kitsukawa <i>et al.</i> , 1995
NRP1^{Sema3a}^{-/-}	Survive until birth with only a few surviving to adulthood. Aberrant guidance of sensory afferent nerves to synaptic targets in CNS, defasciculation of spinal and cranial nerves. No vascular defects.	Gu <i>et al.</i> , 2003
NRP1^{Sema3a}^{-/-}/NRP2^{-/-}	Mice show cardiac defects with persistence of the truncus arteriosus, failure of septation of the cardiac outflow tract and atrial enlargement.	Gu <i>et al.</i> , 2003
NIP^{-/-}	Mice survive to adulthood but are 30% smaller and the number of small arteries is reduced. The last 3 amino acids SEA are important to transduce NRP1 signal to NIP.	Wang <i>et al.</i> , 2006 Chittenden <i>et al.</i> , 2006

Table 1.1: Phenotype of Neuropilin Mutant Mice

This table enumerate several mutant genotypes related to the two neuropilin genes as well as there interacting protein NIP along with their phenotypes and references to the corresponding papers.

chemorepulsive NRP1 ligand, Sema 3A (Karihaloo et al., 2005). Antibodies which specifically block VEGF binding to NRP1 inhibited the migratory response to VEGF, *in vitro* endothelial cell sprouting, and neovascularisation *in vivo* (Pan et al., 2007a). Interestingly, the same study reported that blocking NRP1 antibodies prevents pericyte recruitment to new vessels in mouse neonatal retinal vascularisation and tumour vascularisation models, suggesting a role of endothelial NRP1 in the maturation and stabilisation of developing vessels. The cellular mechanism underlying such a role of NRP1 is unclear, but could occur via one or both of two mechanisms, one in which endothelial NRP1 is important for adhesion or chemoattraction of pericytes to developing vessels, and/or a role for pericyte NRP1 in adhesion and migration of immature VSMC (Pan et al., 2007a). The fact that NRP1 is expressed in some mature VSMC cell types suggests a role for this molecule in migratory and adhesive functions of these cells (Shintani et al., 2006).

Analysis of vascularisation in the developing hindbrain of NRP1^{-/-} mice shows that this molecule plays a key role in the guidance of specialised endothelial tip cells in newly sprouting vessels. Tip cells extend long filopodia, which sense gradients of VEGF, and undergo a stereotypic series of movements during development. In NRP1^{-/-} mice, tip cell filopodia remain associated with radial glia in the subventricular zone of the hindbrain and fail to move laterally across this region, forming characteristic tufts (Gerhardt et al., 2004). These findings indicate that NRP1 may not be essential for endothelial cell migration *per se*, or for elaboration of the cellular migratory apparatus, but rather for determining the trajectories of migrating cells, similar to its pathfinding and homing role in neuronal patterning.

Several studies indicating a role for NRP1 in cell adhesion to extracellular matrix, are suggestive that such a role of NRP1 may be independent of known ligand interactions (Murga et al., 2005; Shimizu et al., 2000). Shimizu et al. (2000) found that recombinant NRP1 proteins supported adhesion of a variety of cell lines, including L cells, HEK293T, COS-7, HeLa, p19, KB and NIH3T3, and identified specific regions in the NRP1 b1 and b2 domains for adhesion, but showed that neither Sema 3A nor VEGF-A₁₆₅ interfered with this activity. Furthermore, siRNA-mediated NRP1 knockdown disrupted endothelial cell adhesion to fibronectin, laminin or gelatin, while silencing of VEGFR2 had little effect, suggesting that an NRP1-mediated adhesive function is independent of VEGF-A signalling through VEGFR2 (Murga et al., 2005). Blocking NRP1 antibodies appeared to have no effect on adhesion of endothelial cells to fibronectin (Pan et al., 2007a), consistent perhaps

with the independence of any adhesive function from VEGF binding.

1.7.7 Receptors and signalling mechanisms

NRPs are not thought to be able to transduce a biological signal or response in the absence of another signalling receptor, but to function as co-receptors that bind extracellular ligands with high affinity and complex with other transmembrane molecules (e.g. VEGFR2 or plexins) to form a holoreceptor. However, this model does not fully explain all the evidence relating to the functions of NRPs in endothelial cells and other cell types. Furthermore, NRP1 has been demonstrated to associate with at least one intracellular PDZ protein, synectin, raising the possibility that NRPs despite having a small cytoplasmic domain may be able to modulate intracellular signalling through protein-protein interactions.

1.7.7.1 *Plexins*

NRP1 mediates the chemorepulsive effects of Sema 3A, but does so by acting as the ligand-binding module of a complex or holoreceptor between NRP1 and specific members of a family of transmembrane receptors called plexins (Rohm et al., 2000; Takahashi et al., 1998; Tamagnone et al., 1999). NRP1 and 2 form complexes with several plexins independently of the presence of their ligand Sema 3A, including plexin A1, plexin A2, plexin A3 and B1 (Cheng et al., 2001; Rohm et al., 2000; Tamagnone et al., 1999). The human plexin gene family comprises nine members divided into four subfamilies: four plexin As (1 to 4), three plexin Bs (1 to 3), plexin C1 and plexin D1 [30]. Plexins are large transmembrane receptors with extracellular regions comprising a Sema domain (homologous to sema domains in Semaphorins), two or three plexin-cysteine-rich regions called Met-related sequences (MRS) domains also found in the MET oncogene family, and three or four immunoglobulin-like domains shared by plexins and transcription factors (IPT), a transmembrane domain, and an intracellular region containing two conserved regions, the functions of which are not yet clearly defined, but show some homology to ras GTPase-activating protein (GAP) domains, and in the case of Plexin B1 has been shown to act as a GAP for R-Ras (Oinuma et al., 2004).

Sema 3A contains both a NRP binding site, and potential sites of interaction with Plexins. Antipenko et al. (2003) have proposed a model based on the crystal structure of the Sema 3A Sema domain and mutagenic analysis, in which Sema 3A binding results in a 2:2:2

complex between Sema 3A, plexin A1 and NRP1, and involving relief of autoinhibition of plexin A1 . This results in activation of the plexin intracellular domain and the transduction of signals essential for chemorepulsion. Deletion of the cytoplasmic domain of NRP1 did not impair Sema 3A signalling (Nakamura et al., 1998), indicating that neuropilins may function solely as the binding entity of the complex while plexins mediate signalling. The cytoplasmic domain of plexins is responsible for downstream signalling induced by semaphorin and resulting in the collapse of neurons (Rohm et al., 2000; Takahashi et al., 1999; Tamagnone et al., 1999). Monomeric GTPases of the Rho family are thought to play a key role in regulating actin-based motility in neuronal cells (Hall and Nobes, 2000), and have been implicated in Semaphorin-mediated growth cone collapse (Negishi et al., 2005; Rohm et al., 2000)]. Recruitment of Rnd to the cytoplasmic Plexin A1 domain triggers cytoskeletal collapse, but this effect is antagonised by RhoD which is also able to bind to Plexin A1 but blocks repulsion of sympathetic neurons (Figure 1.14) (Zanata et al., 2002). Sema 3A-induced cytoskeletal collapse in Cos-7 cells was dependent on co-transfection of Plexin A1 and NRP1, required activation of Rac, but not Rho, and involved a direct interaction between Rac and the plexin A1 cytoplasmic domain (Meyer and Feldman, 2002; Ng and Luo, 2004; Turner et al., 2004). If and how NRP1 regulates Plexin-dependent signalling through small GTPases is unclear.

1.7.7.2 CAM (*Cell-Adhesion Molecule*) L1

The immunoglobulin superfamily cell adhesion molecule, L1, is also a potential partner for NRP1 in mediating chemorepulsive Sema 3A signals (Figure 1.14). L1-deficient mice are smaller, less sensitive to touch and pain, and exhibit lack of hind limb coordination compared to littermate controls, and display a striking reduction in the size of the corticospinal tract and in the association of Schwann cells with axons, (Dahme et al., 1997). That part of the phenotype in these mice results from defective axonal guidance orchestrated by Sema 3A, is suggested by the inability of Sema 3A to repel L1-deficient cortical axons (Castellani et al., 2000), and the formation of stable complexes of L1 and NRP1 mediated by interactions between their extracellular domains (Castellani et al., 2002). NRP2, which is not required for Sema 3A signalling, was unable to associate with L1-CAM (Castellani et al., 2002). Several L1 mutations located in the NRP1 binding region are associated with a spectrum of human neurological disorders, including X-linked hydrocephalus and MASA syndrome (Mental retardation, Aphasia, Shuffling gait, Adducted thumbs). Interestingly, one such mutation, L120V, also disrupts L1 association with NRP1, suggesting

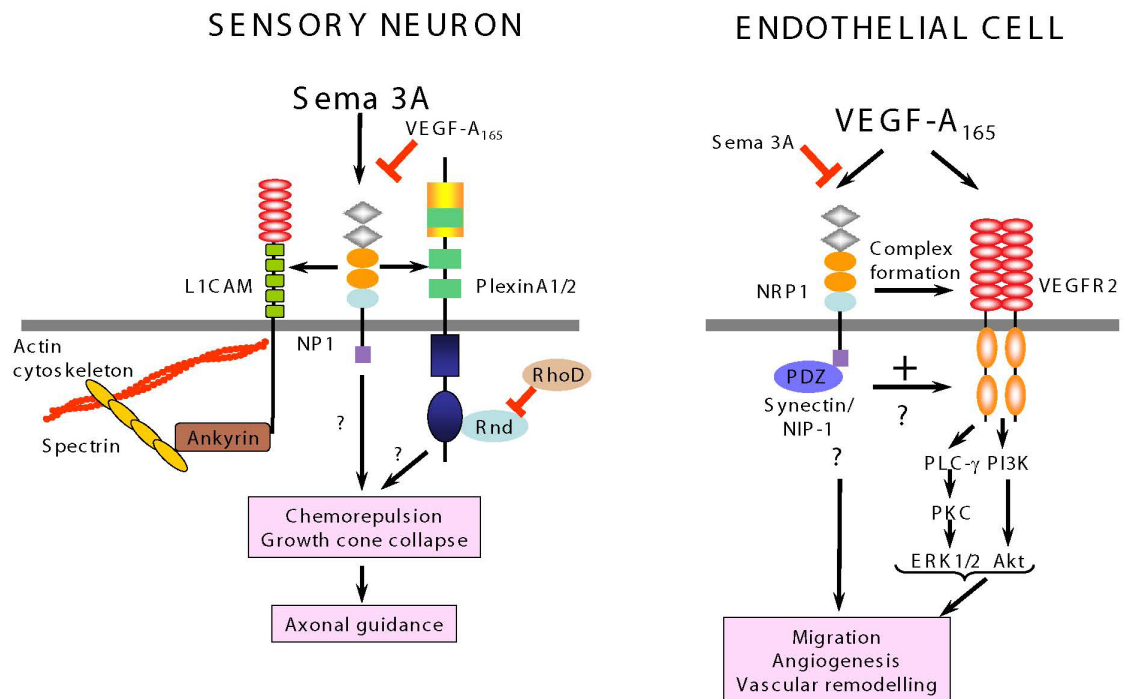


Figure 1.14: **Mechanisms of NRP action**

Left: In sensory neurons, NRP1 can complex with plexin A1 or A2, or with L1-CAM. L1 CAM contains six Immunoglobulin(Ig)-like domains (red ellipses) and five fibronectin type III domains (green squares) in its extracellular region. The L1 intracellular domain associates with ankyrin (rounded brown rectangle), which may link L1 with spectrin (chain of yellow ellipses) and actin (red chain of small red circles), and is implicated in mediating effects of Sema 3A on the cytoskeleton. Plexin A1 activity is regulated by the rho-like GTPases Rnd and its antagonist RhoD (respectively, beige and light blue circles), though how NRP1 regulates this pathway is unclear; the inhibitory effect of RhoD on plexin A1 activity is represented by a red barred line.

Right: VEGF-A₁₆₅ binds to NRP1 and promotes complex formation. The NRP1 carboxy-terminal PDZ domain binding motif associates with the PDZ protein synectin and this is thought to be important for the role of NRP1 in VEGF-A signalling and function mediated via VEGFR2. In some contexts, VEGF-A₁₆₅ and Sema 3A may also competitively bind to NRP1 resulting either in inhibition of Sema 3A binding and downstream signalling, or potentially the converse (indicated by a red bars). NRP1/VEGFR2 complex formation is implicated in optimal activation (indicated by +), via a mechanism that is still unclear (?), of VEGFR2 signalling via phospholipase C-gamma (PLC-γ), leading to activation of PKC and ERK1/2, and PI3K, mediating activation of the serine-threonine kinase, Akt/PKB. Figure adapted from Pellet-Many et al. (2008).

a causal role of defective L1/NRP1 signalling in human neurological disease (Castellani et al., 2002). Soluble L1 extracellular domain is able to convert repulsive Sema 3A signals into chemoattraction by binding in trans to NRP-1 and this conversion is mediated by nitric oxide-dependent activation of guanylate cyclase and consequent cyclic GMP synthesis (Castellani et al., 2002), reminiscent of the role of asymmetric guanylate cyclase distribution in determining chemoattractant properties of Sema 3A in cortical apical dendrites (Polleux et al., 2000). The effects of L1 deficiency do not phenocopy the neural defects of Sema 3A-deficient or NRP1 Sema 3A^{-/-} mice, indicating that L1 regulates Sema 3A signalling via NRP1 in a more restricted set of neurons. It is also unclear yet whether the L1/NRP1 complex is either distinct from, or linked with, the plexin/NRP1 holoreceptor.

1.7.7.3 *VEGFRs*

In endothelial cells, neuropilins are co-receptor for VEGFs by forming complexes with the VEGF protein tyrosine kinase receptor, VEGFR2/KDR. Soker et al. (1998) identified NRP1 as a high affinity receptor for VEGFA₁₆₅ in endothelial and tumour cells and found that NRP1 co-expression with VEGFR2 enhanced VEGF-induced chemotaxis in comparison to cells expressing VEGFR2 alone (Soker et al., 1998). Co-expression of NRP1 with VEGFR2 also enhances VEGFR2 binding to VEGF, phosphorylation, signalling and migration (Mac Gabhann and Popel, 2005; Soker et al., 2002; Whitaker et al., 2001). Whether or not NRP1 increases the affinity of VEGFR2/KDR for VEGF-A remains uncertain. However, the enhanced function of VEGFR2 in the presence of NRP1 has been attributed to enhanced VEGFR2 signalling when it complexes with NRP1, rather than to an increase in the intrinsic affinity of VEGF for its receptors (Whitaker et al., 2001). There are also differing reports as to whether or not VEGF binding is required for NRP1/VEGFR2 complexation. Two groups showed that association between NRP1 and VEGFR2 was dependent on VEGF treatment (Pan et al., 2007a; Soker et al., 2002), whereas Whitaker et al. (2001) and Shraga-Heled et al. (2007) found that co-immunoprecipitation of VEGFR2 and NRP1 in Cos-1 cells co-expressing the two receptors and in HUVECs occurred independently of VEGF-A₁₆₅.

While the precise mechanism mediating the modulation of VEGFR2 signalling by NRP1 is unclear, complex formation does appear to play a crucial role. Complexation between NRP1 and VEGFR2 enhances VEGF binding, and inhibition of complex formation is associated with reduced VEGFR2 phosphorylation, intracellular signalling, mitogenesis, cell migration, and angiogenesis (Oh et al., 2002; Pan et al., 2007a; Soker et al., 2002; Wang

et al., 2003; Whitaker et al., 2001). Since VEGF stimulates biological activities in PAE cells expressing only VEGFR2, signalling via VEGFR2 can be triggered by VEGF-A₁₆₅ independently of NRP1. Furthermore selective inhibition of VEGF-A₁₆₅ binding to NRP1 and NRP1/VEGFR2 complex formation does not prevent either VEGFR2 signal transduction or the stimulation of endothelial biological responses by VEGF-A₁₆₅ (Jia et al., 2006; Pan et al., 2007a). Thus, inhibition of VEGF₁₆₅ binding to NRP1 using a specific peptide antagonist (EG3287) that mimicks the VEGF NRP1 binding domain, attenuated rather than inhibited VEGF-induced VEGFR2 tyrosine phosphorylation, and activation of ERK and phospholipase C- γ , and reduced prostacyclin production, but had less effect on Akt activation and did not reduce the cell survival or proliferative responses to VEGF (Gluzman-Poltorak et al., 2000). Antibodies that specifically block VEGF binding to NRP1 prevented NRP1 complexation with VEGFR2, but had limited effects on VEGFR2 phosphorylation and signalling (Pan et al., 2007a). It seems therefore that NRP1 is not essential for the full spectrum of signalling pathways and biological responses stimulated by VEGF, but is instead required for optimal VEGF-induced VEGFR2 signalling through some pathways and for maximum function in the case of certain biological processes, such as migration.

Several mechanisms could theoretically account for the ability of NRP1 to enhance VEGFR2 signalling. NRP1 could increase the affinity of VEGFR2 for VEGF-A, though previous studies have concluded that NRP1/VEGFR2 complexation does not change the affinity of VEGF for VEGFR2 (Whitaker et al., 2001). However, selective antagonism of VEGF binding to NRP1 also greatly reduced VEGF-A cross-linking to VEGFR2 in human endothelial cells co-expressing the two receptors, though the antagonist had no effect on VEGF-A binding to PAE cells expressing only VEGFR2 (Jia et al., 2006), suggesting that NRP1/VEGFR2 complex formation may stabilise binding of VEGF-A₁₆₅ to VEGFR2, therefore increasing the longevity of VEGFR2 signalling. A second mechanism is that NRP1/VEGFR2 complex formation may stabilise VEGFR2 at the cell surface, by rendering it less labile, and/or less prone to receptor-mediated endocytosis and therefore able to increase the duration and amplitude of receptor activation and downstream signalling. In support of this notion, siRNA-mediated NRP1 knockdown was recently reported to both reduce VEGFR2 expression and attenuate VEGF-induced gene expression in human endothelial cells (Holmes and Zachary, 2005). Thirdly, as discussed in more detail below, NRP1 may itself either transduce intracellular signals, or participate in protein-protein interactions, which enhance the signalling function of the NRP1/VEGFR2 complex relative

to VEGFR2 alone.

NRP1 has been found to bind *in vitro* to the VEGFR1/Flt-1 extracellular Ig-like domains 3 and 4 (Fuh et al., 2000), and it is possible that such a complex may compete for VEGF binding to VEGFR2/NRP1 heterodimers. Recent findings indicate that NRP2 interacts with VEGF-C in a heparin dependent manner and VEGF-D in a heparin-independent manner (Karpanen et al., 2006). Moreover, VEGFR3 and NRP2 displayed colocalisation and co-internalised following stimulation by VEGF-C and D (Karpanen et al., 2006). The formation of complexes between NRP2 and VEGFR2 or VEGFR3 resulted in a lowering of the activation threshold of VEGFR2 and an enhancement of cell survival and migration induced by VEGF-A and the VEGFR3 ligand, VEGF-C (Favier et al., 2006). VEGFR3 and VEGF-C are both strongly implicated in lymphatic vascular development (Adams and Alitalo, 2007), and complex formation between VEGFR3 and NRP2 may help to explain the involvement of NRP2 in lymphangiogenesis as suggested by the phenotype of NRP2 knock out mice (Yuan et al., 2002) and Table 1.1. Co-immunoprecipitation of cross-linked ¹²⁵I-VEGF also revealed the existence of a NRP2/VEGFR1 complex (Gluzman-Poltorak et al., 2001), but the biological relevance of such a complex is not clear.

1.7.7.4 *Other ligands and co-receptors*

Recent evidence indicates that NRP1 may be a receptor for other growth factors in the regulation of non-neuronal and non-endothelial cells (Glinka and Prud'homme, 2008; West et al., 2005). over-expression of NRP1 in pancreatic cancer cells promotes tumour cell invasion and HGF-induced c-Met signalling (Hu et al., 2007; Matsushita et al., 2007). NRP1 can associate with c-Met (Matsushita et al., 2007), and NRP1 and NRP2 bind HGF, and mediate HGF stimulation of endothelial cell migration and proliferation (Sulpice et al., 2008). NRP1 also mediates VSMC motility induced by PDGF secreted by breast cancer cells (Banerjee et al., 2006). The role of NRP1 in HGF and PDGF signalling is presently unclear. The homodimeric animal lectin, Galectin-1, has also been identified as a ligand for NRP1 in vascular endothelial cells and mediate VEGFR2 signalling and endothelial cell migration (Hsieh et al., 2008).

1.7.7.5 *NRP-interacting proteins*

While there is strong evidence that NRP1 functions primarily as a co-receptor without an independent signalling role, some findings suggest that NRPs have functions that are

not dependent on the known NRP ligands and interacting receptors, and further raise the possibility that NRP1 is able to support functional cellular signalling. For example, VEGF elicits biological responses in some cell types that are NRP-positive but express little or no KDR, such as vascular smooth muscle cells (Ishida et al., 2001) and diverse cancer cells (Table 1.2). As discussed above, antibodies and antagonists that selectively block VEGF binding to NRP1 have restricted effects on endothelial VEGF signalling and in *in vivo* models of angiogenesis, which do not replicate the effects of function-blocking antibodies directed against VEGF (Jia et al., 2006; Pan et al., 2007a), suggestive that NRP1 has VEGF independent roles in endothelial cells. Though the cytoplasmic domains of NRP1 and NRP2 are small, they do contain a carboxy-terminal consensus PDZ binding motif that associated with the PDZ protein, NIP1 (Neuropilin Interacting Protein-1), also called synectin or GIPC1 (RGS-GAIP-interacting protein-1) in a yeast two-hybrid screen (Cai and Reed, 1999). A functional role for NRP1 association with synectin in angiogenesis is supported by the finding that expression of NRP1 lacking the C-terminal SEA motif disrupted vessel formation in zebrafish, and that knockdown of synectin in zebrafish produced a similar vascular phenotype to that caused by NRP1 knockdown (Wang et al., 2006). Furthermore, synectin was found to associate with NRP1 in human endothelial cells, and synectin knockdown inhibited NRP1-mediated endothelial migration (Wang et al., 2006). In contrast, the C-terminal PDZ-binding domain does not appear to be important for Sema 3A-mediated neuronal pathfinding functions of NRPs (Nakamura et al., 1998). Synectin/GIPC was originally found to associate with one of the regulators of G protein signalling (RGS) proteins, called RGS19 (also known as GAIP) (De et al., 1998). The GIPC/RGS19 complex is anchored to the cell membrane, localises to clathrin-coated vesicles and has been implicated in endocytosis and intracellular membrane trafficking (Abramow-Newerly et al., 2006). Synectin/GIPC binds to G protein coupled receptors and modulates their signalling (Abramow-Newerly et al., 2006), and can also interact with up to twenty other proteins, including the proteoglycan, syndecan-4 (Gao et al., 2000), the integrin 5 and 6 subunits (El et al., 2002), the transmembrane semaphorin, M-SemaF (Wang et al., 1999), and rho GEF (rho guanine exchange factor or syx1) (Liu and Horowitz, 2006). These findings suggest that synectin has the potential to participate in multimeric protein complexes or scaffolds able to link surface receptors and integrins with intracellular signalling networks. *In vivo* studies show the importance of synectin in the development of a functional vascular system in both the zebrafish and the mouse (Chittenden et al., 2006). Synectin deficient mice are viable, but the mice are smaller

(30% less than littermate controls), and the number of small arteries were significantly reduced, resulting in impaired vascular functions (Chittenden et al., 2006). Arterial cells from synectin^{-/-} mice exhibited reduced angiogenic and endothelial responses *in vitro*, and aberrant cellular distribution of rac1 (Chittenden et al., 2006). The mainly microvascular arterial defects in synectin knockout mice do not phenocopy the embryonic lethality or aberrant macrovascular development of either global or endothelial-specific NRP1 deficiency, indicating that not all developmental functions of NRP1 are dependent on synectin association. However, it is possible that other related molecules, such as GIPC2 or GIPC3, may compensate for loss of synectin (Kato, 2002). Nevertheless, the fact that synectin binds to the C-terminus of NRP1 is suggestive of independent cytoplasmic signalling and suggest the existence of other as yet unidentified interacting proteins.

1.7.8 Neuropilin Functions in Disease and Adult Tissues

1.7.8.1 Cancer

NRP1 and NRP2 are expressed by a wide variety of human tumour cell lines and diverse human neoplasms (Bielenberg et al., 2006; Ellis, 2006; Soker et al., 1998), and are implicated in mediating effects of VEGF and Semaphorins on the proliferation, survival and migration of cancer cells (Bachelder et al., 2001; Chabbert-de et al., 2006; Miao et al., 2000). Table 1.2 summarises the expression of NRPs and other VEGF receptors in a panel of representative carcinoma cells. Over-expression of NRP1 in Dunning rat prostate AT2.1 carcinoma cells increased tumour growth *in vivo* (Miao et al., 2000), while NRP1 knockdown using siRNA inhibited breast carcinoma cell migration (Bachelder et al., 2003), and a peptide targeted to the VEGF binding site of NRP1 induced breast tumour cell apoptosis (Barr et al., 2005). NRP1 is expressed in patient specimens from lung, breast, prostate, pancreatic and colon carcinomas, but not in corresponding normal epithelial tissues (Fukahi et al., 2004; Kawakami et al., 2002; Lantuejoul et al., 2003; Latil et al., 2000; Parikh et al., 2004, 2003; Stephenson et al., 2002). NRP1 has also been found in several other tumours including melanoma (Straume and Akslen, 2003), astrocytoma (Broholm and Laursen, 2004) and neuroblastoma (Fakhari et al., 2002). NRP2 expression was reported in lung cancer (Kawakami et al., 2002; Lantuejoul et al., 2003), neuroblastoma (Fakhari et al., 2002), pancreatic cancer (Cohen et al., 2002), osteosarcoma (Handa et al., 2000) and bladder cancer (Sanchez-Carbayo et al., 2003). It has been suggested that NRP1 is more prevalently expressed in carcinomas (mainly of epithelial origin), whereas NRP2 may

be more frequently expressed in non-carcinoma neoplasms such as melanomas, leukaemias and neuroblastomas (Vales et al., 2007; Bielenberg et al., 2006; Ellis, 2006). However, as Table 1.2 indicates there is no sharp distinction between the types of neoplasms expressing NRPs 1 and 2 (Marcus et al., 2005), and often they are co-expressed. Furthermore, different cell lines derived from the same tumour types, such as glioma (Rieger et al., 2003), may exhibit divergent patterns of NRP1 and NRP2 expression.

	NRP1	NRP2	VEGFR2	VEGFR1	VEGFR3
A549 (lung)	++	+	-	-	-
MDA-MB-231 (breast)	+	?	-	++	-
MCF-7 (breast)	+	?	-	++	-
ACHN (kidney)	+	++	-	-	-
SKOV-3 (ovary)	+	+	-	-	-
DU145 (prostate)	++	-	-	-	-
RT112/84 (bladder)	+	?	-	-	-
BxPC-3 (pancreas)	+	?	-	-	-
SK-MEL-5 (melanoma)	+/-	++	-	-	-
MDA-MB-435 (melanoma)	+	++	-	-	-
SK-N-AS (neuroblastoma)	+	-	-	-	ND
SK-N-DZ (neuroblastoma)	+	-	-	-	ND
U87 MG (glioma)	++	+	ND	ND	ND
Astrocytoma(GBM)	+	?	-	-	-
Leukaemia (AML, various)	+	+	-	+/-	+/-
Leukaemia (CML, various)	+	+	+/-	+/-	-

Table 1.2: **Neuropilins and VEGFRs expression in tumour cells** AML, acute myeloid leukaemia cell lines; CML, chronic myeloid leukaemia cell lines; ND, not determined; ?, very low or uncertain expression; +/-, low expression or no expression in other; +, moderate expression; ++, strong expression. Figure from Pellet-Many et al. (2008).

Clinical studies suggest that NRP1 plays a role in tumour growth and disease progression (Bielenberg et al., 2006; Ellis, 2006; Guttman-Raviv et al., 2006). Over-expression of NRP1 has been demonstrated to be associated positively with the metastatic potential, advanced stage and clinical grade of prostate carcinoma (Latil et al., 2000). NRP1 up-regulation in gastrointestinal carcinomas appears to correlate with invasive behaviour and metastatic potential (Hansel et al., 2004). Co-expression of NRP1 and NRP2 also increased in the progression from dysplasia to microinvasive lung carcinoma, and correlated significantly with tumour progression and poor prognosis in patients with non-small cell lung carcinoma (Kawakami et al., 2002). NRP1 also appears to be preferentially expressed in metastatic cells, and is found, for example in the metastatic breast cancer cell lines MDA-MB-231 and MDA-MB-435 but not in the nonmetastatic cell line MDA-MB-453 or some nonmetastatic tumours (Bachelder et al., 2001; Soker et al., 1998).

Although most studies have indicated a pro-tumourigenic role of NRPs, some reports suggest that NRP1 plays a more complex role in some tumour types. Thus, NRP1 over-expression in Panc-1 cells was found to reduce tumour volume and incidence (Gray et al., 2005), whereas the same group showed that FG pancreatic carcinoma cells expressing NRP1 have increased resistance to anoikis and cytotoxic drugs (Wey et al., 2005). Furthermore, some findings suggest that not all effects of VEGF in tumour cells may be dependent on NRP1. Lee et al. (2007) found that survival effects of VEGF in the breast carcinoma MDA-MB-231 and MCF-7 cell lines were mediated by internally expressed VEGFR1/Flt-1, and were unaffected by NRP1 knockdown.

There is also some evidence pointing to potentially differential or antagonistic roles of NRP1 and NRP2 in tumour cell regulation. Sema 3F, the best-characterised ligand for NRP2 induces a poorly vascularised non-metastatic phenotype in xenografts of melanoma in mice (Bielenberg et al., 2004). Sema 3F and a second NRP2 ligand, Sema 3B, are both localised to the 3p21.3 chromosomal region, which is commonly deleted in human lung cancers (Sekido et al., 1996; Tomizawa et al., 2001). These findings suggest that Sema ligands for NRP2 are potential tumour suppressors.

Recent studies provide direct evidence that NRP1 contributes to tumour cell growth and tumour neovascularisation *in vivo*. A peptide that inhibits VEGF binding to NRP1 has been reported to inhibit angiogenesis and growth of tumour xenografts (Starzec et al., 2006). An antibody targeted to the b1 domain that specifically blocks VEGF-A binding to NRP1 causes a range of effects in endothelial cell cultures including inhibition of

VEGFR2 complex formation, VEGF-induced migration, and vascular sprouting, reduces angiogenesis in a neonatal retinal neovascularisation model, and inhibits tumour growth and tumour vascularisation in mouse xenograft models. A blocking NRP1 antibody alone had a relatively small effect on tumour growth, but produced a strong additive effect when used in combination with the blocking VEGF antibody, bevacizumab or avastin (Pan et al., 2007a), now approved for clinical use in several late stage carcinomas. Interestingly, Pan et al. (2007a) reported that the anti-tumour activity of blocking NRP1 antibodies was not dependent on NRP1 expression in the tumour cell line used in the xenograft model. Furthermore, this study also found no evidence of direct effects of NRP1 antibodies on tumour cell proliferation, suggesting that their anti-tumourigenic effects were mainly due to inhibition or destabilisation of the tumour vasculature. However, any conclusion that the role of NRP1 in tumourigenesis is restricted to vascularisation should be considered in the light of studies, discussed above, implicating NRP1 in cell migration and adhesion. Furthermore, NRP1 plays a role in potentiating the effect of HGF (hepatocyte growth factor)/Scatter factor signalling through the c-Met receptor in both glioma and pancreatic cancer cell lines, regulating tumour progression and invasion (Hu et al., 2007; Matsushita et al., 2007). These findings taken together with the expression of NRPs in diverse neoplasms, suggests a possible role for this molecule in tumour invasion and metastasis in addition to its involvement in tumour vascularisation.

1.7.8.2 Immune system

NRP1 was found to be expressed in naive T-cells and immature dendritic cells (antigen-presenting cells), cell types that interact during the primary immune response in the secondary lymphoid organs (Tordjman et al., 2002), and are essential for triggering the proliferation and differentiation of mature T-cells that will later interact again with antigen-presenting cells to mediate antigen elimination (Wulfing and Rupp, 2002). NRP1 expressed on naive T-cells also mediated their clustering with NRP1-expressing Cos-7 cells, and the stimulation of resting T-cell proliferation by dendritic cells was reduced by $\approx 50\%$ by blocking NRP1 antibodies (Tordjman et al., 2002). T-cell activation was also reduced by Sema 3A (Catalano et al., 2006; Moretti et al., 2006). NRP1 distribution in T-cells was polarised, and NRP1 co-localised with the T-cell marker, CD3, at the interface between dendritic cells and immature T-cells (Tordjman et al., 2002). Although the biological relevance of these findings for the immune response in vivo is unclear, they are consistent with the formation of homophilic NRP1 interactions between dendritic cells and imma-

ture T-cells, which contribute to an early step in formation of the immunological synapse, essential for T-cell maturation (Tordjman et al., 2002).

1.7.8.3 Wound healing

There is evidence that NRP1 is up-regulated in response to tissue injury and may be involved in regeneration and repair. In *Xenopus*, when the optic nerve was crushed and allowed to regenerate, the level of NRP1 increased and remained elevated for weeks before finally declining after healing (Fujisawa et al., 1995). NRP1 was also found to be strongly expressed in the neovasculature during wound angiogenesis in a murine model of dermal wound healing, whereas blocking anti-NRP1 antibody reduced wound vascularization (Matthies et al., 2002). Optic nerve injury in a rat model resulted in cell invasion at the site of injury by microglia, oligodendrocytes and astrocytes associated with induction of Sema 3A and NRP1 (Nitzan et al., 2006). NRP1 may protect neuronal cells against damage resulting from stress or injury. Thus IFN γ (interferon γ) activation of microglia, a cell type that becomes activated following neuronal injury, caused up-regulation of NRP1 and plexin A1 and was associated with induction of microglial apoptosis by Sema 3A, suggesting that a NRP1-mediated pathway may protect neurons against damage caused by activated microglia (Majed et al., 2006). Expression of NRPs, VEGFs and class 3 semaphorins are also up-regulated by cerebral ischaemic injury in different animal models (Ara et al., 2004; Fujita et al., 2001; Gavazzi et al., 2000; Jin et al., 2000; Lindholm et al., 2004; Pasterkamp et al., 1999; Winter et al., 2002; Zhang et al., 2001). For example, in a mouse model of cerebral ischaemia, NRP1 mRNA expression increased rapidly and remained elevated for at least a month after the ischaemic event (Zhang et al., 2001). Interestingly, NRP1 was not only localised to the ischaemic neurons, but also to the endothelial cells of the brain vessels. These findings are suggestive of a dual role for NRP1 in the response to cerebral injury, in the promotion of both neuronal growth and in cerebral angiogenesis.

1.7.8.4 Other functions

NRP1 expression has been reported in other cell types, including bone-marrow-derived progenitor cells (Fons et al., 2004), platelets (Kashiwagi et al., 2005) and the granulosa and theca cells in the follicles of the bovine ovary (Shimizu et al., 2006), although the role of NRP1 in these systems has not yet been defined.

Aims of this thesis

When I first began to study for my PhD, an important role for the VEGF co-receptor, NRP1, in cancer cells and in endothelial cell biology was widely accepted and several colleagues in this lab were working on aspects of these questions. However, little was known regarding the mechanism of action through which NRP1 regulated endothelial cell function. It was also unknown whether NRP1 had a role in other cell types associated with the vasculature. The initial overall aim of my thesis was to characterise the role of NRPs in vascular cells and to elucidate intracellular mechanisms that conferred an important role for NRP in vascular development as indicated by the phenotypes of NRP knockout and transgenic mice. To do this I started to investigate the expression of NRPs in endothelial cells (EC). At the same time, it was also of interest to examine whether NRP1 was also expressed in vascular smooth muscle cells (VSMC), a major constituent of many blood vessels. Interestingly, I observed strong NRP1 expression in human primary VSMC from coronary arteries, and additionally also noted a different pattern of NRP1 expression in VSMC compared with EC, notably, the expression of a novel high molecular weight NRP1 species not present in EC, but also expressed in diverse tumour cells. Since at that time, very little if anything was known concerning expression of NRP1 in VSMC, or about the identity of a high molecular weight NRP1 species, I decided to focus in my thesis on the role of NRP1 in VSMC and the characterisation of this novel NRP1 form. The specific aims of my thesis were to:

- Identify the high molecular weight form of NRP1 expressed in VSMC.
- Examine the possible role of NRP1 in functions of VSMC such as cell migration.
- Determine the role of NRP1 in the biological actions of known growth factors for VSMC, particularly PDGF and VEGF.
- Investigate the role of NRP1 in intracellular signalling in VSMC, particularly signalling linked to cell migration, and compare its role in VSMC signalling with that in EC.

Chapter 2

Materials and Methods

2.1 Materials

NRP1, NRP2 and synectin small interfering ribonucleic acids (siRNAs) were purchased from Ambion and a nucleofection kit was used for transfection of smooth muscle cells (Amaxa). Oligofectamine and lipofectamine 2000 (Invitrogen) were used to transfect HU-VECs and cancer cells with siRNAs and plasmids, respectively. Human recombinant soluble NRP1 was purchased from R&D systems. Recombinant soluble NRP1 b1 domain was a kind gift from Dr Charles Allerston (Centre for Structural Biology, UCL). Tunicamycin, Chondroitinase ABC and Heparinase I and III (heparitinase) were purchased from Sigma. Antibodies were obtained from Santa Cruz Biotechnologies, Cell signaling, Seigaku and BD biosciences (see Table 2.1). ELISA kits for the detection of both total and phospho PDGFR α and β were obtained from R&D. Recombinant VEGF-A₁₆₅ were from R&D systems, and recombinant PDGF-AA and PDGF-BB were from Peprotech. ¹²⁵I-VEGF-A₁₆₅ was purchased from Amersham and ¹²⁵PDGF-BB from Perkin-Elmer. Protease inhibitor cocktail was CompleteTM from Roche and Phosphatase inhibitor cocktail were from Sigma. The protease inhibitor comprises aprotinin, bestatin, calpain inhibitor, chymostatin, leupeptin, pepstatin, PMSF, trypsin inhibitor. Phosphatase Cocktail 1 contains microcystin LR, cantharidin, and (-)-p-bromotetramisole, and phosphatase cocktail 2 contains sodium vanadate, sodium molybdate, sodium tartrate, and imidazole. Products used for the generation of adenoviruses were obtained from Invitrogen and were part of the Gateway[®]cloning system technology. Unless stated otherwise, all other chemicals were obtained from Sigma, Merck or QUIAGEN and were of the highest grade available.

Table 2.1: **Antibodies used in this study**

Antigen	Catalogue number	Company	Host	Dilution
NRP1 N-terminus	A-12	Santa Cruz	Mouse	1:300
NRP1 C-terminus	C-19	Santa Cruz	Goat	1 :750
NRP1 N-terminus	AF566	R&D	Goat	1:500
NRP1 N-terminus	AF3870	R&D	Sheep	1:1000
NRP2 N-terminus	C-9	Santa Cruz	Mouse	1:500
Flk-1	A-3	Santa Cruz	Mouse	1:300
Flt-1	C-17	Santa Cruz	Rabbit	1:300
PDGFR α	A-12	Santa Cruz	Rabbit	1:500
PDGFR β	P-20	Santa Cruz	Rabbit	1:500
Phospho-PDGFR α	Tyr720	Santa Cruz	Goat	1:300
Phospho-PDGFR β	Tyr1021	Santa Cruz	Rabbit	1:300
Synectin	N-19	Santa Cruz	Goat	1:500
GAPDH	V-18	Santa Cruz	Goat	1:750
Heparan Sulphate (clone 10E4)	370255	Seigaku	Mouse	1:500
Chondroitin Sulphate	C8035	Sigma	Mouse	1:500
Phospho-Tyrosine 4G10	9411	Cell signaling	Mouse	1:1000
Akt	4685	Cell signaling	Rabbit	1:1000
Phospho-Akt (Ser473)	9271	Cell signaling	Rabbit	1:1000
p44/42 MAPK (Erk1/2)	9102	Cell signaling	Rabbit	1:1000
Phospho-Erk1/2 (Thr202/ Tyr204)	9101	Cell signaling	Rabbit	1:1000
p130Cas	610271	BD Biosciences	Mouse	1:1000
phospho-p130Cas (Tyr 410)	4011	Cell signaling	Rabbit	1:1000
Phospho-Src	2101	Cell signaling	Rabbit	1:1000
Phospho-CAMKII (Thr286)	3361	Cell signaling	Rabbit	1:1000
Cofilin	3312	Cell signaling	Rabbit	1:1000
Phospho-cofilin (Ser3)	3311	Cell signaling	Rabbit	1:1000

2.1.1 NRP1 antagonists

The neuropilin 1 antagonist EG3287 was synthesised as described previously (Jia et al., 2006) and was the generous gift of Ark Therapeutics Ltd. It is a small peptide of 28 amino acids with the sequence H₂N-SCKNTDSRCKARQLELNERTCRCDKPRR-OH, and was shown to bind to the VEGF binding site of NRP1, subsequently decreasing VEGF signalling in cells co-expressing NRP1 and KDR (Jia et al., 2006). Synthesised later was EG00086 which has the same peptide sequence as EG3287, but is modified at its amino-terminus by addition of an octanoyl group, and has somewhat improved potency compared with EG3287 (Figure 2.1 and 2.2).



Figure 2.1: EG00086 amino acid sequence

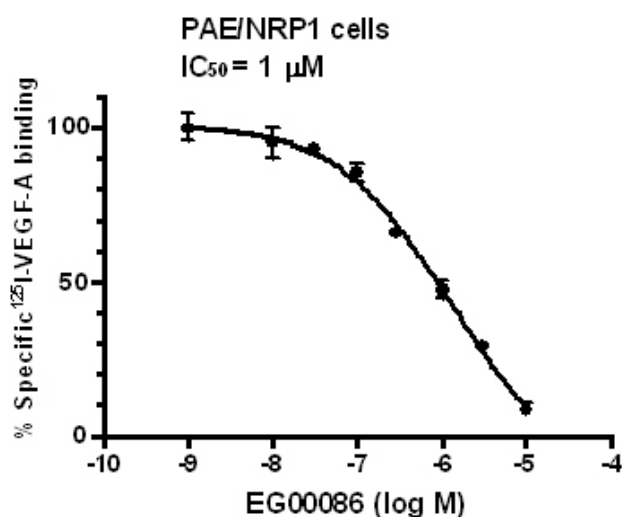


Figure 2.2: EG00086 competitive binding curve

Binding assays were performed in PAE NRP1 cells in the presence of EG00086 at different concentrations (from 10 μM to 1 nM). Results are expressed in percentage of VEGF-A₁₆₅ specific binding in function of the log of EG00086 Molar concentration. The IC₅₀ (inhibitory concentration 50%) which corresponds to the concentration of EG00086 that produces half of the total ¹²⁵VEGF-A₁₆₅-specific binding. Data kindly provided by Haiyan Jia.

2.2 Cell culture

2.2.1 Primary cells

Human coronary smooth muscle cells (HCASMCs; TCS CellWorks) or human aortic smooth muscle cells (HAoSMCs; TCS CellWorks) were grown on cell cultureware in a 37°C, 95% air/5% CO₂ incubator. Smooth muscle cell basal medium (SmBM; TCS CellWorks) supplemented with 0.5 ml antibiotic cocktail with gentamycin (25 µg/ml) and amphotericin B (50 µg/ml) and growth supplement was used to maintain the cells. Human umbilical vein endothelial cells (HUVECs; TCS CellWorks) or human coronary artery endothelial cells (HCAECs; TCS CellWorks) were grown in gelatin-coated cell cultureware in a 37°C, 95% air/5% CO₂ incubator. Endothelial basal medium (EBM; Cambrex BioScience Ltd) supplemented with gentamycin-ampicillin, epidermal growth factor and bovine brain extract (Singlequots; Cambrex) and 10% foetal bovine serum (FBS) was used to maintain the cells. The cells were passaged by trypsinisation and used at no later than passage 6 for endothelial cells and passage 5 for SMC.

2.2.2 Cell lines

Porcine Aortic endothelial cells (PAE), A549 cells (human alveolar basal epithelial cells carcinoma) and ACHN cells (human renal adenocarcinoma cells) were cultured in F12, DMEM and RPMI media respectively (GIBCO), all containing 10% FBS and penicillin-streptomycin (SIGMA, 10,000 units/ml penicillin and 10 mg/ml streptomycin). Lysates from Skov-3 (human ovarian adenocarcinoma cells), Skbr-3 (human breast adenocarcinoma cells), MCF-7 and MDA-MB-231 (both human breast adenocarcinoma cells) were a kind gift from Dr Gianluca Sala.

Human embryonic kidney (HEK) 293A cells (Invitrogen) were grown in cell cultureware in a 37°C, 95% air/5% CO₂ incubator. Cells were maintained in DMEM (Invitrogen) supplemented with 10% FBS, 1% penicillin-streptomycin (Sigma), and passaged by trypsinisation.

Table 2.2: Cells used in this study

Abbreviation	Full name or type	Culture medium
HCASMC	Human coronary artery smooth muscle cell	SMC basal medium
HAoSMC	Human aortic artery smooth muscle cell	SMC basal medium
HUVEC	Human umbilical vein endothelial cell	EC basal medium
HCAEC	Human coronary artery endothelial cell	EC basal medium
PAEC	Porcine aortic endothelial cell	RPMI
PAEC/NRP1	Porcine aortic endothelial cell stably expressing NRP1	RPMI with geneticin
A549	Human alveolar epithelial carcinoma cells	DMEM
ACHN	Human renal adenocarcinoma cells	F-12
Skov-3	Human ovarian adenocarcinoma cells	lysates
Skbr	Human breast adenocarcinoma cells	lysates
MCF-7	Human breast adenocarcinoma cells	lysates
MDA-MB-231	Human breast adenocarcinoma cells	lysates
HEK 293A	Human embryonic kidney	DMEM

2.3 Immunoprecipitation

2.3.1 Principle

Immunoprecipitation consists of the capture of a protein from solution. An antibody directed against a protein of interest binds to its target in a cell lysate. This primary antibody is then bound by a secondary reagent, inert beads conjugated to *Staphylococcus* protein A or G, which bind to the constant region of antibodies. These beads are then separated (along with their complexed antibody/protein) from the cell lysate by centrifugation. Other endogenous binding partners that interact with the target protein are co-purified and might appear in the immunoprecipitate, depending on the stability of these interactions.

2.3.2 Experimental details

For co-immunoprecipitation experiments, cells were washed twice in ice-cold phosphate buffered saline (PBS) and lysed by scraping in lysis buffer containing 20 mM Na₂HPO₂ pH 7.4, 150 mM NaCl, 1% Triton X-100, 5 mM EDTA with protease and phosphatase inhibitor cocktails. Lysis was allowed to continue on ice for 30 minutes, after which the

lysate was clarified by centrifugation (20 minutes, 16000*g*, 4°C) and the insoluble material discarded. The lysate was then pre-cleared by incubating with washed protein A/G plus agarose beads (Santa Cruz) for 30 minutes followed by antibody binding overnight at 4°C. Protein A/G plus agarose beads were added to capture the immunocomplex for 4 hours at 4°C with constant rotation. The IP complex was then dissociated by incubation with 2x SDS-PAGE (sodium dodecyl sulfate polyacrylamide gel electrophoresis) sample buffer (4% SDS, 20% glycerol, 10% 2-mercaptoethanol, 0.004% bromophenol blue, 0.125 M Tris HCl, pH 6.8) and heating at 100°C for 5 mins. Lysates were stored at -20°C until used.

In experiments where NRP1 immunoprecipitates were treated with lyases (chondroitinase and heparitinase), cells were washed twice in ice-cold PBS and lysed by scraping in lysis buffer with protease and phosphatase inhibitor cocktails. NRP1 was immunoprecipitated from the cell lysates by incubating with an antibody directed against the NRP1 carboxy terminus (C-19) overnight at 4°C. Immunocomplexes were captured with protein A/G Plus-Agarose Beads (Santa Cruz Biotechnologies) for 4 hours at 4°C. Immunoprecipitates were then washed, and subjected to various enzyme treatments, as described in paragraph 2.4.2.2, before analysis by western blot.

2.4 Enzyme and drug treatments

2.4.1 Tunicamycin treatment

Tunicamycin is a mixture of antibiotics which inhibit the enzyme N-acetylglucosamine phosphotransferase which catalyses a key rate-limiting step in protein N-linked glycosylation. Tunicamycin blocks the synthesis of all N-linked glycoproteins but also causes cell cycle arrest in G1 phase.

On the day prior to the treatment, fresh medium was added to confluent cells seeded in 6 well-plates. Tunicamycin was added to a final concentration of 5 $\mu\text{g}/\text{ml}$ and incubated at 37°C, for 16 hours. Cells were then lysed with RIPA buffer (30 mM TrisHCl, pH 7.4, 150 mM NaCl, 1% NP-40, 0.5% Deoxycholate, 2mM ETDA) containing protease inhibitors and phosphatase inhibitor cocktails. Lysates were stored at -20°C until used for western blotting.

2.4.2 Chondroitinase and Heparinase treatments

Chondroitinase (produced in *Proteus vulgaris*) and heparinase (produced in *Flavobacterium heparinum*) are lyases that catalyse the degradation of glycosaminoglycans to unsaturated oligosaccharides and unsaturated disaccharides. Their primary substrates are chondroitin sulphate and heparan sulphate proteoglycan, respectively.

2.4.2.1 In intact cells

Chondroitinase was reconstituted in a buffer containing 0.01% BSA and heparinase I and III (heparitinase) solutions were prepared in a buffer containing 20 mM Tris-HCl, pH 7.5, containing 0.1mg/ml BSA and 4 mM CaCl₂. The enzymes were added to the culture medium of the cells (complemented with 3% BSA) at a final concentration of 1 unit per ml. Cells were then incubated for a further 2 hours at 37°C. Cells were then used in further experiments.

2.4.2.2 In immunoprecipitates

NRP1 immunoprecipitates were treated for 2 hours at 37°C with chondroitinase and heparitinase at 1 Unit per ml in 1% NP-40, 0.15 M NaCl, 0.05 mM CaCl₂, 20 mM Tris, pH 7.2. Immunoprecipitates were then washed 3 times with lysis buffer (20 mM Na₂HPO₄ pH 7.4, 150 mM NaCl, 1% Triton X-100, 5 mM EDTA, proteases and phosphatases inhibitors) and finally resuspended in SDS-PAGE sample buffer for protein analysis.

2.5 siRNAs transfection

2.5.1 Principle of siRNA mediated knockdown

RNA interference (RNAi) is a method for silencing gene expression. Two types of RNA molecules, microRNAs (miRNA) and small interfering RNAs (siRNA) are central to RNA interference. Briefly, dsRNAs resulting from transcription are degraded to \approx 20nt short fragments by an endonuclease called Dicer, and then incorporated into a large multiprotein complex, called RISC (RNA induced silencing complex). Cellular RNA complementary to the incorporated RNA fragment is recognised by RISC and then degraded. SiRNAs (and other nucleic acids) are negatively charged and therefore, are not readily taken up by most cells. SiRNAs can be introduced into cells by binding with a cationic liposome-

like reagents such as oligofectamine, which can then cross the cell membrane so carrying the siRNA into the cell. However, some cells such as primary vascular smooth muscle cell are known to be difficult to transfect, in these cases, siRNA can be introduced *via* electroporation. This technique involves applying an electric field to induce the formation of microscopic pores within the cell membrane through which nucleic acids can pass.

2.5.2 Experimental details

Pre-designed siRNAs (small interfering RNA) to NRP1, NRP2, GIPC1 (synectin) and p130Cas were obtained from Ambion (Applied Biosystems); details for the siRNA sequence are in the table below (Table 2.3). Negative control siRNAs with sequence that do not target any gene product were used for determining transfection efficiency and to control the effects of siRNA delivery. These non targeting siRNAs have no sequence similarity to known genes and are validated for use in human, mouse, and rat cells. We used the Silencer® Negative Control siRNA 1 (catalogue number: AM4635).

Table 2.3: Nucleotide sequence of siRNAs used in this study (Ambion)

Target Gene	Catalogue #	Sequence (5'→3')	Locus ID
NRP1	AM16704	GGAUUUUCCAUACGUUAUGtt	8829
NRP2	16704	GGUCGUUUGAAUCCAAAGtt	8828
GIPC1	4392420	AGGACAAAAGGAACCCGGAAtt	10755
p130Cas	s18373	GGUCGACAGUGGUGUGUAU	9564

2.5.2.1 HUVECs and cancer cell lines.

Transfection of HUVECs and cancer cells were performed in 6-well plates and the volumes of reagent used in the following protocol are adapted to this cultureware format. siRNAs and transfection reagents (oligofectamine or lipofectamine 2000, Invitrogen) were diluted in warm optimem as follow:

- Tube A: 176 μ l Optimem medium (GIBCO) + 4 μ l siRNA (final siRNA concentration 200 nM) or 1 μ g plasmid DNA
- Tube B: 10 μ l Optimem medium (GIBCO) + 10 μ l oligofectamine or lipofectamine 2000

Both tubes were incubated for 10 mins at room temperature. Then tube A (siRNA or plasmid DNA) was mixed with tube B (oligofectamine or lipofectamine 2000) to give a total volume of 200 μ l. This solution was incubated for 25 minutes at room temperature with occasional mixing to allow the transfection complexes to form. Meanwhile, the cells were washed with optimem and 800 μ l of warm optimem was added in each well. The 200 μ l of complexed siRNA/DNA was added to the cells (final volume, 1ml) and incubated for 4h at 37°C. After this period, 500 μ l of 30% FBS in optimem without antibiotics was added to each well without removing the siRNA-containing media, to give a final concentration of 10% FBS. The medium was changed back to cell-type specific complete medium and incubated for a further 48-72h and the cells were used for further experiments and knockdown/DNA expression was assessed by Western blot.

2.5.2.2 HEK 293A Cells

The following technique and reagents were used to transfect HEK (Human embryonic kidney) 293A cells in order to produce the crude stock of adenovirus. The following description is for the treatment of a 100 mm dish containing cells at approximately 60% confluence.

8 μ l of Fugene transfection reagent were added to 350 μ l of serum free DMEM and incubated for 5 minutes at room temperature. Then, 4 μ g of DNA was added to this Fugene/DMEM solution and incubated for a further 15 minutes. In the meanwhile, the cell culture medium was replaced and 10 ml of new complete medium was added to the cells. Finally, the DMEM/DNA/Fugene mixture was added to the cells and incubated overnight. The cell medium was replaced the next day with some fresh complete medium, containing 10% FBS and antibiotics. The efficiency of the transfection was evaluated by performing a control transfection with pmaxGFP (Lonza, 0.5 μ g/ μ l) and observing the cells under a fluorescent microscope.

2.5.2.3 VSMC

Electroporation was used for VSMCs transfection with siRNAs (nucleofector kit, Amaxa), as these cells have proven particularly difficult to transfect using other approaches. Briefly, 10⁶ cells were resuspended in 100 μ l of transfecting reagent in the presence of 200 nM siRNA (*e.g.* NRP1, NRP2, synectin, p130Cas and negative control) and transferred to the nucleofector's carousel, in which an electrical field (program A-33) was applied, causing

a significant increase in the electrical conductivity and permeability of the cell plasma membrane, thus allowing entrance of siRNA in the cytoplasm. After treatment, 500 μ l of prewarmed complete medium was added to the cuvette and cells were then transferred to two 6-well plates (one plate was used for further experiments and the other was used to assess protein knock down by Western blotting). The medium was changed the day after nucleofection and cells were used 24 to 72 hours after transfection. The efficiency of the transfection was evaluated by performing a control transfection with pmaxGFP (Lonza, 0.5 μ g/ μ l) and observing the cells under a fluorescent microscope.

2.6 125 I-VEGF-A₁₆₅ and 125 I-PDGF-BB binding assays

2.6.1 Principle of radiolabeled ligand binding assay

A radioligand is a radioactively labeled molecule that can specifically associate with a receptor. The rate and extent of radioligand binding provides information on the number of binding sites on the surface of the cell, and their affinity. Competitive binding experiments measure the binding of a single concentration of this labeled ligand in the presence of unlabeled ligand which compete for the same binding sites. In this study, I aimed to quantify the amount of binding of 125 I-VEGF-A₁₆₅ and 125 I-PDGF-BB to cells in the presence of 100-fold excess concentration of the respective unlabeled growth factor.

2.6.2 Experimental details

HCASMC and EC were seeded in 24-well plates so they were confluent for the day of the binding assay. Cells were then washed twice with cold PBS. Binding medium (DMEM, 25 mM HEPES pH 7.3 containing 0.1% BSA and other agents as indicated) was added (with antagonists in some experiments), followed by addition of 0.1 nM of 125 I-VEGF-A₁₆₅ (1200-1800 Ci/mmol, GE Healthcare Plc) or 0.1 nM of 125 I-PDGF-BB (1825 Ci/mmol, Perkin-Elmer), in the absence or presence of a 100-fold excess concentration of the respective unlabelled growth factor. After 2 hours of incubation at 4°C, the medium was aspirated, and cells washed four times with cold PBS. The cells were then lysed with 0.25 M NaOH, 0.5% sodium dodecyl sulphate (SDS) solution, and the bound radioactivity of the lysates was measured. Non specific binding was determined as the total binding minus binding (non-specific) in the presence of 100-fold excess unlabeled VEGF-A₁₆₅ (R & D Systems) or PDGF-BB (Peprotech).

2.7 Western blotting

2.7.1 Principle of Western blotting

Heating of cell lysates with sample buffer containing SDS, an anionic surfactant, and dithiothreitol (DTT) (or Tris(2-carboxyethyl)phosphine, TCEP) as reducing agent, reduces protein disulphide bonds to thiol groups and disrupt non-covalent bonds in the proteins, denaturing them, and causing the molecules to lose their native conformation. The proteins migrate through a gel which pore size is determined by the concentration of acrylamide in the gel, under the influence of an electric field. Larger proteins migrate slower than smaller proteins, resulting in a separation of proteins largely based on molecular size. The proteins are then transferred onto a protein-binding membrane (typically nitrocellulose or Polyvinylidene fluoride, PVDF) under the influence of an electric field. For antibody detection, protein binding sites on the membrane are blocked by incubating with a protein solution such as milk or BSA, and a protein of interest is then specifically bound with an antibody. This primary antibody is then detected with a secondary antibody conjugated to an enzyme, often a peroxidase. A liquid substrate is then added, which in the presence of the peroxidase enzyme emits light in a chemiluminescent reaction, and this light is detected using photographic film. Western blotting is often described as a semi-quantitative technique, with the amount of protein originally present in the lysate proportional to the amount of peroxidase enzyme bound to protein on the membrane, and so the amount of light generated and area and intensity of the band present on the film. The amount of protein in each sample was assessed before loading, to quantify the amount of protein present in the lysates and thus allowing equal loading. Blotting with an antibody to glyceraldehyde 3-phosphate dehydrogenase (GAPDH), the enzyme that catalyzes the sixth step of glycolysis, was used to visually confirm comparable protein loading.

2.7.2 Protein assay

The Bio-Rad DC protein assay was used to determine the protein concentration of lysates following detergent solubilisation. Similarly to the Lowry assay, two steps lead to color development: the reaction between protein and copper in an alkaline medium, and the subsequent reduction of Folin reagent by the copper-treated protein. The characteristic blue color with a maximum absorbance at 750 nm and minimum absorbance at 405 nm is due to the amino acids tyrosine and tryptophan, and to a lesser extent, cystine, cysteine,

and histidine (Bio-Rad DC Instruction manual). Briefly, 5 μ l of lysate sample were added to 20 μ l of the alkaline copper tartrate solution, and mixed in a 96-well plate with 200 μ l of a dilute Folin reagent. The absorbance of each well at 690 nm was determined. Standards containing 0 to 2 mg/ml BSA were used to construct a (linear) standard curve of protein concentration. The protein concentration of unknown samples was determined by extrapolation from the standard curve.

2.7.3 Experimental details

Cell lysates were separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) on 10% or 4-12% Bis-Tris polyacrylamide pre-cast gels (Invitrogen), and electrotransferred to PVDF membrane. Membrane were blocked with 5% milk in PBST (Phosphate buffered saline with 0.1% Tween-20) or TBST (tris(hydroxymethyl)aminomethane buffered saline with 0.1% Tween-20) then incubated with primary antibodies overnight (dilutions of antibodies as provided by the manufacturer: see table 2.1). Membranes were then washed 5 times in PBST or TBST, incubated for 1 hour with horseradish peroxidase (HRP)-labeled secondary antibody (anti-goat, anti-mouse, anti-sheep and anti-rabbit IgG, all from Santa Cruz), and proteins were detected using the ECL or ECL-plus (depending on signal strength) western blotting detection system and hyperfilm ECL (both Amersham biosciences).

2.8 Enzyme-linked immunosorbent assay: ELISA

2.8.1 Principle

ELISAs were performed to quantitatively evaluate the presence of both total and phospho PDGFR α and β . The DuoSet IC ELISA contains the basic components required for the development of sandwich ELISAs to measure human PDGFR α and β in cell lysates, an immobilised capture antibody specific for human PDGFR α and β binds both tyrosine-phosphorylated and unphosphorylated PDGFR α and β . After washing away unbound material, a biotinylated detection antibody specific for total PDGFR α and β is used to detect both tyrosine-phosphorylated and unphosphorylated receptor, utilising a standard Streptavidin-HRP format, while an HRP-conjugated detection antibody specific for phosphorylated tyrosine is used to detect only tyrosine-phosphorylated receptor, utilising a standard HRP format.

2.8.2 Experimental details

Briefly, the capture antibody was diluted to a working concentration of 4 $\mu\text{g}/\text{ml}$ in PBS, without carrier protein and 100 μl were immediately added to a 96 well microplate for overnight incubation at room temperature. The day after, each well was washed 4 times with washing buffer (0.05% Tween 20 in PBS, pH 7.2-7.4) and blocked for 2 hours with 300 μl of PBS containing 1% BSA. Wells were washed again 3 times before adding 100 μl of lysate prepared in the following diluent: 1% NP-40, 20 mM Tris (pH 8.0), 137 mM NaCl, 10% glycerol, 2 mM EDTA, and 1 mM activated sodium orthovanadate. The same diluent without protein was used as the blank. The plate was left to incubate for 2 hours at room temperature and washed again. 100 μl of detection antibody diluted according to the manufacturer's recommendations in 20 mM Tris, 137 mM NaCl, 0.05% Tween 20, 0.1% BSA, pH 7.2-7.0 was added directly to the well, and left to incubate for a further 2 hours. When measuring total PDGFR α and PDGFR β levels, the following extra step was required: after another 3 washes, Streptavidin-HRP was diluted according to the manufacturer's recommendations in the same buffer as the detection antibody (see above) and 100 μl added to each well for 20 minutes at room temperature. Finally, 100 μl of substrate solution was added to each well for 20 minutes, complemented after this time by 50 μl of stop solution. The absorbance of each well was determined immediately, using a Tecan Genios plate reader at 450nm with a reference wavelength at 595nm.

2.9 Flow cytometry

2.9.1 Principle

Flow cytometry determines the properties of individual cells, including size and the absence or presence of particular fluorescently labeled antibodies associated with the cell membranes, and can be used to sort cells into populations defined by these parameters. A beam of light of a single wavelength is directed onto a hydrodynamically-focused stream of fluid containing cells conjugated with fluorophore probes such as antibodies, which allows the determination of the proportion of cells analysed that express the antigen.

2.9.2 Experimental details

HUVEC and HCASMC were harvested using a non-enzymatic dissociation buffer that chelates Ca^{2+} ions and therefore allows the cells to detach. Because antibodies can bind non-specifically to the FcR (crystallisable fragment receptors) of cells, we blocked these receptors by pre-incubating the cells with an FcR blocking reagent (Miltenyl Biotech). Cells were spun down and resuspended in blocking buffer (1% BSA/HEPES with FcR blocking reagent) to which antibodies to NRP1, VEGFR1 and VEGFR2 (KDR) were added for 20 minutes at 4°C . NRP1 and VEGFR1 antibodies were purchased from Miltenyl Biotech, VEGFR2 was obtained from R&D Systems, all antibodies were conjugated to phycoerythrin (PE), used at a 1:10 dilution and the isotype-matched (mouse IgG_1) antibodies were used as a control. Cells were then washed with PBS containing 0.2% BSA, spun down and resuspended in PBS containing 1% BSA, 20mM HEPES and 7-aminoactinomycin D (7AAD, 1:100 dilution, Sigma Aldrich). Because 7-AAD is a fluorescent chemical compound with a strong affinity for DNA and does not readily pass through intact cell membranes, cells with compromised membranes (dead cells) will stain with 7-AAD, while live cells with intact cell membranes will remain dark. Cells were gated according to forward and side scatter and 10^4 gated events (fluorescent signal emitted from a single live cell) were acquired using a Becton Dickinson FACScan flow cytometer and with CellQuestProTM software.

2.10 Transwell migration assay

2.10.1 Principle

The Boyden chamber assay, was originally developed by Boyden for the analysis of leukocyte chemotaxis (Boyden, 1962). The assay is based on a chamber of two medium-filled compartments separated by a microporous membrane. Cells are seeded in the upper compartment and are allowed to migrate through the pores of a membrane into the lower compartment comprising chemotactic molecules. After an appropriate incubation time depending on the cell type, the membrane between the two compartments is fixed and stained, and the cells that have migrated to the lower side of the membrane are counted. Therefore, the Boyden chamber-based cell migration assay has also been called a filter membrane migration assay, trans-well migration assay, or chemotaxis assay.

2.10.2 Experimental details

This type of transwell assay was used to assess cellular migration through a porous polyethylene terephthalate membrane (Figure 2.3). Transwell membranes were coated with collagen overnight at 4°C (0.01% collagen solution, Sigma), inserted into a 24-well plate, while serum free medium with or without growth factor (*e.g.* VEGF-A at 25 ng/ml, PDGF-AA or PDGF-BB at 30 ng/ml) was added at the bottom of the well, underneath the transwell. Cells were grown to 90% confluence, trypsinised and resuspended in serum free medium before seeding into the transwell at a density of 1×10^5 cells per insert for EC, or 5×10^4 cells per ml for HCASMC (8 μ m membrane pore size, 6.4 mm diameter, BD Falcon). Treatments with the NRP1 antagonist, EG3287, soluble NRP1 or b1 domain were performed by incubating the reagents for 30 minutes with the resuspended cells prior to seeding, and those treatments were also added to the bottom of the well with the growth factors. Cells were then allowed to migrate for 4 hours, after which, the upper surface of the transwell membrane was gently swiped with a cotton bud to remove non-migrated cells and stained with Reastain Quick-Diff Kit (Reagen). The cells that traversed the membrane were counted under bright field microscopy (x200 magnification) using an eyepiece indexed graticule. Eight fields were counted per insert, each treatment was performed in duplicate and each experiment was performed at least three times ($n = 3$). The mean count was used as the count for that insert.

2.11 Immunofluorescence

2.11.1 Principle

Staining of permeabilised cells with fluorophore-conjugated antibodies allows the detection a target protein within a cell. Using confocal microscopy, proteins can be localised to a single plane and co-localisation with other proteins stained with a different dye can be assessed given that the high Z-axis resolution is correctly set.

2.11.2 Experimental details

Cells were grown on gelatin-coated glass cover slips (15 mm diameter) in 24-well plates. After treatment, cells were fixed for 15 minutes in 4% paraformaldehyde and permeabilised to allow penetration of the antibodies for 10 minutes with 0.1% Triton-X. They were then

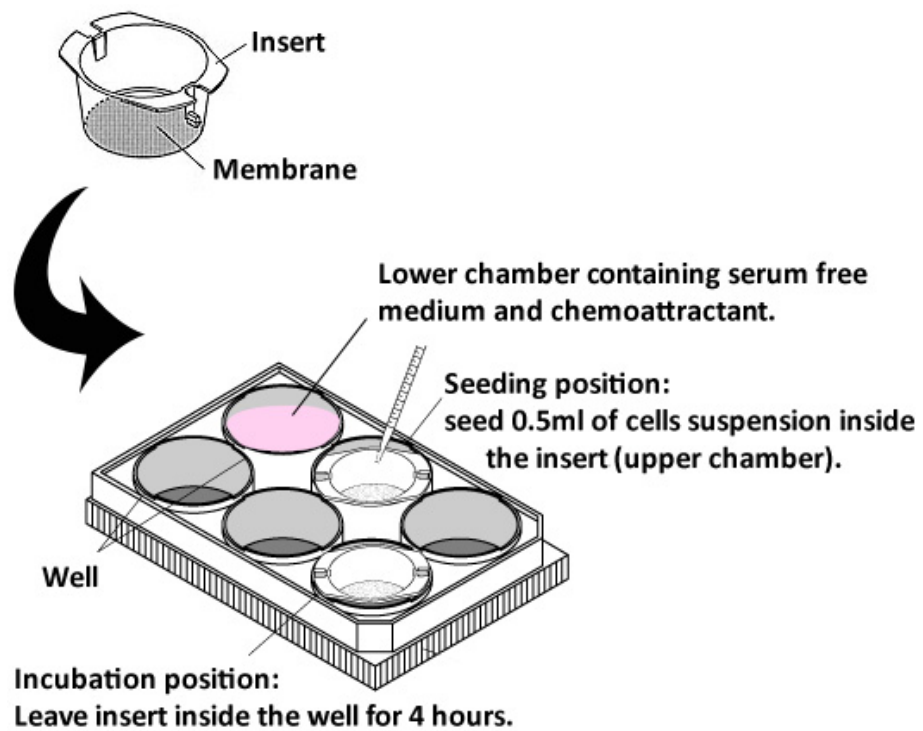


Figure 2.3: **Schematic representation of the modified Boyden chamber system for migration assay**

In the bottom of the well, serum free medium is supplemented with the chemoattractant (PDGF-BB or VEGF₁₆₅) while the resuspended cells are seeded in the upper chamber in serum free medium and left to migrate through the porous membrane for 4 hours. Figure modified from the BD falcon catalogue.

incubated overnight at 4°C with the primary antibody solutions (chondroitin sulphate, heparan sulphate, PDGFR α , PDGFR β and NRP1 antibodies diluted at 1:200 in 0.1% TWEEN 20, 1% BSA in PBS). The secondary antibodies, Alexa fluor 488-conjugated anti-goat and Alexa fluor 555-conjugated anti-mouse (Invitrogen Molecular Probes) were prepared in 0.1% TWEEN 20, 1% BSA in PBS (dilution 1:1000) and applied to the cells for 1 hour in the dark. Cells were then rinsed several times with PBS and finally, the coverslip was mounted on to a slide using Citifluor AF-1TM glycerol based medium (Citifluor Ltd.). Samples were kept in the dark at 4°C until they were analysed by confocal microscopy. Images were acquired using a Leica TCS SP2 confocal microscope (excitation at 488 nm and 543 nm).

2.12 Molecular biology

Adenoviruses were generated using the Gateway system from Invitrogen to study the effect of wild-type (WT) NRP1 and NRP1 mutant lacking the intracellular domain (Δ C) as well as a NRP1 mutant lacking the major glycosaminoglycan (GAG) modification (S612A). The amino acid and mRNA nucleotide sequences for NRP1 are shown in the Appendix, Figures A.1 and A.2.

2.12.1 Adenoviral vectors

Structure and infection

Adenoviruses are medium-sized (\approx 100 nm), non-enveloped, icosahedral viruses composed of a nucleocapsid and a double-stranded linear DNA genome. There are over 51 different serotypes in humans, which are differentiated on the basis of variable regions of their capsid proteins (Russell, 2000). They are able to infect various species of organisms, including humans, and are responsible for several diseases, such as, infections of the upper respiratory tract, infantile gastroenteritis, tonsillitis and conjunctivitis.

Replication

The adenovirus genome is composed of linear, non-segmented double stranded (ds) DNA and around 3038 kilo base pairs (kbp) long, which allows the virus to theoretically carry 30 to 40 genes. Adenoviruses are able to replicate in the nucleus of mammalian cells using the host replication machinery. Entry of adenoviruses into the host cell requires two sets of interactions between the virus and the host. Entry into the host cell is initiated

by the binding of the fibre protein of the viral capsid to the cell receptor. There are two well-described receptors: CD46 for the group B human adenovirus serotypes and the coxsackievirus/adenovirus receptor (CAR) for all other serotypes. CAR is expressed by ECs and proliferating SMCs *in vitro* and its expression can be induced in SMCs *in vivo* after balloon angioplasty in the rat carotid (Nasuno et al., 2004). This is followed by a second interaction between a specialised motif in the penton base protein interacting with an integrin molecule. This interaction stimulates cell signaling and thus induces actin polymerisation resulting in entry of the adenovirus into the host cell within an endosome (Wu and Nemerow, 2004).

Once inside the cell, the virus exits the endosome and with the help of cellular microtubules is transported to the nuclear pore complex whereby the adenovirus particle disassembles. Viral DNA is subsequently released and enters the nucleus via the nuclear pore (Meier and Greber, 2004). After this, the DNA associates with histone molecules and viral gene expression starts. As early as 1-2 hours after infection, viral DNA can be detected in the nucleus. The adenovirus genome has two phases of gene expression, an early and a late phase, separated by viral DNA replication. The early genes are responsible for expressing mainly non-structural, regulatory proteins that alter the expression of host proteins necessary for DNA synthesis and for the inhibition of premature death of the infected cell by the host-immune defenses. Once the early genes have generated the adequate virus proteins, the replication of the adenovirus genome starts. The late phase of the adenovirus life cycle consists of producing the structural proteins to assemble the genetic material produced by DNA replication. Finally, once all the viral components have been generated, the virus is packed into its protein coat. The host cell is then lysed, releasing the virus particle for the next round of infection.

Relevance of adenoviral vectors in this study

Because primary cells and especially vascular smooth muscle cells are particularly difficult to transfect, adenoviruses were chosen as a delivery vehicle for the NRP1 constructs. They have been shown to be a powerful tool to produce strong expression of constructs inserted in their genome in a wide variety of cells including HUVECs (Riccioni et al., 1998) and SMCs (Shintani et al., 2006). The adenoviral vector used in this study and provided in the Gateway System, pAd/CMV/V5-DEST, is unable to synthesise viral proteins and therefore cannot produce a viable virus capable of infecting other cells. This is due to the removal of the E1 and E3 regions responsible for activation of genes necessary for

expression of certain transcription factors that control the expression of viral proteins. The gene coding for a protein of choice, inserted in the adenoviral genome will be expressed under the control of the host cell transcription factors. The expression level of the construct decreases with time and this decline is further accentuated by cell division because adenoviruses do not integrate into the host genome, but are expressed episomally. Accordingly, a multiple of infection (MOI) of 20 was used in this study and cell functions were investigated at 3 to 4 days after infection.

2.12.2 Adenovirus production using the Gateway®cloning system

The Gateway®Technology is a universal cloning method based on the site-specific recombination properties of bacteriophage lambda (Landy, 1989). The Gateway®Technology enables movement of DNA sequences into multiple vector systems for functional analysis and protein expression (Hartley et al., 2000).

The first step of the cloning process requires the introduction of a construct sequence into a universal Entry vector (pENTRTM/D-TOPO®) (Figure 2.4). This reaction is catalysed by the enzyme topoisomerase which allows the ligation of the linearised entry vector comprising a four nucleotide (nt) overhang (attP-containing donor) with a blunt-ended fragment (attB-flanked PCR product) with a 5' CACC sequence (complementary to the overhang sequence). The entry vector comprises a kanamycin resistance gene to select for positive recombinant Entry clones. Once in the Entry vector, the sequence of interest can be transferred to several destination vectors. This is the second step of the cloning process, which is performed by recombination between the attL1/L2 sites in the entry vector and the attR1/R2 sites in the recombination vector.

pAd/CMV/V5-DEST Vector is a Gateway®adapted vector specifically designed to allow transient expression of recombinant fusion proteins in dividing and non-dividing mammalian cells. Key Features of the destination vector include a CMV (cytomegalovirus) promoter for high level of expression of the gene of interest and a C-terminal V5 epitope for detection of recombinant protein using the Anti-V5 antibodies (Figure 2.5). Also, positive recombinants possess an ampicillin resistance to allow for their selection. When transfected to 293A cells, the Pac I linearised pAd/CMV/V5-DEST vector produces E1, E3 lacking adenoviruses containing the sequence of interest (crude adenoviral stock). This cell line, a subclone of the 293 cell line, supplies the E1 (E1a and E1b) proteins required for production of replication-competent adenovirus and exhibits a flattened morphology

to enhance visualization of plaques. If the construct is engineered so it is lacking a stop codon at the end of its nt sequence, the destination vector adds a C-terminus tag. The V5 epitope tag is derived from a small epitope present on the P and V proteins of the paramyxovirus of simian virus 5 (SV5) and contains the 14 amino acids GKPIPPLLGLDST, which adds 4.3 kDa to the protein of interest. No V5 tag is added if the construct includes an in-frame stop codon.

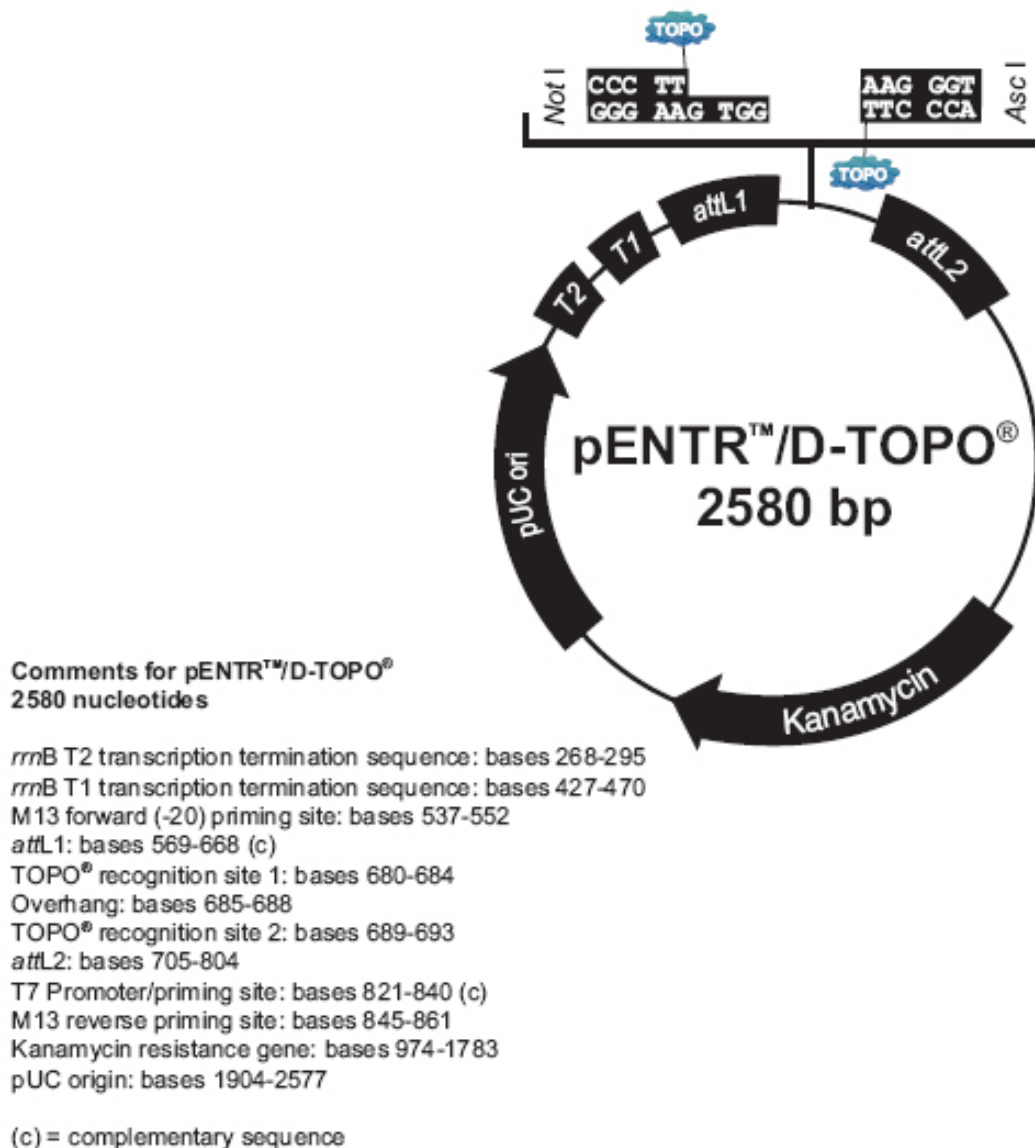
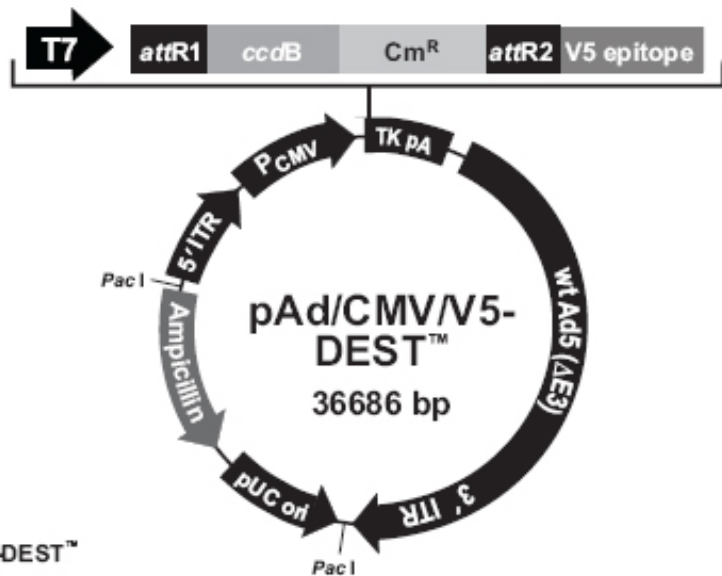


Figure 2.4: Map of pENTRTM/D-TOPO® from the Gateway®Technology manual.



Comments for pAd/CMV/V5-DEST™
36686 nucleotides

Human Ad5 sequences (wt 1-458; includes 5' L-ITR and packaging signal): 1-458
 pAd forward priming site: bases 361-384
 CMV promoter: bases 728-1315
 T7 promoter/priming site: bases 1359-1378
 attR1 site: bases 1407-1531
 ccdB gene: bases 1960-2265 (C)
 Chloramphenicol resistance gene (Cm^R): bases 2607-3266 (C)
 attR2 site: bases 3547-3671
 V5 epitope: bases 3697-3738
 TK polyadenylation signal: bases 3765-4036
 Human Ad5 sequences (wt 3513-35935; E3 region deleted, includes 3' R-ITR): bases 4056-34604
 pAd reverse priming site: bases 4059-4082
 pUC origin: bases 34781-35442 (C)
 Ampicillin (*bla*) resistance gene: bases 35568-36428 (C)
bla promoter: bases 36429-36527 (C)
 Pac I restriction sites: bases 34610 and 36684
 (C) = complementary strand

Figure 2.5: Map of pAd/CMV/V5-DESTTM from the pAd/CMV/V5-DESTTM and pAd/PL-DESTTM Gateway® user manual.

Features of pENTR™/D-TOPO® (2580 bp) contains the following elements. Features have been functionally tested.

Feature	Benefit
<i>rmb</i> T1 and T2 transcription termination sequences	Reduces potential toxicity in <i>E. coli</i> by preventing basal expression of the PCR product.
M13 forward (-20) priming site	Allows sequencing of the insert.
<i>att</i> L1 and <i>att</i> L2 sites	Bacteriophage λ -derived recombination sequences that allow recombinational cloning of a gene of interest in the entry construct with a Gateway® destination vector (Landy, 1989).
TOPO® Cloning site (directional)	Allows rapid, directional cloning of your PCR product.
T7 promoter/priming site	Allows <i>in vitro</i> transcription, and sequencing of the insert.
M13 reverse priming site	Allows sequencing of the insert.
Kanamycin resistance gene	Allows selection of the plasmid in <i>E. coli</i> .
pUC origin of replication (<i>ori</i>)	Allows high-copy replication and maintenance in <i>E. coli</i> .

Table 2.4: Features of pENTR™/D-TOPO® from the Gateway® Technology manual.

Features of the Vector

The pAd/CMV/V5-DESTTM vector (36686 bp) contains the following elements. All features have been functionally tested.

Feature	Benefit
Human adenovirus type 5 sequences (corresponds to wild-type 1–458 and 3513–35935 sequence) Note: The E1 and E3 regions are deleted.	Encodes all elements (except E1 and E3 proteins) required to produce replication-incompetent adenovirus including: <ul style="list-style-type: none"> • Left and right ITRs • Encapsidation signal for packaging • E2 and E4 regions • Late genes
pAd forward priming site	Allows sequencing of the insert.
CMV promoter	Allows high-level expression of the gene of interest
T7 promoter/priming site	Allows <i>in vitro</i> transcription in the sense orientation and sequencing through the insert.
<i>attR1</i> and <i>attR2</i> sites	Bacteriophage λ -derived DNA recombination sequences that permit recombinational cloning of the gene of interest from a Gateway [®] entry clone (Landy, 1989).
<i>ccdB</i> gene	Allows negative selection of the plasmid.
Chloramphenicol resistance gene (Cm ^R)	Allows counterselection of the plasmid.
V5 epitope	Allows detection of the recombinant fusion protein by the Anti-V5 Antibodies
Herpes Simplex Virus thymidine kinase (TK) polyadenylation signal	Allows efficient transcription termination and polyadenylation of mRNA
pAd reverse priming site	Allows sequencing of the insert in the anti-sense orientation.
pUC origin	Allows high-copy replication and maintenance in <i>E. coli</i> .
<i>bla</i> promoter	Allows expression of the ampicillin resistance gene.
Ampicillin resistance gene (β -lactamase)	Allows selection of the plasmid in <i>E. coli</i> .
<i>Pac</i> I restriction sites (positions 34610 and 36684)	Allows exposure of the left and right ITRs required for viral replication and packaging.

Table 2.5: Features of pAd/CMV/V5-DESTTM from the pAd/CMV/V5-DESTTM and pAd/PL-DESTTM Gateway[®] user manual.

2.12.3 Mutagenesis

2.12.3.1 Mutagenesis strategy

First of all, I had to identify which Serine-Glycine (SG) consensus site was the site of GAG attachment. There was a total of nine SG sites in the NRP1 amino acid sequence. Table 2.6 shows the amino acid sequence of human NRP1 and NRP2 aligned with one another and table 2.7 shows the SG sites that are relatively well conserved across vertebrate species and only present in the NRP1 sequence. A total of three SG consensus sites were found to be both exclusive to NRP1 and highly conserved between species. In order to determine the role of these sites in NRP1 O-linked glycosylation, the serine residues were mutated alone or in combination to alanine using the Quickchange II technology (Stratagene).

In order to generate the NRP1 construct lacking the C-terminus domain, I determined the position of the transmembrane domain and the amino acid sequence of the intracellular domain using the SMART database (Figure 2.6). I found that the transmembrane domain extended from aa 857 to aa 879 and the cytoplasmic domain from aa 880 to aa 923. The following 44 amino acid sequence corresponds to the intracellular domain:

YCACWHNGMSERNLSALENYNFELVDGVKLKKDKLNTQSTYSEA

To delete the intracellular domain, I inserted a stop codon after the amino acid in position 879. The amino acid in position 880 is a tyrosine coded by the three nucleotides TAC, we therefore mutated TAC into TAG (codon stop) using site-directed mutagenesis.

2.12.3.2 Mutagenesis principle

Site-directed mutagenesis is used to make point mutations and performed using PfuTurbo DNA polymerase and a temperature cycler. PfuTurbo®DNA polymerase replicates both plasmid strands with high fidelity and without displacing the mutant oligonucleotide primers. The basic procedure utilises a supercoiled double-stranded DNA vector with an insert of interest and two synthetic oligonucleotide primers containing the desired mutation. The oligonucleotide primers, each complementary to opposite strands of the vector, are extended during temperature cycling by PfuTurbo DNA polymerase. Incorporation of the oligonucleotide primers generates a mutated plasmid containing staggered nicks. Following temperature cycling, the product is treated with Dpn I. The Dpn I endonuclease (target sequence: 5' GATC 3') is specific for methylated and hemimethylated DNA and

is used to digest the parental DNA template and to select for mutation-containing synthesized DNA. DNA isolated from almost all *E. coli* strains is methylated and therefore susceptible to Dpn I digestion. The nicked DNA vector containing the desired mutation(s) is then transformed into TOP10 *E. coli*. The small amount of starting DNA template required to perform this method, the high fidelity of the PfuTurbo DNA polymerase, and the low number of thermal cycles all contribute to the high mutation efficiency and decreased potential for generating random mutations during the reaction.

2.12.4 Experimental details

2.12.4.1 Agarose gel electrophoresis and recovery of DNA

Amplified PCR product was mixed with 6X loading buffer (Fermentas) and run on a 1% agarose gel containing ethidium bromide (200 ng/ml) at 100 V, using TAE running buffer (40 mM tris-acetate, pH 8.3; 1 mM EDTA). After adequate separation, the gel was observed under UV illumination and the band of interest was excised and added to a preweighed tube. DNA was recovered from the gel piece using the Qiaquick Gel Extraction Kit (Qiagen) and finally eluted into 50 μ l of double-distilled water. DNA concentration was determined by absorbance at 260 nm on a Biophotometer (Eppendorf).

2.12.4.2 TOPO cloning into pENTR/D-TOPO entry vector

WT full-length Human NRP1 expressed in pcDNA 3.1(+) was available in the laboratory. NRP1 open reading frames (ORF) were sub-cloned into the pENTR/D-TOPO vector by PCR amplification with primers designed according to the manufacturer recommendations (5' CACCATGGAGAGGGGGCTGCC 3' and Rev: 5' TCATGCCTCCGAATAAGTACTCTGTG 3') using TOPO cloning (Invitrogen). Briefly, gel-purified PCR product was added to pENTR/D-TOPO vector with the provided salt solution. The reaction was mixed and incubated at room temperature for 5 mins, and then used to transform competent TOP10 *E. coli*.

2.12.4.3 Transformation and growth of competent E. coli

One-shot chemically competent TOP10 *Escherichia coli* bacteria (Invitrogen) were used for transformations. A vial of TOP10 bacteria was slowly thawed on ice. The plasmid was added and gently mixed with the bacteria for 30 min on ice, after which the bacteria were heat-shocked in a water bath at 42°C for 30s and then placed on ice again for a few minutes.

200 μl SOC medium (Invitrogen) was added to the cells which were incubated for 1 h at 37°C with shaking. The culture was plated on to prewarmed 10 cm dishes containing Lysogeny broth (LB) agar and either 100 $\mu\text{g}/\text{ml}$ kanamycin (for pENTR) or 100 $\mu\text{g}/\text{ml}$ ampicillin (for pAd) as appropriate for the plasmid used for transformation. Plates were incubated overnight at 37°C. The day after, individual colonies were picked and transferred into 14 ml round bottomed tubes containing 3 ml LB broth and the appropriate antibiotic. Cells were grown overnight at 37°C with shaking, and used immediately for minipreps or stored at 4°C until further use.

2.12.4.4 Plasmid DNA minipreps

The QIAprep miniprep procedure (QIAGEN) uses the modified alkaline lysis method of Birnboim and Doly (1979). Bacteria are lysed under alkaline conditions, and the lysate is subsequently neutralised and adjusted to high-salt binding conditions in one step. After lysate clearing, the sample is ready for purification on the QIAprep silica membrane. Briefly, a single colony was picked from a selective plate and used to inoculate 3 ml of LB medium containing the appropriate selective antibiotic which was incubated overnight at 37°C with vigorous shaking. The pelleted bacterial cells were resuspended in 250 μl buffer P1 and transferred to a centrifuge tube, followed by addition of 250 μl of buffer P2 and 350 μl of buffer N3. After centrifugation, the supernatant was applied to the miniprep column and the flow-through discarded. The column was then washed with the application of PB buffer followed by the addition of 0.75ml of PE buffer and finally elution of the DNA in 50 μl of water. DNA concentration was determined by absorbance at 260 nm on a biophotometer.

2.12.4.5 Site-directed mutagenesis

Site-directed mutagenesis was performed using the Quickchange II kit (Stratagene) according to the manufacturers instructions. PCR reactions, containing 30ng pENTR-WT NRP1 template plasmid and 10 pmol of each primer, were performed in a final volume of 50 μl . Cycling parameters (22 cycles) were 1.5 min initial melt at 95°C; melting for 1 min at 95°C; annealing for 1.5 min at 55°C; extension for 5 min at 68°C and final extension for 10 min at 68°C. Dpn I was added to the cooled sample and digestion was allowed to proceed at 37°C for 12 h. 2 μl of the cooled sample was used to transform supercompetent TOP10 *E. coli*, which were plated onto kanamycin-containing agar plates to select for the presence of the pENTR plasmid. The primers used for the nine Serine to Alanine substi-

tutions and the insertion of a stop codon after the transmembrane domain are shown in table 2.8.

2.12.4.6 DNA sequencing

Sequencing was performed by Cogenics (Takeley, Essex). The primers used for sequencing are detailed in table 2.9. The complete integrity of the NRP1 nucleotide sequence was assessed after cloning into the pENTR and pAd vectors, while only the mutated region of NRP1 was sequenced after the site directed mutagenesis. The NRP1 sequence was verified by manually checking the chromatogram supplied by the sequencing service using the Sequencher software package (Gene Codes).

2.12.4.7 Cloning into pAd/CMV/V5-DEST destination vector

NRP1 with the desired mutations were cloned from the pENTR/D-TOPO entry vector into the pAd/CMV/V5-DEST destination vector (Invitrogen) according to the manufacturers instructions. Briefly, the pENTR plasmid containing the NRP1 constructs were mixed with the pAd/CMV/V5-DEST vectors and clonase II enzyme mix (Invitrogen) was added to initiate recombination. Recombination was allowed to proceed at room temperature overnight and was stopped by addition of proteinase K and incubation at 37°C for 10 min. The mixture was then transformed into TOP10 *E. coli* and grown overnight on ampicillin-containing plates. The following day, colonies were picked and grown in ampicillin-containing broth and large quantities of DNA were produced by maxi-preps using the HiSpeed Plasmid Maxi kit (Qiagen). DNA was recovered and its integrity and correct orientation was assessed by sequencing.

2.12.4.8 Preparation of low-titre adenoviral stock

pAd maxipreps were digested by Pac I (New England Biolabs) to linearise the pAd plasmid and expose the viral inverted terminal repeat (ITR) sequences. At the end of the enzymatic reaction, phenol-chloroform extraction was performed to remove the enzyme and other possible contaminants. The pAd vector was transfected into HEK 293A cells using Fugene (see 2.5.2.2). Around 10 days later, the cells rounded up and started to detach from the dish. The cell suspension was transferred to a 50 ml Facon tube and the virus was released from the cells by using three repeated freeze-thaw cycles (30 min at -70°C and 10 min at 37°C). The supernatant containing the virus was obtained by centrifugation (3000g for 15

min), and then stored at -70°C or used immediately to produce a high-titre viral stock.

2.12.4.9 Preparation of high-titre adenoviral stock: Caesium chloride virus purification

100 μl low-titre virus stock were used to infect fifteen 15cm dishes of HEK 293A cells. After a few days, when cells rounded up and detached from the plate, cells were squirted off the dish and centrifuged for 5 minutes at 500g. The cell pellet was resuspended in 8 ml sterile PBS and the suspension was freeze-thawed 4 times before the supernatant was transferred to a fresh tube containing 4.4g caesium chloride. The CsCl/virus mixture was transferred to an Optiseal centrifuge tube (Beckman) and centrifuged in a Ti 70.1 rotor overnight at 60000 rpm at 10°C . The virus particles were concentrated in a discrete band, which was withdrawn using a needle. The virus was then mixed with an equal concentration of sterile 2X freezing buffer (5 mM Tris-HCl pH 8; 50 mM NaCl; 0.05% w/v BSA; 25% glycerol) and stored at -70°C .

2.12.4.10 Virus titration

Viral titre was determined using the QuickTiter Adenovirus Titer Immunoassay kit (Cell Biolabs). Briefly, 293 cells were plated in 24-well plates at $2.5 \cdot 10^5$ cells/ml. One hour after plating cells, virus at 10-fold serial dilutions (10^3 - 10^6) were added to each well. The cells were allowed to grow for 2 days for virus production to occur before being fixed in methanol, blocked with BSA, incubated in anti-hexon antibody and then alkaline phosphatase-conjugated secondary antibody. Virus-containing cells were visualised by incubation in diaminobenzidine (DAB) for 10 minutes, and stored in PBS at 4°C . For most microscopes, a standard 10X objective lens with 10X eyepiece lens has a field diameter (D) of 1.8 mm, then:

$$\text{Area per field} = 3.14 \times (\text{D}/2)^2 = 3.14 \times 0.9^2 = 2.54 \text{ mm}^2$$

For 24-well plate, area of a well is 2 cm^2 , therefore,

$$\text{Fields/well} = 2 \text{ cm}^2 / 2.54 \text{ cm}^2 = 3.8 \text{ cm}^2 / 2.54 \times 10^{-2} \text{ cm}^2 = 79$$

The average number of positive cells was counted in five different fields (100X magnification) and the viral titre determined from the following equation:

$$\text{Viral Titre (ifu/ml)} = \frac{(\text{positive cells/ field}) \times (79 \text{ fields/well}) \times (\text{dilution factor})}{(0.1 \text{ ml})}$$

2.13 Statistical analysis

Results were obtained from at least three separate experiments. Representative Western blots are shown to present protein expression. Other results are represented in bar graphs, where bars represent the average of three experiments and the brackets represent standard error mean. Statistical analysis was performed using Prism (GraphPad) on normalised data by either one- or two-way ANOVA as appropriate, with Bonferroni test for multiple comparisons used to locate differences. Statistical significance was accepted at $P < 0.05$.

Domains within *Homo sapiens* protein NRP1: (data from SMART database)

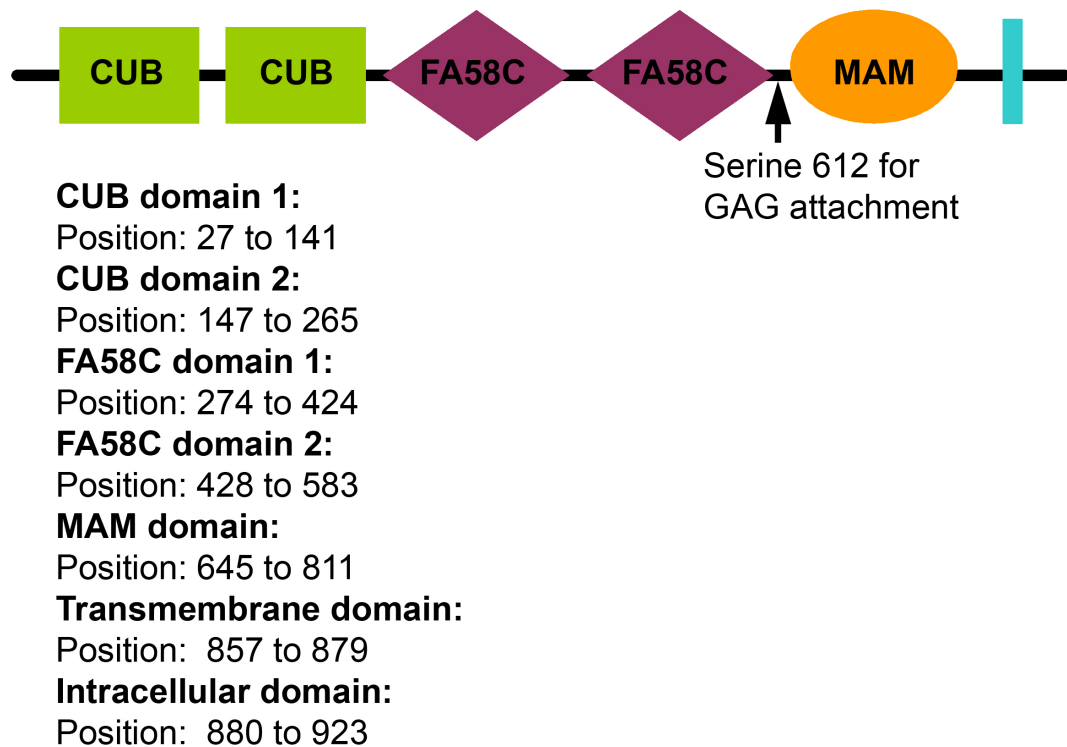


Figure 2.6: NRP1 domain homologies obtained from the SMART database and their corresponding amino acid position.

Table 2.8: Nucleotide sequence of primers used for mutagenesis

Mutation introduced	Codon changed	Primer	Sequence (5'→3')
S115A	from TCA	Forward	CCTCCTCCTGTTGTGTCTGCAGGGCCATTCTTTTATC
	to GCA	Reverse	GATAAAAGAAATGGCCCTGCAGACACAACAGGAGGAGG
S155A	from AGT	Forward	CCAGAACTACACAACACCTGCTGGAGTGATAAAGTCCCC
	to GCT	Reverse	GGGACTTTTATCACTCCAGCAGGTGTTGTGTFAGTCTGG
S240A	from TCG	Forward	GGTCGAATCCGATCCTCAGCGGGCATTCCTCCATG
	to GCG	Reverse	CATGGAGAGAATGCCCGCTGAGGATCGGATTCGACC
S283A	fromTCA	Forward	GAAAGCTTGGGCATGGAAGCAGGAGAAATTCATTTCTGAC
	to GCA	Reverse	GTCAGAATGAATTTCTCCTGCTTCCATGCCCAGAGCTTC
S432A	from TCT	Forward	GATAACAGATTATCCTCGCGCTGGAAATGTTGGGTATGGTG
	to GCT	Reverse	CACCATACCCAAACATTCACAGCGGAGGATAATCTGTTATC
S439A	from TCT	Forward	GGAATGTTGGGTATGGTGGCTGGACTTATTTCTGACTC
	to GCT	Reverse	GAGTCAGAAATAAGTCCAGCCACCATACCCAAACATTC
S469A	from TCT	Forward	GCCTTGGTAACCAGTCCGCTGGCTGGGCACTTCCACC
	to GCT	Reverse	GGTGGAAAGTGCCACGCCAGCGGACTGGTTACCAGGC
S612A	from AGT	Forward	GACCAGGCCAACTGCCACGCTGGAAACAGGTGATGACTTC
	to GCT	Reverse	GAAGTCAATCACCTGTTCCAGCGTGGCAGTTGGCCTGGTC
S729A	from TCT	Forward	CCTTCTGGTATCACATGGCTGGTCCCACGTCGGCAC
	to GCT	Reverse	GTGCCGACGTGGGACCCAGCCATGTGATACCAGAAGG
ΔC	from TAC	Forward	GCTGTCTGTGGGTCGTGCTGTAGTGTGCCCTGTTGGCATAATGG
	to TAG	Reverse	CCATTATGCCAACAGGCCACACTACAGCACGACCCACAGACAGC

Table 2.9: Nucleotide sequence of primers used for sequencing

Number	Sequence (5'→3')
1	ACCTTACATCTCCTGGTTATCCT
2	TCCTGTTGTGTCTTCAGG
3	GGATGTTCTGTCGCTACG
4	TCCTACCGAGAGTGGATACAGGTA
5	CCACTGATAACTCGATTTGTC
6	GAAGCACCGAGAGAACAAGG
7	AACTATGATACACCTGAGC
8	ACGGTCATAGACAGCACC
9	AAGGTGACCACTGGAAGG
10	AGGTGACAAGAACATCTCCAGG

Chapter 3

Characterisation of Neuropilins in vascular cells

When I first started this project, a role for NRPs in endothelial and cancer cell biology was already established and commonly accepted. During the characterisation of NRP expression in these cells, I noticed that NRP1 was expressed as a single band in EC while it was expressed as two separate bands in cancer cells. I also decided to investigate NRP expression in VSMC as these cells interact with EC. NRP1 was found to be highly expressed in SMC, something unknown at that time. As with cancer cells, NRP1 is expressed as two separate species in SMC. I then went on to investigate the nature of these novel NRP1 species and characterise NRP expression in VSMC in more detail.

3.1 Identification of Neuropilins in vascular and cancer cells

Immunoblotting of lysates prepared from Human coronary artery smooth muscle cells (HCASMC), Human aortic smooth muscle cells (HAoSMC), Human umbilical vein endothelial cells (HUVEC) and Human coronary artery endothelial cells (HCAEC) showed that NRP1, NRP2 and synectin, the only NRP1 interacting protein (NIP) known to date, were all expressed in these cells (Figure 3.1). In contrast, the major signalling VEGF receptor (VEGFR2/KDR), could not be readily detected in smooth muscle cells by Western blot. Although a faint VEGFR2 signal was seen in HCASMC, no signal at all was observed in HAoSMC, whereas VEGFR2 was strongly expressed in both endothelial cell types. PDGF receptor expression was not observed in endothelial cells, but was readily detected in both smooth muscle cell types. PDGFR β and PDGFR α were expressed in both cell types, though more PDGFR α was expressed in HAoSMC. Interestingly, in addition to the normal full length NRP1 band of 130 kDa, a high molecular weight NRP1-immunoreactive band was prominently expressed in HCASMC and HAoSMC but was not

seen in endothelial cells.

A similar high molecular weight NRP1 species is also expressed in several cancer cell lines including A549 (pulmonary carcinoma cell line), ACHN (renal carcinoma cell line), Skov-3 (ovarian carcinoma cell line), Skbr3 (mammary carcinoma cell line) and MB-231 (mammary carcinoma cell line), while in comparison, this high molecular weight signal was not detected in HUVEC or in MCF-7 (mammary carcinoma cell line) (Figure 3.2). Moreover, U87 a glioblastoma cell line and DU145, a prostate carcinoma cell line both express both NRP1 species (data observed by colleagues, not shown).

I used flow cytometry to further assess the expression of NRP1, VEGFR1 and VEGFR2 in vascular cells. VEGFR1, VEGFR2 and NRP1 were all expressed significantly in HUVEC while only NRP1 was expressed at significant levels by HCASMC (Figure 3.3). The proportion of cells expressing these receptors are given in table 3.1.

Table 3.1: Percentage of HUVEC and HCASMC expressing VEGFR1 and 2 and NRP1

	VEGFR1	VEGFR2	NRP1
HUVEC	25%	79%	99%
HCASMC	7%	9%	88%

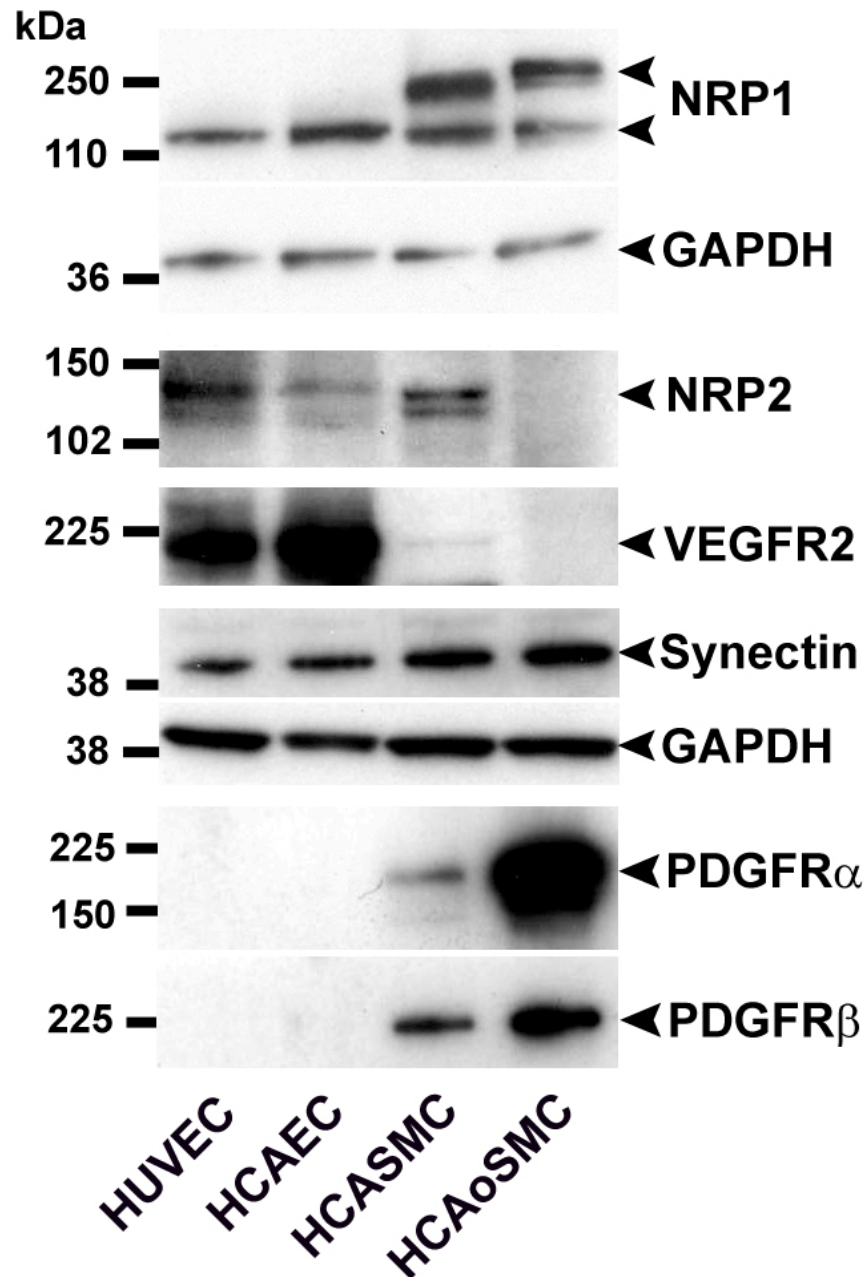


Figure 3.1: NRPs, VEGFR2, synectin and PDGFRs expression in vascular cells
 This Western blot shows NRP1, NRP2, VEGFR2, PDGFR α and PDGFR β expression in vascular cells. Cell lysates were prepared from HUVEC, HCAEC, HCASMC and HCAoSMC run on SDS-PAGE and blotted for NRP1, NRP2, VEGFR2, synectin, PDGFR α , PDGFR β and GAPDH as a loading control. Note the presence of a high molecular species in SMC.

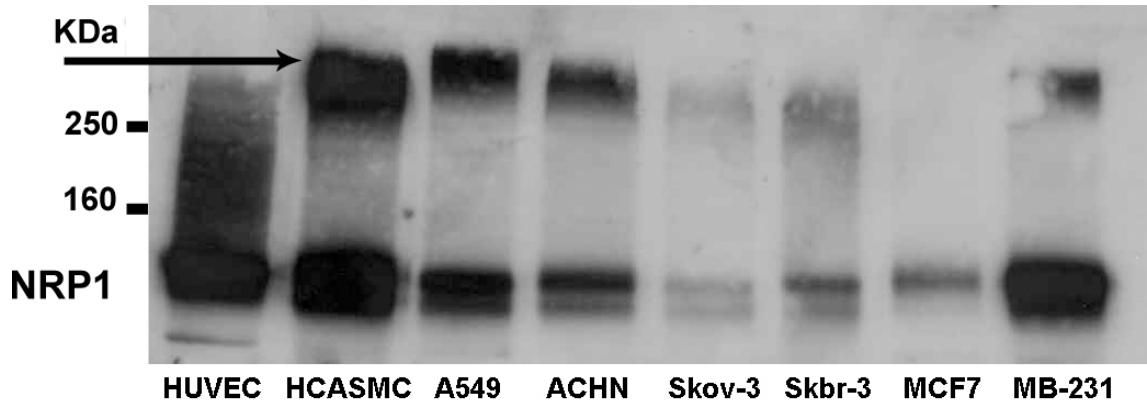


Figure 3.2: NRP1 expression in different cell lines

This Western blot shows NRP1 protein expression in the vascular cells HUVEC and HCASMC, as well as in several cancerous cell lines. Lysates were prepared from HUVEC, HCASMC, and the following cancerous cells: A549: lung cancer, ACHN: kidney cancer, Skov-3: ovarian cancer, Skbr3: breast cancer, MCF-7: breast cancer and MB-231: breast cancer. Note the presence of a high molecular species in some cell lines.

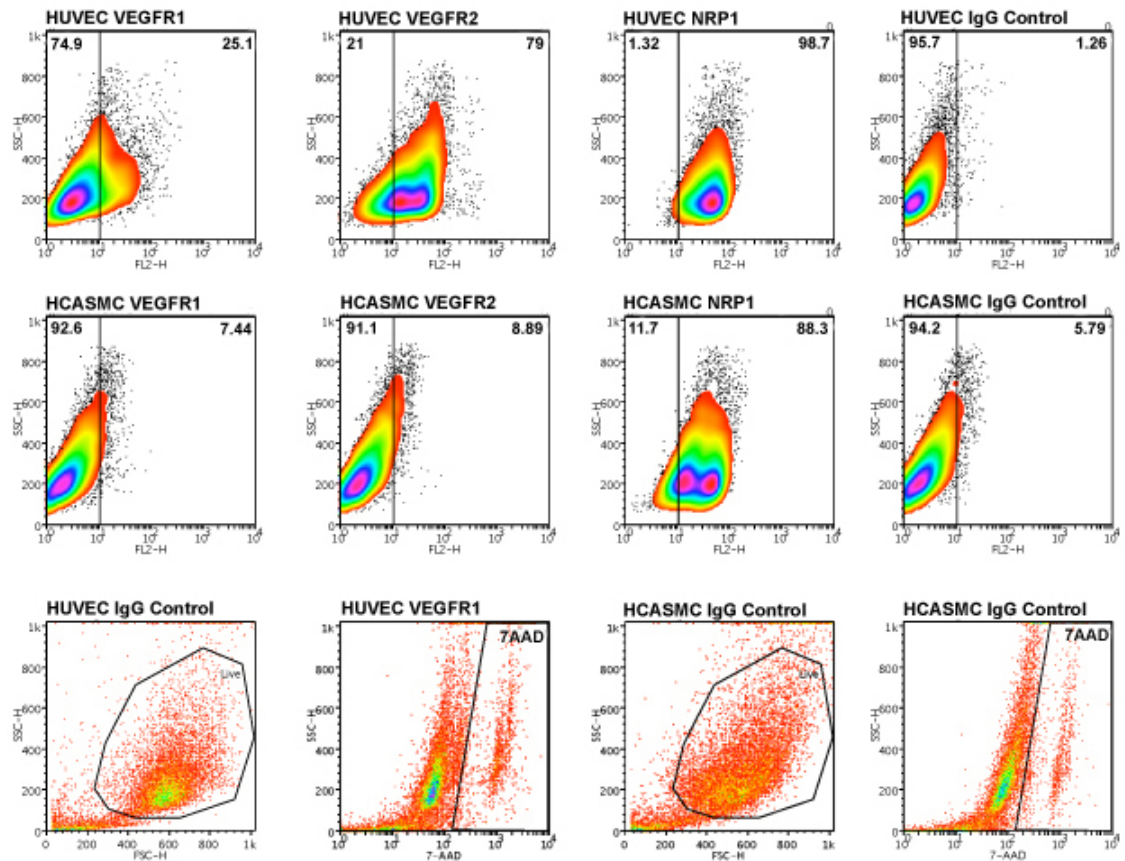


Figure 3.3: Flow cytometry

Flow cytometry data showing the expression of NRP1, VEGFR2 and VEGFR1 at the surface of HCASMC and HUVEC.

3.2 The high molecular weight NRP1-immunoreactive band corresponds to a specific NRP1 species

To assess the specificity of the higher molecular weight band observed in Figure 3.2, siRNA knockdown of NRP1 and NRP2 in A549 cells and VSMC, which strongly expressed both forms of NRP1 was performed.

NRP1 siRNA-mediated knockdown abolished expression of the high molecular weight band in A549 and VSMC, while control and NRP2-specific siRNAs had no effect (Figure 3.4 and 3.5). An antibody against NRP2 did not recognise a similar high molecular weight band in HCASMC (Figure 3.1) and in A549 (Figure 3.4). These results suggested the existence of two NRP1 species: one at the known and predicted molecular weight of 130 kDa, and another species greater than 250 kDa in several cancer cell lines and in VSMC. This >250 kDa form is not expressed in endothelial cells, and does not exist in NRP2.

3.3 Neuropilin 1 and 2 are and N-glycosylated

I addressed the possibility that the high molecular weight NRP1 band in HCASMC could be due to N-linked glycosylation by using the antibiotic tunicamycin, which prevents the linkage of carbohydrates to the amide group of asparagine residues in the protein core of N-glycosylated proteins. SMC and EC were treated with tunicamycin, and lysates were then immunoblotted with NRP1 antibody. After 16 hours of treatment, a clear shift was observed in the apparent 130 kDa molecular weight NRP1 band in EC and of both the high molecular weight and typical full length 130 kDa NRP1 bands in SMC, but tunicamycin did not abolish expression of the high molecular weight species (Figure 3.6). The same lysates were run and blotted for NRP2 protein expression (Figure 3.6). Similar to NRP1, a shift was observed in the NRP2 band, showing that it is also post-translationally modified by N-glycosylation. It was also noted that tunicamycin reduced the level of expression NRP1 and NRP2, an effect which is most likely due to the known inhibitory effect of tunicamycin on protein synthesis.

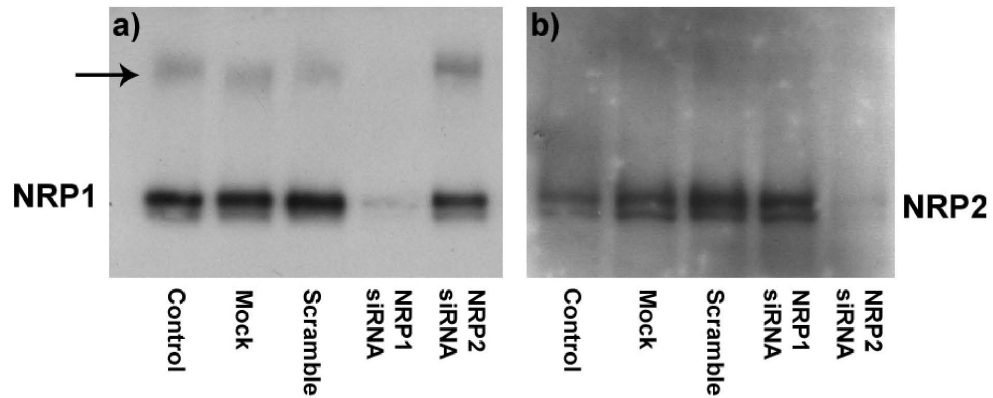


Figure 3.4: **Effects of NRP1 and NRP2 knockdown in A549 cells**

A549 cells were transfected with NRP1 and NRP2 siRNA using oligofectamine 2000. 72 hours after transfection, cells were harvested with RIPA buffer and protein lysates were prepared and 5 μ g of total protein were run on SDS-PAGE, blotted, and probed with the indicated antibodies.

a) Western blot for NRP1 shows the presence of two populations of NRP1. The arrow points out the NRP1 population at 250 kDa that disappears when cells were treated with siRNA. b) Western blot for NRP2, shows no evidence of a high molecular weight form.

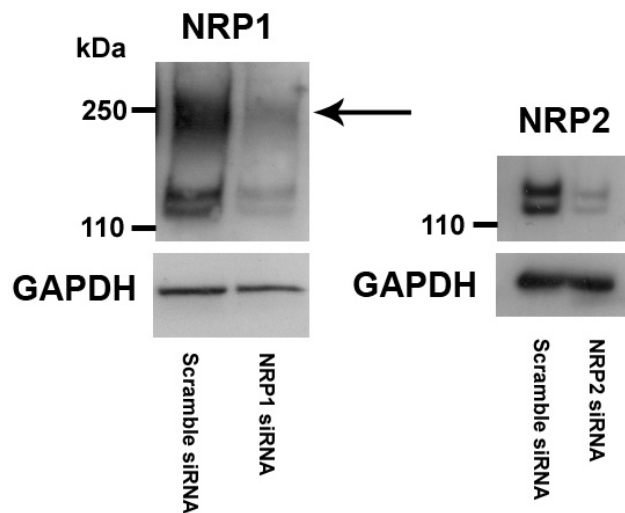


Figure 3.5: **Effects of NRP1 and NRP2 knockdown in HCASMC**

HCASMC cells were transfected with NRP1 and NRP2 siRNA using the nucleofactor kit (AMAXA). 72 hours after transfection, cells were harvested with RIPA buffer and protein lysates were prepared and 5 μ g of total protein were run on SDS-PAGE and probed with the indicated antibodies. On the left panel, the Western blot for NRP1 shows the presence of two populations of NRP1. The arrow points out the NRP1 population at 250 kDa that disappears when cells were treated with siRNA. On the right, the Western blot for NRP2, shows no evidence of a high molecular weight form.

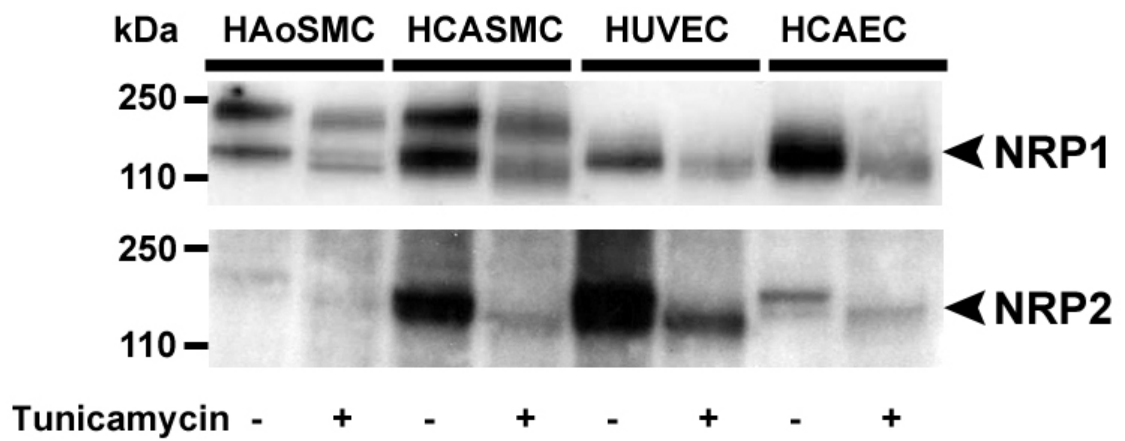


Figure 3.6: NRP1 and NRP2 is modified by N-linked glycosylations in vascular cells

Human aortic and coronary smooth muscle cells and Human umbilical vein and coronary artery endothelial cells were pretreated in the presence or absence of 5 $\mu\text{g}/\text{ml}$ tunicamycin for 16 hours, lysates were then prepared, ran on SDS-PAGE and immunoblotted with antibody to NRP1 and NRP2.

3.4 Neuropilin 1 is O-glycosylated

3.4.1 Enzyme treatment on intact cells

I next investigated whether appearance of the high molecular weight NRP1 band was due to the addition of O-linked glycosaminoglycans (GAG) containing either chondroitin sulphate (CS) or heparan sulphate (HS). Addition of GAG is known to produce very high molecular weight proteoglycan (PG) species and is an important post-translational mechanism in VSMC (see Chapter 1.6). I used chondroitinase and heparitinase (also known as heparinase III), two lyases that catalyse the cleavage of carbon-oxygen bonds of CS- and HS-GAGs respectively, thereby degrading the long polysaccharide chains into short oligosaccharides. Treatment of SMC cultures with chondroitinase caused a marked reduction in the level of the high molecular weight NRP1 band, with a concomitant increase in expression of the 130 kDa band, while heparitinase had a smaller but also significant effect, and a combination of chondroitinase and heparitinase completely abolished the presence of the high molecular weight band (Figure 3.7). It was noted that chondroitinase did not completely remove the >250 kDa species in VSMC, whereas the high molecular weight band was entirely abolished in A549 cells using chondroitinase alone and heparitinase had no effect at all (Figure 3.9). Moreover, treatment of A549 cells and VSMC with chondroitinase and heparitinase did not result in an increase in the 130 kDa population of NRP2, suggesting that NRP2, unlike NRP1, is not O-glycosylated, consistent with Figures 3.1 and 3.4 (Figure 3.10).

3.4.2 Enzyme treatment of NRP1 immunoprecipitates

To further verify the nature of NRP1 post-translational modification, NRP1 immunoprecipitates were also treated with lyases. Treatment of NRP1 immunoprecipitates from HCASMC with chondroitinase resulted in the disappearance of the high molecular weight NRP1, while heparitinase had a smaller effect (Figure 3.8). These results indicate that the high molecular weight NRP1 band in HCASMC results primarily from post-translational addition of CS-containing GAG, while only a small fraction is modified by the addition of HS-containing GAG. Both high molecular weight and 130 kDa NRP1 are additionally modified by some N-linked glycosylations. In contrast to NRP1, NRP2 does not appear to undergo GAG modification, but is also modified by N-glycosylations (Figure 3.6). The efficacy of the enzymes in removing CS-GAG and HS-GAG from VSMC surface was con-

firmed by immunofluorescent staining with specific CS and HS antibodies (Figures 3.13 and 3.14).

I quantified the proportion of glycanated and non-glycanated NRP1 by densitometry using Image J. I also analysed the proportion of each glycanated population (chondroitin sulphate *versus* heparan sulfate). A possible limitation of this method is that it is assumed that the antibody recognises all NRP1 forms with similar affinity.

The non-modified population NRP1 accounted for approximately 54% of the total population, hence the glycanated population (CS- and HS- modified) for 46%. Then I examined the proportion of chondroitin sulphate modified population and heparan sulphate population in function of the appearance of the high molecular weight band intensity and also in function of the appearance of the 130 kDa NRP1 band in response of chondroitinase and heparitinase treatment. I first analysed the disappearance of the high molecular weight band with the treatment of chondroitinase decreasing the signal more significantly than with heparitinase (Figure 3.11 a). Moreover, chondroitinase treatment of HCASMC increased the 130 kDa band more significantly than the heparitinase treatment (Figure 3.11 b). Combining these data, I concluded that the chondroitin sulphate modified NRP1 population accounted for approximately 70% of the glycanated population and therefore the heparan sulphate population, for $\approx 30\%$ (Figure 3.12).

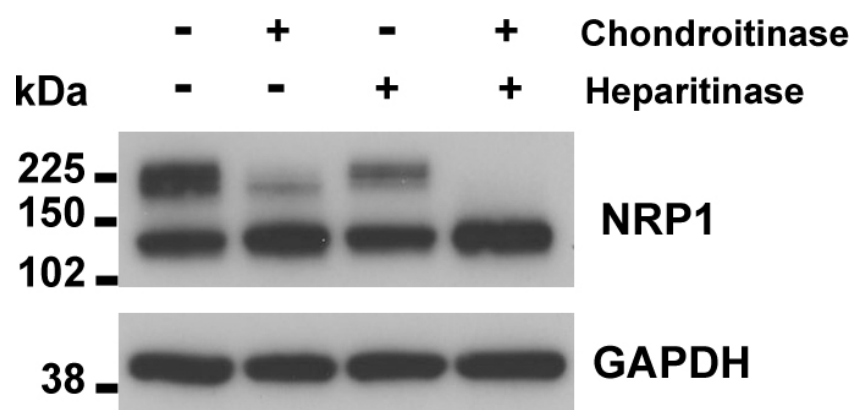


Figure 3.7: **Chondroitinase and heparitinase treatment of HCASMC**

The cells were incubated for 2 hours either with or without chondroitinase and heparitinase III (heparitinase) or with a combination of both (each at 1 Unit/ml), lysates were then prepared and 5 μ g of total protein were run on SDS-PAGE and blotted with NRP1 antibody. Results are representative of three independent experiments.

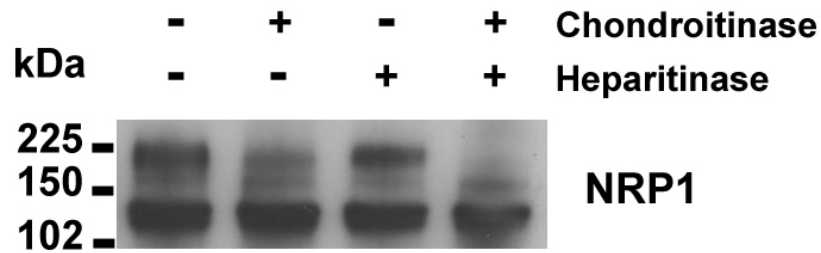


Figure 3.8: **Chondroitinase and heparitinase treatments of NRP1 immunoprecipitates obtained from HCASMC lysates**

NRP1 was immunoprecipitated from HCASMC and incubated for 2 hours with or without chondroitinase, heparitinase, or with a combination of chondroitinase and heparitinase (all at 1U/ml), lysates were then prepared and 5 μ g of total protein were run on SDS-PAGE and blotted with NRP1 antibody (to the C-terminus). Results are representative of three independent experiments.

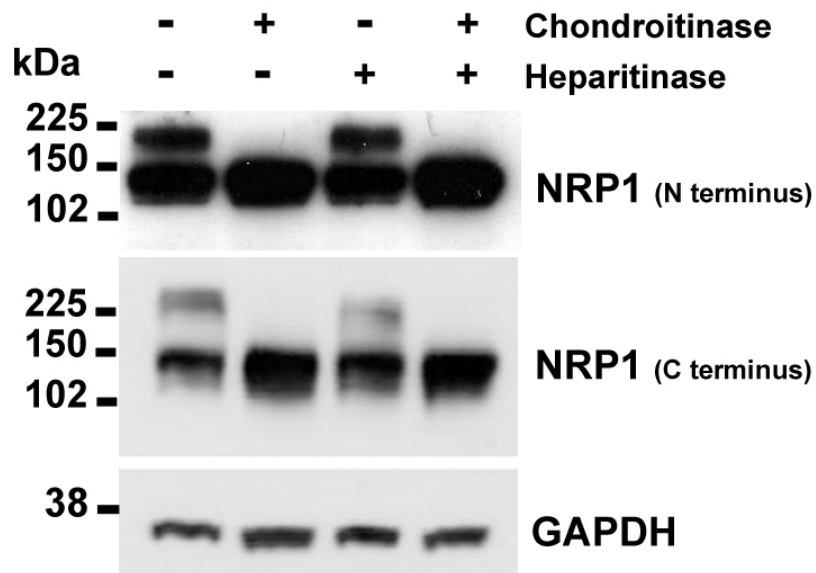


Figure 3.9: **Chondroitinase and heparitinase treatment of A549**

The cells were incubated for 2 hours with either with or without chondroitinase and heparinase III (heparitinase) or with a combination of both (each at 1 Unit/ml), lysates were then prepared and 5 μ g of total protein were run on SDS-PAGE and blotted with NRP1 antibodies raised against both N and C termini.

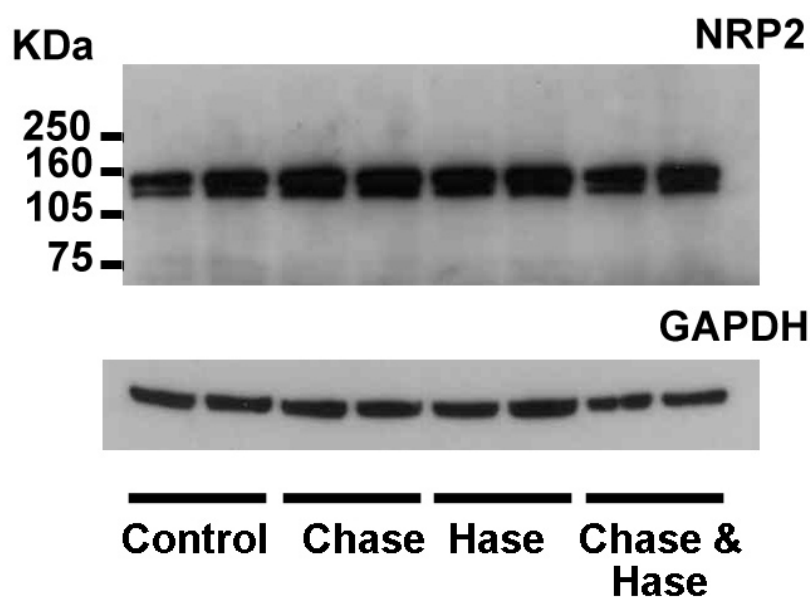


Figure 3.10: **Chondroitinase and heparitinase treatment of A549**

The cells were incubated for 2 hours with either with or without chondroitinase and heparitinase III (heparitinase) or with a combination of both (each at 1 Unit/ml), lysates were then prepared and 5 μ g of total protein were run on SDS-PAGE and blotted with an antibody raised against the N-terminus of NRP2.

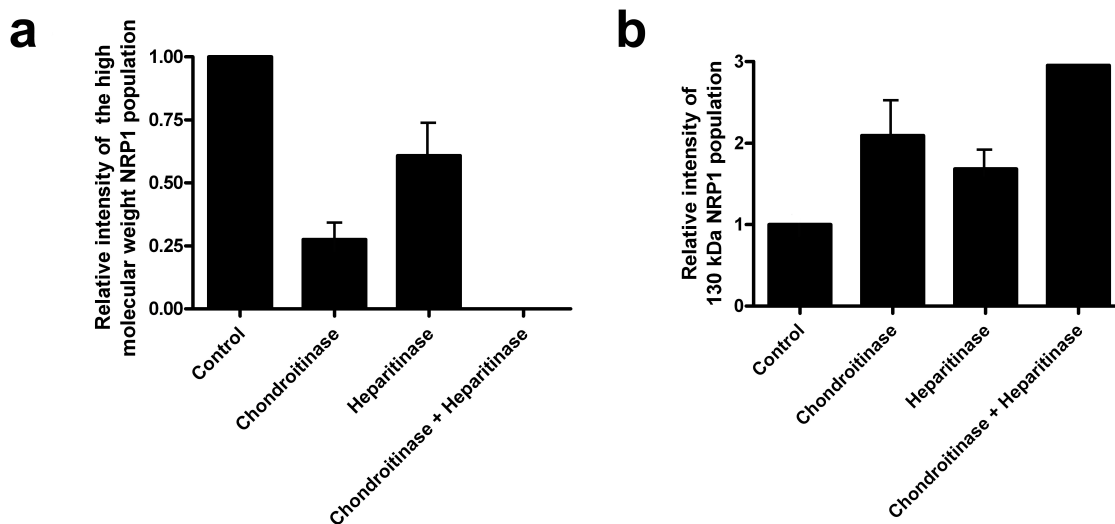


Figure 3.11: **Relative intensity of NRP1 signals in function of lyases treatment**

Western blots of NRP1 in HCASMC that were treated with chondroitinase and heparitinase were analysed by densitometry using Image J. a) The disappearance of the high molecular weight band with the treatment of chondroitinase decreasing the signal more significantly than with heparitinase. b) The chondroitinase treatment of HCASMC increased the 130 kDa band more significantly than the heparitinase treatment. These results are means of values obtained from three independent experiments +/- SEM.

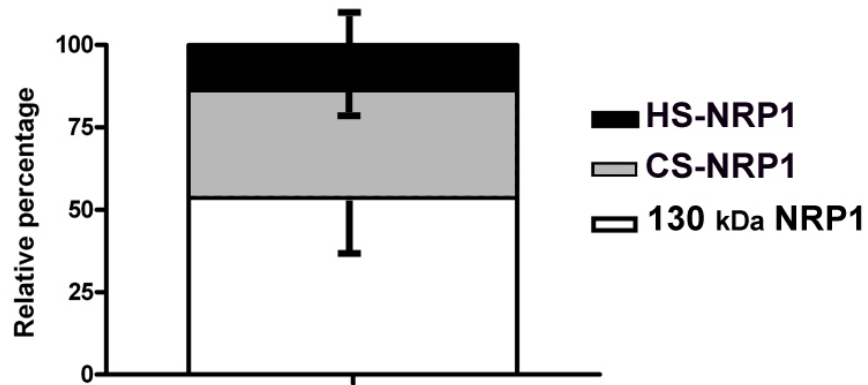


Figure 3.12: **Relative percentage of NRP1 populations**

From Western blot analysis by densitometry using Image J in Fig. 3.11, the relative proportions of glycanated *versus* non-glycanated NRP1 population were determined as 54% *vs.* 46% respectively. CS-modified NRP1 accounted for 70% of the glycanated population of NRP1, 30% for the heparan sulphate modified. These results are means of values obtained from three independent experiments, brackets represent the SEM.

3.4.3 NRP1 colocalises with CS and HS

I next investigated the localisation of chondroitin sulphate (CS) and heparan sulphate (HS) glycosaminoglycans and NRP1 in vascular cells using confocal immunofluorescent microscopy. These studies showed that NRP1 partially colocalised with CS and HS in HCASMC both in the cytoplasm and at the membrane (Figures 3.13 and 3.14). Some NRP1 colocalised with both GAG species (in yellow on the merged confocal images), and another NRP1 pool did not colocalise with CS or HS. The same staining was performed in HUVEC, and, in contrast to HCASMC, little colocalisation of NRP1 with CS could be observed, consistent with the finding that NRP1 is not expressed as a GAG modified form in endothelial cells (Figure 3.15).

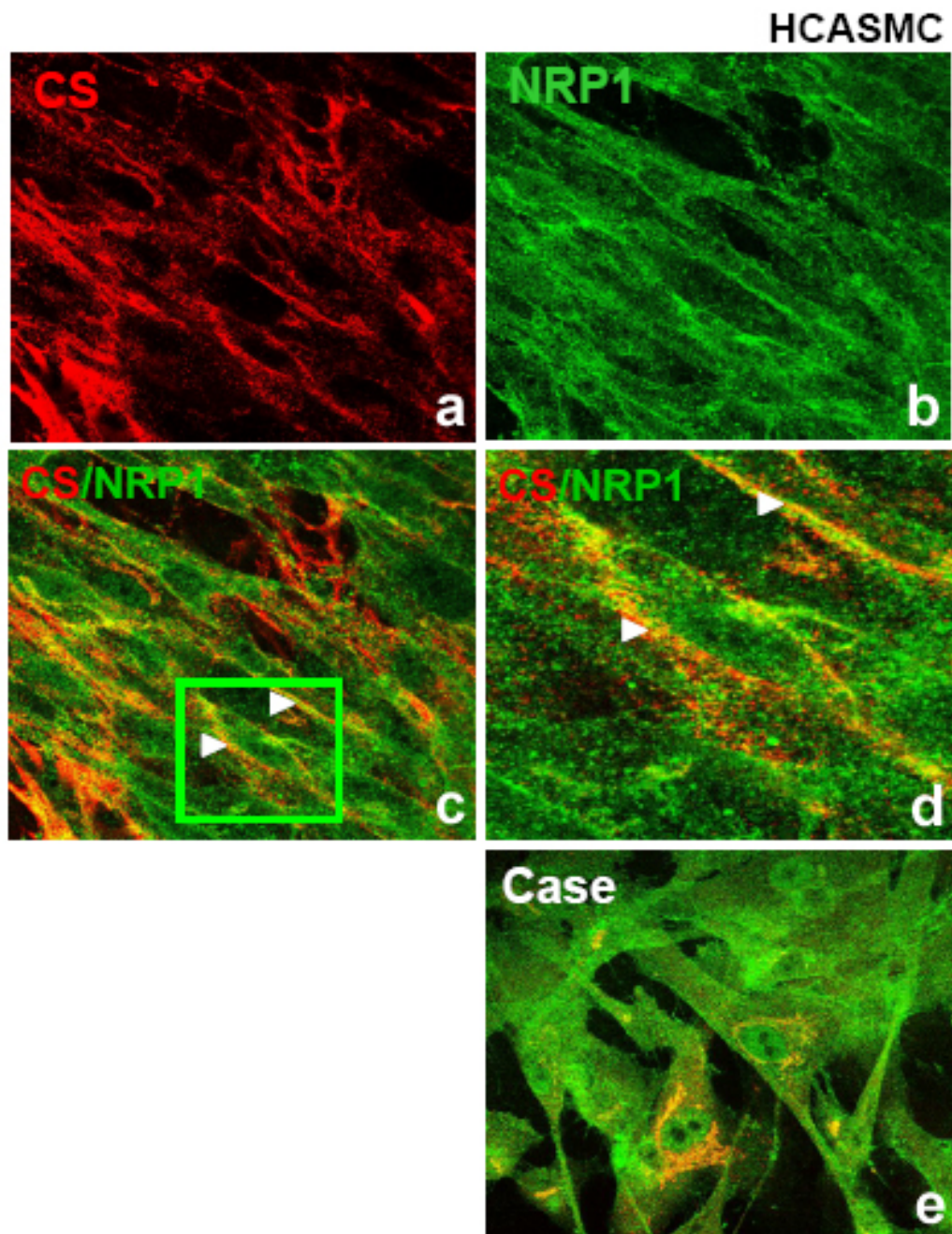


Figure 3.13: NRP1 and CS colocalisation in HCASMC

Immunofluorescent staining of fixed and permeabilised HCASMC was performed using the C-terminus NRP1 antibody with FITC-anti goat as the secondary antibody, CS antibody with Alexa Fluor 555 anti mouse secondary antibody as secondary reagent.

CS is seen on the red channel (a) and NRP1 on the green channel (b). Staining of both NRP1 and CS can be observed in the cytoplasm and at the membrane where they both partially colocalise (c,d). On the merged confocal image, white arrows indicate colocalisation between CS and NRP1, and the region framed in green is magnified to clearly identify colocalisation (d). The confocal image on the bottom left (e) shows the merged images obtained after chondroitinase (Case) treatment of HCASMC. The images are representative of three independent experiments yielding similar results.

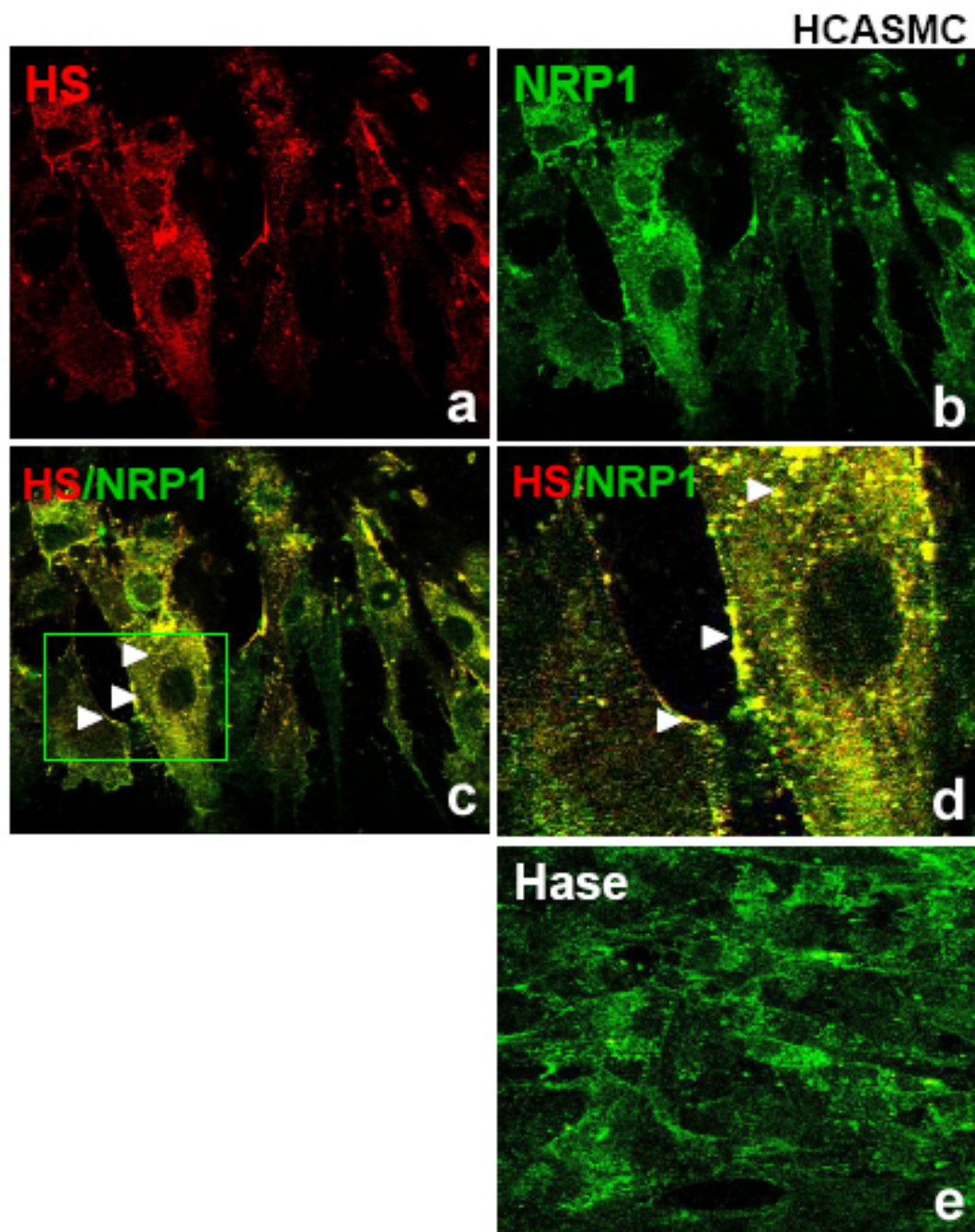


Figure 3.14: **NRP1 and HS colocalisation in HCASMC**

Immunofluorescent staining of fixed and permeabilised HCASMC was performed using the C-terminus NRP1 antibody with FITC-anti goat as the secondary antibody, HS antibody with Alexa Fluor 555 anti mouse secondary antibody as secondary reagent.

HS is seen on the red channel (a) and NRP1 on the green channel (b). Staining of both NRP1 and HS can be observed in the cytoplasm and at the membrane where they both partially colocalise (c,d). On the merged confocal image, white arrows indicate colocalisation between HS and NRP1, and the region framed in green is magnified to clearly identify colocalisation (d). The confocal image on the bottom left (e) shows the merged images obtained after Heparinase (Hase) treatment of HCASMC. The images are representative of three independent experiments yielding similar results.

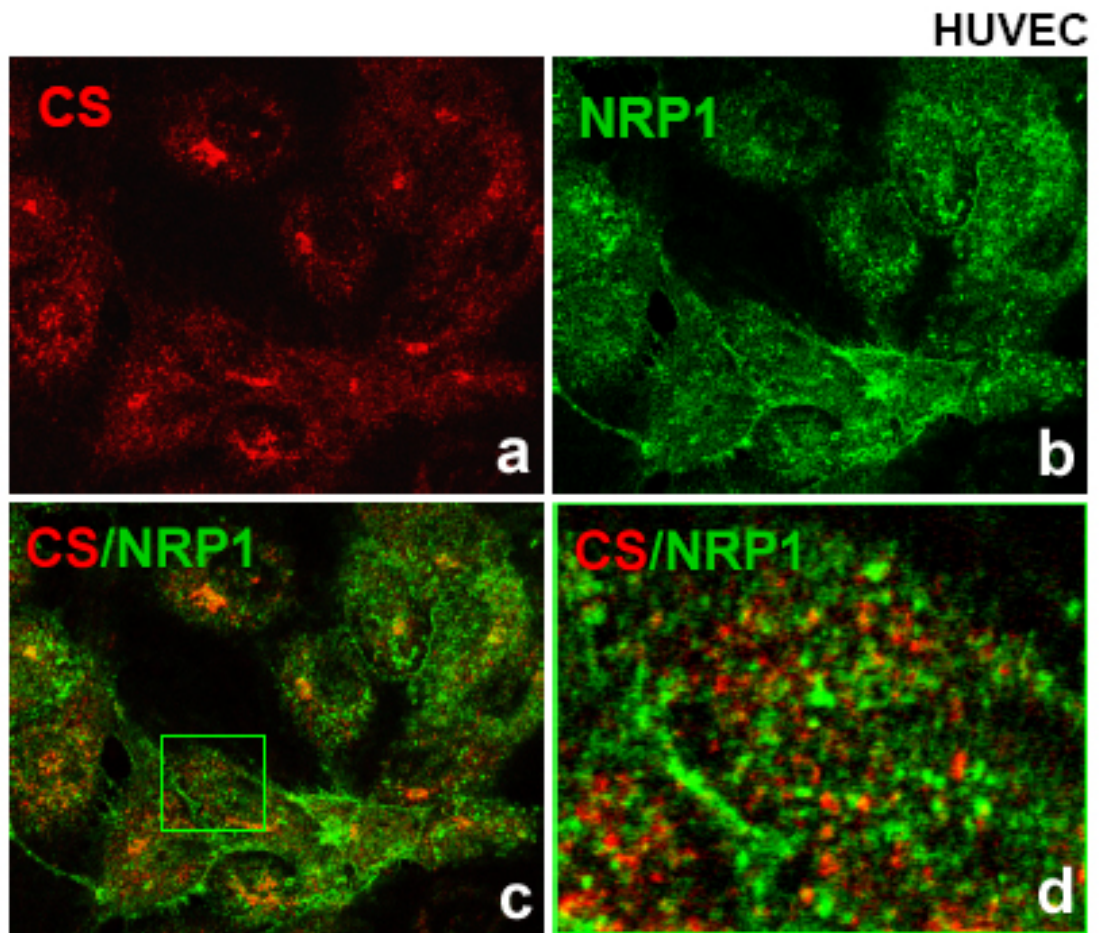


Figure 3.15: Cellular distribution of NRP1 and CS in HUVEC

Immunofluorescent staining of fixed and permeabilised HUVEC was performed using the C-terminus NRP1 antibody with FITC-anti goat as the secondary antibody, CS antibody with Alexa Fluor 555 anti mouse secondary antibody as secondary reagent.

CS is seen on the red channel (a) and NRP1 on the green channel (b). NRP1 can be observed at the membrane and CS mostly in the cytoplasm. On the merged confocal images (c and d), in contrast to HCASMC, little colocalisation between NRP1 and CS can be observed.

3.5 Identification of the site of attachment of NRP1 GAG modification

Both chondroitin and heparan sulphate chains can contain over 100 individual sugar residues, each of which can be covalently sulphated in variable positions and quantities. CS- and HS- GAGs are linked to hydroxyl groups on serine residues of protein cores. Usually, glycosylated serines are often followed by a glycine and have neighbouring acidic residues, but this motif does not always predict glycosylation. I therefore examined the amino acid (aa) sequence of both NRP1 and NRP2 across species and found several NRP1 Serine-Glycine (SG) conserved sites that were highly conserved between species and absent from the NRP2 aa sequence. I used the NRP1 and NRP2 amino acid aligned sequences and the NRP1 aa sequences across species to identify those sites (Tables 2.6 and 2.7). The three serines in positions 240, 432 and 612 were good potential candidates as they were only present in the NRP1 sequence and conserved between vertebrate species. Table 3.2 highlights how the different SG candidates sites were selected.

pcDNA 3.1(+) NRP1 WT and NRP1 with Serine to Alanine substitution at the three SG sites (S240A/S432 double mutant and S612A) were generated *via* site-directed mutagenesis as described in section 2.12.3.1. I transfected Porcine Aortic Endothelial Cells (PAEC) with the NRP1 mutant constructs using lipofectamine 2000 because these cells are known to express no or very little endogenous NRP1 (Figure 3.16). As expected, there was no endogenous NRP1 expressed in PAEC and transfection of the NRP1 WT construct resulted in the expression of both the 130 kDa and high molecular weight forms. The expression of the S240A/S432A mutant also resulted in the expression of both the 130 kDa and high molecular weight species. On the other hand, the single point mutation at position 612 resulted in the expression of only 130 kDa NRP1 with no expression of the high molecular weight NRP1 species.

The other NRP1 mutants, with an alanine at position 115, 283, 439 and 729 were also transfected into PAEC (Figure 3.17). None prevented the expression of the high molecular weight NRP1 species. It was noted, however, that the S240A/S432A double mutant caused some reduction in expression of the >250 kDa NRP1 species compared with NRP1 WT.

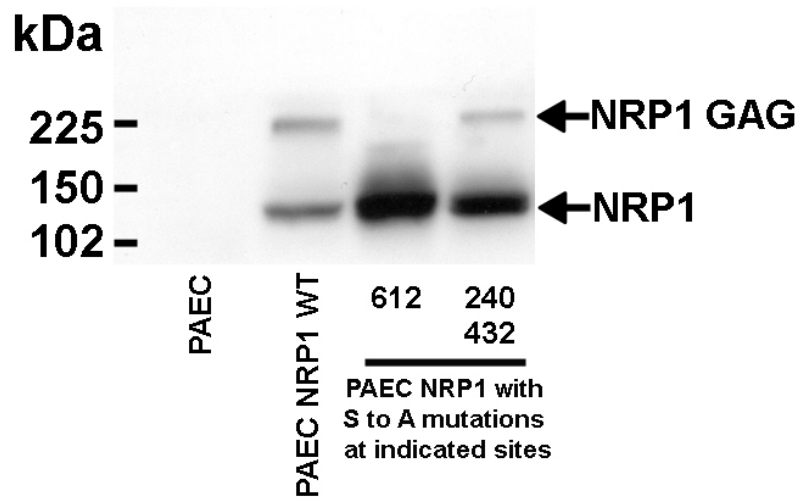


Figure 3.16: Expression of the three candidate NRP1 mutant constructs in PAEC

NRP1 WT pcDNA 3.1 (+) construct as well as two NRP1 mutants with a Serine substituted to an Alanine at the position indicated were expressed in PAEC. Non transfected PAEC were used as control. 48h after transfection, lysates were obtained from the transfected cells and run on SDS-PAGE. NRP1 expression was checked using the NRP1 C-terminus antibody.

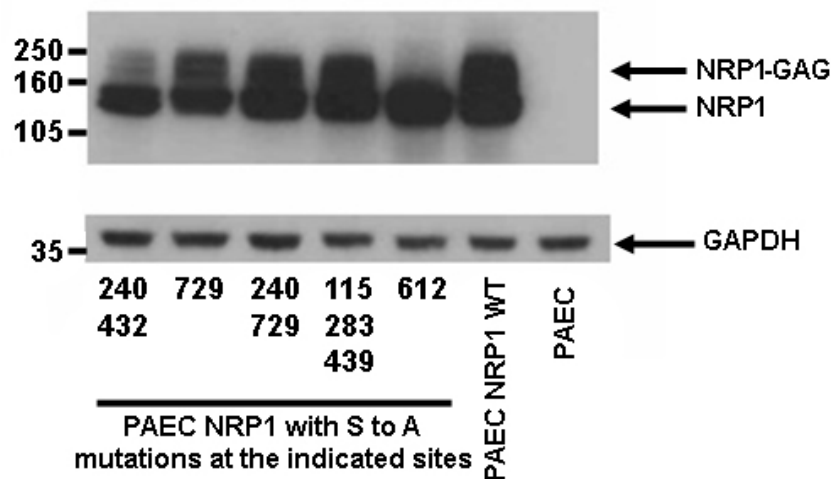


Figure 3.17: Expression of all potential NRP1 mutant constructs in PAEC

NRP1 WT pcDNA 3.1 (+) construct as well as the three NRP1 mutants with a Serine substituted to an Alanine at the position indicated were expressed in PAEC. Non transfected PAEC were used as control. 48h after transfection, lysates were obtained from the transfected cells and run on SDS-PAGE. NRP1 expression was checked using the NRP1 C-terminus antibody.

Table 3.2: Identification of potential SG sites for GAG addition

Mutation number	mutation site	conserved between species	conserved between NRP1 and NRP2	good candidate
1	S115A	+	+	No
2	S155A	-	+	No
3	S240A	+	-	Yes
4	S283A	+	+	No
5	S432A	+	-	Yes
6	S439A	+	+	No
7	S469A	-	+	No
8	S612A	+	-	Yes
9	S729A	+	-	Yes

3.6 Discussion

NRP1 is widely expressed in several cell types, particularly neuronal, endothelial and tumour cells. However, at the onset of my PhD research, nothing was known regarding NRP1 expression in VSMC. The experiments presented in this chapter demonstrate that NRP1 and NRP2 are strongly expressed in HCASMC and HAoSMC. Furthermore, in addition to the 130 kDa NRP1 band corresponding to the major species in endothelial cells, a high molecular weight NRP1 species at around 250 kDa is also expressed in VSMC. This NRP1 high molecular weight band was specific to NRP1 because knocking down the receptor by siRNA silencing resulted in the disappearance of both bands on Western blot, whereas NRP2 knockdown had no effect. Moreover, I observed that a wide range of cancer cells express NRP1 (Appendix ??) and most of these cells also express both NRP1 species (Figure 3.2 and appendix A.3). In contrast, NRP2 was not expressed as a high molecular weight species in any cell type examined, as judged by Western blot.

Subsequent experiments presented in this chapter showed that NRP1 is modified by a combination of N-linked and O-linked glycosylations. Treatment with tunicamycin which specifically blocks N-linked glycosylation caused relatively small shift (≈ 10 -20 kDa) in the position of both 130kDa and >250 kDa NRP1 bands to smaller species, indicative of N-glycosylation of both NRP1 forms. The apparent molecular weight of NRP2 was also shifted by tunicamycin suggesting that it, too, is N-glycosylated. However, tunicamycin did not block expression of the >250 kDa band, indicating that N-glycosylation could not fully account for expression of this NRP1 form. The NRP1 aa sequence contains six potential sites for N-linked oligosaccharide attachment. Two Asparagines occur in a consensus sequence, Asparagine - X - Serine (where X is any aa except Proline), which corresponds to the preferential acceptor motif and are situated in the second CUB (a2) domain. Four Asparagine - X - Threonine consensus sequences were found in the FV/VIII domains (b1/b2) and between the MAM (c) and transmembrane domain. I did not have time to pursue further investigation on the function of post-translational modification by N-oligosaccharides although it is known that their addition to a target molecule is important for the folding of some proteins and also can influence ligand binding affinities (Tift et al., 1992). Further study of the role of NRP glycosylation is warranted.

I then investigated the potential involvement of O-glycosylations in the generation of the high molecular NRP1 species. This type of glycosylation occurs at a later stage during protein processing, probably in the Golgi apparatus and O-linked glycoproteins are usu-

ally large proteins (>200 kDa) that have comparatively less branching than N-glycans. Chondroitinase and heparitinase (also known as heparinase III), are lyases that catalyse the cleavage of carbon-oxygen bonds of chondroitin sulphate- and heparan sulphate glycosaminoglycans respectively, thereby degrading the long polysaccharide chains into short oligosaccharides. These findings demonstrate that the high molecular weight form of NRP1 expressed in VSMC and tumour cells is due largely to O-linked glycosylation either predominantly (as in VSMC) or solely (as in A549 cells) resulting from CS-GAG addition, but with some addition of HS-GAG in VSMC. Treatment of HCASMC with chondroitinase removed most of the >250 kDa species but did not completely abolish it, while heparitinase alone caused a small reduction in this band, and chondroitinase and heparitinase together completely abolished it. Quantification indicated that the non-modified NRP1 form accounted for approximately 54% of the total NRP1 in HCASMC, while CS-modified NRP1 accounted for approximately 70% of the glycanated NRP1 and HS-NRP1 for the remaining 30%, 35% and 15% of the total NRP1 population respectively. However, analysis of NRP1 glycosylation in A549 cancer cells indicated that, the post-translational modification was uniquely due to the addition of chondroitin sulphate moiety to the NRP1 protein core.

As mentioned in the introduction, it is known that O-linked GAGs attach preferentially to consensus sites consisting of serines often followed by a glycine with neighbouring acidic residues. Mutation of candidate consensus sites and expression of these mutants showed that a single Serine at position 612, between the second FV/VIII domain and the MAM domain, was the major site of O-linked GAG attachment to NRP1 (Figure 2.6). However it was noted that expression of a double NRP1 mutation at Serines 240 and 432 also resulted in substantially less expression of the GAG-modified NRP1 species than NRP1 WT. This observation suggests that other potential sites of glycosylation may contribute to O-linked glycosylation, though as the S612A mutation completely abolished the modification, this is clearly the primary site of GAG addition. It is unclear why O-linked glycosylation is different qualitatively and quantitatively in different cell lines. In particular, the apparent absence of GAG-modified NRP1 in HUVEC and HCAEC is puzzling. It is plausible that the lack of GAG-modified NRP1 in endothelial cells may be due to a lack of expression of enzymes essential for this modification, *e.g.* CS synthase. However, this would imply the existence of enzymes with a specific or selective role in NRP1 glycosylation, since it is implausible that endothelial cells are devoid of the enzymatic mechanisms necessary for O-linked glycosylation in general. Alternatively, trafficking of newly synthesised NRP1

may differ in different cell types, routing NRP1 for O-linked glycosylation in some but not in endothelial cells. There is evidence that elongation of O-linked glycans can occur in a protein-specific manner (Moloney et al., 2000). The molecular basis for differential glycosylation of NRP1 in VSMC and EC warrants further study. Regardless of the precise mechanisms involved, the abundant expression and absence of GAG-modified NRP1 in cognate VSMC and EC, respectively, is suggestive of cell-type specific functions of this species. It is also unclear why NRP2 seems to be only post-translationally modified by N-glycosylations, even though its sequence contains consensus sites for O-linked oligosaccharides attachment. Presumably, the three-dimensional structure of NRP2 in which these consensus sites occur do not permit recognition by the enzymes essential for GAG chain initiation or elongation.

Clinical studies suggest that NRP1 expression is related to cancer aggressiveness and plays a role in tumour growth, disease progression, and potentially in metastasis. Recent studies in our group unveiled a role for the CS post-translational modification in cancer cells which will be developed in more detailed in the next chapter. Several biopsies of human gliomas were probed for the expression of NRP1. Although several samples showed strong expression of NRP1 in both low- and high-grade gliomas, little expression of NRP1-CS in all samples was detected. These data suggest that there is a correlation between the highly invasive nature of these tumours, and a low NRP1-CS to NRP1 expression ratio (Frankel et al., 2008).

While this work was in progress, NRP1 expression in human VSMC and NRP1 GAG modification in VSMC were reported by Shintani et al. (2006). The results presented in this chapter are in close agreement with those of Shintani et al. (2006). However, GAG-modification of NRP1 in tumour cells has not been reported by other groups. In addition, NRP1 expression in VSMC has also been reported by two other groups (Banerjee et al., 2006; Liu et al., 2005).

The relevance of NRP1 expression or function in cultured human VSMC is unclear. However, in other work from this laboratory, immunofluorescent staining of NRP1 in injured rat carotid arteries revealed strong NRP1 staining in the neointima (appendix A.4). Furthermore qRT-PCR showed that NRP1 mRNA was upregulated following injury of rat carotid arteries (appendix A.5). These results indicate a potential role for NRP1 in VSMC proliferation and for migration in neointima formation. Whether NRP1 has a role in normal VSMC functions in healthy blood vessels in animals or humans will require generation of

VSMC-specific NRP1 knockouts and analysis of NRP1 expression in human tissues.

Chapter 4

Function of NRP1 in vascular cells

At the beginning of this PhD I focused my attention on the role of NRPs in endothelial cells as it was known that the receptor played an essential role in angiogenesis and therefore in EC migration. The role of NRP1 in endothelial and tumour cell biology as well as its importance in development, had been studied, but several aspects remained unclear.

A novel discovery, described in Chapter 3, was the significant expression of NRP1 in vascular smooth muscle cells. Results in the previous chapter also indicated that NRP1 was GAG modified in VSMC and some tumour cells but underwent little modification in endothelial cells. I therefore wanted to examine the role of GAG modification in ligand binding to NRP1 function in cells, and, in particular, to examine the role of NRP1 in VSMC.

4.1 Role for NRP1 in EC biology

Previous work in this laboratory, led to the development of a NRP1 antagonist called EG3287 (and later, its other more stable version, EG00086) that specifically inhibits VEGF-A₁₆₅ binding to porcine aortic endothelial cells expressing NRP1 (PAE/NRP1) and breast carcinoma cells expressing only NRP1 receptors for VEGF-A₁₆₅, but had no effect on binding to PAE/KDR or PAE/Flt-1. This useful tool as well as the use of siRNAs, soluble recombinant forms of NRP1 and virus over-expression of the receptor allowed me to study the role of NRP in vascular cells. First I decided to investigate the role of NRP1 in EC and whether NRP1 post-translational modification had an effect on VEGF-A₁₆₅ binding to NRP1.

4.1.1 Lyases does not alter ^{125}I -VEGF- A_{165} binding in porcine aortic endothelial cells

Though HUVEC and HCAEC displayed no apparent GAG-modified NRP1, I tested whether chondroitinase and heparitinase could affect VEGF- A_{165} binding to NRP1. Firstly, I used the porcine aortic endothelial cell line PAE NRP1 that expresses exclusively NRP1 and no other VEGF receptor as a tool to study specific binding of VEGF- A_{165} to NRP1. In contrast to HUVEC and HCAEC, PAE NRP1 cells also expressed the GAG-modified NRP1 species (Figure 3.16). The binding assay was optimised and an average of 16.6% was obtained for non specific binding, 83.4% for specific ^{125}I -VEGF- A_{165} binding. Before the binding assay, cells were pre-treated or not (Control) for 2 hours with chondroitinase, heparinase III (heparitinase) or both enzymes. Treating the cells with lyases had no significant effect on ^{125}I -VEGF- A_{165} specific binding (Figure 4.1). These results indicate that, in PAE NRP1 cells at least, the addition of the GAG moiety has no effect on VEGF- A_{165} ability to bind NRP1.

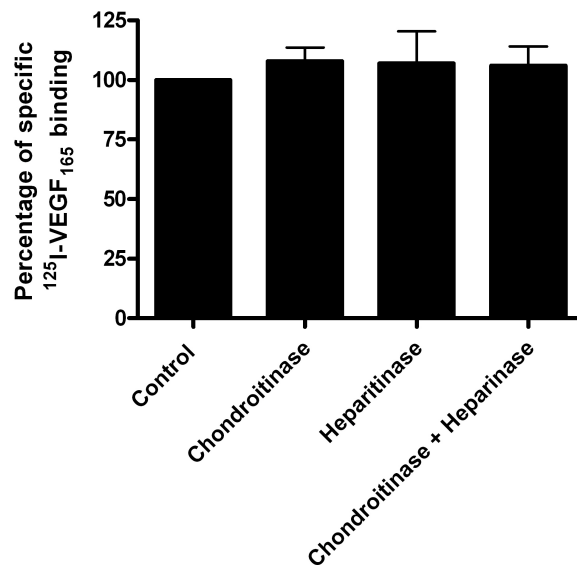


Figure 4.1: **Lyases do not alter ^{125}I -VEGF- A_{165} binding in PAE NRP1 cells**

PAE NRP1 cells were pre-treated or not (Control) for 2 hours with chondroitinase, heparinase III or both at 1 U/ml in complete RPMI medium with 0.1% BSA. After 2 hours incubation, cells were lysed and bound radioactivity measured with a gamma-counter. Bars represent the mean of three independent experiments +/- SEM.

4.1.2 Lyases does not alter ^{125}I -VEGF- A_{165} binding in Human coronary artery endothelial cells

I also used HCAEC that express NRP1 in addition to the other VEGF receptors KDR (VEGFR2) and Flt-1 (VEGFR1). The binding assay produced on average 26% non specific binding and 74% of specific ^{125}I -VEGF- A_{165} binding. Similarly to PAE NRP1 cells, treating HCAEC with chondroitinase or heparinase III had no effect on specific ^{125}I -VEGF- A_{165} binding (Figure 4.2).

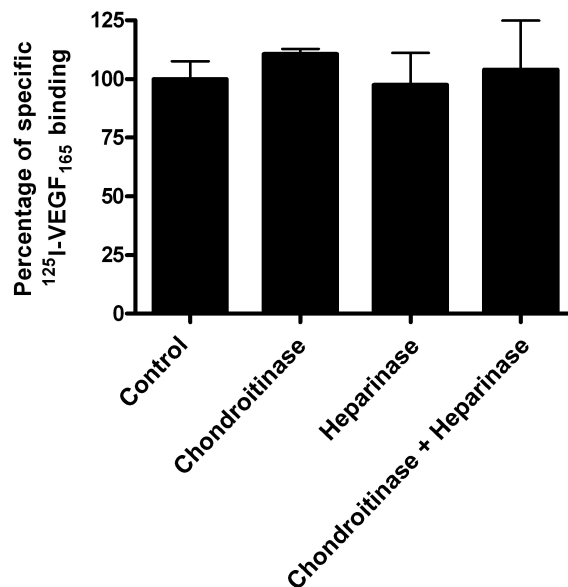


Figure 4.2: Lyases does not alter ^{125}I -VEGF- A_{165} binding in HCAEC

HCAEC were pre-treated or not (Control) for 2 hours with chondroitinase, heparinase III or both at 1 U/ml in complete EC medium with 0.1% BSA. After 2 hours incubation, cells were lysed and bound radioactivity measured with a gamma-counter. Bars represent the mean of three independent experiments +/- SEM.

4.1.3 NRPs knockdown inhibit EC migration towards VEGF-A₁₆₅

A role for NRP1 in EC migration has previously been demonstrated, but has not been examined specifically in HCAEC. The role of NRP1 in the migratory response of HCAEC towards VEGF-A₁₆₅ was examined by siRNA-mediated knockdown of NRP1 and NRP2 expression. Transfection of HCAEC with siRNAs targeted to NRP1 and NRP2 significantly reduced protein expression in HCAEC (Figure 4.3). When siRNA-transfected cells were used to perform a migration assay in response to VEGF-A₁₆₅, NRP1 knockdown caused a significant decrease in migration (Figure 4.4). NRP2 siRNA also significantly inhibited the migratory response to VEGF-A₁₆₅. These results obtained in HCAEC are similar to those obtained using HUVEC and Human microvascular endothelial cells (HMVEC) (Murga et al., 2005; Favier et al., 2006).

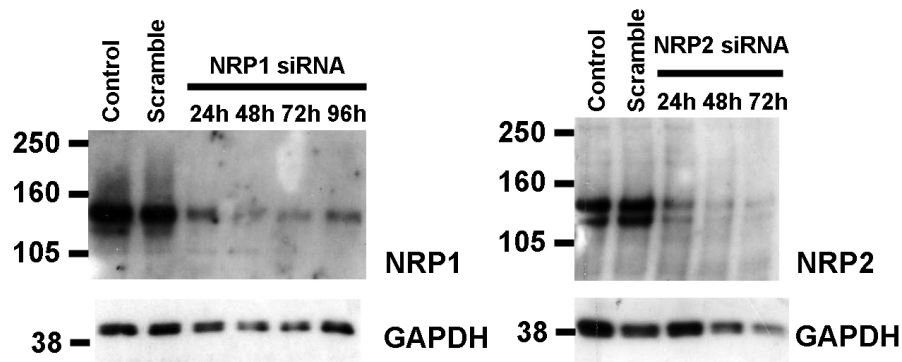


Figure 4.3: **NRP1 and NRP2 protein expression levels after siRNA transfection** HCAEC were transfected with NRP1, NRP2 and control scrambled siRNA using oligo-fectamine and, at the times indicated after transfection, lysates were prepared and 5 μ g of total protein were run on SDS-PAGE and blotted for NRP1 and NRP2. Significant knockdown was obtained for both receptors up to 72 hours after the transfection. The cells were used for migration assays 48 hours after transfection.

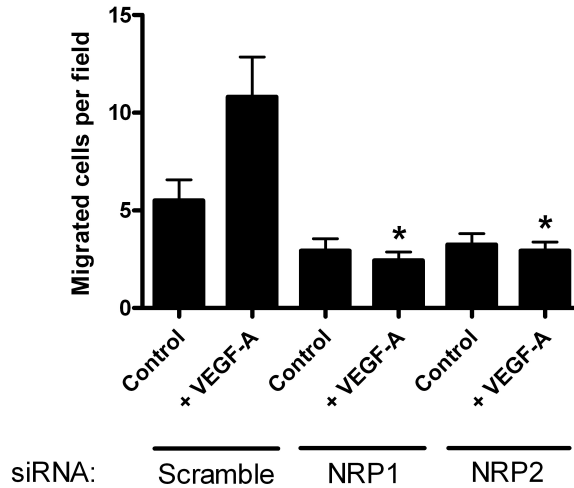


Figure 4.4: **NRP1 and NRP2 knockdown inhibit EC migration towards VEGF-A₁₆₅**

HCAEC were transfected with NRP1, NRP2 and control scrambled siRNA using oligofectamine. Significant knockdown was obtained after 48h and cells were then used for migration assay. After 4 hours, the cells that migrated through the porous membrane were fixed, stained and counted at x200 magnification. Bars represent the means of three independent experiments +/- SEM. *P<0.05 versus VEGF-A₁₆₅ plus scrambled siRNA.

4.1.4 Overexpression of NRP1 Δ C mutant, lacking the C-terminus, decreases VEGF-A₁₆₅ stimulated HUVEC migration

The NRP1 cytosolic domain has previously been implicated in the functions of NRP1 in endothelial cells. I used an adenoviral construct expressing NRP1 extracellular domain and transmembrane domain to further explore the role of the cytoplasmic domain of NRP1 in EC. Protein expression was confirmed by Western blotting using the NRP1 antibody targeting the extracellular domain. Infection of HUVEC with Ad.NRP1 resulted in a significant increase in protein expression above the endogenous level (Figure 4.5), but did not significantly alter the migration of HUVEC towards a gradient of either VEGF-A₁₆₅, compared to control cells infected with Ad.GFP (Figure 4.5). In contrast, Ad.NRP1 Δ C infection, resulting in over-expression of NRP1 lacking the intracellular domain, caused a significant inhibition of cell migration induced by VEGF-A₁₆₅.

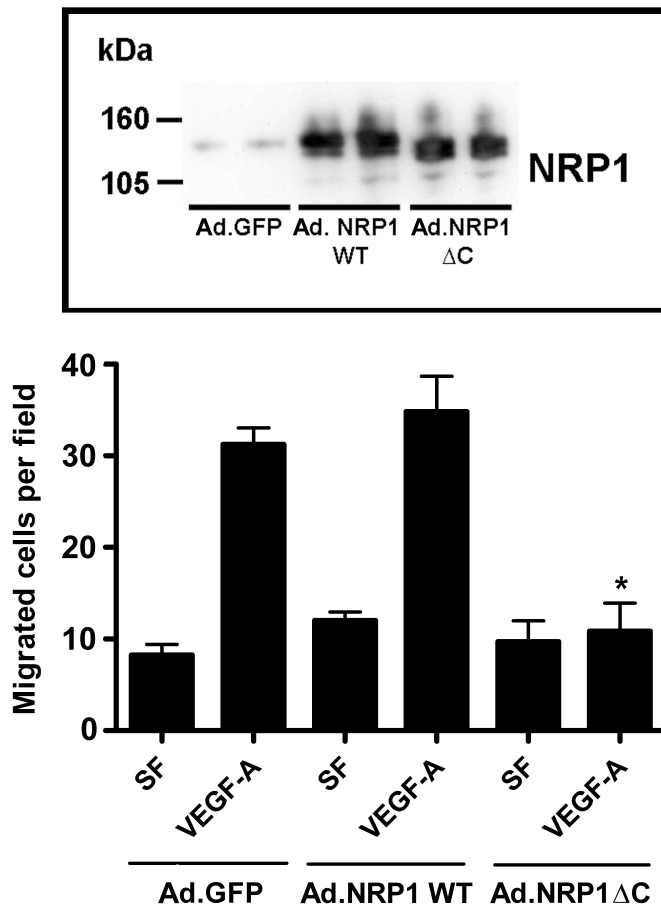


Figure 4.5: Overexpression of NRP1 Δ C mutant, lacking the C-terminus, decreases VEGF-A₁₆₅ stimulated HUVEC migration

HUVEC were infected with the following adenoviral constructs: Ad.GFP, Ad.NRP1 WT and Ad.NRP1 Δ C at a MOI of 20. Three day after the infection, cells were harvested and seeded into a transwell to perform a migration assay. VEGF-A₁₆₅ was the chemoattractant at the bottom of the insert at a concentration of 25 ng/ml. After 4 hours, the cells that migrated through the porous membrane were fixed, stained and counted under the optic microscope at a x200 magnification. Overexpression of the NRP1 constructs was confirmed by western blotting (upper panel). Bars represent the mean of three independent experiments +/- SEM. *P<0.05 versus VEGF-A₁₆₅ plus Ad.GFP and VEGF-A₁₆₅ plus Ad.NRP1 WT.

4.2 Role for NRP1 in VSMC biology

When I initially discovered high expression of NRP1 and its post-translational modification in vascular smooth muscle cells, there was no report in the literature of a role for NRP1 in VSMC biology. Therefore, I decided to further examine the role of NRP1 GAG addition as well as a broader role for NRP1 in VSMC biology.

Several studies previously reported that VEGF-A₁₆₅ is able to stimulate the migration of rat aortic VSMC and other VSMC species (Khurana et al., 2004; Liu et al., 2005). This led me to examine the binding affinity of VEGF-A₁₆₅ in VSMC as well as its ability to trigger VSMC migration.

4.2.1 VEGF-A₁₆₅ response in vascular smooth muscle cells

4.2.1.1 VEGF-A₁₆₅ binds to NRP1 of Human coronary artery smooth muscle cells

Assays of ¹²⁵I-VEGF-A₁₆₅ binding to HCASMC typically produced 35.8% non specific ¹²⁵I-VEGF-A₁₆₅ binding and 64.2% specific binding. In contrast to endothelial cells, treatment of HCASMC with chondroitinase alone or chondroitinase led to a significant increase in ¹²⁵I-VEGF-A₁₆₅ binding while heparinase III (heparitinase) alone did not affect significantly ¹²⁵I-VEGF-A₁₆₅ binding (Figure 4.6).

4.2.1.2 VEGF-A₁₆₅ does not stimulate HCASMC migration

Preliminary experiments shown no significant migration of HCASMC in response to VEGF-A₁₆₅ at a biologically active concentration (25 ng/ml). Investigation of the response of these cells to different VEGF-A₁₆₅ concentrations showed that none of the VEGF-A₁₆₅ concentrations used (25, 50, 100 and 200 ng/ml) were able to stimulate HCASMC migration (Figure 4.7).

As mentioned earlier, pre-treatment with chondroitinase increased specific ¹²⁵I-VEGF-A₁₆₅ binding to HCASMC. I therefore hypothesised that, by treating the cells with lyases, one might be able to trigger HCASMC migration in response to VEGF-A₁₆₅. However, the chondroitinase pre-treatment of HCASMC did not alter their lack of migratory response towards VEGF-A₁₆₅ (Figure 4.8).

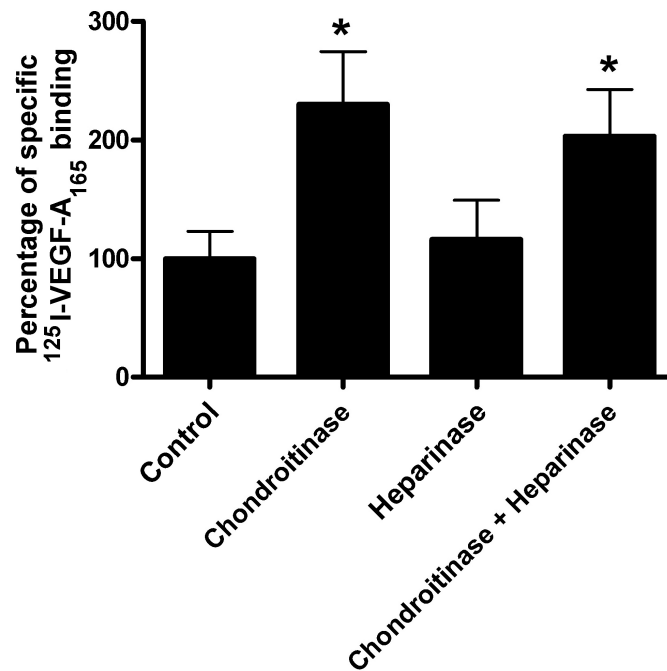


Figure 4.6: **Effects of chondroitinase and heparinase III treatment on ^{125}I -VEGF-A₁₆₅ binding in HCASMC**

HCASMC cells were pre-treated or not (Control) for 2 hours with chondroitinase, heparinase III or both at 1 U/ml in complete SMC medium with 0.1% BSA. After 2 hours incubation, cells were lysed and bound radioactivity measured with a gamma-counter. Bars represent the mean of three independent experiments +/- SEM. * $P < 0.05$ versus control and heparinase treatment.

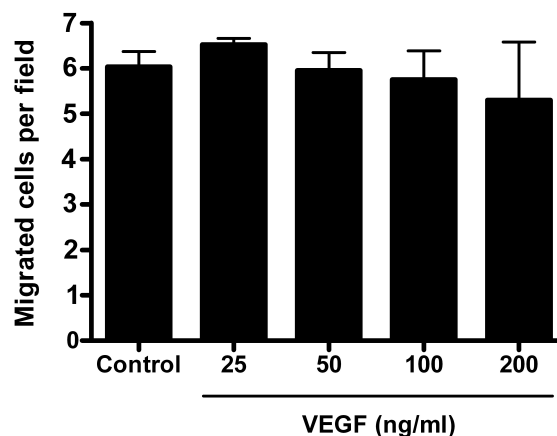


Figure 4.7: **HCASMC migration in response to VEGF-A₁₆₅**

HCASMC were harvested and seeded into transwell to migrate towards VEGF-A₁₆₅ at the indicated concentration for 4 hours. Then, cells that migrated through the porous membrane were fixed, stained and counted at x200 magnification. Bars represent the mean of three independent experiments +/- SEM.

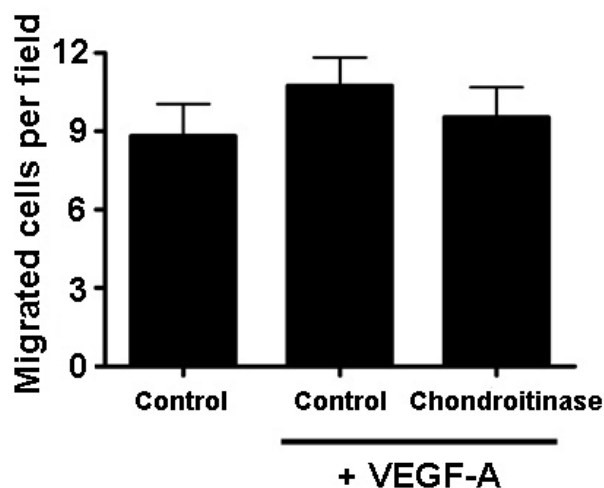


Figure 4.8: **Effect of chondroitinase on HCASMC migration in response to VEGF-A₁₆₅**

HCASMC cells were pre-treated or not (Control) for 2 hours with chondroitinase at 1 U/ml in complete SMC medium with 0.1% BSA. After 2 hours incubation, cells were harvested and seeded into a transwell to perform a migration assay. VEGF-A₁₆₅ was the chemoattractant at the bottom of the insert at a concentration of 25 ng/ml. After 4 hours, the cells that migrated through the porous membrane were fixed, stained and counted at x200 magnification. Bars represent the mean of three independent experiments +/- SEM.

4.2.2 Role of NRP1 in vascular smooth muscle cells functions

4.2.2.1 Radiolabeled PDGF binding to VSMC and PAEC

Other heparin-binding growth factors, including FGF-2 and HGF, have been reported to bind to NRP1. In addition, while this study was ongoing, PDGF-BB was reported to co-immunoprecipitate with NRP1 (Banerjee et al., 2006), suggesting that PDGF-BB, a potent chemoattractant for VSMC, might bind directly to NRP1. I investigated this possibility by determining the specific ligand binding of ¹²⁵I-PDGF-BB to porcine aortic endothelial cells expressing NRP1 (PAE NRP1). While ¹²⁵I-PDGF-BB showed significant specific binding to HCASMC, ¹²⁵I-PDGF-BB exhibited little specific binding to PAE NRP1 cells above the level observed in wild-type PAE not expressing NRP1 (Table 4.1). It was also noted that a high percentage (80-85%) of the total ¹²⁵I-PDGF-BB binding to PAE and PAE NRP1 cells was non-specific.

Cell type	¹²⁵ I-PDGF-BB binding cpm (%Total)		
	Total	Non-specific	Specific
HCASMC	6542 (100, 878)	2600 (40, 383)	3942 (60, 495)
PAE	13899 (100, 1255)	11857 (85, 636)	2042 (15, 723)
PAE/NRP1	13363 (100, 1413)	10626 (80, 728)	2737 (20, 727)

Table 4.1: ¹²⁵I-PDGF-BB binding in PAEC and HCASMC

This table summarises the amount of specific ¹²⁵I-PDGF-BB binding to HCASMC, PAE and PAE/NRP1 cells in count per minutes (cpm) and also as a percentage of specific binding (first item in the parenthesis). HCASMC were incubated with 0.1 nM of ¹²⁵I-PDGF-BB for 2 hours at 4°C. Cells were then lysed with 0.25 M NaOH, 0.5% SDS solution, and the bound radioactivity of the lysates was measured. Non specific binding was determined in the presence of 100-fold excess unlabeled PDGF-BB (Peprotech). The results are expressed as the mean of three independent experiments (the SEM is the second item given in the parenthesis).

4.2.2.2 PDGF stimulates HCASMC migration and signalling

I next investigated whether NRP1 was involved in cell migration stimulated by PDGF. PDGF is a potent mitogenic factor as well as a chemoattractant for VSMC through the activation of its receptors PDGFR α and PDGFR β (Heldin et al., 1985). PDGF occurs as two major homodimeric isoforms, PDGF-AA and PDGF-BB with differing receptor specificities: PDGF-BB binds to both α and β receptors, whereas PDGF-AA recognises only α receptors with high affinity. Since HCASMC expressed both PDGFR α and PDGFR β at significant levels, I initially examined the response of VSMC to PDGF-BB. As expected, PDGF-BB at 30 ng/ml induced HCASMC migration, while VEGF (25 ng/ml) could not (Figure 4.9).

Moreover, stimulation of HCASMC with PDGF-AA and PDGF-BB at 30 ng/ml induced the phosphorylation of the signalling molecules, Akt, ERK 1/2 and p130Cas, while VEGFA₁₆₅ at 25, 50 or 100 ng/ml could not stimulate their activation. In contrast, PDGF-AA and PDGF-BB at 30 or 60 ng/ml could not induce the activation of these intracellular effectors in HUVEC, while VEGF (25 ng/ml) could. These results suggest the specificity of the growth factors for each cell type, probably in accordance with the expression of their corresponding receptors on the cell surface. Moreover, in HCASMC, the lack of activation of intracellular effectors by VEGF-A₁₆₅ is in agreement with the lack of migration in response to a VEGFA₁₆₅ gradient.

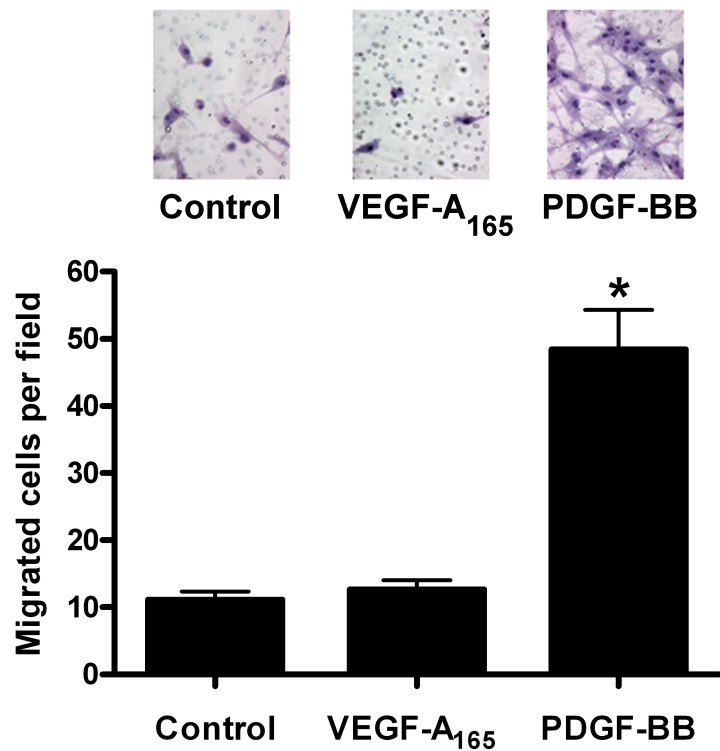


Figure 4.9: HCASMC migration in response to VEGF-A₁₆₅ and PDGF-BB

Cells were harvested and seeded into a transwell to perform a migration assay. VEGF-A₁₆₅ and PDGF-BB were the chemoattractants at the bottom of the inserts at a concentration of 25 ng/ml and 30 ng/ml respectively. After 4 hours, the cells that migrated through the porous membrane were fixed, stained and counted at x200 magnification. Bars represent the mean of three independent experiments +/- SEM. *P<0.05 *versus* control and VEGF-A₁₆₅.

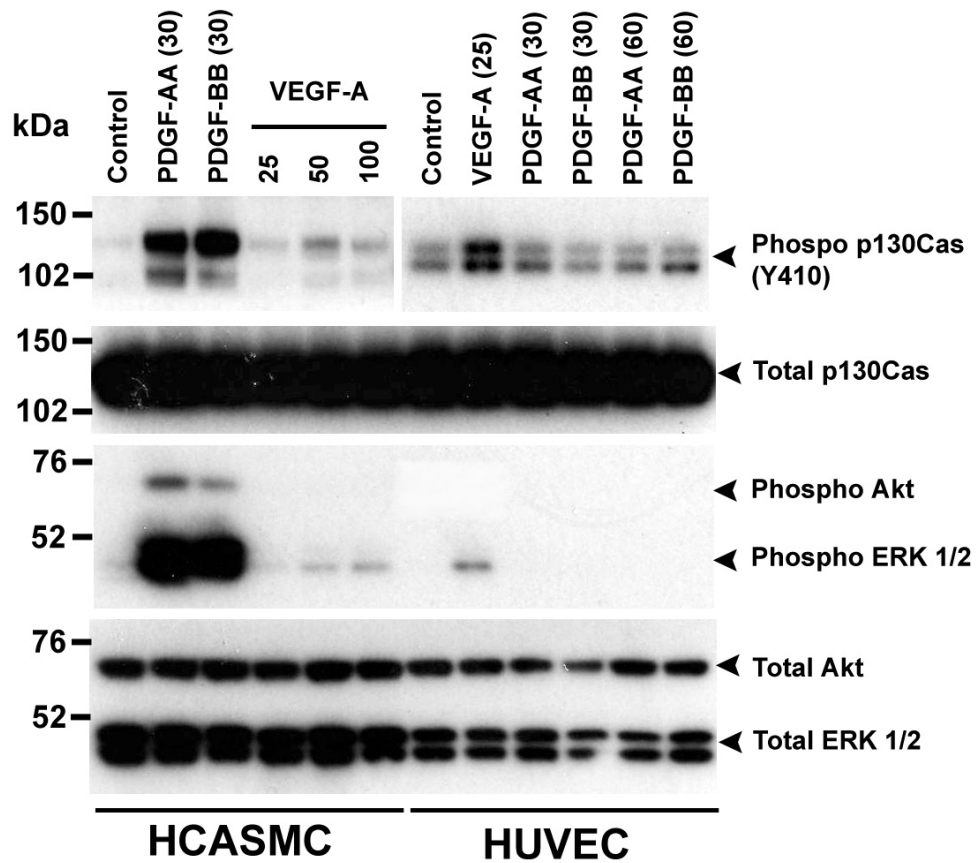


Figure 4.10: **HUVEC and HCASMC stimulation with VEGF-A₁₆₅ and PDGFs**
 HCASMC and HUVEC were stimulated with VEGF-A₁₆₅, PDGF-AA and PDGF-BB for 10 minutes. The concentration of the growth factors used to stimulate the cells are indicated in parenthesis. Cell lysates were then prepared and 5 μ g of total protein were run on SDS-PAGE and blotted for the indicated antibodies.

4.2.2.3 *NRP1 antagonists decrease PDGF-BB stimulated HCASMC migration*

The role of NRP1 in the migratory response to PDGF-BB was addressed by examining the effect of a specific bicyclic peptide antagonist, EG3287, which blocks VEGF binding to the b1 domain of NRP1, and inhibits VEGF-dependent signalling and functions in endothelial cells (Cheng et al., 2004; Jia et al., 2006). Though it was not possible to obtain clear evidence for specific binding of PDGF-BB to NRP1, it was hypothesised that a peptide antagonist of VEGF binding to NRP1 could change the conformation and function of NRP1 by binding directly to the b1 domain even in the absence of a competing natural ligand. Pre-treatment of HCASMC with 100 μ M EG3287 significantly inhibited migration induced by PDGF-BB, by around 30% (Figure 4.11). Amino-terminally modified form of EG3287, called EG00086, which inhibits VEGF-A₁₆₅ binding to NRP1 with a potency similar to that of EG3287 (Figure 2.2) was used in further work. When HCASMC were pre-incubated in the presence of EG00086 at 10, 30 and 100 μ M, the antagonist reduced the chemotactic response of the cells in a dose-dependent manner (Figure 4.13). At a 100 μ M concentration, both EG3287 and EG00086 decreased the migration of HCASMC by around 40%. Also, it was interesting to see that only migration in response to PDGF-BB was significantly reduced by EG00086 as the pre-treatment with the antagonist did not affect the migratory response of HCASMC towards PDGF-AA (Figure 4.12)

4.2.2.4 *Soluble b1 domain and soluble NRP1 decrease PDGF-BB stimulated HCASMC migration*

NRP1 is expressed as a soluble form comprising the a1/a2 and b1/b2 domains, resulting from alternative splicing. Since the soluble form would compete with membrane-associated NRP for binding to its putative ligand(s) in VSMC, I hypothesised that soluble NRP1 might act as an inhibitor of NRP1-mediated effects in VSMC. sNRP1 might also prevent interaction of NRP1 with other receptors, *e.g.* PDGFRs, by forming non-functional dimers with membrane-associated NRP1. Furthermore, since the ligand binding domain resides in part in the b1 domain, soluble recombinant b1 domain might similarly act as an inhibitor of NRP1 dependent cell migration. As shown in Figure 4.14, pre-incubation of HCASMC with soluble NRP1 and soluble b1 domain significantly inhibited migration in response to PDGF-BB with a similar degree of inhibition that was obtained by EG3287 pre-treatment.

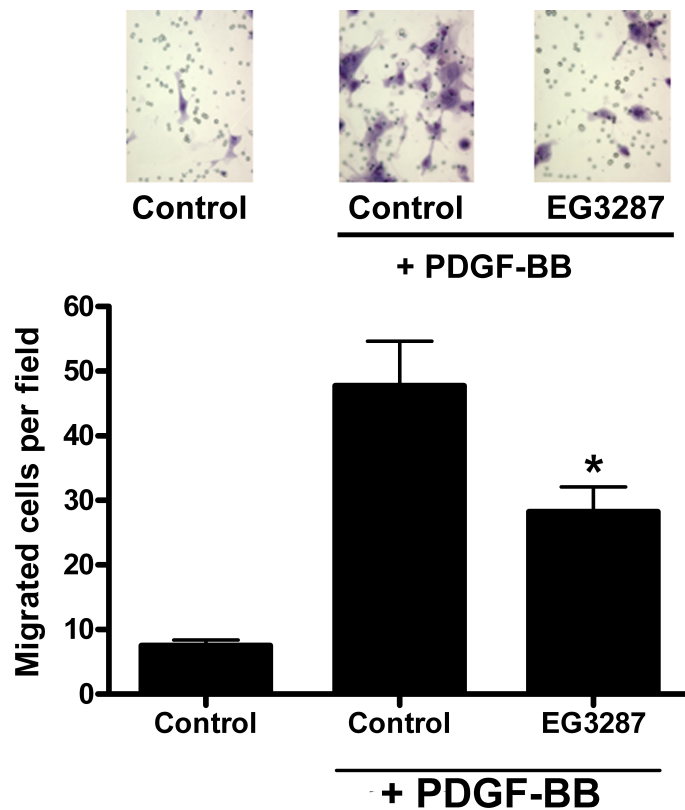


Figure 4.11: The NRP1 antagonist, EG3287, decreases HCASMC migration in response to PDGF-BB

HCASMC were harvested and incubated for 30 minutes with 100 μ M of EG3287, seeded into a transwell to perform a migration assay. PDGF-BB was the chemoattractant at the bottom of the insert at a concentration of 30 ng/ml. After 4 hours, the cells that migrated through the porous membrane were fixed, stained and counted at x200 magnification. Bars represent the means of three independent +/-SEM. *P<0.05 *versus* control plus PDGF-BB.

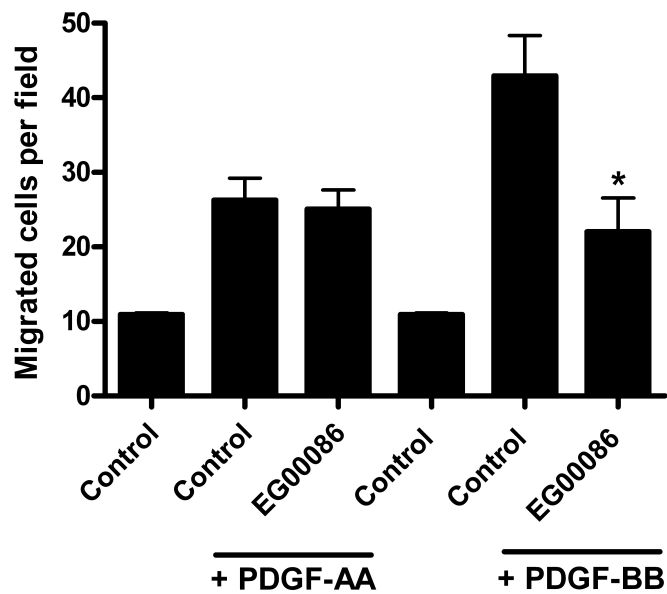


Figure 4.12: The NRP1 antagonist, EG00086, decreases HCASMC migration in response to PDGF-BB but not to PDGF-AA

HCASMC were harvested and incubated or not (Control) for 30 minutes with EG00086 at the $100\mu\text{M}$, then seeded into a transwell to perform a migration assay. PDGF-AA and PDGF-BB was the chemoattractants at the bottom of the insert at a concentration of 30 ng/ml. After 4 hours, the cells that migrated through the porous membrane were fixed, stained and counted at x200 magnification. Bars represent the means of three independent experiments \pm -SEM. * $P < 0.05$ versus control plus PDGF-BB.

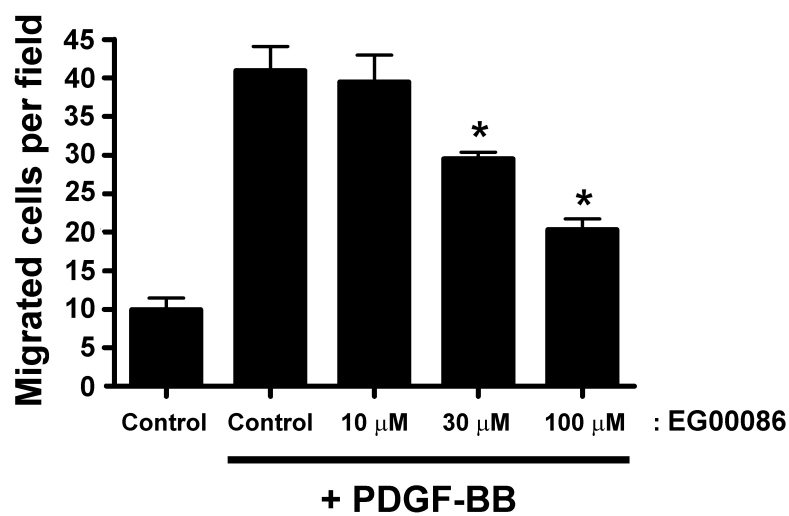


Figure 4.13: The NRP1 antagonist, EG00086, decreases HCASMC migration in response to PDGF-BB

HCASMC were harvested and incubated or not (Control) for 30 minutes with EG00086 at the indicated molarity, then seeded into a transwell to perform a migration assay. PDGF-BB was the chemoattractant at the bottom of the insert at a concentration of 30 ng/ml. After 4 hours, the cells that migrated through the porous membrane were fixed, stained and counted at x200 magnification. Bars represent the means of three independent +/-SEM. *P<0.05 *versus* control plus PDGF-BB.

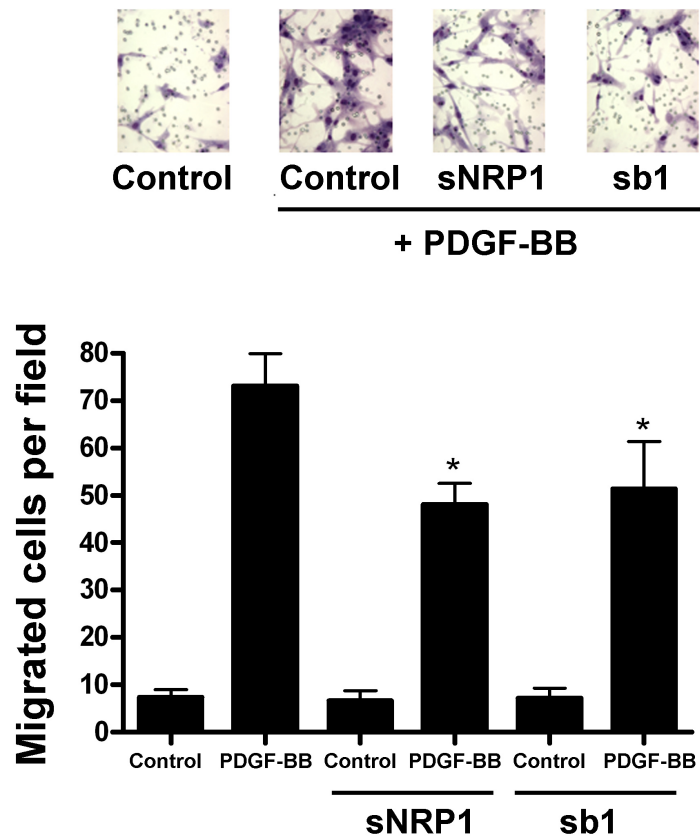


Figure 4.14: sNRP1 and sb1 treatment decrease PDGF-BB stimulated HCASMC migration

HCASMC were harvested and pre-incubated or not (Control) for 30 minutes with sNRP1 and sb1, then seeded in the upper chamber of a transwell to perform a migration assay. PDGF-BB was the chemoattractant at the bottom of the insert at a concentration of 30 ng/ml. After 4 hours, the cells that migrated through the porous membrane were fixed, stained and counted at x200 magnification. Bars represent the means of three independent experiments +/-SEM. *P<0.05 versus control plus PDGF-BB.

4.2.2.5 Effects of NRP1, NRP2 and synectin knockdown on PDGF-BB stimulated HCASMC migration

The role of NRP in the migratory response of HCASMC to PDGF-BB was further examined by siRNA-mediated knockdown of NRP1 and NRP2 expression. Transfection of HCASMC with siRNAs targeted to NRP1, NRP2 and synectin significantly reduced protein expression in HCASMC (Figure 4.15). When siRNA-transfected HCASMC were used to perform a migration assay in response to PDGF-BB, NRP1 knockdown was found to cause a significant decrease in migration (Figure 4.16). NRP2 siRNA also significantly inhibited the migratory response to PDGF-BB though somewhat less so than NRP1, while synectin knockdown tended to reduce the migratory response of HCASMC but the effect was not statistically significant (Figure 4.16).

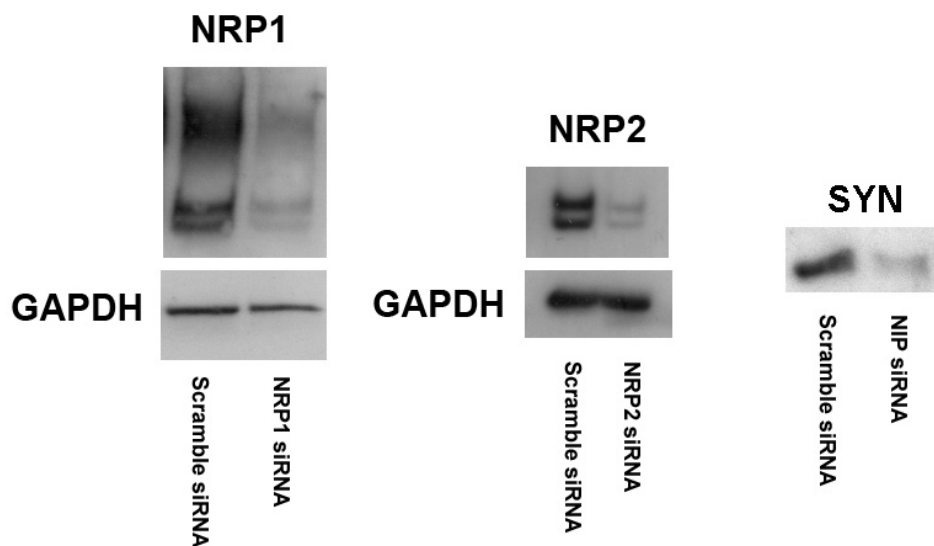


Figure 4.15: **NRP1, NRP2 and synectin knockdown in HCASMC**

HCASMC cells were transfected with NRP1, NRP2 and synectin (SYN) siRNAs using the AMAXA nucleofection kit. 48h after transfection, lysates were prepared and blotted for NRP1, NRP2 and synectin.

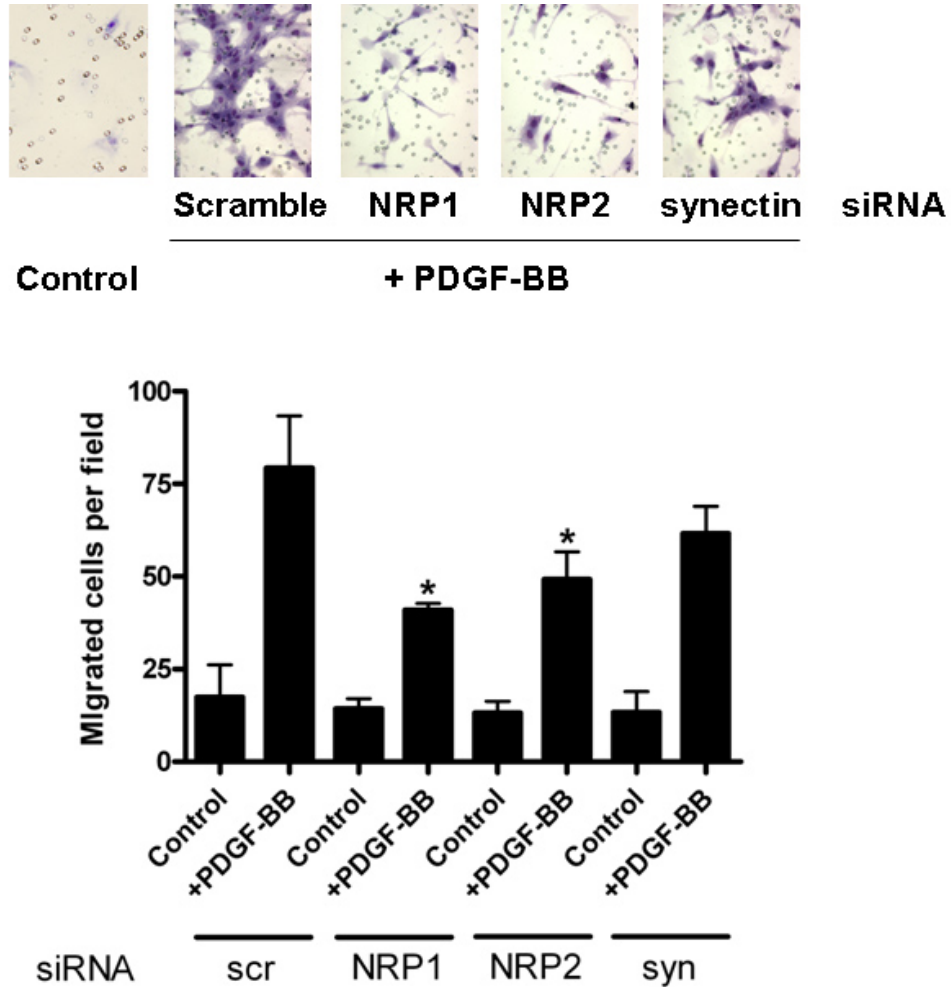


Figure 4.16: **Effects of NRP1, NRP2 and synectin knockdowns on HCASMC migration in response PDGF-BB**

HCASMC cells were previously transfected with NRP1, NRP2, synectin (syn) and scrambled (Scr) siRNAs using the AMAXA nucleofection kit. 48 hours after the transfection, cells were harvested and seeded into a transwell to perform a migration assay. PDGF-BB was the chemoattractant at the bottom of the insert at a concentration of 30 ng/ml. After 4 hours, the cells that migrated through the porous membrane were fixed, stained and counted at x200 magnification. Bars represent the means of three independent experiments +/-SEM. *P<0.05 *versus* control scrambled plus PDGF-BB.

4.2.2.6 *NRP1 knockdown decreases PDGF-AA and PDGF-BB stimulated HCASMC migration*

PDGF-AA also stimulated migration of VSMC, but induced a smaller response compared with PDGF-BB. PDGF-AA-induced migration was also significantly reduced by NRP1 siRNA transfection, by around 40% (Figure 4.17). Therefore, NRP1 also plays a role in PDGF-AA mediated HCASMC migration (Figure 4.17).

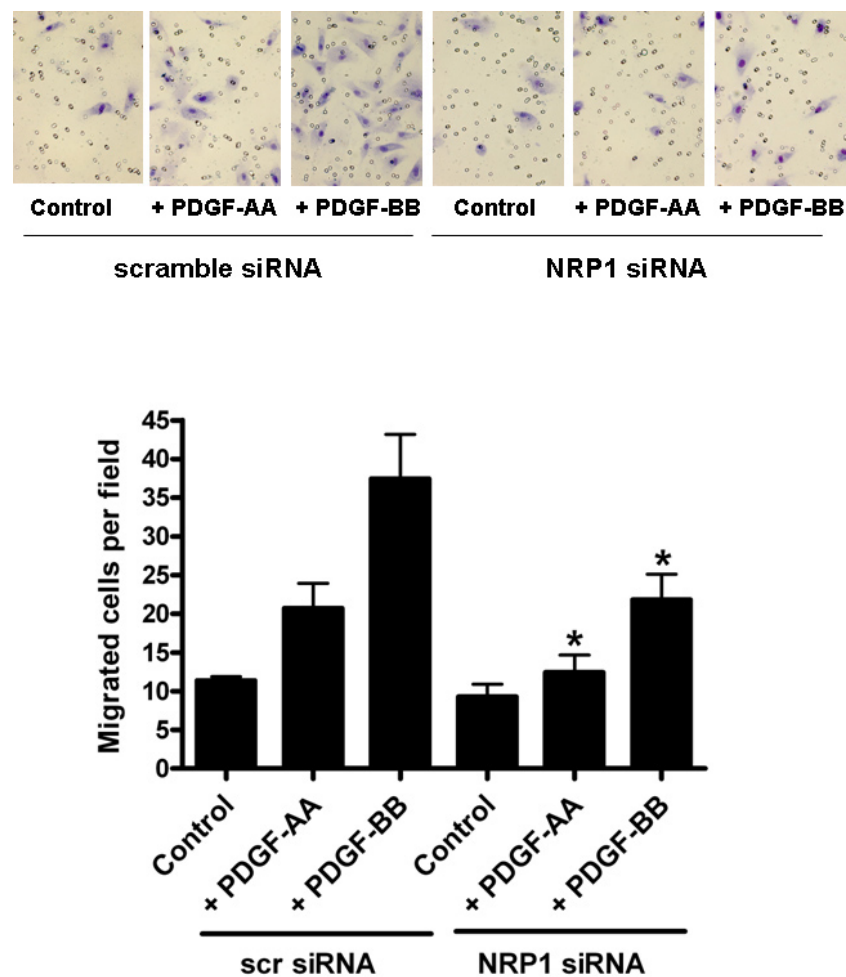


Figure 4.17: NRP1 knockdown decreases PDGF-AA and PDGF-BB stimulated HCASMC migration

HCASMC cells were previously transfected with NRP1 siRNAs using the AMAXA nucleofection kit. 48 hours after the transfection, cells were harvested and seeded into a transwell to perform a migration assay. PDGF-AA and PDGF-BB were the chemoattractants at the bottom of the inserts, both at a concentration of 30 ng/ml. After 4 hours, the cells that migrated through the porous membrane were fixed, stained and counted at x200 magnification. Bars represent the means of three independent experiments +/-SEM. *P<0.05 *versus* corresponding control scrambled plus PDGF-AA and PDGF-BB.

4.2.2.7 Overexpression of NRP1 mutant, lacking the C-terminus, decreases PDGF-AA and PDGF-BB stimulated HCASMC migration

The NRP1 cytosolic domain has been implicated in the functions of NRP1 in endothelial cells, but its precise role remains unclear. To further substantiate the role of NRP1 in the PDGF migratory response in HCASMC, I examined the effects of adenoviral overexpression of wild-type (WT) NRP1 and a mutant NRP1 lacking the cytosolic domain. Expression of Ad.NRP1 adenoviral constructs after infection of HCASMC is shown on Figure 4.18. As expected, the over-expression of Ad.NRP1 Δ C was only detected by an antibody specific for the N-terminus, and not by a C-terminus antibody. Infection of HCASMC with Ad.NRP1 resulted in a significant increase in protein expression above the endogenous level (Figure 4.18), but did not significantly alter the migration of HCASMC towards a gradient of either PDGF-AA or PDGF-BB, compared to control cells infected with Ad.GFP (Figure 4.19). In contrast, Ad.NRP1 Δ C infection, resulting in over-expression of NRP1 lacking the intracellular domain, caused a significant inhibition of cell migration induced either by PDGF-AA or by PDGF-BB, similar to the effect of NRP1 knockdown (Figure 4.19). It was also noted that Ad.NRP1 Δ C over-expression also reduced the basal level of HCASMC migration seen in the absence of PDGF stimulation.

4.2.2.8 Chondroitinase decreases PDGF-BB stimulated HCASMC migration

Chondroitin sulphate chains (CS), which are abundant in the extracellular matrix have been shown to physically interact with growth factors and particularly with PDGF-BB helping the modulation of its biological function. For example, the addition of exogenous free CS chains causes a significant downregulation of the PDGF-BB mediated mitogenic and chemotactic responses in human lung fibroblasts (Fthenou et al., 2008). In this study, the migratory response of HCASMC in response to PDGF-BB was significantly reduced after treatment of intact cells with 1U/ml of chondroitinase (Figure 4.20).

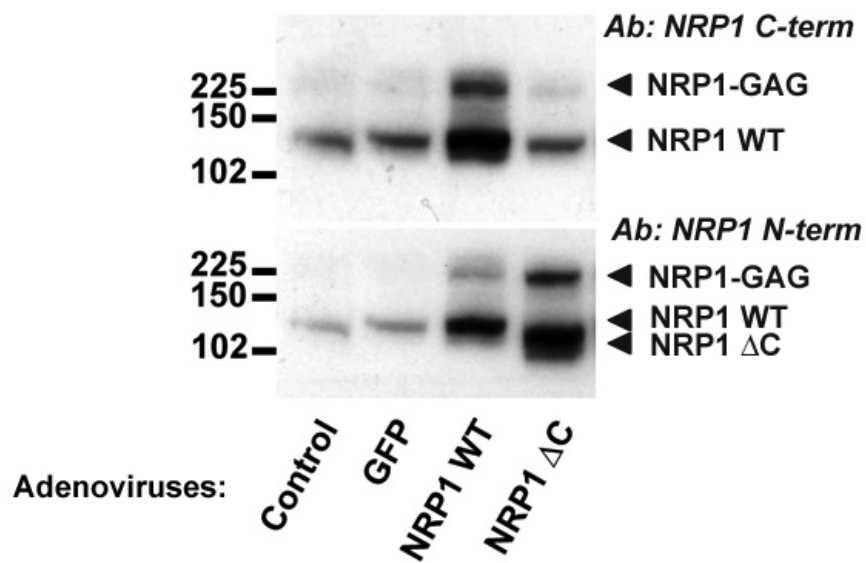


Figure 4.18: **Western blot showing the overexpression of NRP1 WT and Δ C constructs in HCASMC**

HCASMC cells were previously infected with Ad.GFP, Ad.NRP1 WT or Ad.NRP1 Δ C adenoviruses constructs. 48 hours after the transfection, cells were lysed to assess the expression of the NRP1 constructs. The upper panel corresponds to the Western blot obtained using the NRP1 antibody targeting NRP1 C-terminal cytosolic domain (NRP1 C-term), and the lower panel to the Western blot obtained with the antibody targeting the extracellular domain (NRP1 N-term). The position of GAG-modified NRP1 (NRP1-CS), unmodified WT NRP1 (WT NRP1) and NRP1 Δ C are indicated.

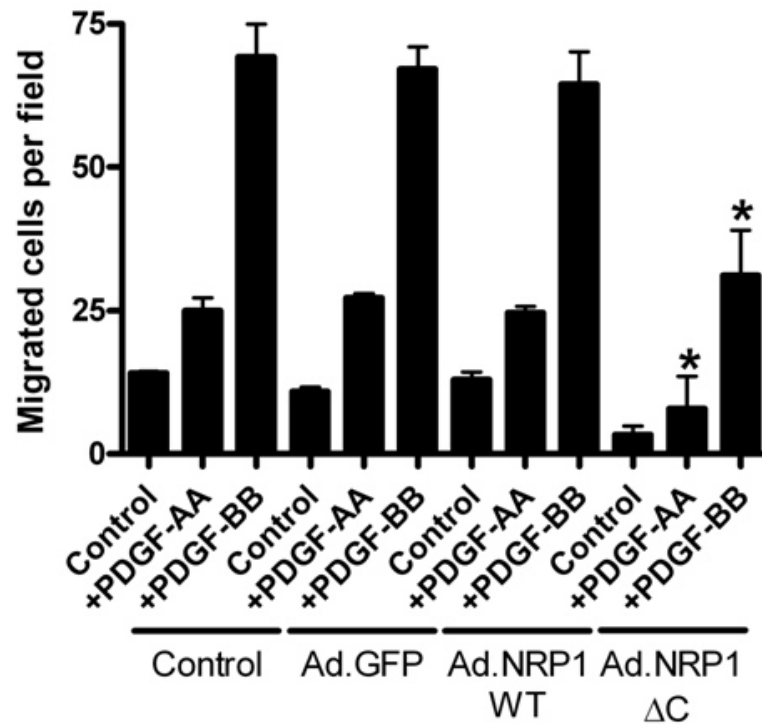


Figure 4.19: **Overexpression of NRP1 Δ C mutant, lacking the C-terminus, decreases PDGF-AA and PDGF-BB stimulated HCASMC migration**
 HCASMC cells were previously infected with Ad.GFP, Ad.NRP1 WT or Ad.NRP1 Δ C adenoviruses constructs. 48 hours after the transfection, cells were harvested and seeded into a transwell to perform a migration assay. PDGF-AA and PDGF-BB were the chemoattractants at the bottom of the inserts, both at a concentration of 30 ng/ml. After 4 hours, the cells that migrated through the porous membrane were fixed, stained and counted at x200 magnification. Bars represent the means of three independent experiments +/-SEM. *P<0.05 *versus* corresponding control, Ad.GFP, Ad.NRP1 WT plus PDGF-AA and PDGF-BB.

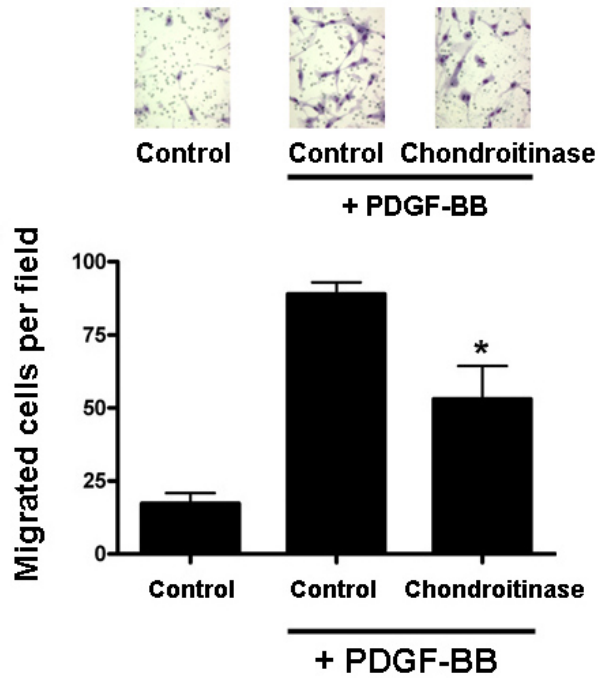


Figure 4.20: **Chondroitinase treatment of HCASMC decreases migration in response to PDGF-BB**

HCASMC cells were pre-treated or not (Control) for 2 hours with chondroitinase at 1 U/ml in complete SMC medium with 0.1% BSA. After 2 hours incubation, cells were harvested and seeded into a transwell to perform a migration assay. PDGF-BB was the chemoattractant at the bottom of the insert at a concentration of 30 ng/ml. After 4 hours, the cells that migrated through the porous membrane were fixed, stained and counted at x200 magnification. Bars represent the means of three independent experiments +/-SEM. *P<0.05 *versus* control plus PDGF-BB.

4.2.2.9 *Overexpression of NRP1 S612A mutant lacking GAG modification does not impair PDGF-AA and PDGF-BB stimulated HCASMC migration*

To study the effect of the GAG modification of NRP1 in HCASMC, I over-expressed an adenoviral NRP1 construct with a Serine substituted to an Alanine in position 612. Two day after transfection, the level of over-expression of the NRP1 construct were assessed, and as expected, only the non-glycosylated form of NRP1 at a molecular weight of 130 kDa was observed on the Western Blot whereas the NRP1 WT construct resulted in the expression of both GAG modified and non-modified (Figure 4.21). In parallel, I used the infected cells to assess migration. A small reduction in the migratory response of HCASMC was observed, but it was not statistically significant altered (Figure 4.22).

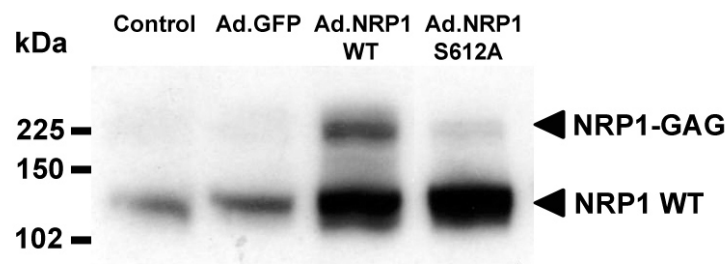


Figure 4.21: **Western blot showing the overexpression of NRP1WT and S612A constructs in HCASMC**

HCASMC cells were previously infected with Ad.GFP, Ad.NRP1 WT or Ad.NRP1 S612A adenoviruses. 48 hours after the transfection, cells were lysed to assess the expression of the NRP1 constructs.

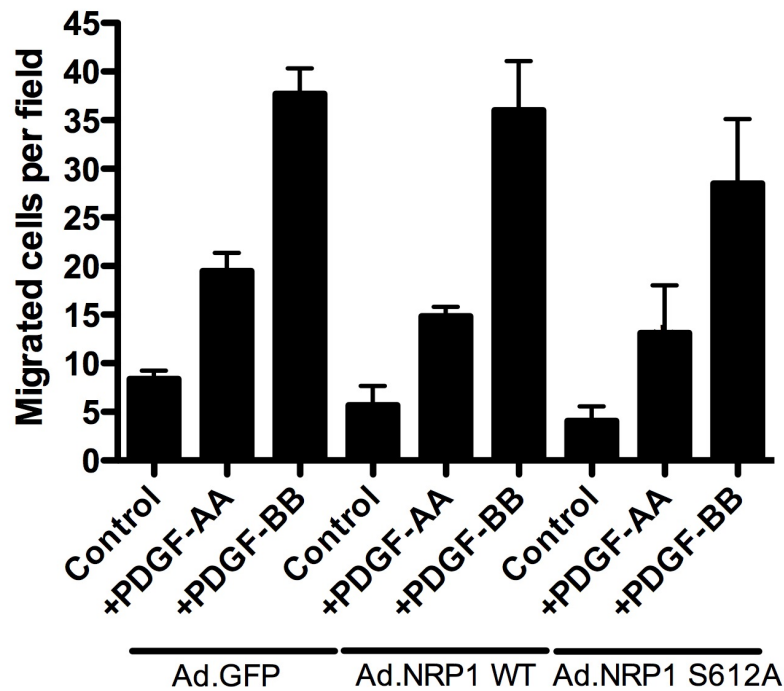


Figure 4.22: **Overexpression of NRP1 S612A mutant, lacking GAG modification, does not impair PDGF-AA and PDGF-BB stimulated HCASMC migration**
 HCASMC cells were previously infected with Ad.GFP, Ad.NRP1 WT or Ad.NRP1 S612A adenoviruses constructs. 48 hours after the transfection, cells were harvested and seeded into a transwell to perform a migration assay. PDGF-AA and PDGF-BB were the chemoattractants at the bottom of the inserts, both at a concentration of 30 ng/ml. After 4 hours, the cells that migrated through the porous membrane were fixed, stained and counted at x200 magnification. Bars represent the means of three independent experiments +/-SEM.

4.3 Discussion

Role for NRP in migration of endothelial cells

In HUVECs and in HCAEC, NRP1 does not appear to be modified by the addition of O-linked GAG and the lyases did not affect VEGF-A₁₆₅ binding to the EC. Nevertheless, VEGF-A₁₆₅ is known to bind to HS with high affinity (Robinson et al., 2006), and in endothelial cells where HS, but not CS, have been enzymatically removed, VEGF-A₁₆₅ signalling is impaired, with lower level of VEGFR2 and ERK1/2 phosphorylation (Ashikari-Hada et al., 2005). VEGFR2 and NRP1 also bind to heparan sulfate, suggesting that the HSPG may play a role in facilitating the formation of the active signalling complex (Jakobsson et al., 2006). In this study, heparinase III (also called heparitinase) that cleaves heparan sulfate exclusively, and does not cleave unfractionated heparin or low molecular weight heparins, had no effect on VEGF-A₁₆₅ binding. This might result from the persistence of other heparan-like GAG or heparin that are not recognised and hence not degraded by the enzyme, but still might play a role in VEGF-A binding.

In PAE NRP1 cells, NRP1 is expressed as both its O-linked GAG form and non-modified form. Nevertheless, the use of chondroitinase and heparinase which results in the removal of the GAG moiety did not alter VEGF-A₁₆₅ binding. This suggest that the GAG moiety does not play any role in VEGF-A₁₆₅ binding to NRP1 in PAEC.

The role for neuropilins in endothelial cell biology is now well established, and both NRP1 and NRP2 have been shown to be important for EC proliferation, migration (Sulpice et al., 2008; Favier et al., 2006) and adhesion (Murga et al., 2005). It therefore not surprising that knockdown of NRP1 and NRP2 *via* siRNA transfection resulted in a significant decrease in migration of HCAEC. Nevertheless, to the best of my knowledge, previous studies have not demonstrated a role for NRP1 in HCAEC. It was also important to demonstrate a role for NRP1 in VEGFA₁₆₅-regulated migration of HCAEC, given that an important goal of this thesis was to examine the role of NRP1 in migration of cognate HCASMC.

Moreover, the results obtained with the over-expression of the NRP1 ΔC adenoviral construct, demonstrate an important role for the cytoplasmic domain in VEGF-A₁₆₅-induced EC migration. The NRP1 ΔC mutant, by exerting a dominant negative effect, possibly prevents interactions between the NRP1 cytoplasmic domain and intracellular binding molecules and could also result in the sequestration of inactive complexes of VEGF, VEGFR and other cellular components important for chemotactic signalling.

Role for NRP in migration of vascular smooth muscle cells

VEGF-A₁₆₅ binds specifically to the surface of HCASMC consistent with expression of VEGFR2 and NRP1 in these cells. Nevertheless, the level of expression of VEGFR2 in HCASMC appears markedly lower than the level of expression of NRP1 (Figure 3.1) and this was further confirmed by flow cytometry which indicated that only 8.89% of the cells expressed VEGFR2 at their surface. Therefore the relevance of the importance of the VEGF receptor is not clear. Pre-treating the cells with chondroitinase resulted in a significant increase of VEGF-A₁₆₅ binding which might be due in part to the removal of the chondroitin sulphate moiety from NRP1. It could also be due to interactions of high affinity between CS and VEGF-A₁₆₅ within the extracellular matrix (ECM), which once abrogated, allow more specific binding between VEGF-A₁₆₅ and its receptors, NRP1 in particular. In VSMC, NRP1 appears to be the main VEGF receptor that is expressed as a high molecular weight -CS or HS- modified form at significant levels, suggesting that any effect of chondroitinase on ¹²⁵I-VEGF-A₁₆₅ binding is due to increased binding to NRP1. However, VEGFR2 is also expressed at low levels (Figure 3.1) and some studies reported VEGFR1 expression in VSMC (Mata-Greenwood et al., 2003). As the lyases act globally on cell surface GAG-modified proteins, it is difficult to attribute their effects to removal of GAG from NRP1 alone.

Several reports in the literature (Banerjee et al., 2008; Grosskreutz et al., 1999; Shintani et al., 2006) have shown a significant role for VEGF-A₁₆₅ in SMC migration in cultured cells. However, in these studies, the effect of VEGF-A₁₆₅ on migration is considerably less than the effects of serum or PDGF, and they should therefore to be interpreted with caution. I could not detect any effect of VEGF-A₁₆₅ on HCASMC migration at concentrations from 25 to 200 ng/ml.

Since VEGF-A₁₆₅ had no effect on HCASMC migration, I decided to investigate a potential role for NRP1 in SMC migration in response to the potent VSMC mitogens, PDGF-AA and PDGF-BB. I first examined if PDGF-BB binds specifically to HCASMC using radiolabelled ligand. There was indeed specific binding of PDGF-BB to HCASMC. PDGF-BB is the ligand for all three possible PDGF receptors dimers, $\alpha\alpha$, $\beta\beta$ and $\alpha\beta$, and PDGF-BB has also been reported to bind NRP1 (Banerjee et al., 2006). Nevertheless, I did not observe significant specific binding of PDGF-BB to PAE cells that stably over-express NRP1, suggesting that PDGF-BB does not bind to NRP1 directly. Moreover, in a cell free competitive binding assay for VEGF to NRP1, PDGF-BB did not displace biotinylated

VEGF-A binding to NRP1, suggesting that VEGF-A and PDGF-BB do not compete for the same binding sites on NRP1 and further confirming the absence of interaction between PDGF-BB and NRP1 (Appendix A.6). It is not possible to preclude the possibility that PDGF binds to NRP1, but I was unable to obtain convincing evidence in support of this conclusion, despite showing that labelled VEGF-A₁₆₅ and PDGF-BB bound specifically to HCASMC.

PDGF are potent mitogenic factor chemotactic factors for VSMC through the activation of their receptors PDGFR α and PDGFR β (Heldin et al., 1985). As expected, PDGF-BB could induce a strong migratory response of HCASMC, of ≈ 5 fold compared to unstimulated and VEGF-A stimulated cells (Figure 4.9). Pre-treating the cells with chondroitinase resulted in a decrease of migration of HCASMC in response to PDGF-BB. In previous studies by Denholm et al. (2001), chondroitinase treatment inhibited melanoma cell invasion and proliferation as well as endothelial cell proliferation and angiogenesis. Other studies shown that chondroitin and/or dermatan sulfate proteoglycans regulate the binding of platelet factor 4, bFGF, HGF and interferon- γ to their respective receptors in a several cell types (Denholm et al., 2001; Hurt-Camejo et al., 1999; Petersen et al., 1998), it is possible that CS could play a role in the binding of PDGF to its receptors. These results therefore suggest a role for CS in SMC migration, but because chondroitinase has a global effect on the cells, we can not draw any conclusion on the role for the chondroitin modification of NRP1 in particular.

Several NRP1 antagonists were developed in our laboratories, EG3287 is a small bicyclic peptide that binds to the VEGF-A₁₆₅ binding domain of NRP1, and later was synthesised EG00086 which has the same peptide sequence as EG3287, but is modified at its amino-terminus by addition of an octanoyl group, and has somewhat improved potency compared with EG3287. EG3287 has no effect on VEGF-A₁₆₅ binding to VEGFR2 and VEGFR1, but inhibited cross-linking of VEGF-A₁₆₅ to KDR in HUVEC, inhibited stimulation of VEGFR2 and PLC- γ tyrosine phosphorylation, activation of ERK1/2 and prostanoid production (Jia et al., 2006). I used these antagonists to study the effects of NRP1 inactivation on HCASMC migration. Pre-treatment of the cells with the antagonist decreased the migratory response to PDGF-BB, while migration towards PDGF-AA was not altered by the antagonist. Given the structural similarities between PDGF and VEGF, and also evidence that VEGF can signal through PDGFR (Ball et al., 2007), it is plausible that the antagonists could inhibit binding of PDGF and VEGF to NRP1 *via* a similar mecha-

nism. However, my data suggest that it is unlikely that VEGF-A₁₆₅ and PDGF-BB were competing against each other for binding to NRP1 at the same site. It is therefore possible that the binding of the antagonists to NRP1 induces a change of conformation that might render NRP1 less active, and thus impairs the response to PDGF-BB. Why the antagonists specifically affect the response to PDGF-BB and have little effect on PDGF-AA-induced migration is unclear, but probably indicates that NRP1 acts to regulate migratory responses to PDGF-BB and -AA *via* different mechanisms. These mechanisms are investigated in greater detail in the next chapter.

NRP1 is known to be expressed as a soluble form comprising the two CUB and two FV/VIII domains, and because the soluble form could potentially compete with membrane-associated NRP for binding to its ligand, I hypothesised that sNRP1 and sb1 domain, the principal VEGF-A binding domain, might act as an inhibitor of NRP1-mediated effects in VSMC. Pre-incubation of HCASMC with sNRP1 and sb1 significantly inhibited migration in response to PDGF-BB, suggesting that sNRP1 or sb1 complexes and sequesters either a ligand or ligands yet to be identified that are important for NRP1 function, and/or with PDGF receptors at the surface of the cell. Alternatively, sNRP1 might complex with membrane-associated NRP1 to form dimers with reduced activity. The key finding of this chapter is that NRP1 plays a significant role in the stimulation of Human VSMC migration in response to the potent chemoattractants, PDGF-AA and PDGF-BB. The conclusion that NRP1 is important for PDGF-induced migration of HCASMC is supported by the finding that siRNA-mediated NRP1 knockdown significantly inhibited HCASMC migration induced either by PDGF-AA or by PDGF-BB.

During the course of this study, work published by Banerjee et al. (2006) also reported the importance of NRP1 in PDGF-BB induced smooth muscle cell migration. They observed that the depletion of NRP1 production by HAoSMC with short hairpin RNA (shRNA) prevents the PDGF-BB dependent migration of VSMC (Banerjee et al., 2006). They also observed a physical interaction between PDGF-BB and NRP1 *via* co-immunoprecipitation. I was not able to show such an interaction, but as described in the following chapter, I found that NRP1 could form a complex with PDGFR α . This result further supports the role of NRP in PDGF-induced migration.

A role for NRP1 in the migratory response to PDGF is also supported by the finding that over-expression of the NRP1 adenovirus construct lacking the cytoplasmic domain (Ad.NRP1 Δ C) inhibited the chemotactic response of HCASMC to PDGF-AA and PDGF-

BB. In endothelial cells, the NRP1 cytoplasmic domain is known to be able to mediate protein-protein interactions and more precisely the C-terminal three amino acids of NRP1 (SEA-COOH) are required for NRP1-mediated angiogenesis (Wang et al., 2006). A NRP1 construct lacking the C-terminus domain caused an inhibition of the migratory response towards PDGF-AA and PDGF-BB which suggests that NRP1 is required for optimal signalling important for migration downstream of PDGF receptors. It was noted that Ad.NRP1 Δ C over-expression had a more marked inhibitory effect on PDGF-induced HCASMC migration than NRP1 knock down *via* siRNA, and also reduced the basal unstimulated level of migration, whereas NRP1 siRNA did not. These results are consistent with the conclusion that the NRP1 Δ C mutant construct exerts a dominant negative effect, possibly *via* prevention of interactions between the NRP1 cytoplasmic domain and intracellular binding partners important for transducing chemotactic signals, and/or sequestration in inactive complexes of PDGF, PDGFR and possibly other cellular components important for chemotactic signalling. One known NRP1-binding molecule is synectin, and the data shows that synectin knockdown reduced PDGF-BB-induced cell migration, though this effect did not reach statistical significance. I hypothesise that other proteins may interact with the NRP1 cytoplasmic domain and contribute to PDGF-induced chemotactic signalling.

Moreover, the O-linked post-translational modification of NRP1 does not seem to play a role in the migratory response of VSMC, as the over-expression of the NRP1 S612A mutant did not alter VSMC migration towards PDGF-AA or PDGF-BB. This result suggests that the GAG modification is not important for PDGFR-mediated migration. In their study, Shintani et al. (2006) showed that VSMC infected with the same S612A adenoviral construct displayed an increased migration in response to VEGF-A₁₆₅. However, because I could not induce migration of HCASMC in response to VEGF-A₁₆₅ and also because of the relatively small induction observed by this group, I believe these results of Shintani et al. (2006) should be considered with caution.

Chapter 5

NRP1 signalling in vascular cells

Many different cytokines are involved in the regulation of chemotactic endothelial and vascular smooth muscle cell migration during angiogenesis. A major promoter of actin-based motility of EC is VEGF, while one of the most potent migratory cytokines for VSMC is PDGF. The activation of small GTPases of the Rho family is involved in regulating EC migration in response to VEGFR2 activation, but NRP1 did not seem to play a role in the activation of the most common molecules involved in chemotaxis in U87 cells such as Akt, Src, Hsp27 or FAK (Frankel et al., results not published). Colleagues in this laboratory found that over-expression of NRP1 in U87MG glioma cells enhanced signalling through p130Cas (Crk-associated substrate), a non-enzymatic docking protein which is strongly implicated in cell migration and invasion (Frankel et al., 2008). The activation of p130Cas was altered in cells depleted of NRP1 by siRNA-mediated knockdown in both EC and VSMC, highlighting a possible role for this protein in regulating vascular cell migration downstream of NRP1. Moreover, they identified the proline-rich tyrosine kinase 2 (Pyk2) as another molecule of which activity is also regulated by NRP1 (Evans et al., MS submitted). This might not appear too surprising in the light of the formation of a stable complex between pyk2 and p130Cas able to transduce signalling in response to stimulation with various growth factors (Kodama et al., 2003) and data showing that VEGF stimulate p130Cas tyrosine phosphorylation (Endo et al., 2003). Given these findings, in this chapter, I investigated the role of a new intracellular signalling pathway downstream of NRP1 involving the docking protein p130Cas in both endothelial cells and VSMC.

5.1 NRP1 signalling in endothelial cells

5.1.1 NRP1 antagonist and NRP1 blocking antibody decrease p130Cas phosphorylation in response to VEGF-A₁₆₅ in endothelial cells

After observing the inhibitory effect of the NRP1 antagonist developed by colleagues in this laboratory on the chemotactic response of vascular cells, I studied the effect of the NRP1 antagonist, EG00086, on the phosphorylation of migratory molecules. A blocking antibody was also used to block VEGF-A₁₆₅ binding to NRP1 and potentially inhibit phosphorylation of effector molecules. Pre-treatment of HUVEC with either the NRP1 blocking antibody (R&D systems, AF566) or the peptide antagonist (EG00086) for 30 minutes, inhibited tyrosine phosphorylation of p130Cas in response to VEGF-A₁₆₅. Pyk2 activation was also reduced (Evans et al., MS submitted), but other signalling molecules were not affected including Akt, Hsp27 and ERK. Quantification of p130Cas phosphorylation shows that when the cells were pre-treated with either reagent, VEGF-A₁₆₅ failed to trigger the activation seen in control cells (Figure 5.1).

5.1.2 NRP1 knockdown decreases p130Cas phosphorylation in response to VEGF-A₁₆₅ in endothelial cells

To further implicate NRP1 as an essential mediator in p130Cas phosphorylation, I transfected endothelial cells with siRNA using Oligofectamine 2000. 72 hours later and after overnight incubation in medium with 0.5% serum, cells were stimulated for 10 minutes with VEGF-A₁₆₅, lysed with RIPA buffer and lysates were blotted for NRP1 expression, to confirm knockdown of the receptor, p130Cas phosphorylation at Tyrosine 410, and the total level of p130Cas. A clear knockdown was obtained in HUVEC as indicated by Western blot (Figure 5.2). Knocking down NRP1 clearly inhibited p130Cas phosphorylation and, quantification of phospho-p130Cas shows a complete lack of activation by VEGF-A₁₆₅ in NRP1 siRNA transfected cells, indicating a role for NRP1 in p130Cas activation in endothelial cells (Figure 5.2).

5.1.3 p130Cas plays a role in endothelial cell migration

To confirm a role for p130Cas in endothelial cell migration, I investigated the level of migratory response of HUVEC in response to VEGF-A₁₆₅ after p130Cas knockdown, as shown in Figure 5.3 (inset). Three days after knockdown, the cells were used to perform a migration assay and cells that had been transfected with p130Cas siRNA displayed

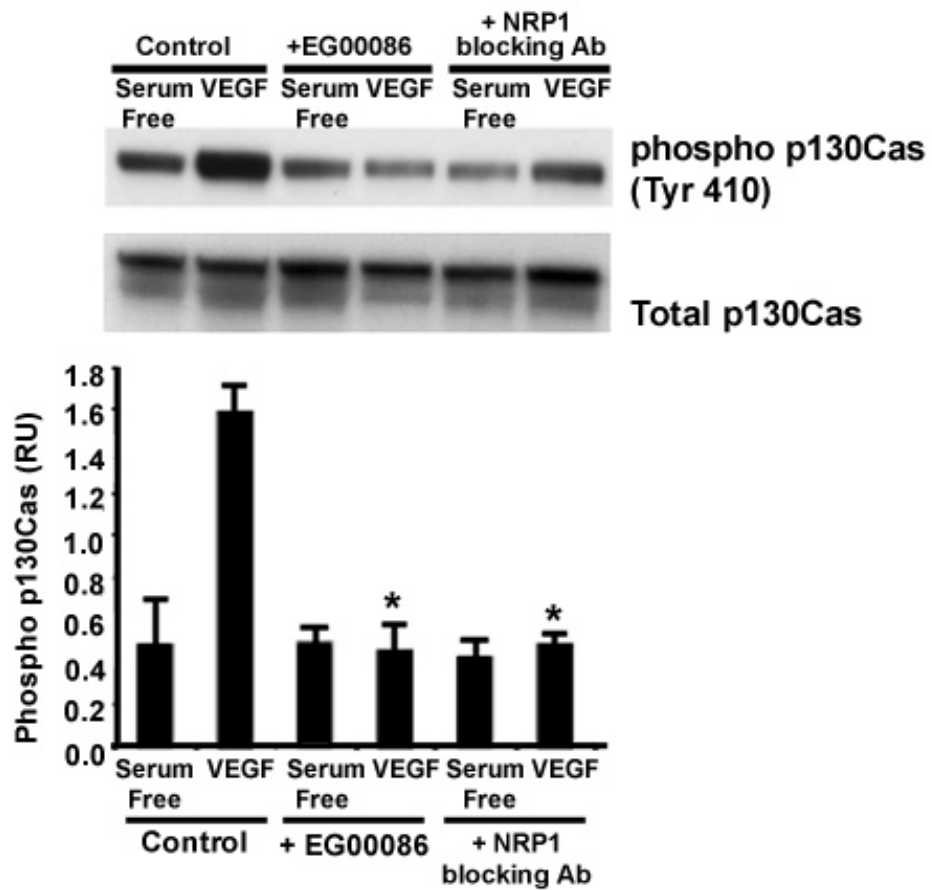


Figure 5.1: NRP1 antagonist and NRP1 blocking antibody decrease p130Cas phosphorylation in response to VEGF-A₁₆₅ in EC

HUVECs were incubated overnight in medium containing 0.5% serum and pre-incubated with 100 μ M EG00086 or NRP1 blocking antibody, and then stimulated for 10 minutes with VEGF-A₁₆₅ (25 ng/ml) or with serum-free medium and lysed in RIPA buffer. Cell lysates were then prepared, blotted, and probed with the indicated antibodies. Quantitation of p130Cas phosphorylation was performed by densitometry using Image J. Data from three independent experiments are presented as p130Cas phosphorylation in relative units (RU) (means \pm SEM) normalised to total p130Cas. *P<0.05 *versus* VEGF control.

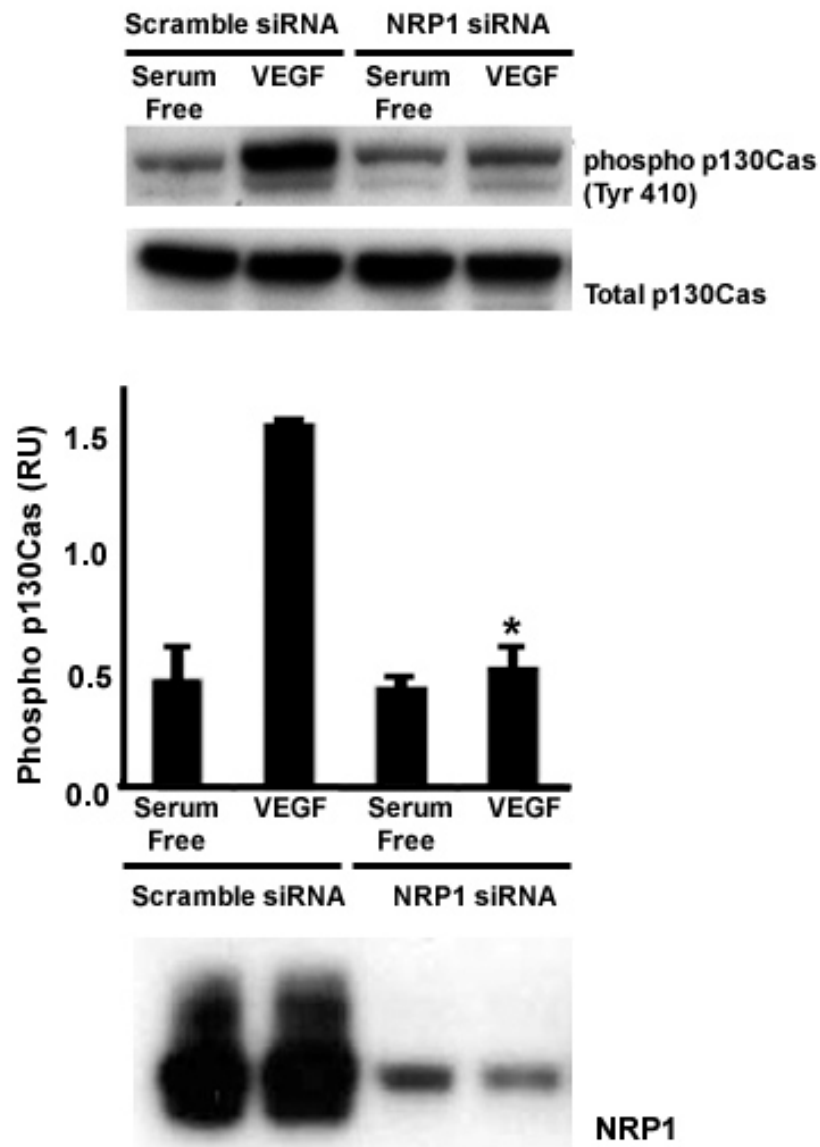


Figure 5.2: NRP1 knockdown decreases p130Cas phosphorylation in response to VEGF-A₁₆₅ in EC

HUVEC cells were previously transfected with NRP1 or scrambled control siRNAs using oligofectamine. After 48 hours, cells were incubated overnight in EC medium containing 0.5% serum. Cells were then stimulated for 10 minutes with 25 ng/ml VEGF-A₁₆₅ or with serum-free medium, and lysed in RIPA buffer. Cell lysates were then prepared, blotted, and probed with the indicated antibodies. Quantitation of p130Cas phosphorylation was performed by densitometry using Image J. Data from three independent experiments are presented as p130Cas phosphorylation in relative units (RU) (means +/- SEM) normalised to total p130Cas. *P<0.05 *versus* VEGF with scrambled siRNA.

a significantly reduced migratory response to VEGF-A₁₆₅ compared to cells transfected with scrambled siRNA. This result strongly implies a role for p130Cas in the migration of endothelial cells.

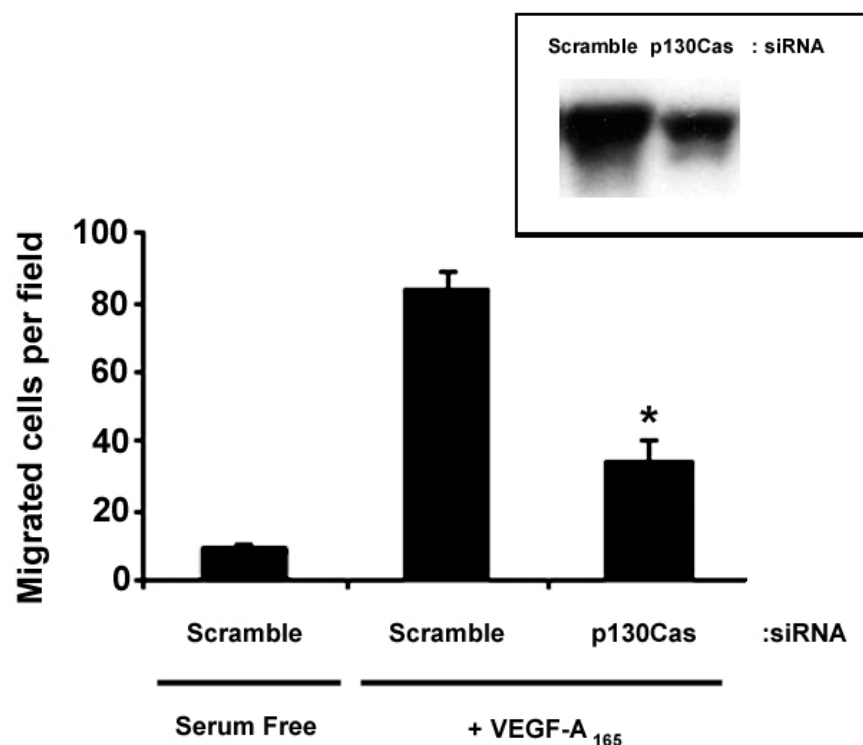


Figure 5.3: p130Cas plays a role in EC migration

HUVECs were transfected with p130Cas or scrambled control siRNA for 72h and then harvested to perform a migration assay in response to VEGF-A₁₆₅ (25 ng/ml). After 4 hours, the cells that migrated through the porous membrane were fixed, stained and counted at a x200 magnification. Bars represent the mean of three experiments +/- SEM. *P<0.05 *versus* VEGF with scrambled siRNA.

Inset: Lysates of HUVEC prepared three days after transfection with scrambled or p130Cas siRNA were blotted with total p130Cas antibody.

5.1.4 NRP1 Δ C mutant overexpression decreases VEGF-A₁₆₅ induced phosphorylation of p130Cas and migration in endothelial cells

As shown in the previous chapter, the NRP1 cytoplasmic domain plays a role in endothelial cell migration, since infection of EC with an adenovirus over-expressing the NRP1 mutant lacking its cytosolic domain results in a significant reduction of migration in response to VEGF-A₁₆₅ (Figure 4.5). It is known that synectin interacts with the cytoplasmic domain of NRP1, regulating in part EC migration, but I further investigated if this decrease in

migration was also correlated with a decrease in phosphorylation of p130Cas. Cells were infected with either GFP, NRP1 WT or NRP1 Δ C mutant for 72h and incubated with medium containing 0.5% serum for the last 12h before being stimulated with 25 ng/ml of VEGF-A₁₆₅. VEGF-A₁₆₅ stimulation resulted in the significant activation of p130Cas at Tyrosine 410 and infection with the adenovirus construct overexpressing NRP1 WT did not alter p130Cas activation. In contrast, over-expression of the NRP1 construct lacking the cytoplasmic domain resulted in a significant decrease of p130Cas activation in response to VEGF-A₁₆₅ (Figure 5.4). This result indicates that, not only NRP1 is important for p130Cas activation, but that its cytosolic domain conveys some signalling input towards this activation.

5.2 NRP1 signalling in vascular smooth muscle cells

5.2.1 NRP1 and PDGFR α co-immunoprecipitate and colocalise at the cell membrane

A mechanism through which NRP1 could regulate migratory responses of HCASMC to PDGF is the formation of heterocomplexes between PDGF receptors and NRP1. As seen in Figure 3.1, HCASMC express similar levels of PDGFRs α and β , either of which can mediate responses to PDGF-BB, whereas PDGFR β only binds to PDGF-BB and does not recognise PDGF-AA with high affinity. To examine association between PDGFRs and NRP1, I attempted to co-immunoprecipitate NRP1 with PDGFRs from HCASMC lysates. Immunoblots of NRP1 in PDGFR α immunoprecipitates detected significant NRP1 compared to IgG controls, indicating that PDGFR α associated with NRP1 (Figure 5.5). However, NRP1 was not detected in PDGFR β immunoprecipitates (5.6), indicating that in contrast to the α receptor, PDGFR β might not interact with NRP1. Co-immunofluorescent staining of NRP1 and PDGFR α in HCASMC also revealed significant colocalisation of these molecules at the cell membrane, consistent with the conclusion that they associate (Figure 5.7). In contrast, co-staining of NRP1 and PDGFR β revealed little significant colocalisation (Figure 5.8).

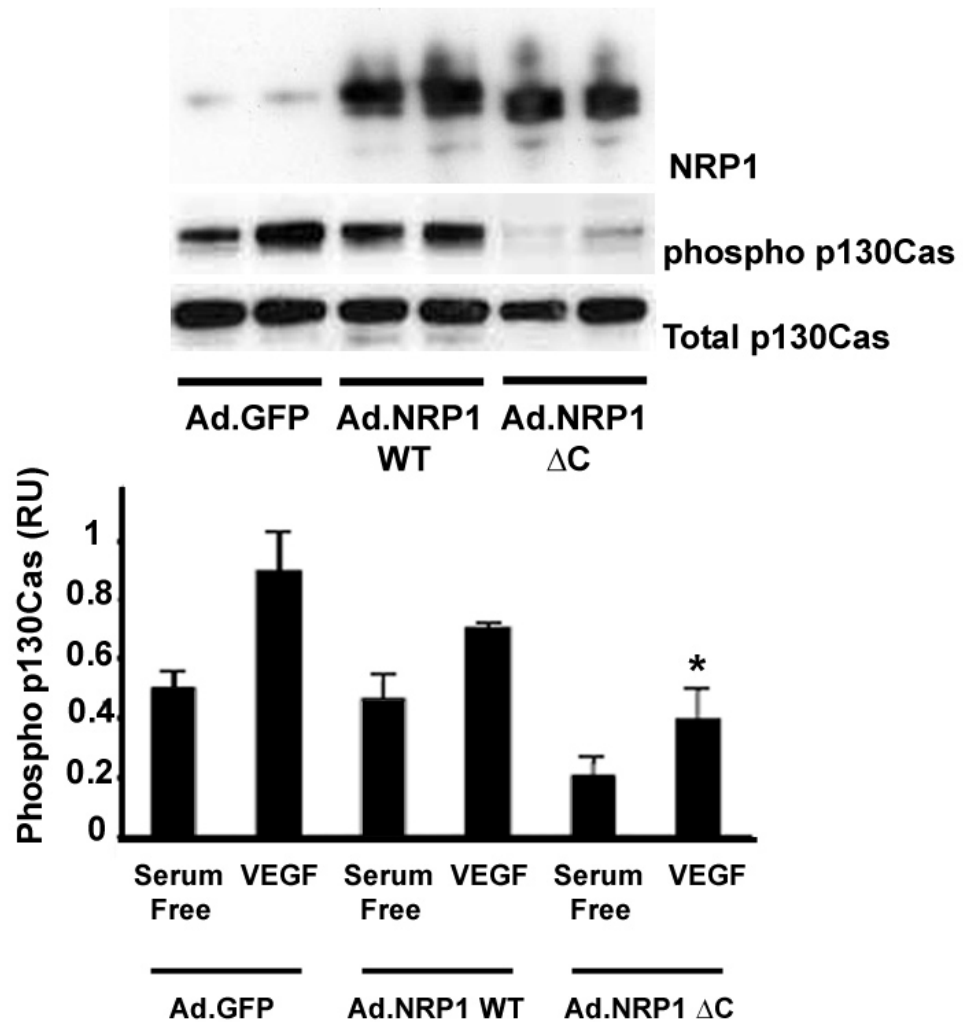


Figure 5.4: NRP1 Δ C mutant overexpression decreases VEGF- A_{165} induced phosphorylation of p130Cas and migration in EC

HUVECs were infected with adenoviral constructs over-expressing either GFP, NRP1 WT and NRP1 Δ C and 48h later were incubated in medium containing 0.5% serum overnight. Cells were then stimulated for 10 minutes with VEGF- A_{165} (25 ng/ml) or with serum free medium and lysed in RIPA buffer. Cell lysates were then prepared, blotted, and probed with the indicated antibodies. Quantitation of p130Cas phosphorylation was performed by densitometry using Image J. Data from three independent experiments are presented as p130Cas phosphorylation relative units (RU) (means \pm SEM) normalised to total p130Cas. * $P < 0.05$ versus VEGF with Ad.NRP1 WT.

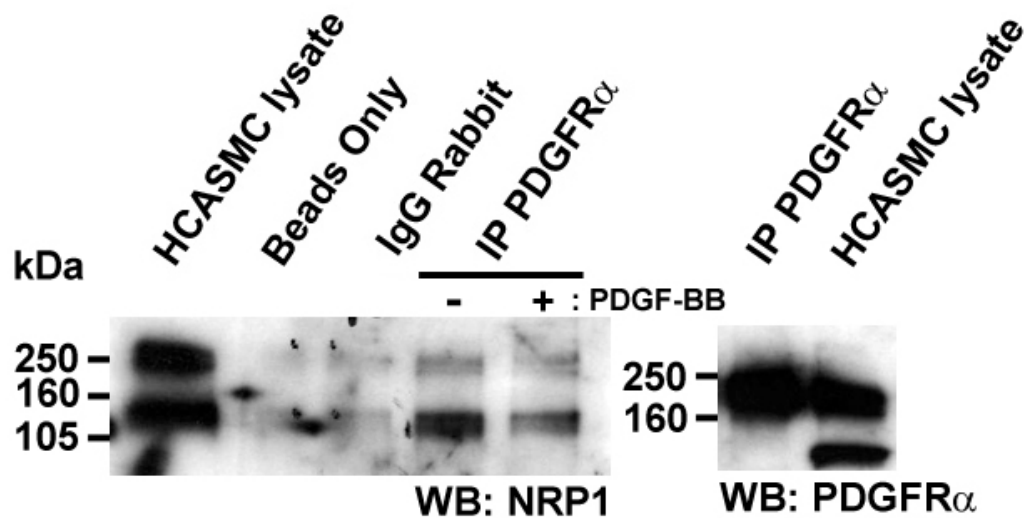


Figure 5.5: NRP1 and PDGFR α co-immunoprecipitate

HCASMC were incubated overnight in serum free medium, incubated with (+) PDGF-BB (30 ng/ml) or with serum free medium (-) and then lysed. Protein lysates were pre-cleared by incubating with washed protein A/G plus agarose beads for 30 minutes followed by antibody binding overnight at 4°C with PDGFR α antibody. Rabbit IgG and agarose beads alone were used as control for non-specific binding of the antibody. The day after, protein A/G plus agarose beads were added to capture the immunocomplex for 4 hours at 4°C in a cold room on a rotator. The IP complex was then dissociated by incubation with sample buffer and heating at 100°C, and run on a 4-12% Bis-Tris gel. Proteins were transferred to membranes which were then incubated with NRP1 antibodies and HRP-labeled anti-goat secondary antibody. The efficiency of the IP was assessed by blotting the immunocomplex for PDGFR α and ran along some HCASMC lysate (right hand blot). The Western blot shown are representative of two independent experiments yielding similar results.

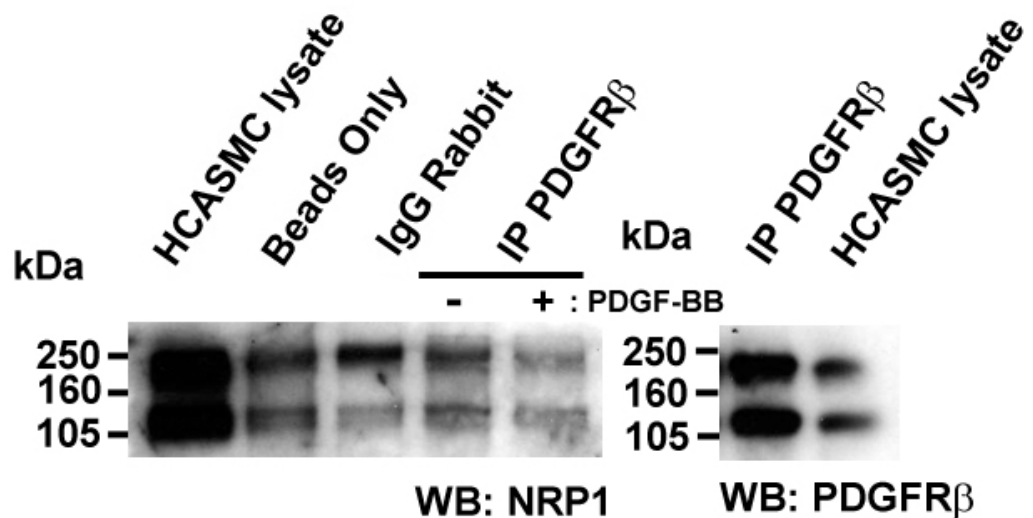


Figure 5.6: **NRP1 and PDGFR β do not co-immunoprecipitate**

HCASMC were incubated overnight in serum free medium, incubated with (+) PDGF-BB (30 ng/ml) or with serum free medium (-) and then lysed. Protein lysates were pre-cleared by incubating with washed protein A/G plus agarose beads for 30 minutes followed by antibody binding overnight at 4°C with PDGFR β antibody. Rabbit IgG and agarose beads alone were used as control for non-specific binding of the antibody. The day after, protein A/G plus agarose beads were added to capture the immunocomplex for 4 hours at 4°C in a cold room on a rotator. The IP complex was then dissociated by incubation with sample buffer and heating at 100°C, and run on a 4-12% Bis-Tris gel. Proteins were transferred to membranes which were then incubated with NRP1 antibodies and HRP-labeled anti-goat secondary antibody. The efficiency of the IP was assessed by blotting the immunocomplex for PDGFR β and ran along some HCASMC lysate (right hand blot). The Western blot shown are representative of two independent experiments yielding similar results.

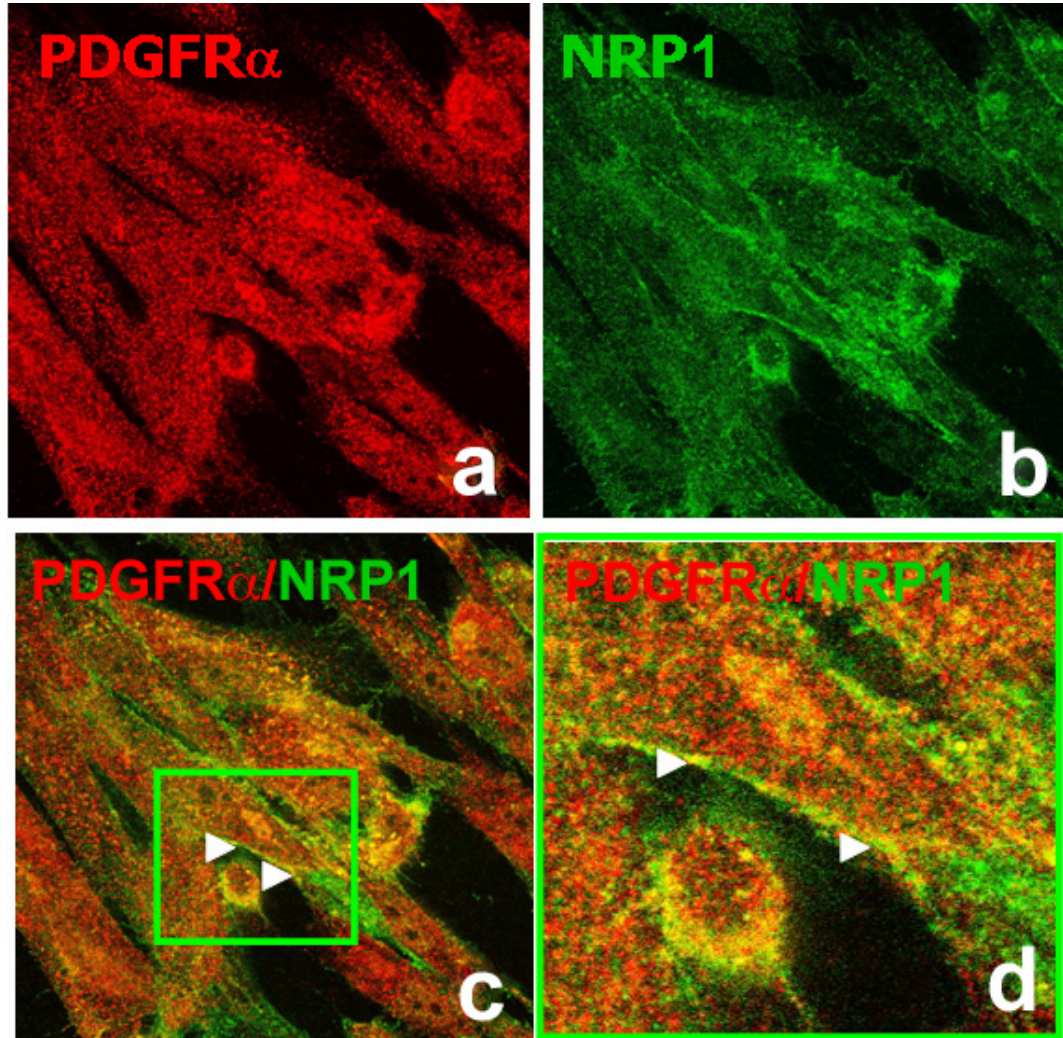


Figure 5.7: NRP1 and PDGFR α colocalisation

HCASMC were fixed with 4% PFA and permeabilised with 0.1% Triton X100. Cells were stained with PDGFR α and NRP1 (C-terminus) antibodies, and then FITC (green, NRP1) anti-goat and Alexa fluor 555 (red, PDGFR α) anti-rabbit were used as secondary reagents. The first panel shows PDGFR α on the red channel (a). On the right, the second panel shows NRP1 staining on the green channel (b). The merged images (c,d) show partial colocalisation between both receptors in the cytoplasm and also at the membrane, as seen in the enlarged panel (d), indicated by the white arrows. Images are representative of three independent experiments.

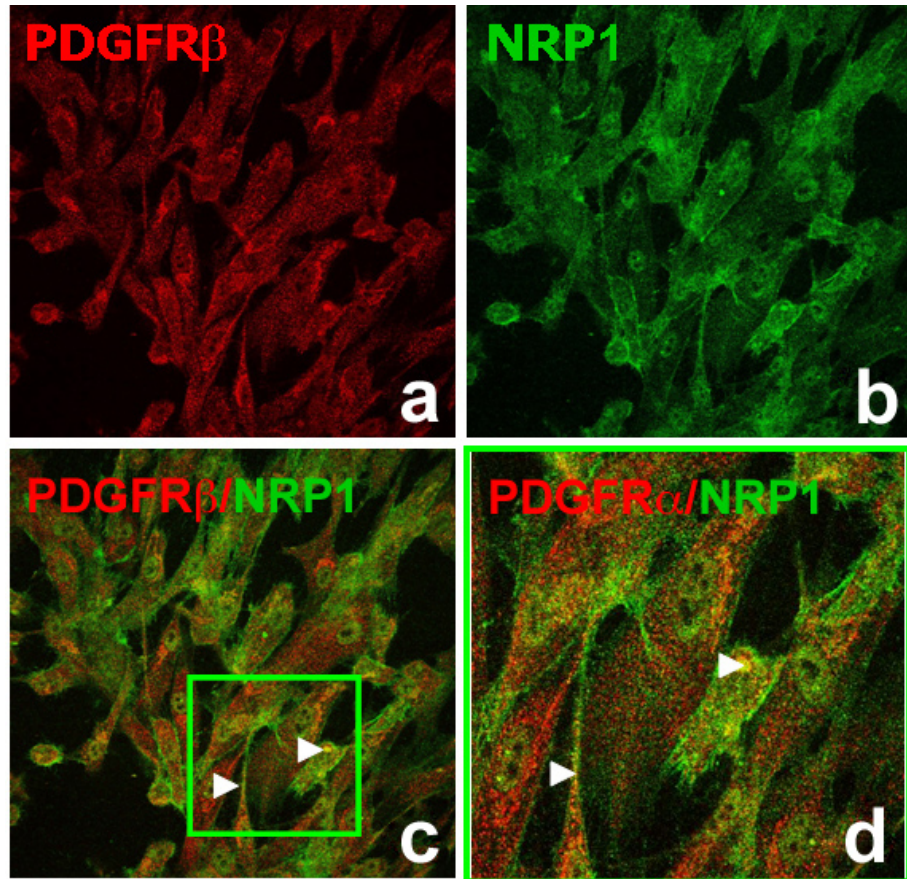


Figure 5.8: NRP1 and PDGFR β colocalisation

HCASMC were fixed with 4% PFA and permeabilised with 0.1% Triton X100. Cells were stained with PDGFR β and NRP1 (C-terminus) antibodies, and then FITC (green, NRP1) anti-goat and Alexa fluor 555 (red, PDFGR β) anti-rabbit were used as secondary reagents. The first panel shows PDGFR β on the red channel (a). On the right, the second panel shows NRP1 staining on the green channel (b). The merged images (c,d) show limited colocalisation between both receptors in the cytoplasm and at the membrane, indicated by the white arrows. Panel d is an enlargement of the boxed area in c. Images are representative of three independent experiments.

5.2.2 NRP1 knockdown decreases p130Cas phosphorylation in response to PDGF in VSMC

PDGF-BB increased p130Cas Tyrosine phosphorylation in HCASMC consistent with this pathway playing a role in the chemotactic response to PDGF-BB (Figure 5.9). Depletion of NRP1 by siRNA-mediated knockdown reduced PDGF-BB-induced p130Cas Tyrosine phosphorylation, but had no significant effect on PDGF-BB-induced activation of ERKs 1/2 or Akt (Figure 5.9). PDGF-AA also stimulated p130Cas tyrosine phosphorylation, though its effect was weaker than that of PDGF-BB, and this response to PDGF-AA was also reduced by NRP1 siRNA (Figure 5.10).

5.2.3 NRP1 Δ C mutant overexpression decreases PDGF-BB induced phosphorylation in VSMC

The role of the NRP1 cytosolic domain in PDGF-induced VSMC p130Cas signalling was examined by over-expressing the NRP1 Δ C mutant in HCASMC. As shown in Figure 5.11, Ad.NRP1 Δ C inhibited PDGF-BB-induced p130Cas tyrosine phosphorylation. Quantification of data from three independent experiments showed that Ad.NRP1 Δ C markedly and significantly reduced the stimulation of p130Cas tyrosine phosphorylation by PDGF-BB (Figure 5.11). In contrast, it was noted that adenoviral wild-type NRP1 over-expression was associated with some enhancement of p130Cas tyrosine phosphorylation, though the effect of Ad.NRP1 WT was not statistically significant (Figure 5.11).

5.2.4 p130Cas plays a role in PDGF-induced VSMC migration

While p130Cas is implicated in the mechanisms underlying cell migration and associated cellular processes such as cytoskeletal organisation (Defilippi et al., 2006), the role of this adapter protein in SMC migration has been little investigated. The role of p130Cas in PDGF-induced cell migration was examined by determining the effect of p130Cas knockdown on chemotaxis. As shown in Figure 5.12, p130Cas siRNA significantly reduced the migratory response of HCASMC to PDGF-BB by an amount similar to the effect of NRP1 knockdown, and also reduced the increase in migration induced by PDGF-AA, though the latter effect was not statistically significant.

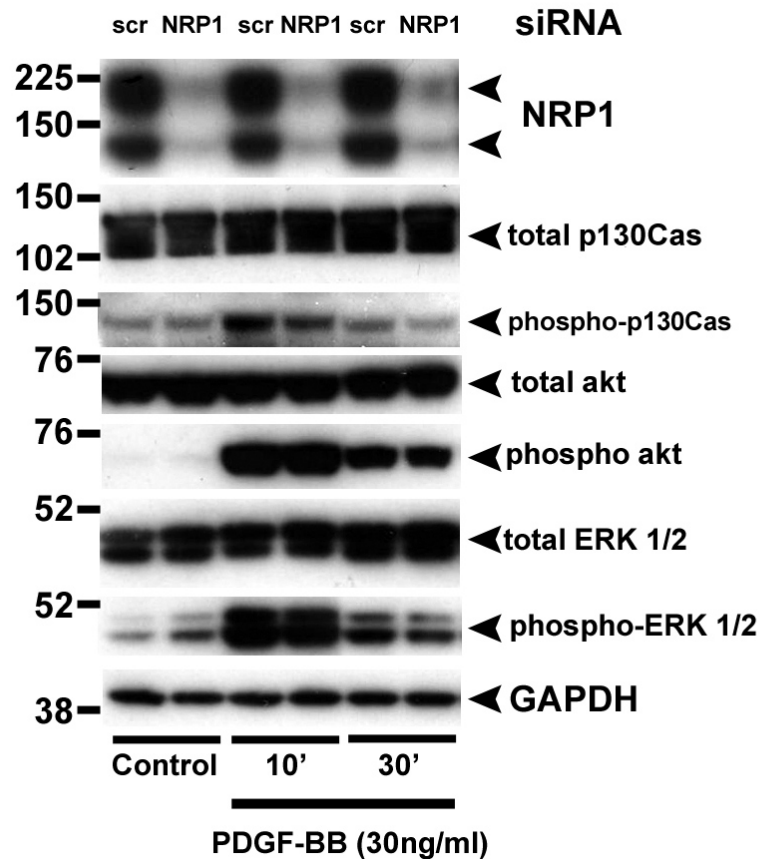


Figure 5.9: NRP1 knockdown decreases p130Cas phosphorylation in response to PDGF-BB in VSMC

HCASMC were previously transfected with NRP1 or scrambled (scr) siRNAs. After 48 hours, cells were incubated overnight in serum free SMC medium. Cells were then stimulated for 10 and 30 minutes with PDGF-BB (30 ng/ml) and lysed in RIPA buffer. Cell lysates were then prepared, blotted, and probed with the indicated antibodies. Results are representative of three independent experiments yielding similar results. Quantitation of p130Cas phosphorylation was performed by densitometry using Image J. Data from three independent experiments are presented as p130Cas phosphorylation relative units (RU) (means +/- SEM) normalised to total p130Cas. * $P < 0.05$ versus PDGF with scramble siRNA at the corresponding time point.

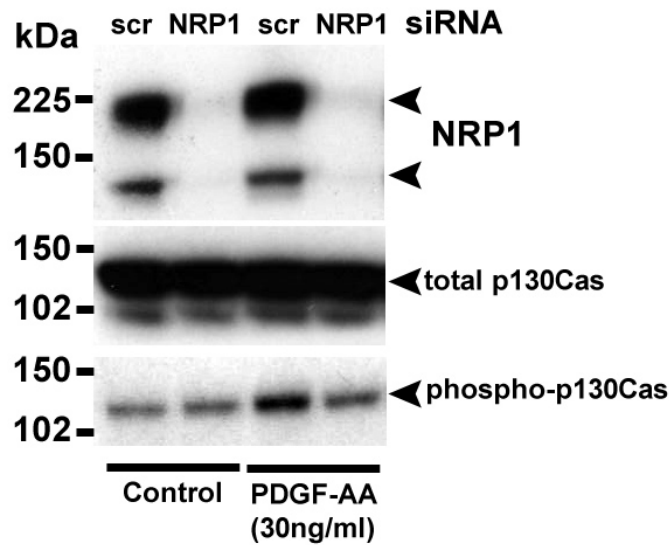


Figure 5.10: NRP1 knockdown decreases p130Cas phosphorylation in response to PDGF-AA in VSMC

HCASMC were previously transfected with NRP1 and scrambled control (scr) siRNAs. After 48 hours, cells were incubated overnight in serum free SMC medium. Cells were then stimulated for 10 minutes with PDGF-AA (30 ng/ml) and lysed in RIPA buffer. Cell lysates were then prepared, blotted, and probed with the indicated antibodies. Results are representative of two independent experiments yielding similar results.

5.2.5 NRP1 modulates PDGFR α activation

In order to further understand the mechanism through which NRP1 regulated PDGF-BB chemotactic signalling, I investigated the contribution of NRP1 to PDGF-BB-stimulated chemotactic cell signalling in HCASMC. I first examined whether NRP1 is required for activation of PDGFRs in VSMC, by determining the effect of NRP1 knockdown on PDGF-induced receptor activation in a quantitative ELISA specific for phosphorylation of PDGFR α and PDGFR β . The results indicate that NRP1 knockdown markedly inhibited PDGF-AA stimulation of PDGFR α activity and significantly reduced PDGF-BB induction of PDGFR α activity, though the effect of NRP1 siRNA on the response to PDGF-BB was noticeably smaller than for PDGF-AA (Figure 5.13, upper). In contrast, NRP1 siRNA had no significant effect on PDGF-BB-induced PDGFR β phosphorylation (Figure 5.13, lower). It was confirmed that PDGF-AA did not stimulate PDGFR β phosphorylation.

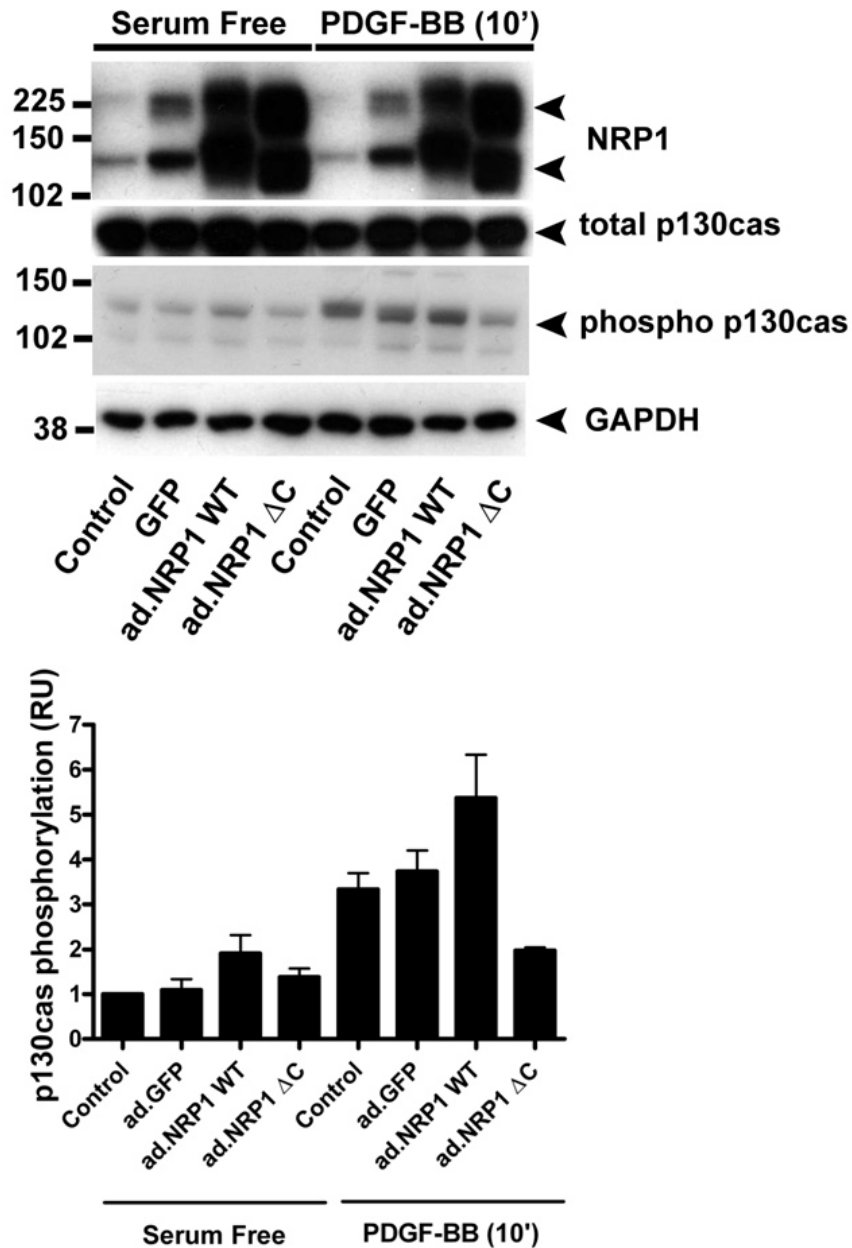


Figure 5.11: NRP1 Δ C mutant overexpression decreases PDGF-BB-induced phosphorylation of p130Cas in VSMC

HCASMC were infected with adenoviral constructs overexpressing either GFP, NRP1 WT or NRP1 Δ C and 48h later were incubated in serum-free medium overnight. Cells were then stimulated for 10 minutes with PDGF-BB (30 ng/ml) and lysed in RIPA buffer. Cell lysates were then prepared, blotted, and probed with the indicated antibodies. Quantitation of p130Cas phosphorylation was performed by densitometry using Image J. Data from three independent experiments are presented as p130Cas phosphorylation relative units (RU) (means \pm SEM) obtained from three independent experiments and normalised to total p130Cas. * $P < 0.05$ versus Control, Ad.GFP and Ad.NRP1 WT.

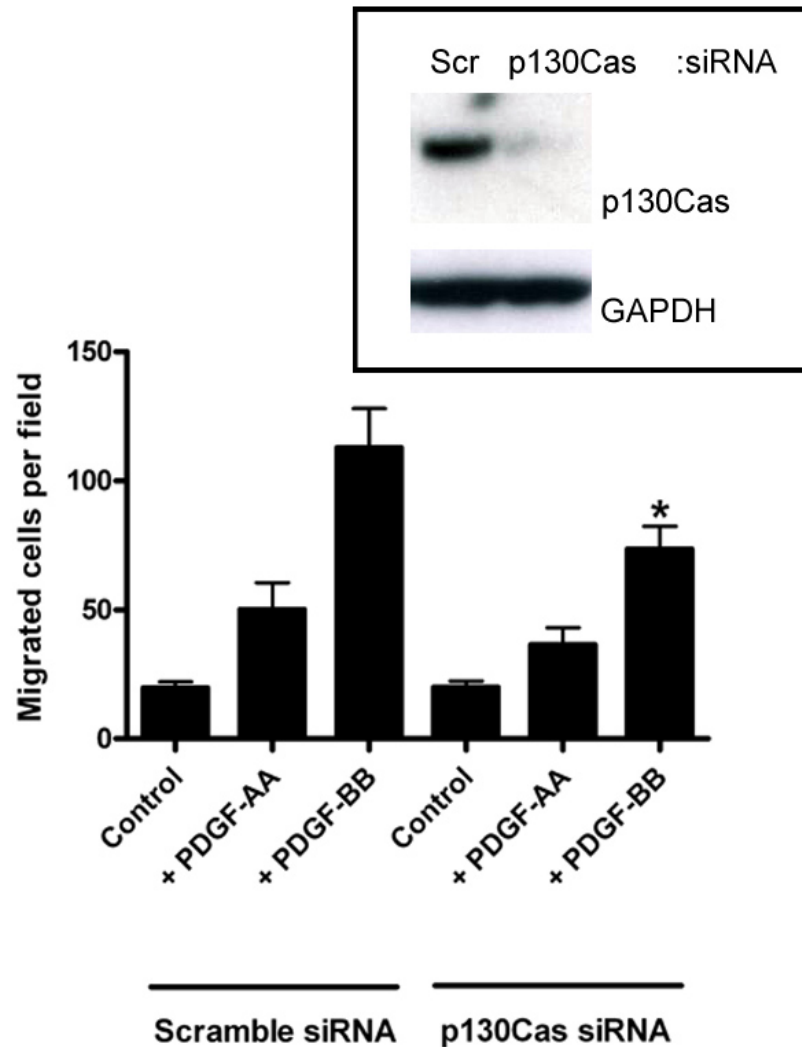


Figure 5.12: p130Cas plays a role in PDGF-induced VSMC migration

HCASMC cells were transfected with p130Cas siRNA for 72h and then harvested to perform migration assays in response to PDGF-AA and PDGF-BB (both 30 ng/ml). After 4 hours, the cells that migrated through the porous membrane were fixed, stained and counted at x200 magnification. Bars represent the means of three independent experiments +/- SEM. *P<0.05 versus PDGF-BB plus scrambled siRNA.

Inset: HCASMC lysates prepared 72h after transfection with scrambled or p130Cas siRNA were blotted with total p130Cas or GAPDH antibodies.

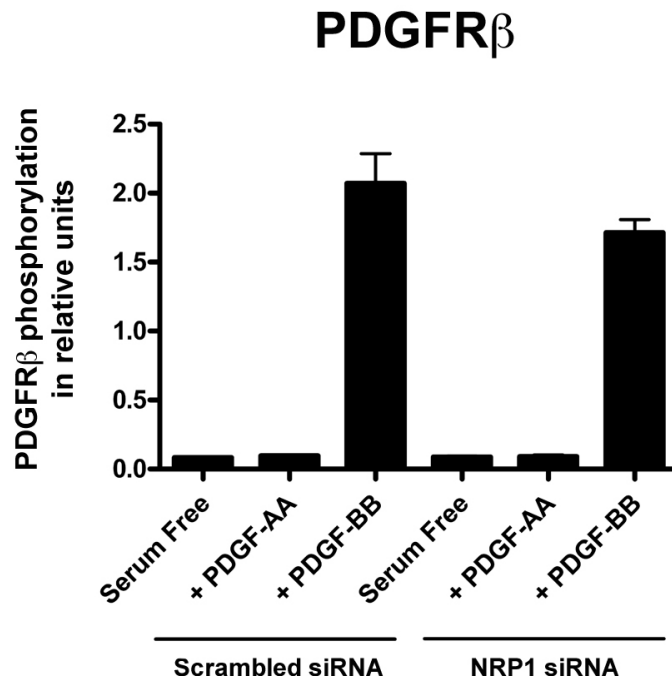
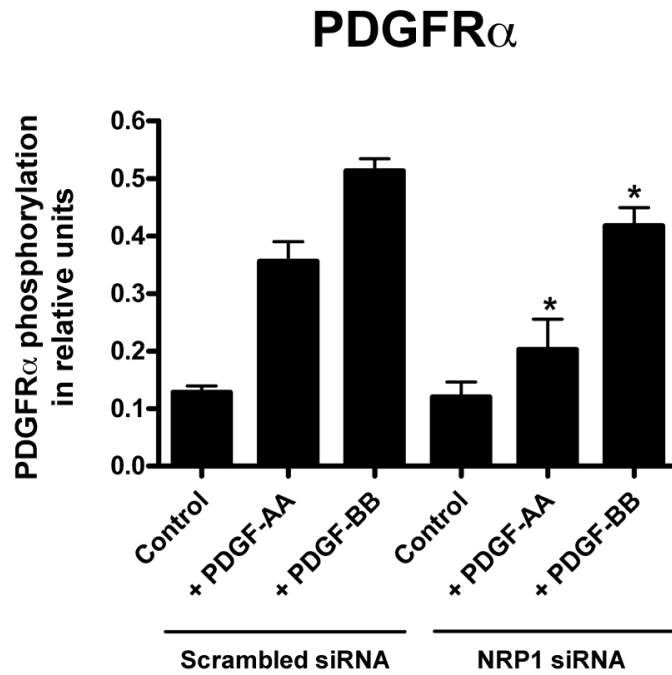


Figure 5.13: Effects of NRP1 knockdown on PDGFRs activity

HCASMC cells were transfected with NRP1 siRNA for 60h and then incubated with serum-free SMC medium for a further 12h. Cells were then stimulated for 10 minutes with PDGF-AA and PDGF-BB (both 30 ng/ml) and lysed in the appropriate ELISA buffer. The protein lysate was then added to 96-well plate pre-coated with phospho- (or total) PDGFR α (upper) and phospho- (or total) PDGFR β antibodies. Detection antibody was finally added and the reaction stopped with appropriate solutions before the absorbance was read using a Tecan Genios plate reader at A450nm with a reference wavelength at 595nm. Bars represent the means of three independent experiments +/- SEM. *P<0.05 *versus* PDGFs plus scrambled siRNA.

5.3 Discussion

5.3.1 Role of NRP1 in VEGF signalling

At the beginning of this study, NRP1 was already known to play an essential role in EC migration by forming complexes with VEGFR2 and further enhancing the VEGF-A₁₆₅ induced signalling response (Soker et al., 2002). Nevertheless, only one molecule, synectin, has been identified as a downstream effector of NRP1; and the receptor is still widely seen as a purely binding entity of the receptor complex, while VEGFR2 is solely responsible for mediating signalling. Here, several approaches were used to inhibit NRP1 function and investigate its role in signalling: the use of a blocking antibody and of a peptide antagonist that both block specifically VEGF-A₁₆₅ and siRNA-mediated knockdown. All these approaches resulted in the decrease of the phosphorylation at Tyrosine 410 of the non-enzymatic docking protein, p130Cas. Moreover, when over-expressing a NRP1 mutant lacking the cytoplasmic domain in EC, phosphorylation of p130Cas was reduced, and the EC migratory response towards VEGF-A₁₆₅ was also decreased. The role of p130Cas was further verified by knocking it down in HUVEC: p130Cas-depleted cells were less able to migrate towards a VEGF-A₁₆₅ gradient, indicating a role for p130Cas in chemotaxis. These findings demonstrate a critical role for NRP1 in mediating stimulation of p130Cas Tyrosine phosphorylation in response to VEGF-A₁₆₅, and further show that this novel NRP1/p130Cas pathway is required for chemotactic migration induced by VEGF-A₁₆₅ in endothelial cells. They also suggest that the NRP1 intracellular domain may itself either transduce specific intracellular signals, or participate in protein-protein interactions, resulting in the activation and/or recruitment of signalling effectors to the NRP1/VEGFR2 complex.

Previous findings have shown that VEGF-A₁₆₅ stimulates p130Cas Tyrosine phosphorylation in human brain microvascular EC (HBMEC) (Avraham et al., 2003) and in HAEC (Endo et al., 2003), but the specific role for p130Cas in VEGF-dependent migration has been little studied, and the mechanisms linking VEGF receptors to p130Cas signalling are unclear. VEGF-A₁₆₅ also activates Focal adhesion kinase (FAK) (Abedi and Zachary, 1997) and p130Cas SH3 domain associates with FAK (Polte and Hanks, 1995) and mediate FAK-promoted cell-migration (Cary et al., 1998). P130Cas also associates with the FAK-related kinase Pyk2 (Ohba et al., 1998) and Pyk2 mediates endothelin-1 signalling in endothelial cells *via* p130Cas (Kodama et al., 2003). VEGF is also known to activate

Pyk2 in HBMEC (Avraham et al., 2003) and in HUVEC (McMullen et al., 2004). Taken together, previous work and the findings presented in this chapter strongly indicate that NRP1 mediates VEGF-A₁₆₅ signalling leading to activation of either FAK and/or Pyk2 and subsequent Tyrosine phosphorylation of p130Cas. Studies by other workers in this laboratory found no effect of NRP1 knockdown on FAK phosphorylation at its major autophosphorylation site, Y397, but showed that NRP1 knockdown inhibits VEGF-induced Pyk2 phosphorylation and that Pyk2 knockdown inhibits VEGF-induced p130Cas Tyrosine phosphorylation. These data suggest that NRP1 is a key mediator of p130Cas signalling *via* Pyk2. However, given the complexity of FAK phosphorylation involving multiple Tyrosine and Serine phosphorylation sites, the possibility is not precluded that NRP1 mediates VEGF-A₁₆₅-stimulated FAK phosphorylation at other sites.

Previous data from this group showed that selective inhibition of VEGF-A₁₆₅ binding to NRP1 using a peptide antagonist partially reduces VEGF-A₁₆₅-induced VEGFR2 phosphorylation at Y1175 and partially inhibits VEGF-A₁₆₅-induced ERK1/2 and Akt activation (Jia et al., 2006). Inhibition of VEGF-A₁₆₅ binding to NRP1 using specific NRP1 blocking antibodies caused only a modest inhibition of VEGFR2 activity and other signalling events (Pan et al., 2007a). Together, previous data indicated that NRP1 made a relatively small contribution to signalling *via* VEGFR2 despite its key role in angiogenesis in development. However, findings presented here show that NRP1 plays a key role in VEGF-A₁₆₅ signalling *via* p130Cas, a molecule with an important role in cell migration, and indicate that NRP1-dependent p130Cas Tyrosine phosphorylation is an important node in VEGF-A₁₆₅-dependent chemotactic signalling. Whether NRP1-dependent p130Cas signalling is important *in vivo* remains to be determined. However, it is of interest that p130Cas knockout mice display aberrant cardiovascular development, similar to the phenotype of NRP1-deficient mice, and that p130Cas is predominantly expressed in the heart and blood vessels at E11.5-E12.5, when the embryonic lethal cardiovascular phenotype is manifested in p130Cas and NRP1 knockout mice (Honda et al., 1998).

The mechanism coupling NRP1 to VEGF-A₁₆₅-induced Pyk2 activation and downstream p130Cas phosphorylation is unclear, but seems to require the NRP1 cytosolic domain. Initial results from co-workers in this lab show that synectin knockdown does not impair VEGF-A₁₆₅-stimulated p130Cas Tyrosine phosphorylation, raising the possibility that other NRP1-associated proteins, yet to be identified, may link NRP1 physically to Pyk2.

5.3.2 Role of NRP1 in PDGF signalling

The conclusion that NRP1 is important for PDGF-BB-induced migration of HCASMC is supported by the effects of NRP1 siRNA and mutant over-expression: NRP1 knockdown significantly inhibited HCASMC migration induced either by PDGF-AA or by PDGF-BB. Overexpression of the NRP1 lacking the cytoplasmic domain (NRP1 Δ C) similarly inhibited the chemotactic response to PDGF-AA and PDGF-BB. Since the NRP1 cytoplasmic domain is known to mediate protein-protein interactions (Cai and Reed, 1999), the fact that a NRP1 construct lacking this domain inhibited the migratory response to PDGF-BB strongly suggests that NRP1 is required for optimal signalling important for migration downstream of PDGFRs. Ad.NRP1 Δ C expression had a more marked inhibitory effect on PDGF-induced HCASMC migration than NRP1 siRNA, and also reduced the basal unstimulated level of migration, whereas NRP1 siRNA did not. These results are consistent with the conclusion that the NRP1 Δ C mutant exerts a dominant negative effect, possibly *via* prevention of interactions between the NRP1 cytoplasmic domain and intracellular binding partners important for transducing chemotactic signals, and/or sequestration in inactive complexes of PDGF, PDGFR and possibly other cellular components important for chemotactic signalling. One known NRP1-binding molecule is synectin, and this study shows that synectin knockdown only tends to reduce PDGF-BB-induced cell migration (Figure 4.16). I therefore hypothesise that other proteins may interact with the NRP1 cytoplasmic domain and contribute to PDGF-BB chemotactic signalling.

Though PDGF-BB has been reported to co-immunoprecipitate with NRP1 (Banerjee et al., 2006), I did not find compelling evidence that PDGF-BB could specifically and with high affinity bind directly to NRP1 either in PAE/NRP1 cells or in a cell-free assay of ligand binding to purified NRP1 b1 domain. These data indicate that PDGF-BB may not be able to bind to the NRP1 extracellular domain with high affinity, though one cannot preclude the possibility that PDGF-BB binding is dependent on other co-factors or co-receptors. The mechanism through which NRP1 regulates PDGF-BB-induced migration in HCASMC may involve protein-protein associations between NRP1 and PDGFRs, similar to the formation of complexes between NRP1 and VEGFR2/KDR, which is thought to be crucial for the role of NRP1 in endothelial responses to VEGF. In support of this hypothesis, I showed that NRP1 co-immunoprecipitates with PDGFR α in HCASMC. However, there was no similar complex formation between PDGFR β and NRP1, indicating that NRP1 may associate preferentially with PDGFR α . Since PDGFR α is a ligand for PDGF-BB

and PDGF-AA homodimers as well as AB heterodimers, these results suggest a possible mechanism in which PDGF-BB and PDGF-AA act through PDGFR α /NRP1 complexes resulting in optimal chemotactic signalling. I further found that NRP1 siRNA-mediated knockdown caused a marked inhibition of PDGFR α activation in response to PDGF-AA, and caused a significant but more modest reduction in PDGF-BB-induced PDGFR α activation. In contrast, NRP1 knockdown had no detectable effect on PDGF-BB induction of PDGFR β activity. The molecular basis for the inhibitory effect of NRP1 knockdown on PDGFR α activity is unclear, but may suggest that the association we found between NRP1 and PDGFR α contributes to receptor activity. Such an effect would also provide an underlying mechanism for the role of NRP1 in PDGF responses in VSMC mediated through PDGFR α . It is important to note that selective NRP1 inhibition in endothelial cells, using either antibodies or antagonistic peptides that specifically block VEGF binding to NRP1 (Jia et al., 2006; Pan et al., 2007a), causes only small or partial reductions in VEGFR2 activity, indicating that NRP1 is not required for VEGFR2 activation in endothelial cells.

Recently, we reported that over-expression of NRP1 in U87 glioma cells is able to regulate tyrosine phosphorylation of p130Cas, an adapter protein, strongly implicated in signalling pathways important for cell migration (Frankel et al., 2008). Here, I have shown that PDGF-BB stimulates p130Cas tyrosine phosphorylation and that this response is partially inhibited by both NRP1 knockdown and by expression of the NRP1 Δ C mutant in vascular smooth muscle cells. PDGF-AA also stimulated p130Cas tyrosine phosphorylation more weakly and this effect was also reduced by NRP1 knockdown. As far as we are aware, these findings demonstrate for the first time that the NRP1 cytosolic domain is required for a specific signalling pathway leading to a functional cellular response. In contrast, NRP1 siRNA had little significant effect on other PDGF-BB-stimulated signalling pathways, including activation of ERK, Akt and FAK. P130Cas is an adapter protein, which is both a substrate for FAK-directed phosphorylation and known to associate with FAK, and FAK is thought to be one of the major distal pathways regulating the activity of p130Cas (Defilippi et al., 2006). Though we observed no detectable change in FAK phosphorylation status at Tyr 397 with NRP1 knockdown, FAK is phosphorylated at multiple sites, and we cannot preclude that NRP1 may also mediate PDGF-dependent FAK phosphorylation important in turn for p130Cas phosphorylation. However, regardless of the precise mechanisms leading to its phosphorylation in HCASMC, p130Cas is a good candidate for mediating PDGF-BB dependent signalling pathways leading to actin cytoskeletal reorganisation and

cell movement. In support of this conclusion, I further demonstrated that p130Cas is essential for the PDGF-BB migratory response, since p130Cas knockdown inhibited migration in response to PDGF-BB. There are no previous reports showing PDGF-BB regulation of p130Cas tyrosine phosphorylation in VSMC or other cell types; indeed, PDGF-BB was shown to increase tyrosine phosphorylation of the Crk-associated substrate (CAS) family member, HEF-1, but not p130Cas, in glioblastoma cells (Natarajan et al., 2006). It remains unclear exactly how NRP1 is able to regulate p130Cas phosphorylation, and investigation of the molecular mechanisms involved warrants further experimental work.

Chapter 6

Discussion

Neuropilins are co-receptors for Semaphorins that were initially discovered in the nervous system and later identified as co-receptors for members of the VEGF family in the vascular system. In the nervous system, they play a role in axonal guidance during embryonic development by mediating growth cone collapse of neurons when binding to their ligands. In the vascular system, the analysis of NRP1 transgenic and deficient mice established an essential role for NRPs in vessel development (Gu et al., 2003; Kawasaki et al., 1999; Kitsukawa et al., 1997; Lee et al., 2002). Moreover, expression of NRPs and functional studies by cancer cells revealed their role in tumour growth and migration, and recent evidence also implicates NRP1 in mediating the primary immune response. All these findings strongly suggest that NRPs are multi-functional co-receptors with essential roles in both the nervous and cardiovascular systems as well as additional roles in other physiological and disease-related settings (Pellet-Many et al., 2008).

At the beginning of this PhD study, I decided to investigate the expression of NRPs in vascular cells and noticed a different expression pattern in HCAEC and HCASMC. Other EC and VSMC, such as HAoSMC and HUVEC also showed similar differences in NRP1 expression typified by expression of a single 130 kDa species in EC and the additional expression of a form of >250 kDa in VSMC. This high molecular weight species was found to correspond to a CS- and HS-GAG modified form expressed in VSMC and, in the case of CS-GAG modified species, also in cancer cells.

My further studies led to the discovery of a role for NRPs in PDGF-stimulated migration of SMC and provided evidence for the colocalisation of NRP1 with PDGFR α , and the occurrence of a PDGFR/PDGF/NRP1 complex in SMC.

Finally, I went on to investigate the possible mechanism(s) underlying the role of NRP1 in VSMC migration. This studies indicated that the phosphorylation of the non-enzymatic docking protein, p130Cas, was reduced in cells depleted of NRP1. Moreover, by using

a NRP1 mutant lacking the cytoplasmic domain, I also showed the importance of the NRP1 cytoplasmic domain in the mediation of PDGF-induced signalling in SMC. Similar findings were obtained in EC and other colleagues in this laboratory further validated this mechanism in cancer cells. Moreover, in SMC, the phosphorylation of PDGFR α was decreased in NRP1 depleted cells indicating the importance of NRP1 in the mediation of PDGFR α signalling in these cells.

6.1 Post-translational modifications

In this thesis, I have shown that a substantial proportion of NRP1 in Human VSMC undergoes post-translational modification by the addition of chondroitin sulphate (CS) and heparan sulphate (HS) glycosaminoglycans (GAG). This modification does not occur for the structurally-related molecule NRP2 or in the two Human endothelial cell types tested (HUVEC and HCAEC). Moreover several cancer cell lines exhibited a similar pattern of glycosylation but involving only CS-GAG (Frankel et al., 2008). While this research was in progress, Shintani et al. (2006) reported modification of NRP1 by both HS- and CS-GAGs in VSMC and EC. In contrast to their findings, I could only detect the addition of CS- and HS-GAG to the core protein in VSMC and did not identify such glycosylation in endothelial cells. The GAG moiety is added to the Serine residue in position 612 which is followed by a Glycine, SG motifs being the preferred sites of GAG attachment, in the linker region between the b2 and MAM domains. This Serine residue is conserved amongst mammals, but it is not present in the amino acid sequence of NRP2. Similar SG sites exist in the amino acid sequence of NRP1 and are potential sites of attachment of GAG. Nevertheless, a single point mutation to Serine 612 resulted in the complete disappearance of the high molecular weight NRP1 species on Western blots. However, it was observed that expression of a double NRP1 mutant at Serines 240 and 432 resulted in a decreased expression of the GAG-modified NRP1 species compared to NRP1 WT. This observation suggests that other sites of glycosylation may contribute to O-linked glycosylation, though as the S612A mutation completely abolished the modification, this is clearly the primary site of GAG addition.

It is unclear why O-linked glycosylations are different in different cell lines: either CS- or HS-modified in HCASMC and HAoSMC, CS-modified in several cancer cell lines or non-modified in HUVEC and HCAEC. It is possible that the expression of the enzymes responsible for GAG addition and elongation are differentially expressed in the different

cell types. It seems unlikely, however, that EC lack the enzymatic machinery required for GAG addition to core proteins. An alternative explanation for the lack of GAG-modified NRP1 in EC is that NRP1 follows an itinerary through the Golgi apparatus which through some undefined mechanism, prevents its post-translational modification. It is also plausible that EC possess mechanisms for rapid removal of GAGs, such that GAG-modified NRP1 is expressed at an undetectable level. There might also be a possible interaction of NRP1 with other co-receptors that make the NRP1 glycosylation site inaccessible for the transferase to elongate the GAG chain; since EC express different co-receptors for NRP1 than VSMC, it might explain their difference in glycosylation. This is one hypothesis that I will look further into by expressing NRP1 with its co-receptors in PAE cells. Data from Shintani et al. (2006) suggest that NRP1 expression and its GAG modification post-transcriptionally regulate VEGFR2 protein expression, nevertheless, I would like to further test if the presence of VEGFR2 influences the expression of the NRP1 GAG modification. It is also unclear why NRP2 does not undergo GAG-modification. However, one possibility is that the tertiary structure of NRP2 might hide potential consensus sites and therefore prevent recognition by the enzymes essential for GAG chain initiation or elongation.

The fact that the same serine residue can be modified by different GAG is also unusual. Indeed, other GAG-modified proteins (for example, syndecan 1) contain distinct sites of unambiguous attachment of specific GAG (Kokenyesi and Bernfield, 1994). Clearly, the selection of the nature of the GAG might not be dictated by the amino-acid sequence of the core protein only but by some other factors such as the activity of the protein synthases, and the nature of other co-receptors present at the cell surface, and/or the cell extracellular matrix.

In addition, both NRP1 and NRP2 appear to be modified by asparagine (N)-linked glycosylations as indicated by the effect of tunicamycin-mediated inhibition of N-glycosylation on the apparent molecular weight of NRPs (Figure 3.6). This type of post-translational modification is relatively common and occurs in many proteins. N-linked glycosylations can profoundly affect protein folding, oligomerisation and stability (Mitra et al., 2006). Neither the site(s) nor function of NRP1 N-glycosylations are known. In future work, site-directed mutagenesis of predicted sites for N-glycosylation, corresponding to the tripeptide sequences Asn-X-Ser, Asn-X-Thr or Asn-X-Cys, where X can be any amino acid except Proline, may be performed to identify the site and function of this modification.

Specific VEGF-A₁₆₅ binding was not altered by pre-treatment with lyases in endothelial

cells but, interestingly, was increased in smooth muscle cells treated with chondroitinase. Since GAG modification of NRP1 occurred in SMC but not in EC, and that other receptors for VEGF were not significantly expressed in VSMC, these findings suggest that the addition of the CS chain to NRP1 in SMC inhibits VEGF-A₁₆₅ binding, maybe by blocking accessibility of the growth factor to its specific binding site.

Though VEGF-A₁₆₅ was able to bind specifically to SMC, no migratory response towards a VEGF-A₁₆₅ gradient was observed, and this was not altered by chondroitinase pre-treatment. The lack of responsiveness to VEGF in HCASMC may be due to the low expression of VEGFR2/KDR, the major VEGF-A₁₆₅ signalling receptor. In order to study the role of NRP1 in migration of HCASMC, I investigated its contribution to the effects of PDGF-BB, known to be a strong chemoattractant for SMC. Chondroitinase resulted in a decreased migratory response of HCASMC towards PDGF-BB, suggesting that CS-GAG NRP1 is important for PDGF-BB chemoattraction.

However, treatment of cells with chondroitinase and heparitinase (heparinase III) to remove GAGs does not address the role of protein-specific GAG modification, since many proteins are modified in this way, and removal of GAGs from different proteins may have diverse and sometimes confounding effects on cell function. In general, binding of growth factors to a proteoglycan (PG) could play a role in four different functions: (1) determining the concentration of the growth factor near the cell membrane and its interaction with other cell surface receptors; (2) participation of the PG in a co-receptor complex with different abilities to recognise ligands or transduce signalling relative to the more simple growth factor/receptor complex; (3) inhibition of growth factor diffusion to create a more localised signal; and (4) formation of a pool of growth factor that can be released by proteolysis of the GAG moiety or the growth factor (Iozzo, 2005). However, defining GAG function in the VEGF-A₁₆₅/VEGFR2/NRP1 complex is highly problematic since each component is itself able to bind heparin and HS, and because NRP1 can be GAG-modified (Shintani et al., 2006).

To probe the function of NRP1 GAG modification, I generated a mutant S612A NRP1 adenovirus construct for high efficiency transduction of VSMC and EC *in vitro* and *in vivo*. In U87MG human glioma cells, transient expression of this non-modifiable NRP1 mutant (S612A) resulted in enhanced invasion in three dimensions, whereas wild-type NRP1 had no effect (Frankel et al., 2008). In contrast, in HCASMC, the over-expression of the S612A NRP1 construct did not result in any significant change in the cell migratory response.

This result suggests that the O-linked post-translational modification is not important for PDGFR-mediated intracellular signalling and migration. Shintani et al. (2006) found that cells infected with the NRP1 S612A adenovirus displayed an increased migration in response to VEGF-A₁₆₅. However, in the light of the absence of migration of HCASMC in response to VEGF-A₁₆₅ in my hands, I believe these results of Shintani et al. (2006) should be considered with caution.

It is known that NRP1 can form an active complex with VEGFR2 on adjacent cells (Cbe-Suarez et al., 2008). Moreover, an interesting study by Jakobsson et al. (2006) showed that VEGF signalling in endothelial cells is supported by HS expressed in *trans* by adjacent perivascular smooth muscle cells. Transactivation of VEGFR2 led to prolonged and enhanced signal transduction due to HS-dependent trapping of the active VEGFR2 signalling complex (Figure 6.1). I suspect that an analogous model of transactivation of VEGFR2 (in EC) by O-linked GAG NRP1, might be relevant to interactions between VSMC and EC and appropriate to explain the relatively small effect of the NRP1 S612A mutant in SMC. Thus, while the modification could have an effect in *trans* to activate VEGFR2 on adjacent EC, it may not affect signalling in VSMC. This is a possibility that warrant further investigation.

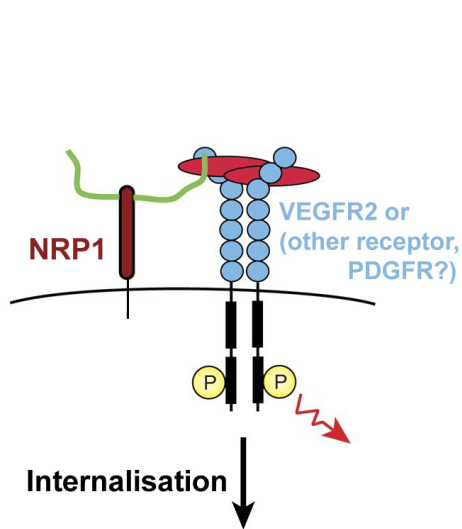
6.2 NRP1 functions in vascular cells

6.2.1 Role of NRP in endothelial cells

The results obtained in EC confirmed previous studies conducted by this and other groups, showing that both NRP1 and NRP2 play an essential role in EC proliferation, migration (Sulpice et al., 2008; Favier et al., 2006) and adhesion (Murga et al., 2005). However, the function of NRPs in HCAEC had not been previously reported, and I confirmed by knockdown experiments using siRNA transfection, that NRPs were also important for migration of HCAEC towards VEGF-A₁₆₅. The anti-angiogenic effect caused by the inhibition of NRPs was also shown by other groups using blocking antibody (Pan et al., 2007a; Sulpice et al., 2008), peptide antagonists (Jia et al., 2001) or with the use of the other NRP ligands Sema3A and Sema3F that compete with VEGF-A₁₆₅ for binding to NRP1 (Miao et al., 1999) and NRP2 (Favier et al., 2006), respectively.

Evidence from Cai and Reed (1999) and Wang et al. (2006) attributed to the NRP1 cytoplasmic domain a role in angiogenesis through interaction with synectin. Indeed, in this

A Trapping in *Cis*



B Trapping in *Trans*

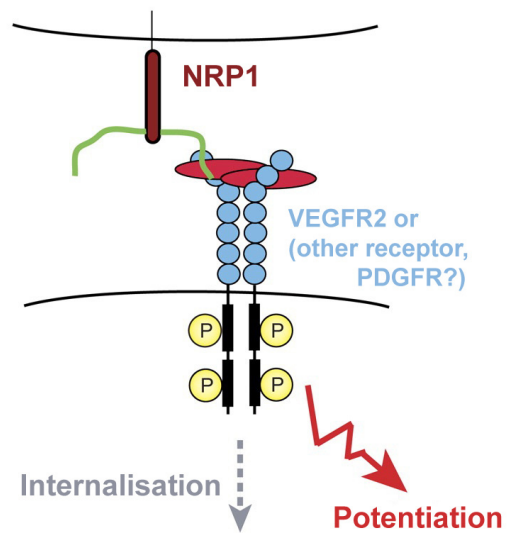


Figure 6.1: Proposed model for a role for NRP1 SMC GAG modification in the transactivation of VEGFR2

This schema represents the model proposed by Jakobsson et al. (2006) to explain the transactivation of VEGFR2 by HSPG in *trans*, adapted to the hypothesis that NRP1 GAG modification might similarly play a role in the VEGFR2/NRP1 complex formation between adjacent cells. Figure adapted from Jakobsson et al. (2006).

study, the over-expression of a NRP1 adenoviral construct lacking its intracytoplasmic domain, exerted a dominant negative effect, resulting in decreased endothelial cell migration. The expression of the ΔC NRP1 receptor probably prevents interactions between the NRP1 cytoplasmic domain and intracellular binding proteins, and also could result in the sequestration of inactive complexes of VEGF, VEGFR and other cellular components important for chemotactic signalling, which are then unable to link with other signalling molecules *via* the NRP1 cytosolic domain. More work is required to define the role of this domain in EC migration and its intracellular partners.

6.2.2 Role of NRP in vascular smooth muscle cells

At the beginning of this work, NRP1 had not been characterised in VSMC. When I discovered the high expression of NRP1 in these cells, several questions were raised: What is the specific ligand for NRPs for this cell type? If NRP1 acts as a co-receptor, what is the nature of its partners in VSMC? What is the function of NRP in VSMC?

Although some reports in the literature had shown effects of VEGF-A₁₆₅ on VSMC *via* the expression of VEGFRs (Liu et al., 2005; Shintani et al., 2006; Grosskreutz et al., 1999; Ishida et al., 2001), I could not detect significant effects of VEGF-A₁₆₅ on migration or signalling in HCASMC and decided instead to investigate the role of NRP in PDGF-induced VSMC migration. PDGF-BB was shown to bind specifically to the surface of HCASMC but did not display convincing specific binding in PAEC or PAE cells stably expressing NRP1 WT. Therefore, PDGF-BB might not be able to bind directly to NRP1 but they both might be part of a receptor/ligand complex. Moreover, results from cell-free assay for VEGF binding to NRP1 suggests that VEGF and PDGF-BB did not compete for the same binding site on NRP1.

PDGF is a dimeric molecule consisting of structurally similar A- and B-polypeptide chains, exerting their cellular effects by binding to and activating the two related protein tyrosine kinase receptors, PDGFR α and PDGFR β (Heldin and Westermark, 1999). The specificity of interaction between the two ligands and their receptors, as well as the fact that the receptors also exists as heterodimers, complicates the interpretation of studies of PDGF. Nevertheless, NRP1 and NRP2 knockdown resulted in a significant decrease of PDGF-AA and PDGF-BB-stimulated SMC migration, suggesting an essential role for NRP in the migratory response of HCASMC. A reduction in migration was also observed following synectin knockdown but was not statistically significant. Synectin is, to date, the only

protein known to interact with the cytoplasmic domain of NRP1. The slight reduction in migration seen with synectin knockdown suggests some role for this intracellular molecule in HCASMC migration but cannot account for the complete effect observed with the NRP1 knockdown. Thus, there may be either other, as yet unknown, molecules which interact with the NRP1 cytoplasmic domain or, alternatively, NRP1 interacts with other receptors whose intracellular signalling is altered in the absence of NRP1.

Pre-incubation of SMC with the specific bicyclic peptide antagonist EG3287, also reduced the migratory response of the cells towards a PDGF-BB gradient. Moreover, another specific antagonist which is an EG3287 derivative, EG00086, had a similar and dose-dependent effect. Interestingly, those effects were only observed when cells were stimulated with PDGF-BB, and the antagonists did not affect PDGF-AA-stimulated migration. If, as my binding data suggest, PDGF does not bind to NRP1 itself, it is possible that the binding of the antagonist alters the conformation of NRP1 which, in turn might impair its ability to form an active complex with PDGF receptors or other signalling components, hence causing a reduced intracellular response. Since effects of antagonists were observed specifically in response to PDGF-BB, presumably the antagonists selectively impact upon signalling *via* PDGFR β and for signalling of PDGF-BB mediated via α and/or β receptors.

To summarise, these experiments show that inhibition of NRP1 function using two independent approaches - siRNA and peptide antagonists - had similar inhibitory effects on PDGF-BB-induced HCASMC migration. My results strongly suggest that NRP1 plays an important role in the chemotactic response to PDGF-BB.

NRP1 is expressed naturally in soluble isoforms comprising the a1/2 and b1/2 domains of the extracellular region (sNRP1). These soluble forms can act by binding and sequestering ligands such as VEGF and thereby inhibit VEGF-dependent biological processes such as tumour growth and angiogenesis (Gagnon et al., 2000). Though we were unable to detect a stimulatory effect of VEGF in HCASMC, I postulated that NRP1 could mediate PDGF-BB induced cell migration by interacting with an extracellular ligand, either PDGF-BB itself or another as yet unidentified ligand. This possibility predicts that sNRP1 might inhibit responses mediated via membrane-associated cell surface NRP1 by competing with endogenous or exogenous ligands. Incubation of HCASMC with sNRP1 significantly inhibited migration in response to PDGF-BB with a similar degree of inhibition as was obtained using EG3287 or NRP1 siRNA. As the NRP1 ligand binding domain for VEGF resides primarily in the b1 NRP1 domain, I therefore also examined the effect of recombinant b1

domain. Addition of the b1 domain also significantly decreased the migratory response of HCASMC to PDGF-BB with a similar degree of inhibition as sNRP1. Thus, the presence of sNRP1 or sb1 might result in the formation of complexes with PDGF receptors at the surface of the cell which lack intracellular signalling downstream of NRP1, which, in turn, result in the decrease in SMC migration in response to PDGF-BB.

In endothelial cells, the NRP1 cytoplasmic domain is able to mediate protein-protein interactions and more precisely the C-terminal three amino acids of NRP1 (SEA-COOH) are required for NRP1-mediated angiogenesis in zebrafish (Wang et al., 2006). Moreover, the overexpression of NRP1 adenovirus construct lacking the cytoplasmic domain (Ad.NRP1 Δ C) inhibited the chemotactic response of EC in response to VEGF-A₁₆₅. Similarly, in HCASMC, the NRP1 Δ C construct overexpression resulted in a reduction of cell migration in response to PDGF-AA and PDGF-BB, which suggests that NRP1 is required for optimal signalling important for migration downstream of PDGF receptors. NRP1 Δ C exerts a dominant negative effect, possibly by preventing the interactions between the NRP1 cytoplasmic domain and intracellular binding molecules important for transducing chemotactic signals, and/or sequestration in inactive complexes of PDGFR and possibly other cellular components important for chemotactic signalling. I hypothesise that synectin and possibly other proteins may interact with the NRP1 cytoplasmic domain and contribute to PDGF-induced chemotactic signalling.

All the approaches used to inhibit NRP1, block binding of ligands to NRP1, and stop intracellular signalling downstream of NRP1 cytoplasmic domain, reduced PDGF-BB-induced migration and, except in the case of peptide antagonists, also reduced PDGF-AA induced migration. These results strongly suggest a novel role for NRP1 in the mediation of PDGF-induced migration of VSMC.

6.3 NRP1 signalling in vascular cells

Though NRP1 has been extensively studied for the last 10 years in different settings such as in the nervous, cardiovascular, and immune systems and also in cancer, very little is understood about the role of NRP1 in cell signalling and only one molecule, synectin, has been identified as a potential downstream effector of NRP1. Generally, NRP1 has been regarded as a passive binding entity in the complex formed with VEGFR2 and plexins, which are acting as the signalling moieties. This may well be true in the case of the

complex NRP forms with plexins, as deletion of the cytoplasmic domain of NRP1 does not impair Sema3a signalling (Nakamura et al., 1998).

Although inhibition of NRP1 did not impair the activation of several signalling molecules in VSMC and EC, it did result in the decrease of Tyrosine phosphorylation of the non-enzymatic adapter protein, p130Cas. p130Cas (Crk-associated substrate), is a major Src substrate localised at sites of cell adhesion to the extracellular matrix (ECM). It is a non-enzymatic docking protein consisting of multiple protein-protein interaction domains including an N-terminal SH3 domain, a substrate domain, a Src-binding domain near the C terminus and a highly conserved C-terminal region. The substrate domain contains the major sites of p130Cas tyrosine phosphorylation which create docking sites for recruitment of SH2-containing signalling effectors (Sakai et al., 1994a,b). Notably, recruitment of Crk adapter proteins to the substrate domain sites has been implicated in the promotion of Rac activation and cell motility (Sharma and Mayer, 2008). Moreover, the overexpression of the NRP1 ΔC in EC, similarly reduced p130Cas activation, concomitant with a decrease migratory response towards VEGF-A₁₆₅. A role of p130Cas in cell motility was confirmed by siRNA mediated knockdown in HUVEC as p130Cas depleted cells migrate less efficiently towards VEGF-A₁₆₅.

All these observations point towards a critical role for p130Cas in VEGF-A₁₆₅ stimulated signalling and migration downstream of NRP1. Moreover, the NRP1 cytoplasmic domain seems to be required for transducing intracellular chemotactic signalling induced by VEGF-A₁₆₅ in endothelial cells (Figure 6.2). By using the yeast two hybrid system, colleagues in this laboratory found a novel Neuropilin 1-associated protein capable of binding to the cytoplasmic domain of NRP1. The role of this NRP1-associated protein, upstream of p130Cas is currently being investigated. Interestingly, synectin knockdown does not appear to impair VEGF-induced p130Cas phosphorylation (Evans et al., MS submitted).

Similar to the findings in endothelial cells, NRP1 plays a role in PDGF-AA and PDGF-BB-induced migration of HCASMC. NRP1 siRNA-mediated knockdown and NRP1 ΔC overexpression both inhibited HCASMC migration induced either by PDGF-AA or by PDGF-BB, suggesting that NRP1 and its cytoplasmic domain are important for chemotactic signalling downstream of PDGFRs. Interestingly, the over-expression of the NRP1 ΔC mutant inhibited PDGF-induced HCASMC migration to a greater extent than the siRNA knockdown, as well as reducing the basal level of migration. These observations suggest that the NRP1 cytoplasmic domain is important for the interaction with intracellular

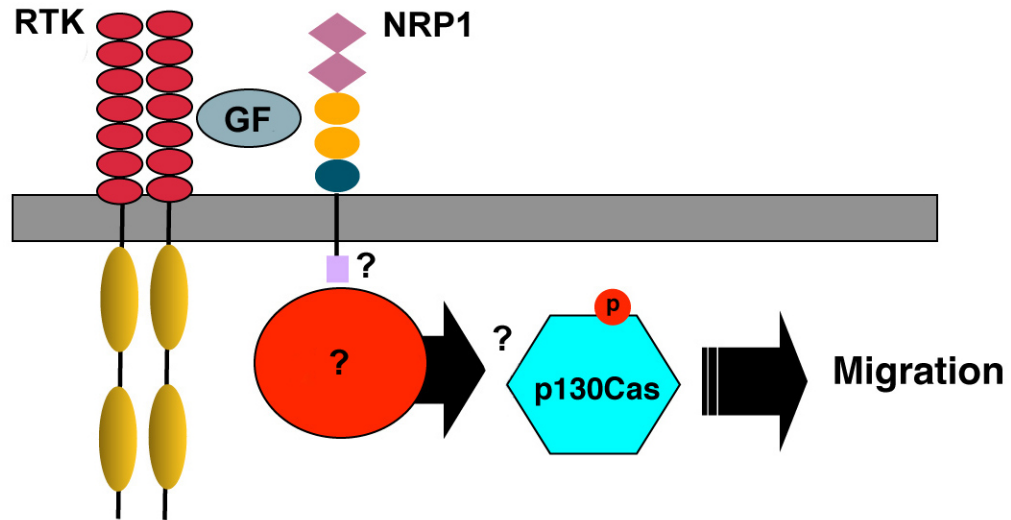


Figure 6.2: NRP1 signals through its cytoplasmic domain to activate a NRP1-associated protein and downstream p130Cas to stimulate migration

This schema represents the possible pathway by which VEGF-A₁₆₅ or PDGF-BB and HGF (this thesis and data observed by colleagues for the later) activate downstream chemotactic signalling molecules leading to the activation of a NRP1-associated protein, p130Cas Tyrosine phosphorylation and cell motility.

molecules important for migration. Moreover the NRP1 mutant might act by sequestering PDGFRs in inactive complexes, disrupting signalling with several intracellular molecules, one of them being synectin. Since synectin knockdown reduced, but not to a significant level, the level of HCASMC migration, other NRP1 intracellular domain binding partners must contribute to PDGF-induced migration.

As observed in endothelial cells (this thesis) and in cancer cells (Frankel et al., 2008), p130Cas Tyrosine phosphorylation is stimulated *via* NRP1 in VSMC. In this study, I have shown that both PDGF-AA and PDGF-BB are able to stimulate p130Cas activation and that this response is partially inhibited by NRP1 knockdown and by the expression of the NRP1 ΔC mutant in HCASMC. In contrast, other intracellular signalling molecules involved in survival and proliferation such as FAK, Akt, or ERK were not significantly affected by the lack of NRP1 at the cell surface. It is surprising that FAK activity was not altered as p130Cas is known to both associate with FAK and to be a substrate for FAK-directed phosphorylation (Defilippi et al., 2006). Nevertheless, as I probed only one major site of phosphorylation, I cannot exclude the possibility that NRP1 activates other FAK phosphorylation sites that might be more important for the mediation of p130Cas activation. Little work has been done on the role of p130Cas in PDGF-dependent sig-

nalling and migration in VSMC. Nevertheless, p130Cas seems to play a role in growth factor-dependent recruitment of adaptors and effectors that activate downstream pathways, resulting in cell survival and increased cell motility (Defilippi et al., 2006). In support of a role for p130Cas in VSMC migration, I further demonstrated that p130Cas is essential for the PDGF-AA and PDGF-BB migratory response using siRNA-mediated knockdown. The identity of the kinase directly responsible for PDGF-stimulated p130Cas Tyrosine phosphorylation in VSMC and EC is unknown, though as discussed above, FAK is an obvious candidate. Another candidate is the FAK related kinase, Pyk2. Recent findings from this laboratory show that NRP1 is required for VEGF-induced Pyk2 activation in HUVECs and that Pyk2 mediates VEGF-stimulated p130Cas Tyrosine phosphorylation (Evans et al., MS submitted). Moreover, Pyk2 can associate with p130Cas (Kuwabara et al., 2004). In future work, I would examine the role of Pyk2 in PDGF-induced p130Cas phosphorylation in VSMC.

I hypothesised that, similar to EC where NRP1 modulates VEGF signalling through its association with VEGFR2/KDR, NRP1 might form a complex with PDGFRs in order to modulate PDGF signalling. Although one study by Banerjee et al. (2006) reported the physical interaction between NRP1 and PDGF-BB, I could not reproduce these findings. However, I showed that NRP1 co-immunoprecipitates with PDGFR α in HCASMC. My results indicated that PDGF treatment did not significantly alter the extent of co-immunoprecipitation of PDGFR α and NRP1. This appears to differ from the situation in endothelial cells in which VEGF stimulates VEGFR2 complex formation with NRP1. However, it is important to note that studies of VEGFR2/NRP1 complexation have produced conflicting results. Thus, some groups have concluded that VEGF is required to promote NRP1 association with VEGFR2 (Pan et al., 2007a; Soker et al., 2002), whereas others have reported that NRP1/VEGFR2 association occurs constitutively and is largely independent of ligand stimulation (Shraga-Heled et al., 2007; Whitaker et al., 2001). Therefore, it remains controversial whether VEGFR2/NRP1 complex formation is constitutive or ligand-dependent, or a combination of the two. Further work is required to establish whether the association between PDGFR α and NRP1 in VSMC is regulated by PDGFs, and the role this association plays in VSMC function. However, there was apparently no similar complex formation between PDGFR β and NRP1, indicating that NRP1 may associate preferentially with PDGFR α . Since PDGFR α is able to bind both PDGF-AA and PDGF-BB homodimers as well as AB heterodimers, the interaction between NRP1 and PDGFR α suggests a possible mechanism in which both

growth factors act through PDGFR α /NRP1 complexes resulting in optimal chemotactic signalling. Interestingly, PDGFR α activation in response to PDGF-AA and PDGF-BB was reduced in HCASMC depleted of NRP1 *via* siRNA-mediated knockdown. In contrast, NRP1 knockdown had no detectable effect on PDGF-BB induction of PDGFR β activity, possibly reflecting on the absence of interaction of NRP1 with the β receptor. It was also noteworthy that the inhibitory effect of NRP1 knockdown on PDGF-BB-induced PDGFR α activation was modest. These results suggest that the activation of PDGFR α by PDGF-AA and PDGF-BB is differentially dependent upon NRP1. This might reflect the ability of PDGF-BB to bind and activate PDGFR $\alpha\beta$ heterodimers, which might be less susceptible to inhibitory effect of NRP1 siRNA, whereas PDGF-AA activate only PDGFR $\alpha\alpha$ heterodimers. However, due to assay limitations, it is unclear whether the assay of PDGFR α can detect phosphorylation in heterodimers. Interestingly, a study by Rolny et al. (2002) showed that PDGF-BB can specifically affect phosphorylation of PDGFR α and downstream signalling without affecting PDGFR β activation. These investigators used exogenous heparin to potentiate PDGF-BB-induced PDGFR α phosphorylation without altering the phosphorylation state of PDGFR β in heparan sulphate deficient Chinese hamster ovary (CHO) 677 cells. Moreover, the potentiation of PDGFR α signalling by heparin was not induced by PDGF-AA. These findings suggest that PDGF-AA and PDGF-BB can differentially affect activity of the same receptor. Such a differential mode of action of PDGFs on PDGFR α could be applied to explain the greater inhibitory effect of NRP1 knockdown on PDGF-AA-induced, as compared to PDGF-BB-induced, PDGFR α activity.

In future work I would like to investigate in much greater detail the role of NRP1 in PDGFR activation. These studies would include examination of NRP1 association with PDGFRs using FRET analysis, and investigation of PDGFR activation in heterologous cells expressing different combination of NRP1, PDGFR α and PDGFR β , in order to clarify NRP1-dependent regulation of $\alpha\alpha$, $\alpha\beta$ and $\beta\beta$ receptors dimers. I will also investigate the role of the NRP1 GAG-modification in complex formation between NRP1 and PDGFR using adenoviral over-expression of the S612A construct in HCASMC.

Given the importance of PDGF and VSMC migration for neointima formation, those findings suggest a potential role of NRP1 in aberrant VSMC migration in cardiovascular proliferative disorders, including restenosis, graft failure and atherosclerosis. One of my principal future aim would be to characterise the expression of NRP1 in arteries *in vivo*,

particularly in the rat carotid balloon injury model and to analyse the role of NRP1 in the migration of the VSMC into the neointima. Decreasing the expression of NRP1 in these cells *via* adenoviral delivery, could potentially result in the decrease of the neointimal lesion in the lumen following the injury. Also I will try to characterise the importance of NRP1 and its GAG post-translational modification in the interaction between endothelial cells and smooth muscle by generating spheroids which consist of a sphere formed by a single layer of EC covered by a layer of SMC (Korff et al., 2001). I would expect a disruption between the two layers when cells are depleted of NRP1 and/or lacking the GAG-modification, since NRP1 is important for cell adhesion and the GAG-modification might play a role in the *trans* interaction of receptors between the two cell types. It will also be of interest to examine the role of NRP1 in VSMC by generating VSMC-specific NRP1-deficient mice, possibly by crossing floxed NRP1 mice with mice expressing Cre under the control of a VSMC dominant promoter, such as the smooth muscle cell-specific myosin heavy chain. Future studies might also examine the role of NRP1 in atherosclerotic plaque formation by generating mice doubly deficient in NRP1 in VSMC (if these mice were viable) and ApoE mice.

In conclusion, the investigation of NRP1 in vascular cells led me to the discovery of a different expression pattern in VSMC and EC, with the expression of a single 130 kDa species in EC and the additional expression of a form of >250 kDa in VSMC. This novel high molecular weight species observed in VSMC corresponds to a CS- and HS-GAG modified species. I discovered a role for NRPs in PDGF-stimulated migration of SMC and shown the colocalisation of NRP1 with PDGFR α . I also shown that NRP1 could regulate PDGFR α in VSMC, further reinforcing the role of NRP1 in PDGF-mediated signalling in these cells, probably *via* the formation of a NRP1/PDGF/PDGFR α receptor complex. Finally, investigations of the possible mechanisms responsible for NRP1 role in VSMC led to the discovery of the role of the docking protein, p130Cas, in mediating NRP1 cytoplasmic domain downstream signalling in EC and VSMC.

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Appendix A

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>gi|66912184|ref|NP_003864.3| neuropilin 1 isoform a  
[Homo sapiens]
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PGYLTSPGYPHSYHPSEK  
CEW  
LIQAPDPYQRIMINFNP  
HFDLEDRDCKYDYVEV  
FDGENENGHFRGKFCGK  
IAPPPV  
VSSGPFLFIKFSYDYE  
THGAGFSIRYEVFKRG  
PECSQNYTTPSGVIKSP  
GPFPEKYP  
NSLECTYIVFAPKMSEI  
ILEFESFDLEPDSNPP  
GGMFCRYDRLEIWDGFP  
DVGPHI  
GRYCGQKTPGRIRSS  
SGILSMVFYTD  
SAIAKEGFSANYSVLQ  
SSVSEDFKCMEALG  
MESGEIHS  
DQITASSQYSTNWSA  
ERSRLNYPENGWTPG  
EDSYREWIQVDLGL  
LLRFV  
TAVGTQGAISKETK  
KKYYVKTYKIDVSS  
NGEDWITIKEGNKPV  
LFGNTNPTDVVV  
AVFPKPLITRFVRI  
KPATWETGISMRFEV  
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SSNQGDRNWMPENI  
RLVTSRSGWALPPA  
PHSYINEWLQIDL  
GEEKIVRGI  
IIQGGK  
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YSNNGSDWKMIMDD  
SKRKAKSFE  
GNNNYDTP  
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FIRIYPERATHG  
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VDECD  
DDQANCHSGTGD  
DFQLAGGTTVL  
ATEKPTVIDSTIQ  
SEFPTYGFNCE  
FGWGS  
HKTFCHWEH  
DNHVQLK  
WSVLT  
SKTGPIQDHTG  
DGNFIYSQADEN  
QKGVARLVSPV  
VYSQNSAHCMT  
FWYHM  
SGSHVGT  
LRVKLRYQKPEE  
YDQLVWMAIGHQ  
GDHWKEGRVLL  
HKSLKLYQVIF  
E  
GEGE  
IGKGNLGGIAVDD  
ISINNHISQEDCA  
KPADLDKKNPEI  
KIDETGSAPGYE  
GEGEGD  
KNISRKPGNV  
LKTLDPILITII  
AMSALGVLLGAV  
CGVVLYCACW  
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STYSEA
```

Figure A.1: NRP1 amino acid sequence from the Protein FASTA database (NCBI)

>gi|66912183|ref|NM_003873.3| Homo sapiens neuropilin 1 (NRP1), transcript variant 1, mRNA

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Figure A.2: NRP1 mRNA sequence from the Gene database (NCBI)

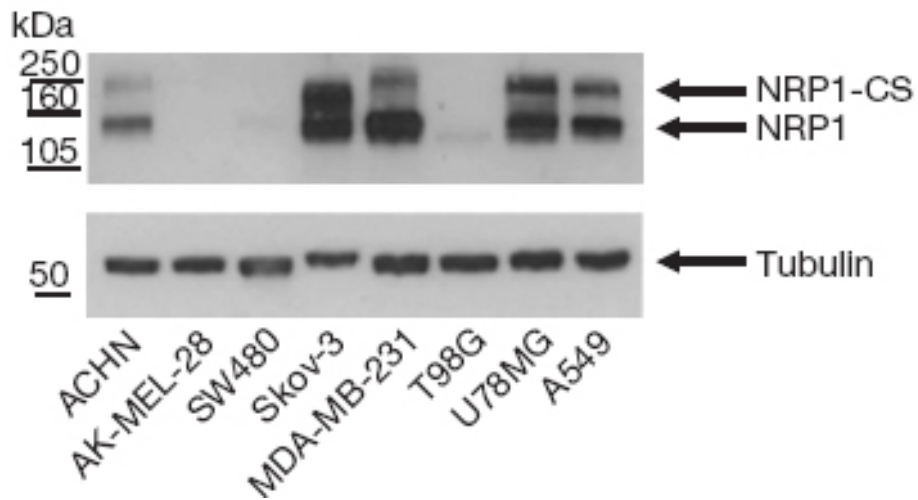


Figure A.3: NRP1 protein expression in tumour cells

The high molecular weight NRP1 species is expressed in several human tumour cell lines: ACHN (renal carcinoma cell line), Skov-3 (ovarian carcinoma cell line), MDA-MB-231 (breast adenocarcinoma cell line), U78 MG (glioblastoma cell line), A549 (pulmonary carcinoma cell), from Frankel et al. (2008).

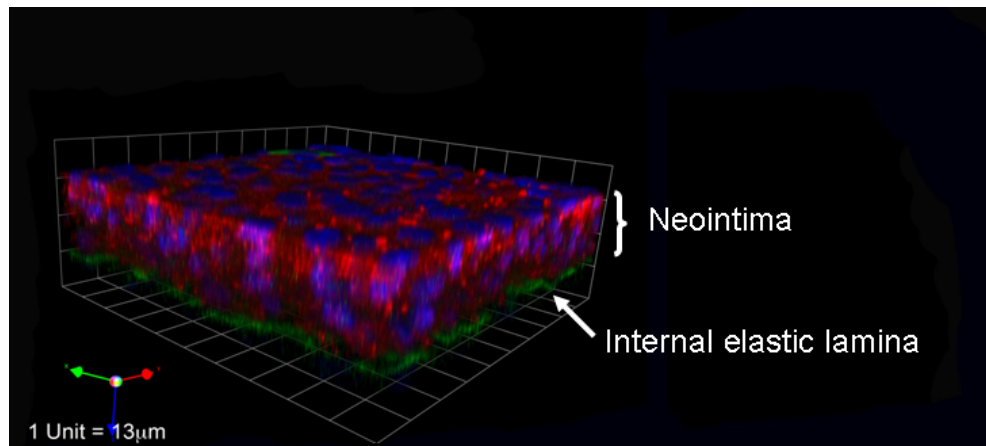


Figure A.4: NRP1 protein expression in SMC in neointimal hyperplasia

3D reconstruction of the rat carotid artery 14 days after balloon injury using the velocity software (document kindly provided by Dr. Manfred Junemann-Ramirez). The smooth muscle cell of the neointimal hyperplasia (with blue nuclei stained with DAPI), on top of the internal elastic lamina (on the green channel), strongly express NRP1 (on the red channel).

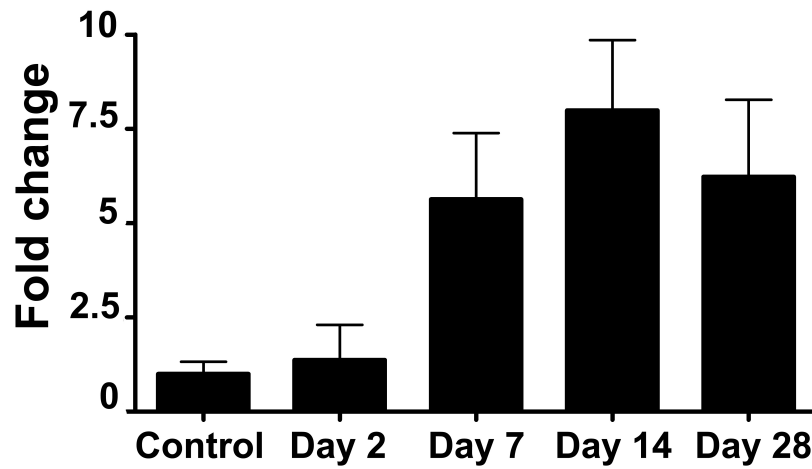


Figure A.5: **NRP1 mRNA expression in SMC in neointimal hyperplasia**
 NRP1 is upregulated by the rat carotid artery smooth muscle cells after balloon injury, with a peak at 14 days after the surgery (data obtained from Dr. David Sanz-Rosa).

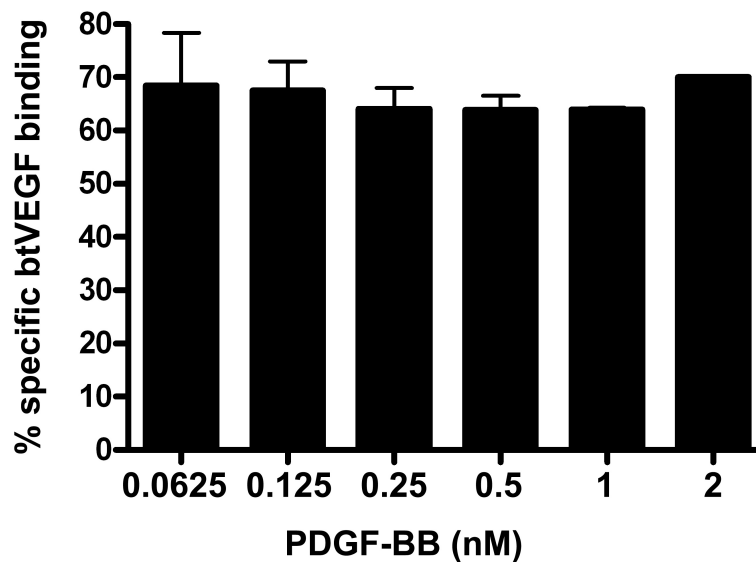


Figure A.6: **Cell free competitive binding assay for VEGF to NRP1**
 Increasing concentrations of PDGF-BB were left to compete for 2 hours with biotinylated VEGF (btVEGF), there was no dose-response observed with the increasing amount of VEGF suggesting that they do not compete for the same binding site on NRP1 (data obtained from Dr. Malini Menon).