Growth factor dependent co-receptor function of Neuropilins in breast carcinoma

by

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Abstract

Neuropilin (Nrp) overexpression is correlated with increased invasion and metastasis in many epithelial carcinomas including breast cancer. The exact molecular mechanism of how Nrp promotes cancer cell tumourigenicity is unknown. Nrp is a coreceptor for VEGF, hepatocyte growth factor (HGF), and also shown to activate TGF-β on tumour cells. We hypothesize that binding of Nrp potentiates growth factor (GF) signalling and results in GF-dependent aggressive phenotype in breast cancer. In the current study, Nrp was shown to potentiate HGF signalling *in vitro* in MCF-7 cells by increasing phosphorylation of the MET receptor. However MDA-MB-231 cell line failed to show any differences after Nrp knockdown, due to constitutively activated MET. Nrp is also shown to increase the number and size of cancer stem cell (CSC) enriched mammospheres through NF-κB pathway activation. These results suggest a novel function of Nrp in CSCs and identify it as a potential target for effective cancer therapy.

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phosphorylation levels

List of Abbreviations

ABC transporters	ATP-binding cassette transporters
AHR	Aryl hydrocarbon receptor
AMD	Age-related macular degeneration
BSA	Bovine serum albumin
Bz	Bevacizumab
CUB domain	Complement binding factors C1s/C1r, embryonic sea Urchin protein fibropellin and Bone Morphogenetic protein 1 domain
CNV	Choroidal neovascularization
CSC	Cancer stem cell
CXCR	Chemokine, CXC Motif, Receptor
dpc	Days post-coitus
DMSO	Dimethyl sulfoxide
DSC	Drug surviving cells
EC	Endothelial cell
ECM	Extracellular matrix
EDTA	Ethylenediaminetetraacetic acid
EMT	Epithelial to mesenchymal transition
ErbB2/ HER2$^+$	Epidermal growth factor receptor 2/ Human Epidermal growth factor Receptor 2
FBS	Fetal bovine serum
FGF	Fibroblast growth factor
Flk1/ KDR/ VEGFR1	Felat liver kinase 1/ Kinase insert domain receptor/ Vascular endothelial growth factor 1
Flt-4/ VEGFR3	Fms-related tyrosine kinase 4/ Vascular endothelia growth factor3
FV/VIII type-C domain	Domain with homology to C-terminal of coagulation factors V and VIII
GAG	Glucose aminoglycan
GAP	GTPase-activating protein
GF	Growth factor
GIPC	RGS-GAIP-interacting protein
G-SCF	Granulocyte colony-stimulating factor
HBSS	Hank's Buffered Salt Solution
Hh	Hedgehog pathway
HGF/SF	Hepatocyte growth factor
iDC	Immature dendritic cells
IκB	Inhibitor of kappa b
IKK	Kinase for IκB
kDa	Kilo Dalton
LAP	Latency-associated propeptide
LTBP	Latent TGF-β binding protein
Lin	Epithelial cell Lineage markers
MAM domain	Meprin/ A5-protein/ PTP μ (mu)
mA	Milli amps
MDR	Multiple drug resistance
MESC	Mammary epithelial stem cells

MMP	Matrix metalloproteinases
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide
NFκB	Nuclear Factor kappa B
nM	Nano Molar
Nrp1	Neuropilin 1
Nrp2	Neuropilin 2
NOD/ SCID mice	Non obese diabetic/ Severe Combined Immunodeficient mice
OD	Optical density
PARP	Poly (ADP-ribose) polymerase
PDZ motif	Post synaptic density protein (PSD95), Drosophila disc large tumor suppressor (DlgA), and Zonula occludens-1 protein (zo-1) motif
PIGF	Placenta growth factor
PMSF	Phenylmethylsulfonyl fluoride
PTP	Protein tyrosine phosphatase
PVDF	Polyvinylidene Fluoride
Rpm	Rotations per minute
RIPA	Radioimmunoprecipitation assay buffer
RPE	Retinal pigment epithelium
RTK	Receptor tyrosine kinase
RT	Room temperature
SCGF-b	Stem Cell Growth Factor-beta
SEA tripeptide	Serine, Alanine, Glutamic acid tripeptide
SEMA-3A	Semaphorin class 3, type A
Smad	Mothers against decapentaplegic homolog 2
sNrp	Soluble Neuropilin isoform
SP	Side population
TGF-β1	Transforming Growth Factor beta isoform 1
TGFβRI/ Alk- 5	TGF beta receptor 1, Activin receptor-like kinase 5
TGFβRII	TGF beta receptor 2
Treg	Regulatory T-cells
V	Volts
VEGF165	Vascular Endothelial Growth Factor isoform 165
vSMC	Vascular smooth muscle cell

1 Background

1.1 Rationale

Neuropilin-1 (Nrp1) and Neuropilin-2 (Nrp2) are single pass transmembrane receptors known to bind two structurally disparate families of ligands: class 3 semaphorins to mediate axonal guidance and members of the angiogenic vascular endothelial growth factor (VEGF) family. Apart from its expression in neurons and endothelial cells, Nrp is shown to be overexpressed in epithelial tumour cells suggesting that its functions in cancer cell biology are beyond VEGF-mediated angiogenesis of the tumour endothelium. Interestingly, Nrp is shown to bind several growth factors and form signalling complexes with their corresponding canonical receptor tyrosine kinases (RTK), or other signalling receptors, on the tumour cell surface. Aberrations in GF signalling pathways such as dysregulated expression of GFs or components of their signalling networks are inextricably linked to cancer biology and several oncogenes are products of RTKs. Therefore, the evidence suggests that Nrp as part of the signalling complex can regulate key aspects of GF signalling and play a role in the induction and progression of cancer.

Nrp is shown to bind and activate latent transforming growth factor beta (TGF-β) cytokine, which has been linked to breast cancer metastasis to the lungs (Glinka and Prud'homme et al., 2008; Padua et al., 2008). Furthermore, Nrp can bind hepatocyte growth factor (HGF) and its receptor MET, as well as to fibroblast growth factors (FGFs), which are all relevant to tumour progression (Matsushita et al., 2008; West et al., 2005). Most of the previous work on Nrp in cancer has focused on its role in binding VEGF and angiogenesis in endothelial cells, and the importance of other interactions remains unknown. Here we aim to characterize the function of Nrp in HGF/MET pathway in luminal and basal mammary carcinoma cell lines *in vitro*. The HGF/MET pathway was selected because a) HGF is a well- known ligand of Nrp (Hu et al., 2007 and Matsushita et al., 2008); b) the MET pathway is relevant to breast cancer metastasis and the invasive nature of the cancer cells (Jedeszko et al., 2009; Parr and Jiang et al., 2001); and c) the role of Nrp1 as a HGF co-receptor has not been studied in breast cancer previously.

Nrp has also been shown to promote sonic hedgehog (Shh) expression, and sustain undifferentiated cancer stem cell (CSC) phenotype in renal carcinomas (Cao et al., 2008). The CSCs are a small population within a tumour and share many characteristics with stem- cells such as: indefinite ability to divide, self-renew and ability to differentiate into cell types from multiple lineages. In addition, CSC have low mitotic rate making them radiotherapy resistant and overexpress ATP-dependent efflux (ABC) transporters to pump out chemotherapeutic drugs. These characteristics make CSCs highly tumorigenic and they have been identified as the major cause for recurrence and poor patient prognosis. CSCs could be the main target for cancer therapy in the near future and an effective strategy to eradicate cancers currently resistant to conventional therapy; however, not much is known about the CSC biology.

NF-κB has recently been found to be activated in mammary CSC population and shown to induce the expression of breast tumour CSC markers Nanog and Sox (Liu et al., 2010). NF-κB pathway is responsive to activation by many stimuli including growth factors (GF). In addition, some studies revealed that CSCs express high levels of cytokines (Levina et al, 2008). This likely assists them with seeding and survival in new areas after metastasis, as well as to resist drug-induced apoptosis (Levina et al., 2008). Therefore, we evaluated the role of GF co-receptor Nrp in NF-κB activation in mammary CSC population.

In addition, we examined changes in the expression levels of Nrp, in response to the drug tranilast. Tranilast is a drug recently identified to target CSCs by binding the aryl hydrocarbon receptor (AHR) and exerting AHR agonistic function (Prud'homme et al., 2010). AHR is a major transcription factor overexpressed in CSCs, and capable of regulating the expression of many genes. Futhermore, to determine more directly whether Nrp has a role in CSC biology and potentially its relevance to therapy, we examined the effects of Nrp knockdown on tumour sphere formation by CSCs.

In this study, we aimed to address the current issues in cancer biology and showed that Nrp1 and Nrp2 are important because they enhance signalling through several pathways relevant to cancer growth, invasion and metastasis.

1.2 Hypothesis

We hypothesize that Nrp is a growth factor (GF) co-receptor and its presence sensitizes the cell to its GF ligand and results in increased GF signalling and GF-dependent aggressive phenotype in breast cancer

1.3 Objectives

I. Investigate the role of Nrp in HGF mediated activation of the MET pathway in breast cancer cells

   a. Induce human mammary cell lines with HGF and evaluate changes in MET phosphorylation at key tyrosine residues, necessary and sufficient for MET pathway activation, in the presence or absence of Nrp

   b. Conduct functional assays to evaluate changes in the following cancer cell properties in the presence or absence of Nrp with or without HGF: Proliferation, Migration and Apoptosis

II. Evaluate the role of Nrp in mammary CSC population

   a. Enrich for CSC using mammosphere assay and observe changes in mammosphere size and number in the presence or absence of Nrp

   b. Evaluate changes in NF- KB activation in mammospheres in the presence or absence of Nrp

   c. Investigate whether the AHR agonist and CSC targeting drug, tranilast alters Nrp expression *in vitro* in murine mammary cell line and *in vivo* in syngeneic mouse model

2 Introduction

The Neuropilin (Nrp) family is implicated in pivotal roles in axon guidance, angiogenesis, cell adhesion and wound healing. The multi domain extracellular region of Nrp allows it to bind an array of growth factor (GF) ligands and their cognate receptors as part of signalling complex. Absence of Nrp from these systems affects efficient and effective signalling key to normal cell function and maintenance. As a GF co-receptor, Nrp plays a role in fine tuning the signalling pathway by increasing ligand receptor affinity, stabilizing the receptor complex on the receptor cell surface or potentiating the receptor tyrosine kinase (RTK) phosphorylation (section 2.4). Apart from its roles in normal cell physiology, Nrp as GF coreceptor participates in the progression of several malignant neoplasms and ocular diseases.

2.1 Structure and domain organization

Neuropilin (Nrp) family is composed of single-pass transmembrane glycoproteins with multi- domain structure (Figure 1). The N-terminus of Nrp encodes a large extracellular region (850 amino acid residues), followed by short membrane-spanning domain (24 residues) and a cytoplasmic domain (40 residues) on the C-terminus. The extracellular region is further divided into 3 subdomains that are homologous to motifs from functionally diverse proteins suggesting domain- shuffling events during evolution. The 3 subdomains are CUB or 'a' domain, FV/VIII type- C domain or 'b' domain and MAM or 'c' domain.
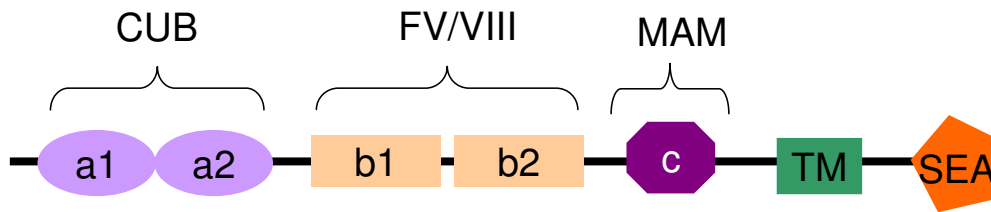
Figure 1 Schematic representation of Nrp primary structure

Nrp is composed of a large extracellular region subdivided into double repeat CUB domains or a1/a2, double repeat FV/VIII domains or b1/b2 and MAM or c domain. CUB and FV/VIII domains are involved in ligand binding. The b1 domain is essential for both SEMA-3 and VEGF-A$_{165}$ binding. The c or MAM domain is shown to play a role in Nrp dimerization which is a requirement in Sema-3 signalling. The Transmembrane (TM) domain is also shown to play roles in receptor oligomerization. The cytoplasmic domain lacks kinase activity but contains a short conserved Serine (S) - glutamic acid (E) - alanine (A) motif or PDZ binding domain known to interact with the multi-domain scaffolding protein GIPC or synectin. The SEA motif is essential for Nrp dependent endothelial cell migration and adhesion. Figure adapted from Pellet Many C. et al., 2008.

2.1.1 CUB domain

The CUB domain shares homology with the motifs found in complement binding factors C1s/C1r, embryonic sea urchin protein fibropellin and bone morphogenetic protein 1 (BMP1). CUB domains are mostly found in proteins involved in developmental processes such as embryogenesis or organogenesis (Bork and Beckmann, 1993). Based on the presence of four conserved cysteine residues and the predicted arrangement of the disulfide bridges, an anti-parallel β barrel topology similar to immunoglobulins has been proposed for the CUB domain (Bork and Beckmann, 1993). Nrp contains a double repeat of CUB domain called 'a1' and 'a2' domains, each 110 residues long and display high degree of similarity between Nrp1 and Nrp2 (Lee et al., 1993). The CUB domain in Nrp binds the N-terminal portion of the semaphorins and is necessary for semaphorin growth cone collapse functions (Vander Kooi et al., 2007). Sempahorins (Sema-3A and Sema-3F) failed to mediate their chemorepulsive functions in the presence of anti- panNrp[A] antibody raised specifically against the CUB domain (Appleton et al., 2007). X-ray crystallography studies have identified a1 domain as

highly flexible in comparison to the a2b1b2 core suggesting that it can undergo conformational changes with respect to the remainder of Nrp in solution or upon receptor binding (Lee et al., 2003).

2.1.2 FV/VIII type- C domain

FV/VIII type-C domain or 'b' domain is 150 residues long and displays homology to the C-terminus of the coagulation factors V and VIII (Toole et al. 1984; Jenny et al. 1987), the extracellular part of the receptor tyrosine kinase DDR (Discoidin domain receptor) and the discoidin-1 subunit found in soil-living amoeba *Dictyostelium discoideum* (Fig. 2) (Johnson et al. 1993). The FV/VIII type- C domain containing proteins play roles in cell adhesion and cell surface-mediated regulatory events. Coagulation factors V and VIII, freely circulating in the plasma bind to the surface of activated platelets (exposed anionic phopsholipids) by means of their FV/VIII type-C domain to initiate secondary hemostasis (Lanzer and Topol, 1950; Davie and Kane, 1988; Kemball-Cook et al., 1988). FV/VIII type-C domain in discoidin-1 subunit mediates cell-substratum attachment and ordered cell migration (Springer et al., 1984). Nrp1 contains a double repeat of the FV/VIII type-C domain and plays a role in both ligand binding and adhesion functions.

The FV/VIII type-C domain is topologically classified as a distorted jelly-roll β barrel with spikes or loops extending on one pole and these spikes constitute the ligand-binding site (Lee et al., 2003). Structural studies by X-ray crystallography conducted on b1 domain of Nrp identified an electronegative pocket created by the spikes in the b1 domain of Nrp (Vander Kooi et al., 2007; Lee et al., 2003). The b1 domains of Nrp1 and Nrp2 are nearly indistinguishable, however the b2 domains superimpose less well, mostly due to differences in the conformation of the spikes (Lee et al., 2003). Perhaps this subtle dissimilarity in structure reflects the ability of Nrp1 and Nrp2 to recognize different ligands from the same family (see section 2.4).

There is strong evidence that $VEGF_{165}$ with its positively charged tail binds to the electronegative pocket in Nrp (Vander Kooi et al., 2007). The basic C-terminus of

semaphorin is also shown to bind the b1 domain; this interaction is not obligate for semaphorin function but it stabilizes Nrp and semaphorin complex. It is important to note that although highly basic, the C-terminus of semaphorin cannot bind the electronegative pocket of Nrp1 on the b domain, because the last amino acid on semaphorin is a hydrophobic valine and not an arginine like $VEGF_{165}$. Therefore $VEGF_{165}$ and semaphorins do not compete with each other for binding Nrp and shown to bind Nrp simultaneously (Appleton et al., 2007).

Presence of a free carboxyl group on C-terminal arginine (R) or lysine (K) in a consensus context R/KXXR/K is a strict requirement to bind Nrp1 and is termed the C-end rule (CendR) (Teesalu et al, 2009). The C-terminus of VEGF encoded by exon-8 contains the motif KPRR (von Wronski et al., 2006). Proteins or peptides ending in R/KXXR/K have a unique property of penetrating deep into extravascular tissue (beyond the blood vessels) and transporting payloads up to the nanoparticle size scale in Nrp dependent manner (Sugahara et al., 2009). These peptides are different from the conventional RGD peptides that deliver the cargo only upto the blood vessels (Arap et al., 1998; Ruoslahti, 2003). Such peptides conjugated to small molecules, nanoparticles, or monoclonal antibodies can bind to Nrp on the tumor cell and can be used for targeted delivery of drug and diagnostic imaging agents into the tumour parenchyma (Feron et al., 2010).

Apart from ligand binding role FV/VIII type-C domain in Nrp is shown to display cell adhesion functions. This region is non-competitive with the $VEGF_{165}$ and semaphorins and therefore indicates a distinct region within the FV/VIII type-C domain to mediate cell adhesion functions (see section 2.5.3).

2.1.3 MAM domain

Nrp1 contains a single 170 amino acid long MAM or 'c' domain. The MAM domain shares homology with Meprin metalloproteinases and the N-terminal domain of the receptor tyrosine protein phosphotases such as PTPμ, PTPκ, and PCP-2 (Figure 2). MAM domain in these proteins functions as a mediator of oligomerization (Zondag et al.,

1995; Marchand et al., 1996). Likewise in Nrp, MAM domain is shown to play a role in Nrp1 dimer or multimer formation and also to hetero-oligomerize with Nrp2 (Giger et al., 2000). In sympathetic neurons Nrp1 on the cell surface binds to sema-3A as a homodimer and Nrp2 binds sema-3F in a similar conformation (Chen et al., 1998). Deletion of the MAM domain resulted in reduction of sema-3A binding to Nrp1 suggesting a reduction in Nrp oligomerization and reduced cooperative binding of ligands (Hang et al., 2000). However MAM domain by itself is not sufficient for Nrp oligomerization suggesting that other domains can mediate Nrp multimerization (Giger et al., 2000).

Apart from roles in oligomerization, MAM domain acts as a signalling interface and signal transducer of semaphorin signalling to plexin receptors. MAM domain deleted Nrp1 mutant was unable to transmit sema3A signalling in embryonic chick sympathetic neurons and antibody directed against the MAM domain of Nrp2 abrogated sema3F-induced growth cone collapse of sympathetic ganglion cells (Giger et al., 1998; Renzi et al., 1999).
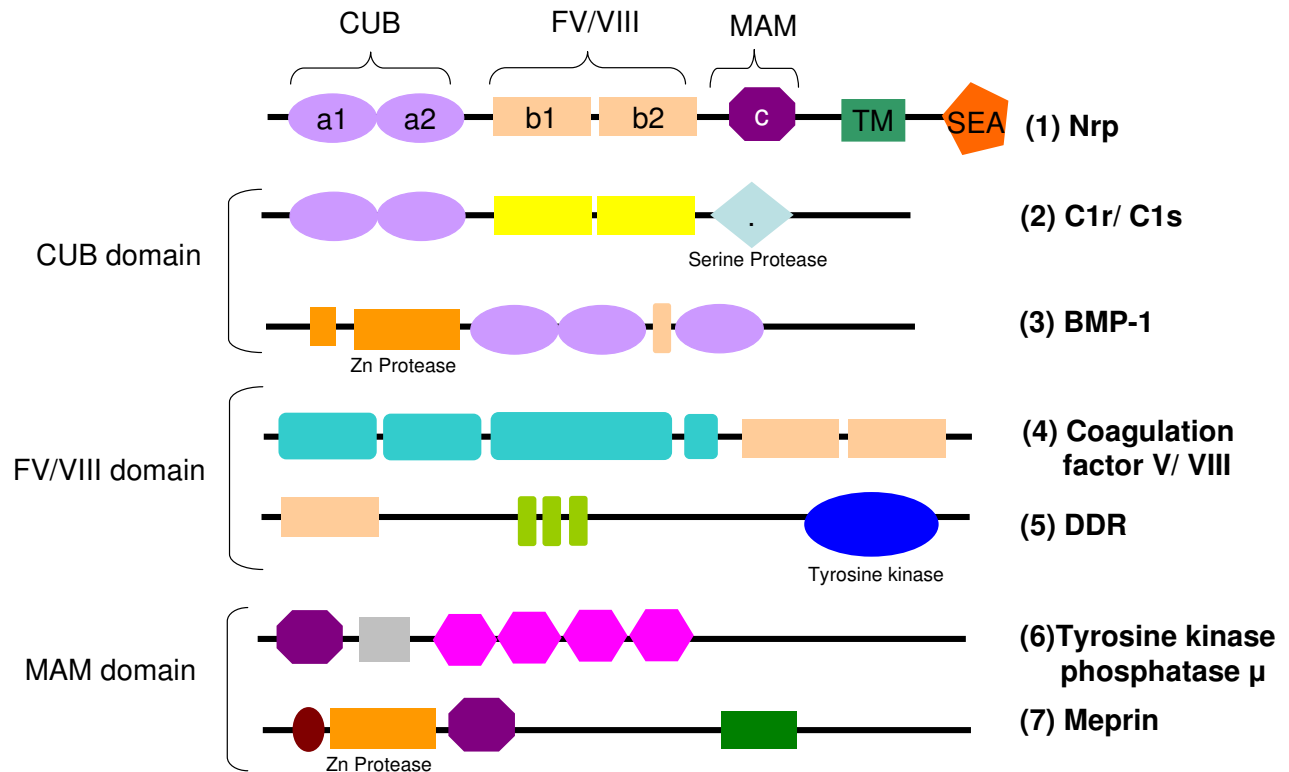
Figure 2 Schematic representation of motifs in functionally diverse proteins resembling extracellular sub domains of Nrp

The CUB domain of Nrp shares homology with developmentally regulated proteins such as the C1r and C1s complement proteins (2) and Bone Morphogenetic Protein-1 (3). Coagulation factor V and VIII (4), Discoidin Domain receptor (5) contain motifs similar to the FV/VIII type- C domain of Nrp and participate in cell adhesion and cell surface-mediated regulatory events. The MAM domain plays a role in receptor homo or hetero oligomerization and is found in protein tyrosine phosphatases (6) and Meprin metalloproteinases (7). Figure adapted from Fujisawa et al., 1997.

2.1.4 Transmembrane domain of Nrp

Nrp has a short transmembrane domain (24 residues) and is shown to contain a putative dimerization GxxxG motif (Roth et al., 2008). Presence of the transmembrane domain motif and receptor dimerization was shown to be prerequisite for sema-3A signalling (Roth et al., 2008). In addition the trans-membrane region of Nrp is believed

to recruit various partners such as members of the plexin family (Tamagnone et al., 1999), L1, NrCAM adhesion molecules (Castellani et al., 2000), VEGFR1, VEGFR2, MET tyrosine kinase receptors (Soker et al., 1998; Bagnard et al., 2001b; Winberg et al., 2001), or integrins (Pasterkamp et al., 2003) and form oligomeric receptor complexes (Roth et al., 2008) with Nrp.

2.1.5 Cytoplasmic domain of Nrp

The cytoplasmic intracellular region of Nrp does not contain any intrinsic kinase activity. However Nrp contains a conserved Serine (S)- Glutamic acid (E) - Alanine (A) motif on its C-terminal and is known to bind PDZ (Post synaptic density protein (PSD95), Drosophila disc large tumor suppressor (DlgA), and zonula occuldens-1 protein (ZO-1)) motif on synectin (Cai and Reed et al.,1999). Synectin or GIPC (RGS-GAIP-interacting protein) is a multi-domain scaffolding protein known to play a role in internalization of cell surface receptors and participate in clathrin-coated vesicular trafficking (Cai and Reed et al., 1999). Significance of SEA motif is indicated in *in-vivo* zebrafish model where the three amino acids on the C-terminus are required for VEGFR-2 binding and Nrp mediated angiogenesis (Wang et al., 2006). SEA motif bound to GIPC is also shown to be essential for α5β1 integrin mediated endothelial cell adhesion (see section 2.5.3; Serini et al., 2006). As a scaffolding protein GIPC assists in multimeric protein complex formation and connects the surface receptors with intracellular signalling networks. The ability of Nrp to participate in diverse signalling pathways might be mediated in part by its intracellular interactions with GIPC.

2.2 Genomic organization and transcriptional control

2.2.1 Nrp1 isoforms

Nrp1 is encoded by 120kb genomic region (chromosomes 10p12) composed of 17 exons (Rossignol et al., 1999). Nrp1 has multiple splice variants; one transmembrane and four soluble forms have been identified and characterized (Figure. 3). The

transmembrane NRP1 isoform encodes 923 amino acid long, full length Nrp1 protein (Figure. 1). The soluble (sNRP1) isoforms are truncated splice variants that do not contain the transmembrane and the cytoplasmic region and their C-terminus is encoded by intron-derived sequences. The soluble isoforms are s_{11} NRP1 (truncated at exon 11), s_{12} NRP1 (truncated at exon 12), s_{III} NRP1 and s_{IV} NRP1 (Rossignol et al., 2000 and Cackowski et al., 2004). The sNRP1 contain the ligand binding a1, a2 and b1 domains and are speculated to act as decoy receptors that bind and sequester the ligands away from the full length NRP1 isforom. sNrp1 bound to the ligand cannot form signalling complexes because they lack the SEA motif containing cytoplasmic domain, MAM and transmembrane domains. It has been suggested that sNrp1 can dimerize with the assistance of heparin binding to the b1 domain (Vander Kooi et al., 2007). s_{III}Nrp1and s_{IV}Nrp1 inhibit VEGF-A$_{165}$ binding *in vitro* in breast cancer cell lines and suppress VEGF-A$_{165}$-induced cell migration and proliferation (Cackowski et al., 2004). s_{12}Nrp1 displayed antagonistic effects by inducing apoptosis and decrease in the number of blood vessels in the tumour upon overexpression in a rat prostate carcinoma model (Gagnol et al., 2000). However the level of expression of sNrp1 is very low in comparison to the full length Nrp1 isoform and to what extent they have an effect on full length Nrp1 functions *in-vivo* is unknown.
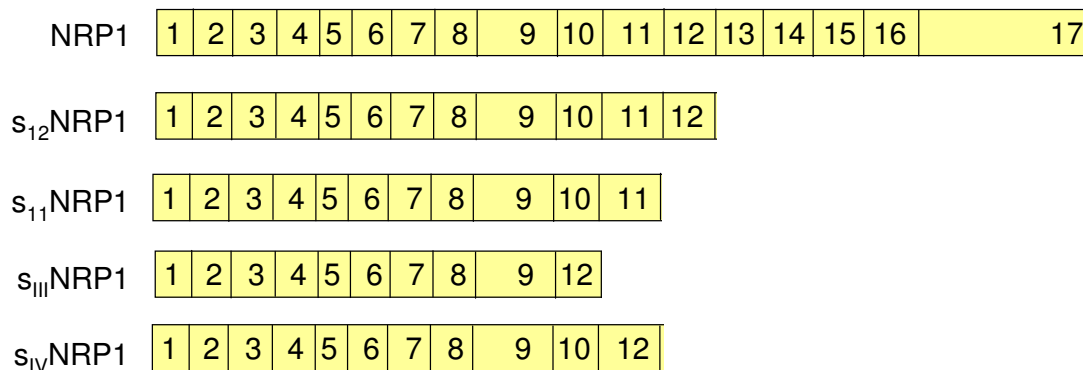
Figure 3 Schematic representation of NRP-1 genomic DNA, cDNA and soluble NRP1 (sNRP1) isoforms

Genomic DNA encoding full length Nrp1 is composed of 17 exons and 16 introns (not shown. NRP1 transcript can undergo splicing at multiple sites and four soluble isoforms have been identified to date: s_{11}NRP1 is truncated at exon 11, s_{12}NRP1 is truncated at exon 12, s_{III}NRP1 and s_{IV}NRP1 after exon 9 contain exon 12 and/ exon 10. Figure adapted from Cackowski et al., 2004.

2.2.2 Nrp2 isoforms

NRP-2 gene is composed of 17 exons (112kb in length) and maps to chromosome 2q34 (Rossignol et al., 1999). Five of the exons are identical in size between the two Neuropilins, in addition strong similarities in exon- intron organization, and position of the splice sites suggest that Nrp1 and Nrp2 arose by gene duplication (Rossignol et al., 2000). Nrp2 has two transmembrane isoforms, NRP2a and NRP2b and three soluble splice variant, sNRP2. The two transmembrane isoforms have identical extracellular domains but have divergent transmembrane and cytoplasmic domains (Rossignol et al., 2000). NRP2a isoform is closer to NRP1 in sequence identity whereas the NRP2b isoform lacks the SEA motif on the cytoplasmic tail of the C-terminus. Therefore NRP2b might interact with molecular adapter other than GIPC for signalling. NRP2a (placenta, lung, liver, heart and kidney) and NRP2b (skeletal muscles and heart) are expressed in a variety of tissues mostly in a nonoverlapping manner (Rossignol et al., 2000). The NRP2 isoforms truncated at the b2 domain are released by cells upon tranlastion as soluble NRP2 (sNRP2). Three sNRP2 isoforms are s12NRP1, s11NRP1 and s9NRP2. The soluble isoforms behave in an antagonistic fashion and sequester ligands away from the full length Nrp1/2 (Cackowski et al., 2004). Presence of sNrp1/2 might be a regulatory mechanism to control ligand concentration and activity of full length Nrp receptor.

2.3 Expression of Nrp

2.3.1 Nrp expression in embryo and adult organism

Nrp was first identified as a neuronal cell-surface protein involved in nerve fiber growth, guidance or fasciculation in the optic tectum of *Xenopus leavis* tadpoles (Takagi et al., 1986). The expression of Nrp is temporally and spatially regulated in specific neuronal circuits (sympathetic and sensory neurons) during development after which its expression is lost in the post-neonate and adult nervous systems (Chen et al., 1998). For instance, in the mouse dorsal root ganglion (DRG), Nrp expression begins 9 days post-coitus (dpc) and rapidly decreases after 15.5 dpc (Kawakami et al., 1996). An

exception is the expression of Nrp in the olfactory nerve in the adult organism because of the frequent replacement and sprouting of the olfactory fibres (Kawakami et al., 1996). In addition, Nrp also plays a role in nerve fibre regeneration after injury to re-establish the neural circuitry as demonstrated during the repair of the damaged optic tectum of Xenopus (Fujisawa et al., 1996). Nrp is expressed exclusively on the cell processes (axon and dendrites) and not on the cell body of a neuron (Fujisawa et al., 1997). Consistent with its semaphorin dependent axon pathfinding function, Nrp is predominantly detected on the neurites, filopodia and lamellipodia of growth cones (Takagi et al., 1995).

Nrp also plays key roles in VEGF mediated endothelial- tip cell migration and vessel development during embryogenesis. Both Nrp1 and Nrp2 are expressed in the earliest vascular structures, the blood islands. Once arteries and veins begin to differentiate their expression pattern becomes more selective. In the embryo, Nrp1 is preferentially expressed in the arterial endothelial cells and Nrp2 on the lymphatic endothelium and small capillaries (Moyon et al., 2001; Herzog et al., 2001, Stalmans et al., 2002). These differences are less clear-cut in adult vasculature. In the adult organism Nrp1 is expressed in high levels in the heart and placenta, moderate levels in lung, liver and kidney and low levels in the brain (Gagnon et al., 2000; Bilenberg et al., 2009). In addition, Nrp is shown to be expressed in human endometrium and shown to regulate vascular remodeling and angiogenesis during the menstrual cycle (Hess et al., 2009). Nrp1 is also expressed in the human decidua and trophoblast suggesting a role for Nrp1 in mediating VEGF dependent angiogenesis required for blastocyst implantation and placentation during pregnancy (Baston-Buest et al., 2010). Vascular smooth muscle cells (vSMC) also express Nrp1 suggesting a possible role in endothelial cell and vSMC interactions (Ishida et al., 2001).

Nrp expression is reported in many other cell types such as dendritic cells and T-cells (Tordjman et al., 2002; Sarris et al., 2008; Curreli et al., 2007), osteoblasts (but not in the more matured osteocytes) (Harper et al., 2001), platelets (Kashiwagi et al., 2005), mesangial cells of the kidney (Thomas et al., 2000), neuroendrocrine cells (Cohen et al., 2001) and multipotent cells such as the neural crest cell (Eickholt et al., 1999), bone

marrow derived progenitor cells (Fons et al., 2004) and mesenchymal stem cells (Dhar et al., 2010). Many of these cell types do not bind or process signalling through the VEGF or Sema pathways suggesting functions of Nrp beyond neuronal guidance and angiogenesis.

2.3.2 Regulation of Nrp Expression

Several transcription factors and growth factors tightly regulate NRP gene transcription to achieve proper neuronal patterning and branching of the vascular system. Prox-1 and COUP-TFII transcription factors play a role in determination of lymphatic and venous vascular identity respectively by repressing Nrp1 and preventing endothelial cells from acquiring arterial phenotype (Hong et al., 2002; You et al., 2005). In contrast, Nrp2 is upregulated by Prox-1 transcription factor to acquire lymphatic endothelial cell fate (Hong et al., 2002). dHAND transcription factor is a positive regulator of Nrp1 expression in the developing vascular mesenchyme and the differentiated vSMCs derivative (Yamagishi et al., 2000). The expression of dHAND and Nrp1 in mice overlapped at E9.5 in the developing yolk sac, aorta and the arch arteries. Nrp1 is severely downregulated in blood vessels of dHAND null mice and display similar defects in the arterial arch development, suggesting a requirement for dHAND- dependent Nrp1 expression for proper cardiovascular development. While dHAND regulates Nrp1 expression in vSMCs, Ets1 transcription factor induces Nrp expression in ECs in VEGF-dependent manner and under hypoxic conditions (Watanabe et al., 2004).

Tumour Necrosis Factor- alpha (TNF-α), an inflammatory cytokine and promoter of angiogenesis is shown to upregulate Nrp1 expression in dose- and time-dependent manner in vascular endothelial cells (Giraudo et al., 1998). The effect of TNF-α is cell type specific as Nrp1 expression in pancreatic cancer cells was unchanged after TNF-α treatment (Parikh et al., 2003). Instead Epidermal growth factor (EGF) is shown to stimulate Nrp1 expression in human pancreatic (Parikh et al., 2003), colon (Parikh et al., 2004) and gastric cancer cell lines (Akagi et al., 2003). Butyrate, a natural histone deacetylase inhibitor (found in fermented fibre), causes downregulation of Nrp1 in colon cancer cell lines by reducing acetylation and decreasing affinity of Sp1 transcription

factor (Yu et al., 2010). Phorbol ester (a plant derived terpene with tumour promoting properties) is shown to recruit Sp1 transcription factor to Nrp1 promoter region and increase NRP1 mRNA levels in podocytes (Bondeva et al., 2009). AP-1 element and the CCAAT box were other regulatory elements identified in Nrp1 promoter characterization studies using Phorbol esters (Rossignol et al., 2003).

Apart from growth factor and transcription factor regulation Nrp expression is regulated by changes in oxygen concentrations in the blood vessels during injury or under pathological conditions. Both Nrp1 and Nrp2 are upregulated in endothelial cells (EC) in the presence of ischemia. Cerebral ischemia due to occlusion of the cerebral artery induced Nrp mRNA expression within 1 to 6 hours and upto 28 days after the focal ischemia occurred. Nrp upregulation was observed in EC, neurons and astrocytes surrounding the infarct (Fujita et al., 2001). Mice deficient in Nrp displayed significantly reduced neovascularization following ischemia in their retinas (Shen et al., 2004). VEGF is strongly induced under hypoxic conditions and is shown to upregulate Nrp expression in HUVECs (Oh et al., 2002). Perhaps the induction of Nrp under hypoxic conditions is VEGF- dependent or on other growth factors that play a role in angiogenesis (FGF, HGF and PDGF (Platelet-derived growth factor)) and are ligands for Nrp (West et al., 2005; Ball et al., 2010). The induction of Nrp under hypoxic conditions suggests important functions of Nrp in tumour angiogenesis (section 2.5.4.2).

2.4 Neuropilin ligands

2.4.1 Semaphorins

Developmental neurobiologists in 1980s observed that optic nerve possesses the ability to recognize and bind specific target neurons, in order to form a neuronal connection. The mechanism of target recognition was attributed to the binding of optic nerve to specific cell surface receptors on the neuron. Antibodies were raised against the optic tectal neurons of *Xenopus leavis* and a screen was conducted to identify the cell surface receptors (Takagi et al., 1987). cDNA cloning revealed a novel type 1 membrane protein with a molecular mass of about 130 kDa and was named neuropilin

for its restricted expression in specific neuropils of the nervous system (Takagi et al., 1991).

Several years after its discovery, Nrp was identified as a high affinity receptor for Collapsin-1/ Semaphorin 3 (Sema- 3) repulsive guidance cue molecules in embryonic rat sensory neurons (He et al., 1997 and Kolodkin et al., 1997). Sema-3 is one of the eight classes of Semaphorin superfamily and is the only soluble class expressed exclusively in vertebrates (Kolodkin et al., 1993). Sema-3 family is further subdivided into types A to G. Antibodies used to block Nrp abrogated the ability of sema-3A to repel sensory axons and to induce growth cone collapse (He et al., 1997). These studies identified Nrp1 as an obligate receptor or co-receptor for sema-3A signalling and accurate neuronal pathfinding. Nrp2 was also identified as a high affinity receptor and necessary for sema- 3F mediated growth cone collapse (Gammill et al., 2007). Nrp bind all members of the sema-3 family but with different affinities (Rohm et al., 2000).

By domain deletion analysis, a1, a2 and b1 domain of Nrp were identified as essential for sema-3 growth cone collapse (Figure. 4) (Appleton et al., 2007). However, Nrp bound to semaphorin is not sufficient to transduce signals from the extracellular surface to the cytoplasmic side as Nrp lacks catalytic activity on the intracellular domain (section 1.1.5) (Fujisawa et al., 2004). The Plexin receptors were identified as the signalling component of the semaphorin signalling complex (Rohm et al., 2000). There are four subclasses of the Plexin family, A to D, each binding a specific combination of semaphorin and Nrp as part of a signalling complex (Zhou et al., 2008; Kruger et al., 2005). Class 3 semaphorins mainly interact with type A plexin receptors; sema-3A and Nrp1 complex with plexin A4 and sema-3F and Nrp2 complexes with plexin A3 (Suto et al., 2005 and Yaron et al., 2005). However sema-3 do not directly bind plexin receptors and their interaction is mediated through Nrp. Nrp binds to sema-3, localizes them on the cell surface and make them available in a proper conformation for binding by plexin for signalling (Appleton et al., 2007).

The Plexins have highly conserved intracellular domains that share homology with Ras GAPs (GTPase-activating proteins). In hippocampal neurons, plexins are shown to have functional and intrinsic GAP activity towards R-Ras (Oinuma et al., 2004). To mediate

growth cone collapse, plexins bind sempahorins in Nrp-dependent manner and induce R- ras inactivation. R-Ras primarily functions to regulate integrin activity and has limited effect on ERK/MAPK pathway unlike other Ras-family members. The increase in GDP-bound (inactive) R-Ras leads to a decrease in integrin-mediated attachment to the ECM. However current literature suggests the involvement of many other players inaddition to the monomeric G-proteins such as MICAL redox protein, several kinases and collapsin-response-mediator phosphoprotein (CRMP) family in sempahorin signal transduction pathway and requires further inspection of the sempahorin intracellular signalling pathway (Schmidt et al., 2007 and Hung et al., 2010).
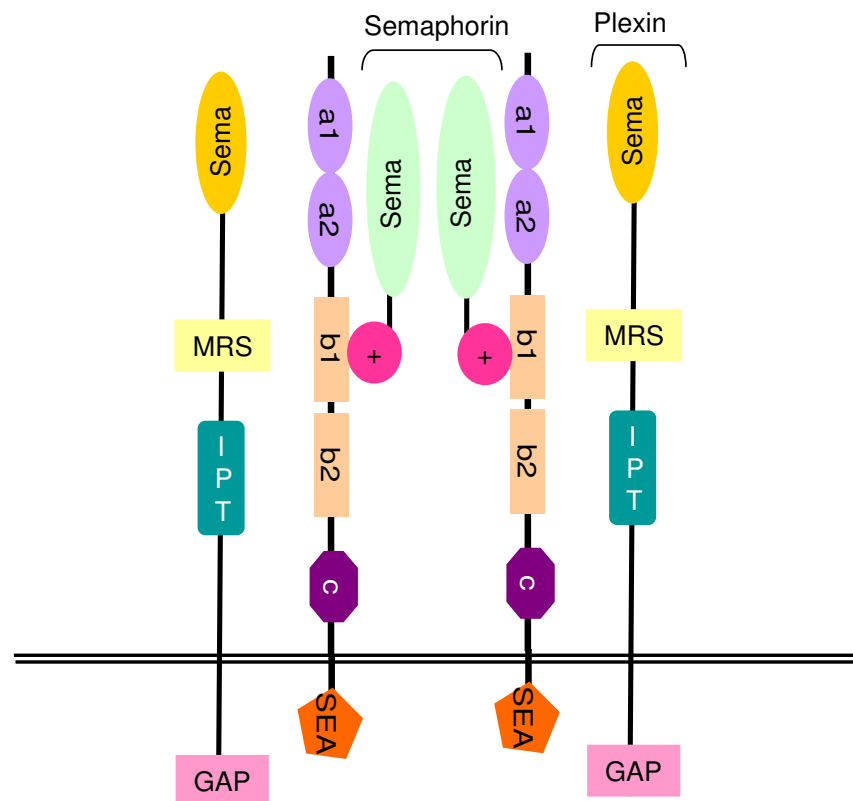
Figure 4 SEMA-3A, Nrp1, PlexA tripartite complex formation mediates axonal growth cone collapse

Semaphorin family contain a conserved 400 amino acid 'Sema' domain that interacts with the a1 and a2 domains on the N-terminus of Nrp1. The basic/ positively charged C-terminus of semaphorin (pink oval '+' sign) interacts with the b1 domain of Nrp. Sema-3 family does not interact with plexins directly. The Plexin receptor family also contains a

'Sema' domain that may interact with Nrp1 b1/b2 domains (not shown). In addition plexins contain a cysteine- rich MRS motif, IPT (Ig-like, plexins and transcription factors) domain and cytoplasmic domains. The cytoplasmic domain of plexin has intrinsic GAP (GTPase-activating protein) activity and transduces signals as a tripartite complex with Nrp and Sema in order to mediate axonal repulsion. Figure adapted from Pellet Many et al., 2008.

2.4.2 Vascular Endothelial Growth Factor (VEGF)

Very soon after its characterization as a high affinity receptor for Semaphorins, Nrp1 was identified on the cell surface of endothelial cells (EC). On the EC surface Nrp was shown to bind a specific isoform of Vascular Endothelial Growth Factor, isoform165 ($VEGF_{165}$) (Soker et al., 1996; Soker et al., 1998). VEGFs are 40-45KDa homodimers that belong to the cystine-knot platelet-derived growth factor (PDGF) family. The VEGF family has five subclasses VEGF-A, VEGF-B, VEGF-C, VEGF-D and PIGF (Placenta-derived Growth Factor). The VEGF-A subtype is the master regulator of angiogenesis, vasculogenesis and vascular permeability during development, injury and pathology. VEGF- A exists as five different splice-variants, isoform $VEGF_{121}$, $VEGF_{145}$, $VEGF_{165}$, $VEGF_{189}$ and $VEGF_{206}$, each named for the number of amino acids (Holmes and Zachary, 2005). $VEGF_{121}$ and $VEGF_{165}$ are the most abundant isoforms of VEGF and they differ in their ability to bind the extracellular matrix. $VEGF_{121}$ isoform does not contain exon 7 that encodes the heparin binding domain and therefore exists as a soluble growth factor. $VEGF_{165}$ is partially membrane bound and is the most potent mitogen for endothelial cells (EC). All isoforms bind to their cognate tyrosine kinase VEGF receptors, VEGFR-1/ Flt-1(fms-like tyrosine kinase) and VEGFR-2/ KDR(kinase domain region), or VEGFR-3/ Flt-4 (fms-related tyrosine kinase receptor 4) for signal transduction.

Nrp1 was pulled down as an intracellular complex with KDR in the presence of $VEGF_{165}$ from ECs (Figure. 5) (Soker et al., 2002). KDR is a major transducer of VEGF signal in EC and results in actin reorganization, mitogenicity and VEGF-mediated chemotaxis (Yoshida et al., 1996; Ferrara and Davis-Smith, 1997). The presence of Nrp1 enhanced the binding of $VEGF_{165}$ to KDR to about 4-fold and increased EC chemotaxis towards VEGF, 2.5-fold greater than KDR alone (Soker et al.,1998). Nrp can also bind $VEGF_{121}$,

however Nrp cannot form functional signalling complexes with this isoform as $VEGF_{121}$ lacks residues encoded by exon 7, which function as a bridge between Nrp and KDR (Pan et al., 2007). Similar to Nrp1, Nrp2 also participates in VEGF dependent functions but in lymphatic endothelial cells. Nrp2 binds to the lymphangiogenic VEGF-C and VEGF-D isoforms and their cognate receptor VEGFR-3 as part of an active signalling complex (Kärpänen et al., 2000). These studies provide clear evidence that Nrp is a VEGF co-receptor and regulates both angiogenic and lymphangiogenic functions in VEGF-dependent manner.

Identification of Nrp as a VEGF co-receptor interested researchers to evaluate its role in pathological angiogenesis. Nrp was shown to co-operate with KDR and VEGF in tumour angiogenesis. Overexpression of Nrp1 by prostate tumour cells *in vivo* resulted in 2.5 to 7 fold increase in tumour size, increased microvessel density and notably less tumour cell apoptosis (Soker et al., 2002; Miao et al., 2000). Similar results were observed in xenografts of human colon cancer cells overexpressing Nrp in nude mice (Parikh et al., 2004). Hepatocellular carcinomas treated with Nrp siRNA led to both reduction in tumour liver volume and inhibition of tumour vascular remodeling (Bergé et al., 2010). Bevacizumab (Bz) (also known as Avastin®, Genentech) is a VEGF-neutralizing antibody approved for patients with HER2 negative metastatic breast tumours (Sachdev et al., 2008). It functions by preventing VEGF from binding to VEGFR1 and VEGFR2 and therefore reduces tumour angiogenesis (Los et al., 2007). However tumours eventually overcome the effect of Bz, but when used in combination with Nrp1 blocking anti-bodies the efficacy of the drug is maintained (Staton et al., 2008).

VEGFRs are associated primarily with EC, however Nrp was shown to be expressed on tumour cell surface. In the breast cancer cell line MDA-MB-231, Nrp1 was expressed in relatively high copy number $1-2x10^5$ receptors per cell, 10- fold higher than on EC surface (Soker et al., 1996). The Nrp on the tumour cell surface was shown to participate in juxtracrine interactions with the KDR on the EC cell in the presence of the ligand (Soker et al., 2002). Also expression of Nrp on tumour cells is shown to participate in VEGF dependent autocrine survival loop in MDA-MB-231 breast cancer cell line (Bachelder et al., 2001). Apart from VEGF-mediated functions on the tumour

cell, Nrp also binds several other growth factors including TGF-β1 and HGF and its involvement in these pathways is discussed in the next section.
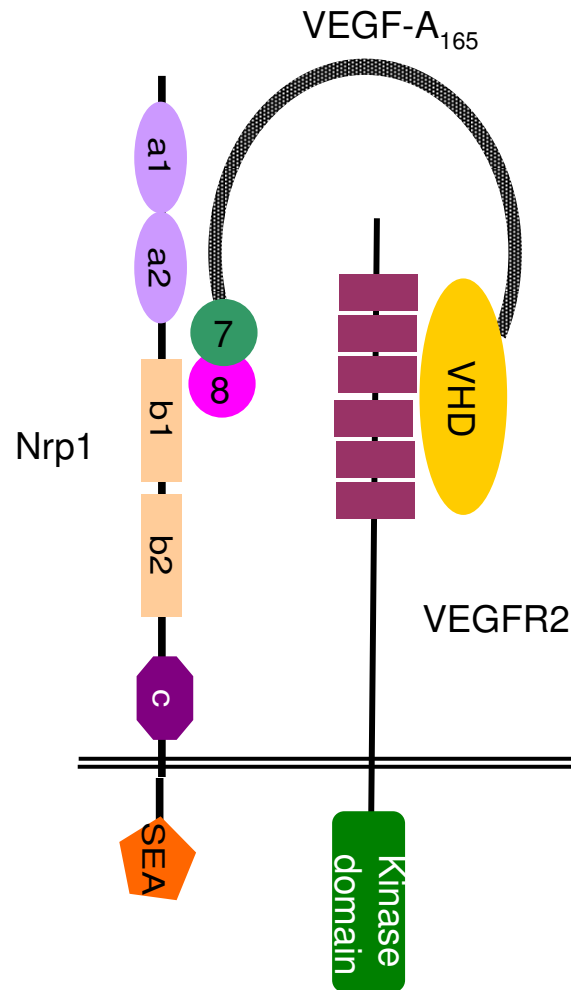
Figure 5 VEGF, Nrp1, KDR/ VEGFR2 tripartite complex formation mediates endothelial cell chemotaxis

VEGF-A$_{165}$ homodimer binds VEGFR2 with its vascular homology domain (VHD). The exon 8 of VEGF$_{165}$ encodes positively charged motif KPRR (residues162 to 165), which binds to the electronegative pocket of the b1 domain in Nrp. The exon 7 encoded residues stabilize the signalling complex by forming a bridge between Nrp and VEGFR2. The kinase domain and Ig-like domain of VEGFR2 are also shown above. Figure adapted from Pellet Many et al., 2008.

2.4.3 Transforming Growth factor beta -1 (TGF-β1)

TGF-β1 is a plieotropic cytokine with diverse functions in cell proliferation, differentiation, migration and apoptosis. Deregulation of TGF-β1 expression or signalling has been implicated in the pathogenesis of a variety of diseases, including cancer, fibrosis and auto-immune diseases. The major functions of TGF-β1 include inhibition of cell proliferation, enhancement of extracellular matrix production and inhibition of the immune system (Hyytiainen et al., 2004). *In vivo* TGF-β1 is secreted from cells as a latent complex consisting of non-covalently attached latency-associated propeptide (LAP) and covalently attached latent TGF-β binding protein (LTBP). The function of LTBP is to allow the delivery of the latent TGF to target locations in the ECM. TGF is activated upon cleavage of LAP by furin-like endoproteinases. Other activators of TGF-β include integrins, matrix metalloproteinases (MMPs), plasmin and thrombospondin-1 (Kaminska et al., 2005). Upon activation TGF-β1 dimer binds to its canonical receptor Type II TGFβ receptor (TGFβRII), Type I TGFβ receptor (TGFβRI) and mediates signalling through the Smad pathway (Figure 6).

Nrp1 is identified as a high- affinity receptor for latent and active TGF-β1 on regulatory T (T_{reg}) cell surface (Glinka and Prud'homme et al., 2008). LAP-TGF-β1 has an arginine rich C-terminal motif (QSSRHRR) that competed with $VEGF_{165}$ for binding to Nrp1 in ELISA assays. Nrp1 was also shown to activate LAP-TGF-β1 on the cell surface of MDA-MB-231 breast cancer cell line. The b2 domain of Nrp1 contains an RKFK motif analogous to Thrombospondin-1 and may play a role in changing the conformation and affinity of LAP to the mature TGF-β. Conventional T cells (CD4^{+} CD25^{-} Nrp1^{-}) coated with Nrp1-Fc, activated LAP-TGF- β1 and acquired strong suppressive T_{reg} activity (Glinka and Prud'homme et al., 2008). Therefore Nrp1 appears to mediate TGF- β1 effects on the cells of the immune system and may play a role in T_{reg} mediated poor tumour immunity (Zou et al., 2006).

In addition, Nrp1 expressed on the surface of MDA-MB-231 was shown to capture and activated LAP-TGF-β1 in Nrp1-dependent manner (Glinka and Prud'homme et al., 2008). Nrp1 associated with TGFβRII and TGFβRI in the presence of TGF-β1 and shown to augment Smad2/3 signalling *in vitro* in breast cancer cell lines (Glinka et al.,

2010). Nrp2 was also identified to play similar role in the TGF-β1 pathway. Within the context of the breast cancer, TGF-β1 is known to have a biphasic nature (Prud'homme, 2007). It has tumor-suppressive effects during the early stages of breast cancer and tumor-promoting role as the disease progresses. It is identified as a key player in epithelial-to-mesenchymal transition (EMT), assisting tumour cell extravasation and breast cancer metastasis to lungs, brain and bone. Therefore Nrp as a TGF-β1 co-receptor may participate in breast cancer cell proliferation, EMT and metastasis.
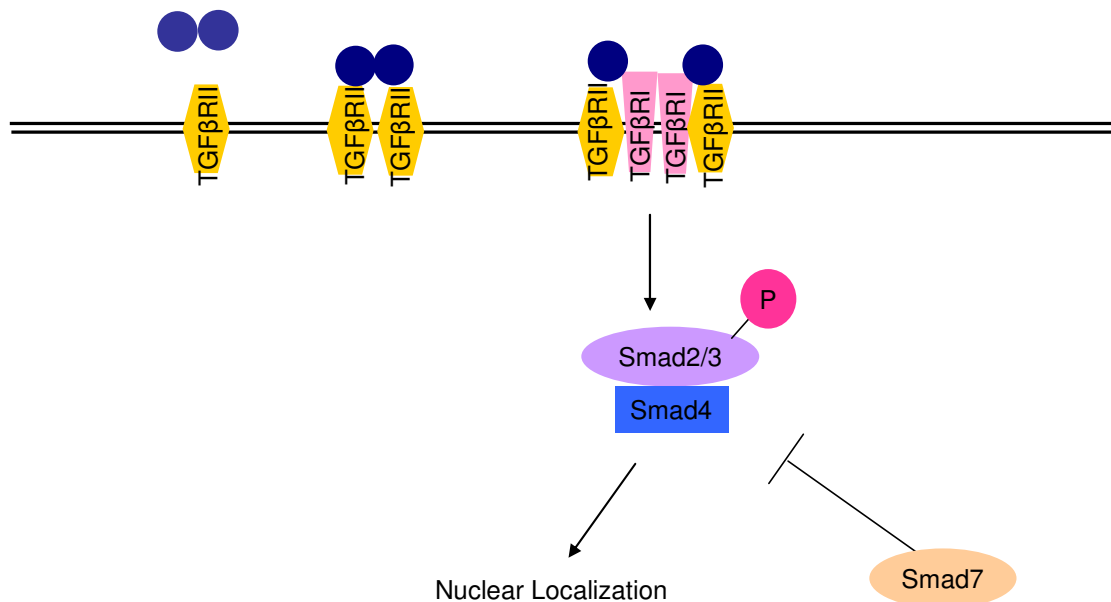
Figure 6 TGF- β1 signalling pathway

The activated TGF-β1 dimer binds to its canonical receptor Type II TGFβ receptor (TGFβRII). Type I TGFβ receptor (TGFβRI) follows and forms a large ligand- receptor complex consisting of the ligand dimer and four receptor molecules. Binding of TGFβ1 brings the intracellular kinase domain of TGFβRII in close proximity to TGFβRI allowing phosphorylation and activation of TGFβRI, which subsequently phosphorylates the Smad 2/3 proteins. Phosphorylated Smad2/3 associate with Smad4 and form a hetero-oligomeric complex, which translocates to the nucleus, binds TGFβ-1 responsive elements and regulates their transcription. Smad7 functions as an inhibitor in a negative feedback loop in the TGF-β1 pathway. Figure adapted from Hui and Friedman, 2003.

2.4.4 Hepatocyte Growth Factor (HGF)

Hepatocyte growth factor (HGF)/ Scatter Factor (SF) is secreted by stromal cells (fibroblasts, macrophages, smooth muscle cells) and acts on the epithelial cells in a paracrine fashion (Matsumoto and Nakamura, 2001). HGF was identified initially as a potent hepatotrophic factor responsible for liver generation, however its plioetropic effects on other cell types such as epithelial, endothelial and haematopoietic cells are well evident (Fautso et al., 1986; Gao et al., 2005). HGF stimulates epithelial cell proliferation and motility. HGF is also essential for branching morphogenesis, tubulogenesis and organ development in the lung, kidney and breast. In the embryo, the HGF/MET pathway is required for hematopoiesis, angiogenesis, muscle and nervous system development, In the adult organism HGF/MET pathway mostly plays a role in wound healing and repair functions (Trusolino et al., 2010). Nrp participates in similar functions during embryo development and in the adult organism (section 2.5) and may participate as a HGF-coreceptor in these processes. In fact, Nrp was recently identified as a HGF co-receptor on Endothelial cells (EC) and shown to potentiate HGF- mediated EC proliferation, migration, and angiogenesis *in vivo* in a mouse model (Suplice et al., 2008).

Apart from mediating normal physiological functions, the MET/ HGF pathway is associated with a wide range of epithelial and mesenchymal cancers like breast cancers, melanoma, fibrosarcoma (Steffan et al., 2010; Weon-kyoo you et al., 2008). In the mammary gland, MET/HGF pathway controls the balance between normal tubulogenesis and development of malignant adenocarcinoma cells (Mettucci et al., 2009; Firon et al., 2000). Over expression of MET contributes to an invasive phenotype during the progression of breast cancer (Beviglia et al., 1997). Specifically, MET expression was elevated at the advancing margins in the DCIS (Ductal carcinoma in-situ) suggesting a strong association between MET signalling and transition to invasive carcinoma (Edakuni et al., 2001; Tuck et al., 1996; Beviglia et al., 1997). Deregulated HGF/Met system triggers a program for epithelial-to-mesenchymal transition conferring invasive growth to carcinomas (Elliott et al., 2002; Mettucci et al., 2009). The HGF/MET pathway promotes invasiveness and metastasis by activating expression of extracellular

matrix degrading urokinase-type plasminogen activator (uPA) and its receptor (uPAR) and also activates cell adhesion molecules required for tumour cell extravasation (Birchmeier et al,. 2003). It was recently shown that HGF secreted by infiltrating fibroblasts (Invasive carcinoma) activates the HGF/MET dependent proteolytic pathways in mammary epithelial cells of DCIS and assists in transition to invasive carcinoma (ICS) (Jadeszko et al., 2009). It is unknown if Nrp plays HGF co-receptor functions and co-operates with the HGF/MET pathway in the progression of breast cancer and therefore is the focus of the current study.

HGF is secreted in an inactive form bound to a 21 amino acid long pro-peptide. The HGF precursor undergoes cleavage after the fourth kringle domain by serine proteases and forms a bioactive heteriodimeric mature HGF molecule (Figure. 7a) (Mizuno et al., 1992). HGF mediates mitogenic, motogenic and morphogenic actions on its target cells via its canonical MET tyrosine kinase receptor. Met receptor is a 190kDa heterodimeric transmembrane protein, composed of a 45 kDa extracellular alpha (α) subunit linked by disulfide bonds to a 145kDa membrane spanning beta (β) subunit endowed with tyrosine kinase activity (Figure. 7b) (Bardelli et al., 1994). Upon binding active HGF, the MET receptor undergoes dimerization and phosphorylation on multiple residues within the juxtamembrane, catalytic core and cytoplasmic tail domains. Phosphorylation of the tyrosine residues 1349 and 1356 on the β-subunit is both necessary and sufficient for HGF pathway activation and generates a multidocking site for downstream activators (Weon-kyoo you et al., 2008). The intracellular signalling cascade transduces the extracellular signal elicited by HGF and results in changes in cell proliferation, migration, survival and adhesion.
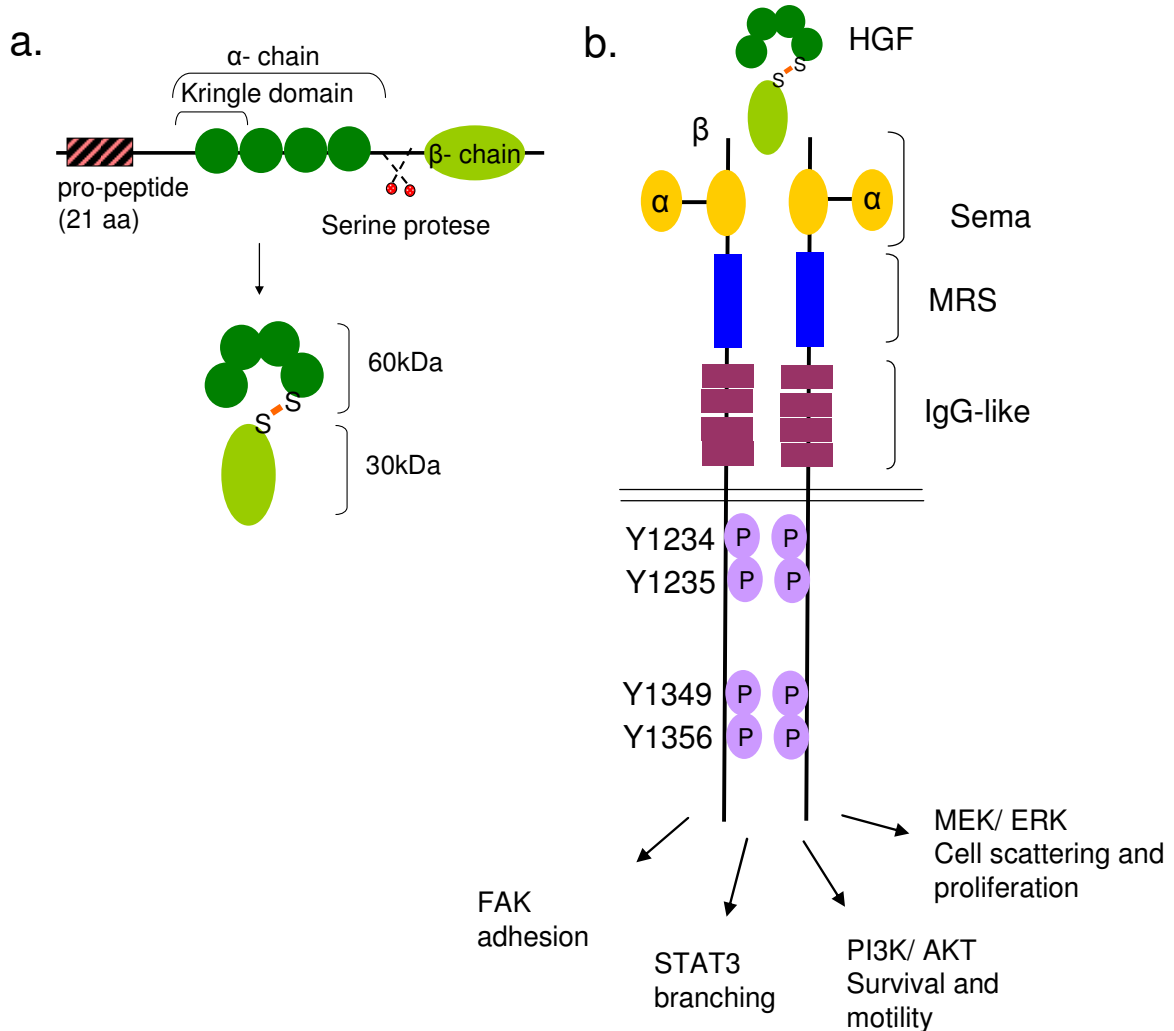
Figure 7 HGF propeptide processing and signalling through MET pathway

a). Human HGF is secreted as an inactive 728 amino acid (aa) single chain propeptide. It is cleaved after the fourth Kringle domain by a serine protease to form bioactive disulfide linked HGF with a 60 kDa α chain and 30 kDa β chain. Figure adapted from You and McDonald, 2008.

b). HGF heterodimer binds to the MET receptor (not shown) and initiates receptor dimerization (not shown). The extracellular region of MET receptor is composed of a Sema domain (homologous to Semaphorins), MRS (Cysteine-rich MET-related sequence) motif and four IgG like domains. Phosphorylation of tyrosine 1234 and 1235 in the β- subunit activates the MET catalytic activity and leads to activation of tyrosine 1349 and 1356. As a result a mutlisubstrate docking sites becomes available and binding of Grb2 and GAB1 adaptor proteins. These adaptor proteins activate a variety of signal transducers including FAK (Focal adhesion kinase), STAT3, AKT, MEK/ ERK and result in the appropriate cell effect. Figure adapted from You and McDonald, 2008.

2.4.5 Other Growth factor co-receptors

Co-receptors are a common phenomenon in many signalling pathways and are one of the most essential multifunctional regulators of signalling. In addition to the Neuropilin family, there are at least seven principal families of signalling co-receptors: interleukin family co-receptors, glypicans, syndecans, betaglycan, CD44, glial-cell-line-derived neurotrophic factor (GDNF) family, and low-density lipoprotein (LDL) receptor-related proteins (Mythreye and Blobe, 2009). These co-receptors share many structural and functional characteristics which are evolutionarily conserved across species. They have an indispensible function in development demonstrated by the lethal phenotypes of the coreceptor knockout mutants (Kirkbride et al., 2005; Mythreye and Blobe, 2009). Co-receptors can be either GPI (glycosylphosphotidylinositol) anchored or are transmembrane receptors (Kirkbride et al., 2005). They modulate ligand binding, promote the formation of signalling complexes and regulate cell signalling of the cognate ligand receptor. The multi-ligand binding ability of co-receptors allows them to participate in diverse range of functions including wound healing, migration, invasion, and adhesion (Orian−Rousseau and Ponta, 2008). They are frequently mutated or have an altered expression in human disease and therefore serve as prime targets for drug development.

2.5 Functions of Nrp in physiology and pathophysiology

2.5.1 Nervous System development and nerve injury repair in adult

Nrp1 null mutants die *in utero* between E10 and E13.5 (E stand for embryonic day) with extensive neuronal defects in central and peripheral nervous system (Kitsukawa et al., 1997). Nrp with semaphorins and plexins orchestrates the accurate pathfinding and homing of axons during embryogenesis. Nrp was shown to be essential for the formation of cranial and spinal nerve projections, guidance of central projections of the cutaneous sensory neurons and guidance of peripheral projections of the vestibular ganglion (Gu et al., 2003). It was recently shown to regulate migration of the multipotent neural crest cells. Specifically Nrp and sema axis prevents intermingling of the hyoid

and trigeminal neural crest streams in the hind brain (Schwarz et al., 2008). Neural crest cells determine the position of sensory neuron cell bodies and therefore Nrp not only plays a role in axonal pathfinding but also in cell body patterning (Begbie and Graham, 2001). Nrp has a more restricted distribution in the postanatal and adult nervous system.

Apart from its roles in the embryo, Nrp is suggested to participate in regeneration of adult sensory neurons after injury (Gavazzi et al., 2001; Pasterkampf et al., 2001; Fujisawa et al., 1996). Williams-Hogarth et al. (2000) observed an increase in Nrp expression in regenerating olfactory nerves after unilateral olfactory bulbectomy. Motor neurons in the spinal cord downregulated Sema and Nrp expression following peripheral nerve lesion and the expression was increased in regenerating axons (Pasterkampf et al., 1998). Approximately 70% of the neurons in dorsal root ganglion upregulated Nrp mRNA following sciatic nerve injury (Gavazzi et al., 2000). Therefore Nrp with semaphorins direct the formation of neural connections in the adult organism following injury.

2.5.2 Cardiovascular development, angiogenesis and wound repair

In addition to displaying neural defects Nrp null mice also display severe vascular anomalies. Nrp1 knock out mice display disorganized vessels in the yolk sacs with a sparse capillary network, fail to develop the branchial arch, great vessels and dorsal aorta, display transposition of the aortic arch and insufficient septation of the *truncus arteriosus* (separation of the aorta and the pulmonary artery) (Kawasaki et al., 1999). Nrp2 null mice exhibit a severe reduction of small lymphatic vessels and capillaries but display no obvious cardio-vascular abnormalities and survive to adulthood (Yuan et al., 2002).

Consistent with the Nrp knockout phenotype, Nrp was identified as a co-receptor for VEGF-A$_{165}$ (section 2.4.2) and a promoter of angiogenesis. Nrp is shown to potentitate VEGFR2 phosohorylation on endothelial cells (EC) and mediate VEGF$_{165}$ dependent EC chemotaxis. In newly sprouting vessels, Nrp assisted specialized endothelial tip

cells to sense local VEGF-A$_{165}$ gradients and undergo chemotaxis (Gerhardt et al., 2004). Antibodies blocking b1 domain of Nrp, which is known to interact with VEGF, inhibited endothelial cell sprouting and neovascularization *in vivo* (Pan et al., 2007). In addition, the Nrp blocking antibody, prevented EC adhesion to pericyte and effected the maturation and stabilization of the developing vessels (Pan et al., 2007).

Healing after an injury requires VEGF mediated angiogenesis to initiate new blood vessel formation. In a murine model of dermal wound repair, Nrp was abundantly expressed on new vasculature in healing wounds. In addition, mice treated with anti-Nrp antibody exhibited a significant decrease in vascular density within these wounds (Matthies et al., 2002). In human surgical wounds Nrp was upregulated early in the wound healing process and its expression was decreased after the tissue was repaired (Kumar et al., 2009). Nrp is also upregulated under ischemic conditions and leads to VEGF dependent neovascularization in cardiac and cerebral ischemia (see section 2.3.2).

2.5.3 Cell adhesion

Cell to cell and cell to extracellular matrix (ECM) adhesive interactions play pivotal role in both normal and tumour cell functions. E-cadherin, a Cell Adhesion Molecule (CAM), expressed on epithelial cells allows their organization in defined structures to accomplish highly specialized functions. Endothelial cells use integrin receptors to adhere to the ECM protein fibronectin (FN) inorder to develop a properly patterned network of blood vessels during development. The immune system requires adhesion of activated lymphocytes to target tumor cells. Apart from normal cell functions, tumour cells utilize cell adhesion during several steps of the metastatic process. Extravastion or escape from the microvasculature is the rate limiting step in the metastatic process (Lester et al., 1992). It requires the adhesion of the tumour cell to the endothelial cell monolayer to survive sheer stress and transmigrate through the basement membrane. Nrps are shown to play a role in both cell to cell and cell to ECM adhesion.

2.5.3.1 Nrp1 and Endothelial Cell to ECM adhesion

Nrp1 has recently been shown to mediate endothelial cell (EC) adhesion to FN, independent of VEGF. Nrp1 binds to and regulates vesicular trafficking of the Fibronectin (FN) receptor, α5β1 integrin (Valdembri et al., 2009). Integrins are heterodimeric transmembrane receptors expressed on EC surface that allow ECs to bind and interact with the extracellular matrix (ECM). Therefore by modulating integrin conformation and trafficking, blood vessel formation can be regulated (Serini et al., 2006).

Nrp1 tightly associates with the active form of α5β1 integrin at adhesion sites by using its extracellular b1/b2 domains. On the cytoplasmic side, Nrp1 PDZ binding domain encoded by the tri-peptide SEA (Serine, Glutamic acid, Alanine) interacts with RGS-GAIP-interacting protein (GIPC). GIPC is a homomultimeric endocytic receptor, which binds the molecular motor Myo6 and Rab5/ Rab-21 GTPases, and selectively allows endocytosis of active α5β1 integrin bound to Nrp1. The active α5β1 integrin is then recycled back to newly forming adhesion sites. Nrp1 allows EC adhesion by promoting efficient endocytosis and recycling of active α5β1 integrin and maintaining steady state adhesion sites between EC and the ECM.

Nrp1 was shown to synergistically cooperate with integrin-β1 to promote cell adhesion to matrix proteins in non-small-cell lung cancer, renal, pancreatic and prostate cancer cell lines *in- vitro* (Jia et al., 2010; Fukasawa et al., 2007). The interaction between integrin and Nrp1 might also allow crosstalk between growth factor receptors that Nrp1 binds. Such interactions have been observed on ECs between VEGFR, Nrp1 and integrin αvβ3 and αvβ5 to regulate EC adhesion, repulsion and/ migration (Byzova et al, 2000; Eliceiri, 2001; Streuli and Akhtar, 2009). Nrps bind multiple ligands, interact with the several CAMs and GF receptors, therefore the cellular response produced (cell migration, repulsion or adhesion) is cellular and subcellular context dependent.

2.5.3.2 Nrp1 and cell to cell adhesion

Nrp1 is also shown to play a role in homo and heterophilic cell adhesion. Fibroblasts transfected with the Nrp1 cDNA acquired cell adhesiveness in a cell aggregation assay.

This adhesion was shown to be mediated by heterophilic interaction between Nrp1 and certain protein molecules different from VEGF or SEMA on fibroblasts (Takagi et al., 1995; Shimizu et al., 2000). It has also been suggested that Nrp1 functions as a cell adhesion molecule during the formation of certain neuronal circuits *in vivo*. An 18– amino acid long region within the b1/ b2 domain, well conserved across species, is reported to play a role in Nrp1 adhesive functions (Shimizu et al., 2000).

Nrp1 on regulatory T cells (T_{reg}) was shown to interact with dendritic dells and formed stabilized immunological synapse suggesting a role of Nrp in maintaining effector T-cell tolerance towards tumour-associated antigens (Tordjman et al., 2002; Sarris et al., 2008). Similarly, Nrp2 expressed on monocyte-derived human dendritic cell surface was also shown to participate in homophilic interactions with T lymphocytes leading to T-cell activation and proliferation (Curreli et al., 2007). In addition Nrp1 is expressed on both endothelial and tumour cells, and might participate in homophilic interactions between the two cell types mediating key steps during angiogenesis and metastasis.

2.5.4 Nrp in Neoplasms and retinopathies

Nrp plays key roles in physiological angiogenesis during development and is upregulated in tissue repair and under ischemic conditions. Nrp also participates in pathological angiogenesis in tumour neovascularization, diabetic retinopathy and age-related macular degeneration (AMD).

2.5.4.1 Retinal vascular diseases

AMD is the major cause of visual impairment in aging adults. Choroidal neovascularization (CNV) leads to abnormal blood vessel growth and permanent damage to the photoreceptors resulting in vision loss in AMD. Choroid is the vascular layer behind the retina and supplies blood vessels to the macula (centre of the retina). The pathophysiology of CNV is not well understood but it may be considered as a wound healing response to damage to the retinal pigment epithelium (RPE) (Ohno-Matsui et al., 2003). Of the five human isoforms of VEGF, the most abundant and perhaps important for stimulation of ocular choroidal neovascularization (CNV) is

VEGF$_{165}$ (Otani et al., 2002). As a coreceptor for VEGF$_{165}$, Nrp1 was identified to play an active role in choroidal neovascularization (CNV) in patients with AMD. Immunohistochemistry revealed an increase in Nrp1 staining in choroidal membranes in areas with high vascularity. Specifically there was strong colocalization between Nrp1 and endothelial cell marker CD-31 (Cui et al., 2003; Lim et al., 2005). Nrp1 was also expressed in transdifferentiated RPE cells, which are the principal nonvascular stromal cells of both vascular (active) and non-vascular (fibrotic) areas in CNVMs (Lim et al., 2005). Nrp1 with VEGFR2 is implicated in RPE proliferation, with VEGF acting as an autocrine GF (Guerrin et al., 2005).

In diabetic retinopathy, blood vessels of the retina loose their structural integrity, specifically amongst the endothelial cells (EC), and pericytes are also lost. The EC develop microaneurysms, become leaky and sometimes occluded. As a result ischemic conditions arise and neovasculariation is induced in the retina. VEGF has been recognized as a predominant factor to induce the ischemic retinal neovascularization (Takagi et al., 2007). In the retinal endothelial cells, Nrp1 was shown to be upregulated by VEGF under hypoxic conditions. Nrp1 is a VEGF co-receptor, suggesting a positive feedback mechanism between Nrp1, VEGFR and VEGF to mediate neovascularization in the retina (Takagi et al., 2007). In addition to its roles in neovascularization, Nrp1 is also shown to participate in scar tissue formation (fibrovascular proliferation) in diabetic retinopathy that may lead to retinal detachment (Ishida et al., 2000)

Nrp2 is also identified to play a role in retinal neovascularization. Under ischemic conditions Nrp2 deficient mice suppressed VEGF-induced vascularization in the retina (Shen et al., 2004). However Nrp1 present in these mice did not compensate for the absence of Nrp2 suggesting different roles of Nrp1 and Nrp2 in retinal neovascularization. Targeting the Nrp family may result in decreased VEGF bioactivity and reduced neovascularization in the ocular tissues involved.

2.5.4.2 Nrp in tumour angiogenesis and other functions

Nrp1 is implicated in tumour neovascularization as a VEGF coreceptor (discussed in section 2.4.2). Nrp2 like Nrp1 can bind members of the VEGF family, specifically the lymphagiogenic members VEGF-C and VEGF-D, and the VEGFR3 on the lymphatic

endothelial cells. Nrp2 null mutants display abnormal lymphatic development with severe reduction in small lymphatic vessels and capillaries. The importance of lymphatic vasculature to metastasis is widely accepted and lymph node analysis is a common clinical practice to diagnose cancer metastasis. Antibodies blocking VEGF binding domains (b1 and b2 domains) of Nrp2 resulted in inhibition of lymphangiogenesis and a reduction in functional lymphatics associated with the tumour *in vivo* (Caunt et al., 2008). Anti-Nrp2 antibody also reduced lung metastasis in murine mammary carcinoma model (Caunt et al., 2008).

Apart from mediating VEGF-dependent angiogenic roles, Nrp play direct roles in tumour cell function. Nrp is expressed in a wide variety of human tumour cell lines and in tumour biopsies in prostate, kidney, bladder, pancreas, skin, colon, lung, ovarian and mammary carcinomas (Pellet-Many et al., 2008; Bielenberg et al., 2007). They are also expressed in glioblastomas, neuroblastoma, osteosarcoma and melanoma cell lines (Straume et al., 2003; Broholm et al., 2004; Fakhari et al., 2002). Increased expression of Nrp correlates with tumour aggressiveness, disease stage or poor prognosis in prostate, lung, ovary, colon carcinoma and gliomas (Pellet-Many et al., 2008; Bielenberg et al., 2007). Nrp2 expression is correlated with lymph node metastasis and associated with reduced overall survival in breast cancer. In addition, both Nrp1 and Nrp2 are identified as independent prognostic factors for long-term survival in breast cancer (Yasuoka et al., 2009; Gosh et al., 2008).

Nrp is implicated in tumour cell migration, invasion and apoptosis. Nrp1 regulates tumour progression and invasion in glioma and pancreatic cancer cell lines through HGF/MET pathway (Hu et al,. 2007; Matsushita et al., 2007). Blocking b1 domain of Nrp1 in kidney, prostate and lung carcinoma inhibited tumour cell migration, cell adhesion and increased cytotoxic effects of chemotherapeutic agents in the presence of VEGF (Jia et al., 2010). Nrp binds and activates TGF-β1 on the breast cancer cell surface and may participate in several TGF-β1 dependent functions including epithelial-to-mesenchymal transition and secretion of matrix metalloproteinases (Glinka et al., 2010). Therefore there is ample evidence for Nrp functions beyond tumour endothelium vascularization. Nrp as a growth factor coreceptor on tumour cell surface appears to be a key player in cancer progression.

2.6 Nrp and Cancer Stem cells (CSC)

There have been several studies aimed at characterizing the function of Nrp in cancer cell lines *in vitro* or in tumour mouse models *in vivo*, but there is a lack of studies testing Nrp function in the cancer stem cell population.

2.6.1 What are CSCs?

Cancer is a disease that arises due to accumulation of genetic mutations over time. In order to achieve malignant transformation a cell needs to undergo a series of mutations. However these mutations are rare and many mutations are required before it develops into a cancer. It is unlikely for a differentiated somatic cell with limited replicative lifespan and low mutation rate to acquire all the essential mutations to develop into a cancer. Adult stem cells are more long-lived and can accumulate more mutations. Cancer Stem Cell (CSC) or hierarchy model suggests that a cancer is composed of heterogeneous cell population organized in a hierarchy with a cell subset similar to adult stem cells at the apex of the neoplastic system (Figure 8). This sub- population is distinct from the rest, possesses indefinite ability to divide, self-renew and proliferate without senescing and is termed the Cancer Stem cell (CSCs) or Tumour Initiating cell (TICs) population. A second model called the stochastic model has also been proposed. Contrary to the CSC model, the stochastic model suggests that a tumour is made up of homogenous population of cells and all cells have equal potential to be tumorigenic, when exposed to random intrinsic or extrinsic factors. It is difficult to test the stochastic model as one cannot predict when and which intrinsic or extrinsic factors turn a cell into CSC (Dick, 2009).

The idea that cancer arises from stem cells dates back to 1858 when Rudolf Virchow observed that teratocarcinomas (cancer of the germ cells) are composed of totipotent embryonal cells. Later Cohnheim and Durante formulated the 'embryonal rest' theory based on the histological similarities between cancer and embryonic tissues (Sell, 2004). The Embryonal rest theory is the precursor to the current CSC theory and proposes that cancers develop from a remnant population of embryonal cells that remain dormant in the adult tissues and have the potential to become cancerous which we know as the adult stem cells. However it is unclear if CSC originates from pre-

existing adult stem cells or do the more differentiated cells undergo mutations to acquire the CSC phenotype (Singh et al., 2010).
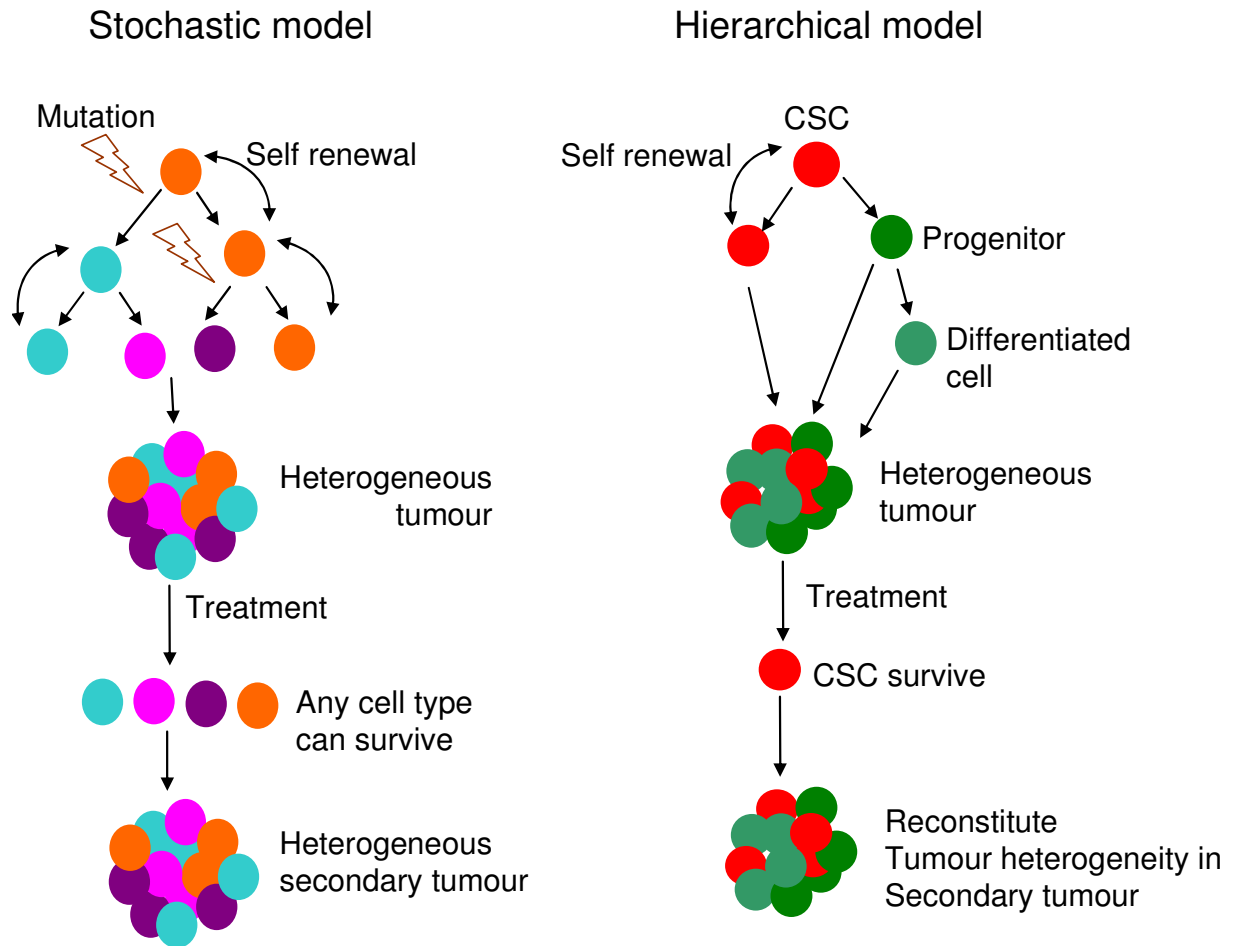
Figure 8 Stochastic and hierarchical models of cancer

According to the hierarchical model, the cancer stem cell (CSC) is at the apex driving the rest of the tumour population. The CSC (red) undergoes assymetrical division to give rise to itself by self-renewal (red) and give rise to a progenitor cell (green). The progenitor cells are committed to give rise to specific cell lineages and lack tumorigenic potential. They undergo proliferation and differentiation to form specialized cells (sea green). The bulk tumour is made up of all these cell types, however upon selection (after chemo or radiotherapy) only the CSC survives, has multilineage differentiation potential and forms the secondary tumour (relapse). The stochastic model suggests that all cells within the tumour have equal potential to form a new tumour upon exposure to random events (eg: mutation). Figure adapted from Kai et al., 2010.

2.6.2 Mammary Cancer stem cells

The mammary gland is a unique organ that has the ability to replenish differentiated myoepithlial, luminal and alveolar cells during cycles of pregnancy, lactation and involution, suggesting the presence of a stem cell population (Figure 9). However, no definitive identification of an adult mammary epithelial stem cell (MESC) has been made. Also the idea of a fixed MESC population is suggested to be oversimplified as the stem-cell like behaviour can be gained or lost based on its interactions with the microenviroment or stem-cell niche (Smalley and Ashworth, 2003). The classical definition of a stem cell is a cell with self-renewal capacity and ability to produce all cell lineages found in the mature tissue. Based on this definition a candidate population has been identified as the MESC. The MESC are identified to be suprabasal, located at the base of the luminal epithelial layer, next to the myoepithelium and not contacting the lumen or the basement membrane (Chepko et al., 1997; Alvi et al., 2002, Chepko et al., 1999). The MESC due to their undifferentiated state do not express the markers of the differentiated myoepithelial and luminal epithelial cells, although combination of certain markers (cytokeratin 19, vimentin, ER and integrin-α6) is proposed (Chepko et al., 1997; Pechoux et al., 1999; Stingl et al., 1998; Stingl et al., 2001; Gudjonsson et al., 2003).

Evidence for the presence of stem cell-like population in human mammary tumours was also discovered (Al- Hajj et al., 2003). This population was identified as CD44$^+$ CD24$^{-/low}$ and was negative for the differentiated breast epithelial cell lineage markers (Lin$^-$). This population displayed an increased ability (upto 10- 50 fold) to form tumours in xenografts in comparison to the rest of the tumour population. This population shared two key features with stem cells, their ability to self-renew and their ability to differentiate into many cell lineages and recapitulate the entire tumour population. CD44$^+$ CD24$^-$ Lin$^-$ cell population derived tumours phenocopied the parent tumour, as shown in the histology sections of xenotransplants and secondary tumours from metastatic sites (Al- Hajj et al., 2003).

In addition to these properties, CSCs are quiescent and have low mitotic rate making them radiotherapy resistant. CSCs also express high levels of ATP-dependent efflux transporters called ATP Binding Cassette (ABC) transporters that pump out xenobiotics

and chemotherapeutic drugs from the cell. CSC cell numbers have been shown to increase following neoadjuvant therapy, and survival of CSCs results in multiple drug resistant (MDR) tumours and cancer relapse.
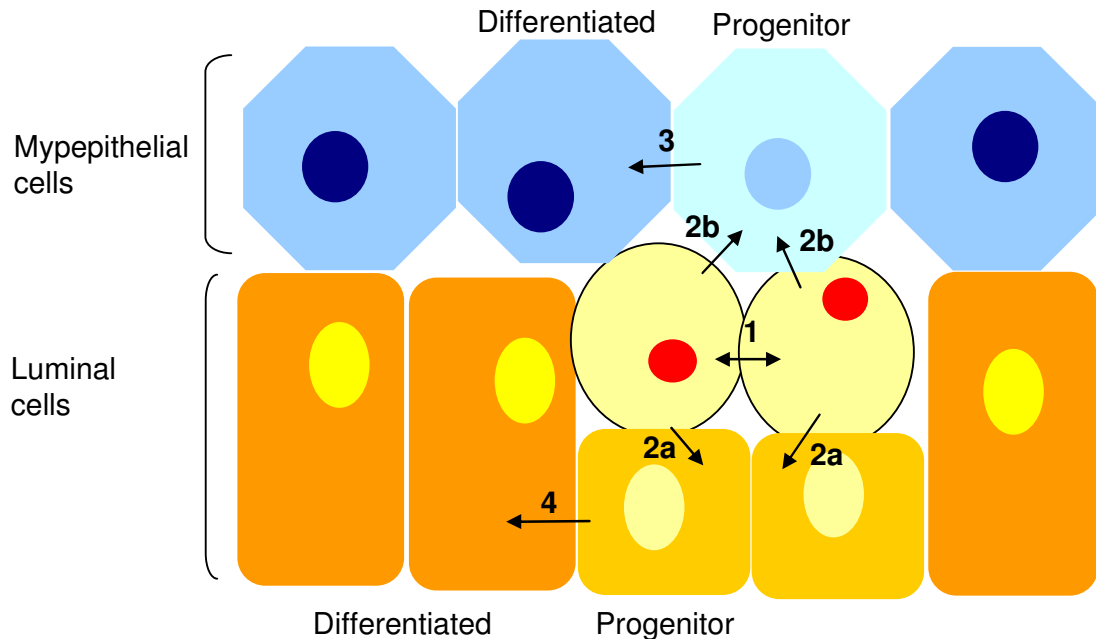
Figure 9 Hierarchical organization of the mammary epithelium

1) Adult mammary stem cells undergo asymmetric cell division to maintain their population. 2) They also generate the transit-amplifying or progenitor cells in the luminal and mypepithelial lineages. It is difficult to distinguish a stem cell from a progenitor/ tranist cell in its early stages as it gradually acquires the lineage markers. 3,4) The lineage committed progenitor/ transit cell undergoes proliferation and differentiation to give rise to the corresponding myoepithelial or lumial cell lineages. The alveolar cell lineage is thought to arise from invasive transit cell, which digests the extracellular matrix and then differentiates into myoepithelial or luminal cells (not shown). Figure adapted from Smalley and Ashworth, 2003.

2.6.3 Isolation and characterization of CSCs

To study CSCs we need to fractionate them from the rest of the population. There are two major approaches to enrich for CSC, candidate and operational approaches. Candidate approach uses cell surface markers such as $CD44^+$ $CD24^-$ to sort for CSC by FACS or magnetic bead sorting (Winquist et al., 2010). However sorting by $CD44^+$

CD24⁻ phenotype does not result in a bonafide CSC population and some cells within this population are non-tumorigenic (Winquist et al., 2010). Including a third marker ALDH1 (aldehyde dehydrogenase), in addition to the CD44$^+$ CD24$^-$ enriched for more CSCs (Winquist et al., 2010; Charafe-Jauffret et al., 2009). Therefore, currently it is not very clear which combination of markers would yield a pure CSC population.

The operational approch utilizes the innate properties of CSC for their enrichment. The side population (SP) assay uses the ability of CSC to pump out chemotherapeutics and xenobiotics. SP assay uses a dye called Hoechst 33342 which is retained by the differentiated population while the CSCs stain negative (Alvi et al., 2003; Hambardzumyan et al., 2006). A SP isolated from the MCF-7 breast cancer comprised 2% of the bulk population and was able to reconstitute the heterogeneity of the cell line in NOD/ SCID mice xenografts (Kondo et al., 2004; Patrawala et al., 2005). But the highly toxic nature of Hoechst 33342 effects cell viability in subsequent functional assays *in vitro* and *in vivo*, and therefore is not the preferred method for CSC enrichment (Charafe-Jauffret et al., 2009).

CSCs are also endowed with drug resistance and are responsible for cancer relapse. Levina et al. (2008) treated human cancer cell lines with the conventional chemotherapeutics, cisplatin, doxorubicin or etoposide and enriched for CSC which they called drug surviving cells (DSCs). The DSCs have also been enriched in breast cancer cell lines by using Mitoxantrone (Prud'homme et al., 2010). These cells maintained self-renewal capacity demonstrated by the mammosphere assay (discussed next), possessed multi-lineage differentiation potential, and were highly tumorigenic and metastatic upon inoculation in SCID mice (Levina et al., Prud'homme et al., 2010). In addition the DSCs were shown to contain two-to-three-fold higher levels of angiogenic and growth factors (VEGF, FGF, IL-6, IL-8, HGF, PDGF-BB, G-CSF, and SCGF-b). DSCs also showed elevated levels of expression of human VEGFR2, FGFR2, CXCR1, 2 and 4 receptors (Levina et al., 2008). *In vivo* the implanted CSCs stimulated the murine stroma to produce increased levels of angiogenic and growth factors (Levina et al., 2008). The angiogenic and growth factors are suggested to be critical for stem cell survival and for their metastasis and establishment at a secondary site. Nrp is a growth factor co-receptor suggesting a possible involvement of Nrp in CSC biology.

Another operational approach is the mammosphere formation assay, which is dependent on the self renewal and anchorage independent growth properties of stem cells. When cultured on nonadherent surfaces in the presence of growth factors (such as Fibroblast Growth Factor) without serum, human mammary epithelial cells formed spherical colonies, which are termed "nonadherent mammospheres" (Dontu et al., 2003). The idea for mammosphere assay came from a major advance in neural stem cell research where undifferentiated multipotent stem cells were found enriched in neurosphere growth suspensions (Reynolds and Weiss 1996; Weiss et al. 1996). The mammospheres are able to differentiate along the three mammary epithelial lineages and reconstitute the mammary gland *in vivo* in mice (Dontu et al., 2003).

The mammosphere assay is widely used to enrich for CSCs from invasive tumour samples and cell lines. The mammospheres enriched from the tumour population are called tumourspheres although the term mammosphere is used interchangeably (Dontu et al., 2003). Mammospheres are shown to contain a more enriched population of stem cells and therefore a more efficient tool to study the CSC population. Mammospheres have 27% SP^+ cells in comparison to the 1-2% SP^+ cells found in the bulk tumour population (Dontu et al., 2003). The mammospheres also show an increase in $CD44^+$ $CD24^-$ Lin^- phenotype, expressed putative stem cell marker OCT4, and displayed high tumorigenic potential in NOD/SCID mice (Ponti et al., 2005). The mammosphere assay is also an effective tool to study pathways critical to both normal and neoplastic stem cells discussed in the next section.

2.6.4 Pathways implicated in stem cell maintenance and function

The Hedgehog pathway (Hh), BMI1/polycomb, NOTCH signalling have been shown to act on stem cell and their progenitors during human mammary development. These pathways are also shown to be critical for breast tumorigenesis and recurrence, suggesting their involvement in CSC biology (Dontu et al., 2004; Dreesen et al., 2007). The formation of mammospheres from pre-invasive ductal carcinoma *in situ* was reduced significantly when NOTCH signalling was inhibited (Farnie at al., 2007). The Hh pathway is shown to play an essential role in the maintenance of the $CD44^+$ $CD24^-$

subpopulation and SP+ population. Knockdown of Gli1 transcription activator of the Hh pathway, suppressed the proliferation of both populations (Tanaka et al., 2009). Although not very well characterized, Nrp is also shown to play some roles in the CSC biology. In an aggressive renal carcinoma cell line (RCC), Nrp1 was shown to play a role in maintaining an undifferentiated phenotype through the hedgehog pathway. Upon Nrp1 knockdown RCC displayed a more differentiated phenotype and expressed the epithelial-speciifc (E-cadherin) and kidney-specific cadherins (Ksp-cadherin). In addition the expression of mesenchymal markers Snail and α- SMA was decreased in Nrp1 knockdown cells (Cao et al., 2008).

Apart from these pathways the NF-κB signalling was recently identified to play a crucial role *in vivo* in mammary CSC biology (Lui et al., 2010). NF- κB is a family of transcription factors and has five members divided into two sub-classes. The p50 and p52 members function as the DNA binding subunit/ domain (DBD), while the c-Rel, p65/RelA, RelB members have activation subunit/ domain (AD) functions. These subunits oligomerize in different combinations to generate a functional transcription factor, containing one DBD and one AD. The most common combination is the p50 and p65 heterodimer (Brasier et al., 2006). The NF-κB signalling is regulated by IκB or inhibitor of κB, which sequesters p65/ p50 dimer in the cytosol. Upon presentation of an appropriate stimulus such as a growth factor, the kinase for IκB (IKK) gets activated (Figure 10). IKK phosphorylates IκB on two key serine residues, which is followed by ubiquitination and degradation of IκB. The active NFκB p65/p50 complex translocates to nucleus for gene transcription of targets including cytokines, chemokines, cyclins and cell adhesion molecules (Gilmore, 2006).

Activation of NF-κB-dependent target genes has been identified in a variety of solid tumors and hematologic malignancies (Zhou et al., 2008). Activated NF-κB is shown to affect cell proliferation, allows evasion of apoptosis and promotes anchorage-independent growth in breast cancer (Ramaswamy and Perou, 2003). Rel A or p65/ p50 heterodimer is shown to be activated in 86% of ER- and ERB2/ HER2+ breast tumours (Biswas et al., 2004). NF-κB is shown to be constitutively activated in acute myelogenous leukemia cells (Guzman et al., 2001) Blocking NF-κB signalling with pathway specific inhibitors such as parthenolide (PTL) and pyrrolidine dithiocarbamate

(PDTC) was shown to specifically inhibit the leukemic stem cell population (Guzman et al., 2005; Schreck et al., 2001). Similar studies conducted in the MCF-7 breast cancer cell line with the NF-κB inhibitors resulted in reduction in mammosphere formation and SP⁺ cell population (Zhou et al., 2008). Recently Lui et al. (2010) have identified a role for activated NF- κB in mammary CSC expansion. Inhibition of the NF- κB pathway with IκB reduced tumour- associated macrophages and tumour neoangiogenesis *in vivo* in mice. In addition the IκB also inhibited the CSC markers, Nanog and Sox2, in transgenic tumours and reduced stem cell expansion *in vitro* (Liu et al., 2010). Inhibition of NF-kB activity reduced mammosphere number by greater than 60%. These studies highlight the significance of NF- κB pathway in CSC biology and identify it as a potential therapeutic target.
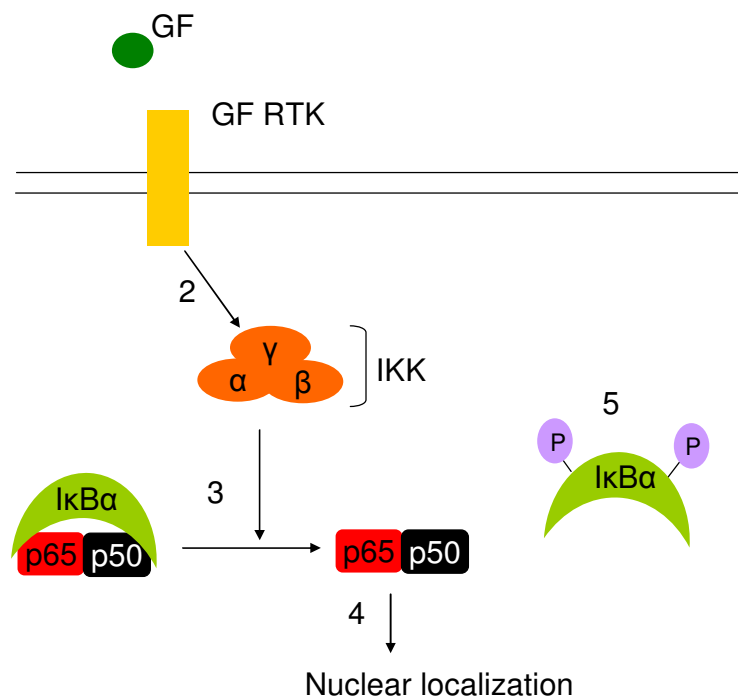
Figure 10 NF-κB signalling pathway

1) NF-κB pathway is activated in response to growth factors. 2) The stimulus activates the kinase for the inhibitor of κB (shown here as the trimeric complex: Iκκα, Iκκβ and Iκκγ/ NEMO). 3) The IκB bound to the NF-κB p65/ p50 dimer, gets phosphorylated and releases the p65/ p50 subunit. 4) The p65/ p50 translocate to the nucleus and bind NF-κB consensus sequences 5) The IκB undergoes proteolytic degradation.

2.6.5 Tranilast, a drug targeting CSC population

CSCs are endowed with properties that allow them to survive the current non-selective therapies and initiate cancer relapse (section 2.6.4). There has been some progress in the development of drugs targeting the CSC population, mostly in non-solid tumours. Parthenolide, a sesquiterpene lactone, has been shown to inhibit the NF-κB pathway and target apoptosis in CSC of both acute and chronic myelogenous leukemia (AML and CML) (Guzman et al., 2005). Sesquiterpene lactones (SLs) are plant-derived compounds often used in traditional medicine against inflammation and cancer (Ghantous et al., 2010). Targeting the PI3K/AKT/mTOR signalling network has also been shown to be effective against CSC in AML (Martelli et al., 2009).

Recently, Tranilast (N-[3, 4-dimethoxycinnamoyl]-anthranilic acid] was shown to specifically target breast cancer stem cells (Prud'homme et al., 2010). The molecular target of tranilast was identified as the aryl hydrocarbon receptor (AHR) (Prud'homme et al., 2010). AHR is a transcription factor known to be activated by polycyclic aromatic hydrocarbons, mostly toxins, such as 2, 3, 7, 8-tetrachlorodibenzo-p-dioxin (TCDD). However tranilast was identified as a non-toxic agonist of AHR, which induces AHR translocation to the nucleus and activated CYP1A1 expression. AHR is expressed at a higher level in CSC and siRNA knockdown of AHR was shown to inhibit the anti-proliferative and anti-mammosphere activity of tranilast. Tranilast was effective at inhibiting CSCs in both triple negative (MDA-MB-231, SUM159, SUM149) and triple positive mammary cancer cell lines (BT474), suggesting that it is an effective therapy for several breast cancer subtypes.

Tranilast is used clinically in Japan as an orally administrated drug, which acts on mast cells to control allergic responses (Konneh, 1998; Komatsu et al., 1988; Azuma et al., 1976; Koda et al., 1985). Tranilast is also used to control fibrotic disorders as it inhibits collagen synthesis and fibroblast proliferation (Suzawa et al., 2002; Fukayama et al., 1996; Prud'homme, 2007; Yamada et al., 1994). Tranilast has been shown to exert antiproliferative and antitumour effects in mammary carcinomas by inhibiting the TGF-β pathway. Specifically, tranilast significantly inhibited TGF- β dependent Smad phosphorylation and epithelial-to-mesenchymal transition (EMT) (Chakrabarti et al.,

2008). Similar effects were observed in gliomas, uterine leiomyoma cells, pancreatic carcinomas and oral squamous cell carcinomas (Platten et al., 2001; Shime et al., 2002; Hirori et al., 2002; Noguchi et al., 2003). In the highly metastatic 4T1 mouse mammary cell line tranilast reduced metastasis to the lungs by 90% in the syngeneic BALb/c mouse model (Chakrabarti et al., 2008). In addition tranilast exerted a broad suppressive effect on immune cells. Tranilast inhibited cytokine production by T cells including TGF- β and IL-17, which have been linked to tumour progression (Chakrabarti et al., 2008). Therefore, tranilast also has an effect on the stromal cells by inhibiting lymphocyte infiltration, reducing fibroblast proliferation and extracellular matrix production.

3 Materials and Methods

3.1 Breast cancer cell lines and culture conditions

Human mammary cell lines MDA-MB-231, MCF-7, SKBr-3, BT474 and MDA-MB-453 were purchased from American Type Culture Collection (Maine, USA) and cultured in Dulbecco's modified Eagle medium (DMEM) (4.5g/L glucose, L-glutamine and sodium pyruvate) supplemented with10% fetal bovine serum (FBS), Penicillin (100 IU), streptomycin (100ug/ml) and Fungizone (0.5ug/ml). MCF-7 cell line was in addition supplemented with insulin (0.01mg/ml). SK-N-SH glioblastoma cell line was a kind gift from Dr. Bhavnani (St. Michael's Hospital, Toronto, Canada) and was cultured in complete DMEM and used for Nrp2 antibody optimization.

4T1 is a murine mammary cell line derived from mammary epithelial carcinoma from BALB/c mice and cultured in DMEM with supplements mentioned above (ATCC, Virginia, USA).

SUM149PT and SUM159PT cells were purchase from Asterand (Detroit, USA) and cultured in Ham's F12 nutrient mixture with 5% FBS, insulin (5ug/ml), hydrocortisone (1ug/ml), HEPES (10mM), Penicillin (100 IU) and streptomycin (100ug/ml).

All cell lines were grown at 37ºC in a 5% CO_2 environment. For long term storage cell lines were cryogenically frozen in their specific culture medium in 5% DMSO.

3.2 *In vitro* knockdown of Nrp1 and 2 using siRNA in breast cancer cell lines

In a six well tissue culture plate, $1x10^5$ MDA-MB-231 or $1.5 x10^5$ MCF-7 cells per well were seeded in their growth medium. Next morning cells were rinsed twice in serum and antibiotic free DMEM before siRNA transfection. 3 ul Oligofectamine reagent (Invitrogen, Cat # 12252-011) was diluted in 13 ul Opti-MEM (Gibco, cat# 31985-070) and incubated for 5 minutes at room temperature (RT). 0.9 uM Nrp1 (santacruz, cat #sc-36038) siRNA diluted in Optimem was added to the diluted transfection reagent and incubated for 20 minutes at RT for complex formation. Complexed siRNA was further

diluted to 65 nM in DMEM, mixed, gently overlaid onto the previously rinsed cells and incubated for 6 hours at 37°C in a CO_2 incubator. MCF-7 cell line was transfected with 35 nM of complexed Nrp1 siRNA. Cells were then returned to DMEM containing 10% FBS and antibiotics 6 hours post transfection. Cells transfected with oligofectamine only without any siRNA served as the negative control in these experiments. To generate double knockdown, MDA-MB-231 cell line was sequentially transfected with 65 nM Nrp2 siRNA (santacruz, cat #sc-36040) using the method described above, 24 hours post transfection with Nrp1 siRNA.

3.3 Protein extraction and quantitation

Cells were rinsed twice in 1X PBS, scraped and collected on ice in lysis buffer (50 mM Tris; pH 7.6, 150mM NaCl, 0.1% NP-40) containing the cocktail of protease inhibitors: PMSF (1mM diluted in isopropanol, Sigma, cat# P-7626), Leupeptin and Pepstatin (5ug/ml), Aprotinin (5ug/ml, Sigma, cat# A6279). Phosphorylated protein lysates were further supplemented with a mixture of tyrosine, serine/threonine, acid and alkaline diluted phosphatase inhibitors (1:100) (Sigma, Cat # P5726 and P2850). Cells were lysed by using Sonic Dismembrator (Fischer, model #300) with three 10 second pulses set at relative output of 0.2. Total protein lysates were collected as supernatant after spinning at 13,200 rpm in an Eppendorf 5415R Micro Centrifuge for 20 minutes at 4ºC and stored at -80ºC.

Protein concentration was estimated by using Bradford assay (Bradford, 1976). Protein samples were diluted (1:1000) in distilled water and (1:5) Bradford dye reagent (Biorad, Cat# 500-0006), incubated for 20 minutes at room temperature and quantified using Spectrophotometer- DU 800 (Beckman Coulter). Protein standard curve ranging from 0 to 5 ug/ ml was generated using Bovine Serum Albumin (BSA) (sigma, cat# P0914) and used to estimate the concentration from sample OD_{595}.

3.4 Western Blotting

Concentrated (5x) Laemmli sample buffer (60 mM Tris; pH 6.8, 5% β-mercaptoethanol, 10% glycerol, 3% sodium dodecyl sulfate (SDS) and bromophenol blue) was added to 50 ug cell lysates. Samples were incubated for 5 minutes in boiling water bath, vortexed and loaded onto a 10% SDS-polyacrylamide gel and ran at 30 mA. The gel was transferred to PVDF memrbrane (Millipore, Cat# IPVH0010) at 11V.

The membranes were blocked in 5% skim milk in Tris- buffer with 0.1 % Tween 20 (Sigma, Cat # P1379) (TBS-T) for 1 hour at RT and incubated over night at 4ºC with the appropriate primary antibody (Table 1). The membranes were rinsed in TBS-T for 10 minutes and the washes repeated three times. The membranes were then incubated with the appropriate secondary horseradish peroxidase conjugated antibody for 1 hour at RT (Table1) and washed again in TBS-T. Proteins were visualized using enhanced Luminol Chemiluminescence substrate (Perkin Elmer, Cat # NEL101001) and autoradiography film (Kodak X-OMAT Blue, Cat # 1776699).

 When required the membranes were stripped in 1X stripping buffer (Chemicon, Cat # 2502) for 20 minutes at RT, rinsed twice in 5% skim milk in TBS-T and incubated with appropriate antibodies.

Table 1. Primary and secondary antibodies used for immunoblotting

Antibody	Source	Supplier	Working Dilutions	Predicted MW
Primary				
MET	Rabbit polyclonal	Santa Cruz, sc-10	1:200	Met Precursor: 170 kDa
				β- subunit: 145kDa
phospho Tyr1349 MET	Rabbit polyclonal	Santa Cruz,sc-34086	1:200	131kDa
Neuropilin-1	Goat polyclonal	Santa Cruz, sc-7239	1:200	130 kDa
Neuropilin-2	Mouse monoclonal	R & D, MAB567	2ug/ml	130kDa
PARP, cleaved PARP	Rabbit polyclonal	Cell signalling, 9546	1:200	PARP: 116kDa
				Cleaved form: 89KDa
β- actin	Mouse monoclonal	Abcam, ab8226	1:20,000	42kDa
Secondary				
anti rabbit- HRP	Goat polyclonal	Promega, W4011	1:1000	
anti mouse- HRP	Goat polyclonal	Promega, W4021	1:1000	
anti goat- HRP	Donkey polyclonal	Genscript, A00178	1:1000	

3.5 MTT cell proliferation assay

3×10^3 MDA-MB-231 cells were seeded in DMEM containing 10% FBS without antibiotics in a 96-well plate. Cells were rinsed and transfected with either Nrp1 siRNA alone or both Nrp1 and 2 siRNA as described previously (section 3.2). Cells sham transfected with oligofectmaine served as the negative control for the assay. MTT assay was performed by adding 20% MTT reagent to cells and incubated for 2 hours at 37ºC (Mosmann et al., 1983). Cells were solubilized in acidified (0.04N HCl) isopropanol for 1 hour and optical density was determined at 540 nm using a plate reader. Wells containing medium and MTT dye without cells served as the blank for absorbance readings. Assay was conducted in triplicates over 48, 72, 96 and 144 hours post-transfection. The OD_{540} subtracted from the blank was used as a measure of cell proliferation and any significant differences between Nrp knockdown and sham-transfected groups was statistically evaluated (see section 3.13).

3.6 Scratch wound cell migration assay

1.5×10^5 MDA-MB-231 cells were seeded per well of a 6-well plate and treated with Nrp1 siRNA or sham transfected as described previously (see section 3.2). 48 hours post-transfection cells formed a confluent monolayer. Three parallel scratches were made across the monolayer perpendicular to the line of reference (Figure 11). Cell migration was photographed at points of intersection between the line of reference and wound at 0 and 6 hours using a phase contrast microscope (10x magnification). Percentage of wound closure was evaluated using TScratch software as described previously (Gebäck et al., 2009). Assay was conducted in triplicates per experiment and repeated three times. When required the cells were induced with 5 ng/ml HGF in DMEM containing antibiotics without serum. To minimize the confounding effect of proliferation on migration, the assay was conducted in the absence of FBS. Absence of FBS did not affect cell viability as determined by trypan blue staining at the end of 6 hours.

Points of intersection

Line of reference
(drawn using a marker at
the bottom of the plate,
prior to cell seeding)
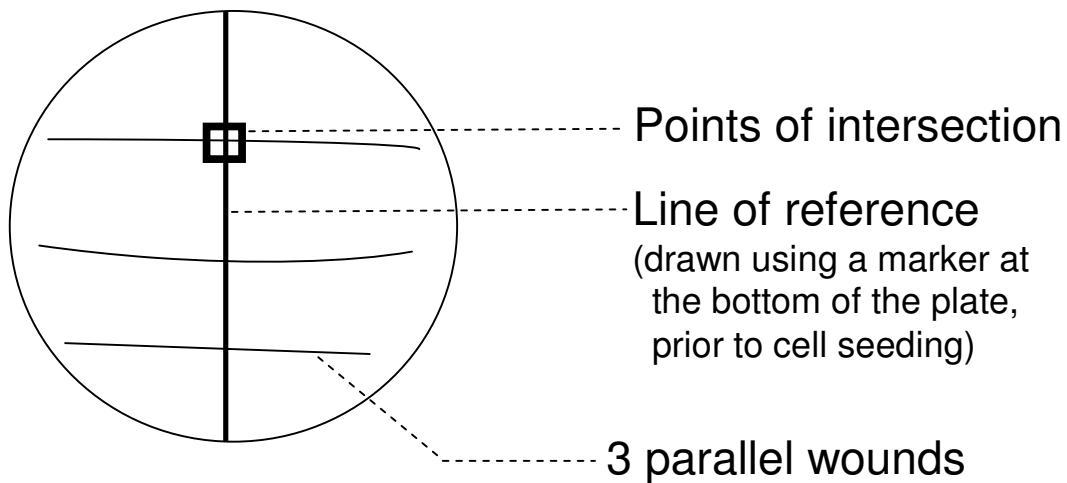
3 parallel wounds

Figure 11 Schematic representation of the scratch wound migration assay

Indicated are the three parallel wounds generated using a P200 yellow pipette tip perpendicular to the line of reference. Indicated by the black square is the point of intersection at which measurements were taken regularly and cell migration was evaluated by microscopy (section 3.6).

3.7 Apoptosis assay

MDA-MB-231 cells treated with Nrp1 siRNA or sham control (48 hours post-transfection) were induced to undergo apoptosis using 25uM Etoposide (Sigma, Cat # E1383) or DMSO (solvent for Etoposide) for 24 hrs in DMEM containing 1% FBS with antibiotics. Total cell lysates were prepared (see section 3.3) and probed for cell apoptosis marker, cleaved PARP, by immunoblotting (see section 3.4).

3.8 *In vitro* activation of MET pathway using recombinant Hepatocyte Growth Factor (HGF)

MDA-MB-231 cell line transfected with Nrp1 siRNA or both Nrp1 and 2 siRNA or sham transfected were serum-starved overnight in DMEM 48 hours post-transfection. Next morning the cells were rinsed twice in DMEM and treated with 0, 1 or 5 ng/ml recombinant HGF (R and D Systems, cat # 294-HGN) for 15 minutes at 37°C. Cells were rinsed in 1X PBS, lysed in cold lysis buffer containing phosphatase, protease inhibitors and probed for total MET, p-MET (Tyr-1349), Nrp1, Nrp2 and β- actin as the loading control (section 3.3 and 3.4).

MCF-7 cell line was transfected with Nrp1 specific siRNA or sham transfected, induced with recombinant HGF for 15 minutes at 37°C and cell lysates were prepared using the same procedure.

3.9 Mammospheres

1.5×10^5 MDA-MB-231 cells were seeded per well of a 6-well plate in complete medium. Next morning cells were transfected with Nrp1 siRNA and then sequentially with Nrp2 siRNA (see section 3.2). 24 hours post transfection with Nrp2 siRNA, cells were rinsed twice in 1X PBS and collected non-enzymatically in Hanks' Balanced Slat Solution (HBSS) buffer containing EDTA (0.5 mM) and incubated for 30 minutes at 37ºC. Cells were counted, spun and resuspended in CnT-27 mammosphere medium (Cell n TEC, Cat# CnT-27) and growth additives as described previously (Dontu et al., 2003). 1000 cells/ well were seeded in 100ul CnT-27 media in ultra-low attachment 96-well plate (Coring, Cat# 3474).

Self-renewal capacity of the CSCs was determined by passaging primary mammospheres 7 days post-initial seeding when they were approximately 60μm in diameter as recommended by the manufacturer. To generate secondary mammospheres, primary mammospheres were harvested and spun at 300 x g for 10 minutes, the supernatant was aspirated and cells resuspended in 0.1 %Trypsin-EDTA. Trituration was then used to dissociate mammospheres into single cell suspensions.

The cell suspension was rinsed in cold HBSS, spun at 300 x g for 10 minutes and resuspended in CnT-27 medium. Cell viability was determined using Trypan blue (1:1) and cells were seeded at 1000 cells/ well in 100ul CnT-27 medium in a 96-well plate to generate secondary mammospheres. The size and numbers of mammospheres were quantitated using Image J software (NIH, USA). Cell aggregates equal to or greater than 60 μm in diameter were considered mammospheres.

3.10 NFκB p65 ELISA assay

Protein cell lysates from Nrp double knockdown or sham-transfected MDA-MB-231 cells were prepared in cold RIPA buffer (1X PBS, pH 7.4, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, phosphatase and protease inhibitors (see section 3.3). Protein extract was collected as the supernatant after spinning for 20 minutes at 13,000 rpm at 4℃. To measure the active form of NFκB p65 in the cell lysates an ELISA kit (Stressgen, Cat # EKS-446) was used.

NFκB p65 ELISA plate was purchased pre-coated with streptavidin bound NFκB biotinylated-consensus sequence (Figure 12). Protein extract was added to 1X binding buffer per well and incubated with mild agitation for 1 hour at RT. 40 pmoles NFκB competitor duplex was used as a negative control and to ensure signal specificity. The competitor duplex was added to the plate before addition of the protein extract. 2ul of TNFα activated HeLa cell Nuclear Extract was used as the positive control. After incubation for 1 hour with protein extract and/ appropriate controls the wells were washed in 200ul of 1X wash buffer three times and incubated with 100 ul of 1:1000 NFκB p65 primary antibody for 1 hour at RT without agitation. Washes were repeated as described previously. Wells were then incubated with 100ul of 1:10,000 diluted HRP-conjugated secondary antibody for 1 hour at RT without agitation and washes repeated as described previously. Luminol enhancer solution and Peroxidase solution were mixed in 1:1 ratio, 100ul of this reagent was added per well and incubated for 30 sec. The Chemiluminescence was measured by using a luminometer (Lumat LB 9507) in relative luminescence units (RLU).
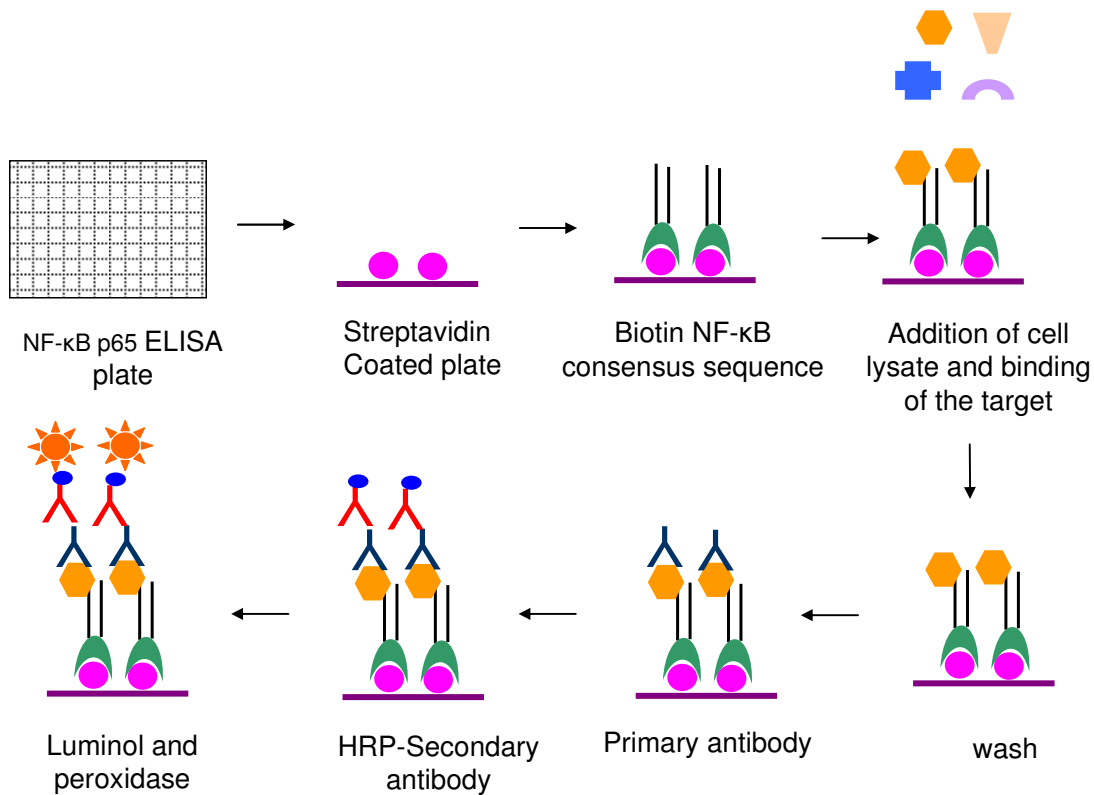
Figure 12 Schematic representation of NFκB p65 ELISA assay
1) NFκB p65 ELISA plate was purchased pre-coated with streptavidin (Stressgen Biotechnologies). 2) NFκB biotinylated- consensus sequence (yellow triangle with duplex) binds to streptavidin (green circle) on the plate. 3) Cell lysate or TNFα activated HeLa cell Nuclear Extract (positive control) is then added. 4) Washes are performed to remove any unbound fraction or non-specific binding, bound and activated NFκB p65 is indicated as blue cross. 5) NFκB p65 primary antibody is then added (red Y-shaped symbol). 6) Following incubation with primary antibody secondary HRP- conjugated antibody is added (blue Y-shaped symbol with red circle). 7) Luminol (chemiluminescent substrate) and Peroxidase (oxidizing agent) are mixed and added to every well and 8) chemiluminescence is detected by luminometer.

3.11 Tranilast treatment of 4T1 murine cell line

1×10^6 4T1 cells per well were seeded in a 6-well plate in DMEM supplemented with 10% FBS and antibiotics for 24 hours. Cells were then G_0 arrested by using serum-free medium for 24 hours. Cells were then released by the addition of serum and treated with 800 µmol/L Tranilast (a kind gift from Dr Richard Gilbert, St. Michael's Hospital,

Toronto, Ontario, Canada) dissolved in 0.8% DMSO. 0.8% DMSO solvent (vehicle) was used as the negative control. Cell lysates were prepared at the end of 24 and 48 hours after treatment with tranilast or vehicle and immunoblotted for Nrp1.

3.12 Preparation of mouse tumour lysates for immunoblotting

Cryogenically frozen breast tumours from female BALB/c mice were kindly provided by Dr. Subramaniam (St. Michael's Hospital, Toronto, Canada). Six week old female BALB/c mice were inoculated with syngeneic 4T1 cells ($5x10^5$ cells/ pad) in mammary fat pads and treated with 300 mg/kg dose of Tranilast or $NaHCO_3$ (sodium bicarbonate) vehicle by gavage daily until 28 days. Tumour lysates were prepared by mechanical homogenization on ice using a mortar and pestle in cold RIPA buffer (50 mM Tris-HCl, pH 7.4, 150 mM sodium chloride, 1 mM EDTA, 1% Triton X-100, 1% Sodium Deoxycholic acid, 0.1% SDS and protease inhibitors (see section 3.3)). Protein lysates were collected as the supernatant fraction after centrifugation, quantitated and probed for Nrp1 expression by immunoblotting (see section 3.4).

3.13 Statistical Analysis

Microsoft Excel 2003 or GraphPad Prism 3.0 program (GraphPad Software Inc., USA) were used for statistical analysis in MTT proliferation, migration, mammosphere and NFκB p65 ELISA assay. To determine if significant differences exist between the experimental and control groups two- sided Student's t- test was used. P-value of 0.05 was used as significant in all experiments and results are expressed as mean ± stdev.

4 Results

4.1 Expression of Nrp1 in human breast cancer cell lines

To characterize the function of Nrp1 in breast cancer cell biology, expression of Nrp1 in various cell lines was evaluated. Cell lines with gene expression patterns representative of various breast cancer subtypes were selected. SUM159, MDA-MB-231, MDA-MB-453 cell lines represented the basal-like or triple negative (ER-, PR-, ERBB2/HER2-) subtype, BT474 and MCF-7 represented the hormonal luminal subtype (ER+, HER2-) and SKBr3 represented the ERBB2/ HER2 amplified subtype. Total protein cell lysates were prepared for these cell lines and probed for Nrp1. MDA-MB-231 cell line expressed the highest level of Nrp1, indicated by 130 kD band (Figure 13; lane 1). MCF-7 and SUM159 also expressed Nrp1 but to a much lower extent (Figure 13; lane 2 and 5). MDA-MB-231 cell line was selected as the best candidate for *in vitro* knockdown studies because it endogenously expresses high levels of Nrp1, is highly invasive and metastatic in nature, and is a prototype for hormone-independent, triple-negative, breast cancer subtype for which there is a lack of effective therapies.
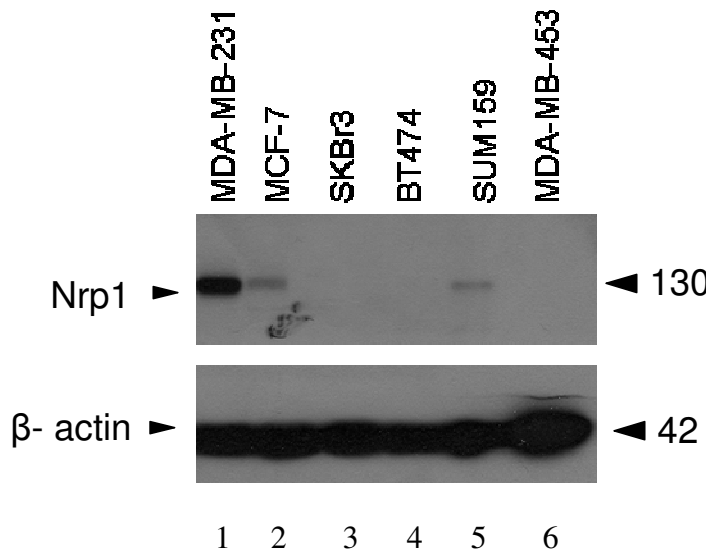
**Figure 13 Nrp1 expression in human breast cancer cell lines**

Western blot depicting the expression of Nrp1 in various breast cancer cell lines. MDA-MB-231, MCF-7 and SUM159 endogenously expressed Nrp1 (30kDa) at various levels (lanes 1, 2, 5 repectively). β- actin is used as the loading control.

4.2 siRNA knockdown of Nrp1 in MDA-MB-231 cell line

To knockdown Nrp1 in MDA-MB-231 cell line, short-interfering RNA (siRNA) specifically targeting Nrp1 mRNA sequence was used. Nrp1 knockdown was optimized by using different transfection reagents (Oligofectamine and Fugene) and different siRNA concentrations (20, 65, 80 and 150nM). Two controls were included; cells were either transfected with scrambled siRNA or sham-transfected with the transfection reagent without any siRNA (Figure 14a; lane 5 and 6). Whole cell lysates were prepared and the levels of Nrp1 knockdown were evaluated by immunoblotting. β-actin was used as the loading control. The minimal effective siRNA concentration required to knockdown Nrp1 was 65nM (Figure 14a; lane 2).

The time interval over which Nrp1 knockdown is maintained was examined next. Cells were transfected with 65nM siRNA and cell lysates were collected at 72, 96 and 144 hours post-transfection and compared to the corresponding sham- control time point. Transient Nrp1 Knockdown using 65nM siRNA was effective upto 144 hours in MDA-MB-231 cell line, allowing sufficient time for *in vitro* functional assays to be conducted (Figure 14b; row 1, lane 2). Sham transfected cells represent the endogenous levels of Nrp1 expression at each time point (Figure 14b; row 2, lanes 1 to 3).

Changes in cell morphology of MDA-MB-231 cells treated with Nrp1 siRNA was evaluated by light microscopy. Both Nrp1 knockdown and sham-transfected control cells displayed the classical mesechymal or fibroblast-like phenotype known for this cell line (Figure 14c) (Liu and Feng et al., 2010). In addition there were no observable changes in cell-to-cell or cell-to-matrix interactions in MDA-MB-231 Nrp1 siRNA treated cells.

a.

b.

c.

Nrp1 siRNA

Sham transfected

4X   **10X**
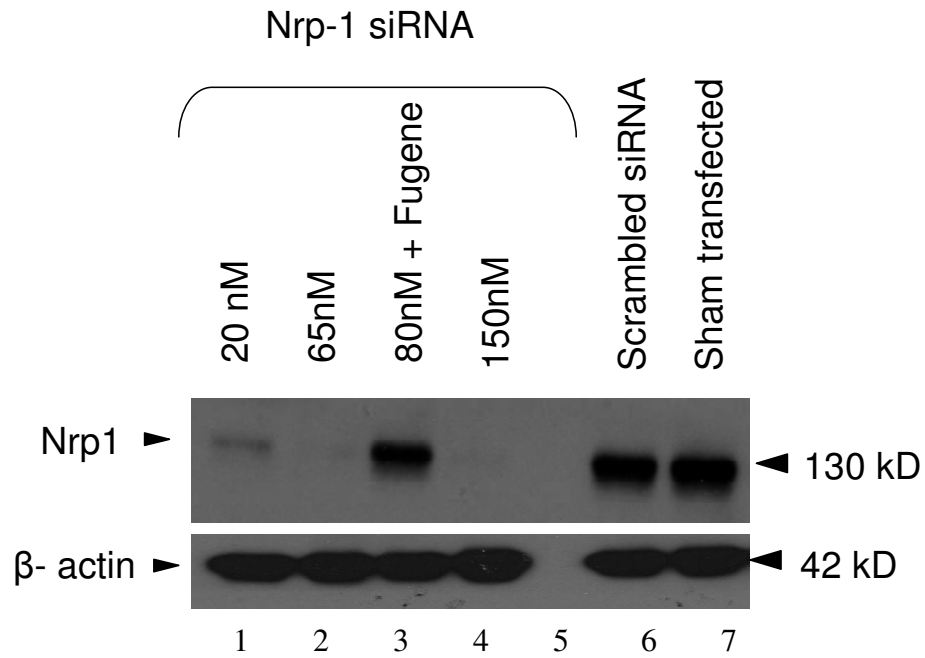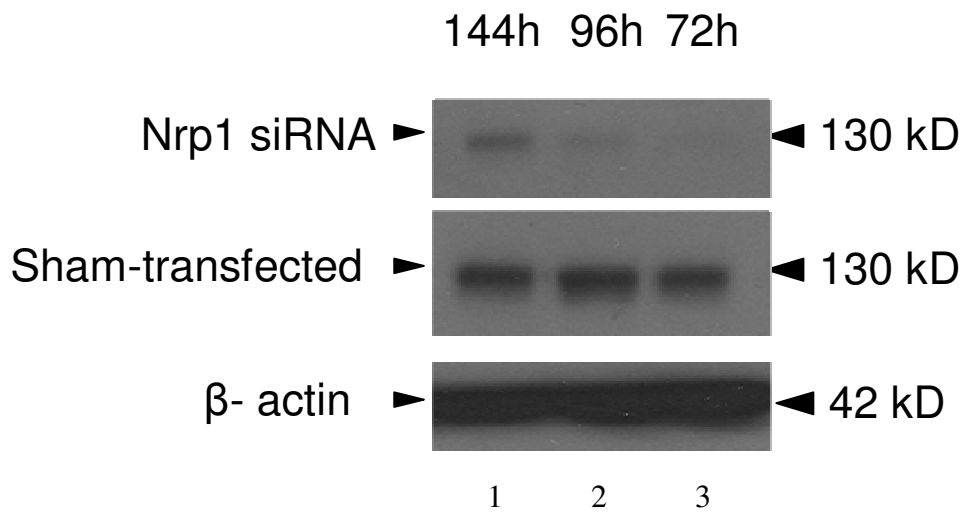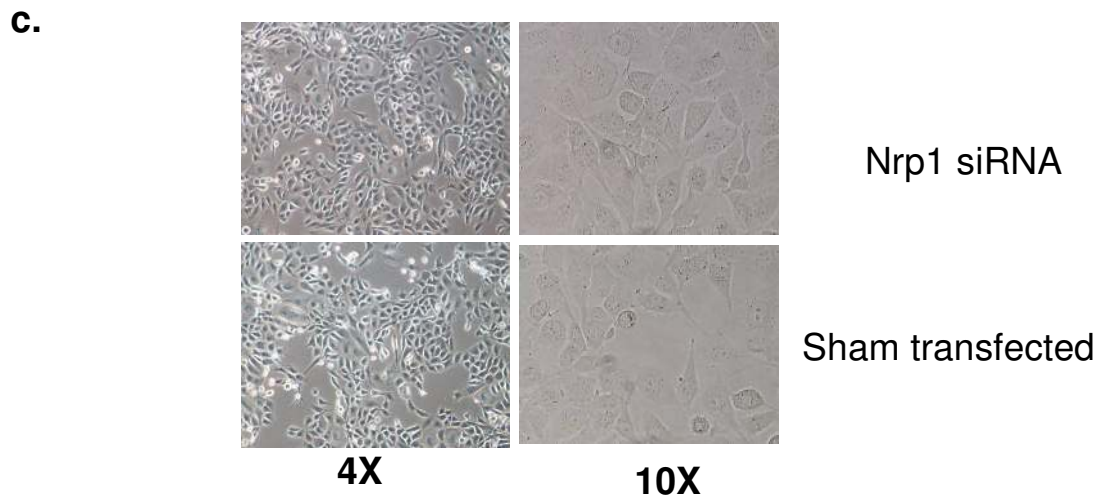
Figure 14 Optimization of Nrp1 siRNA knockdown in MDA-MB-231

a) MDA-MB-231 cells were transfected with various concentrations (20, 65, 80 and 150nM) of Nrp1 specific siRNA, scrambled or sham-transfected (lane 5 and 6 respectively) and Nrp1 levels (130 kD) were evaluated using western blot.

b) Whole cell lysates were prepared at 72, 96 and 144 hours post transfection with 65nM siRNA and Nrp1 knockdown was evaluated. Nrp1 transient transfection was maintained over 96 hours (row 1, lane 2) in comparison to the corresponding sham-transfected control (row 2, lane 2).

c) 72 hours after siRNA treatment, MDA-MB-231 cells were photographed using 4x or 10x magnification on a light microscope. The cell line did not display any observable morphological differences in comparison to the sham control.

4.3 Knockdown of Nrp1 does not significantly affect cell proliferation *in vitro*

There were no phenotypic differences between Nrp1 knockdown or sham- transfected control cells (section 4.2); however to answer if functional differences exist between these two groups, *in vitro* functional assays were conducted. The role of Nrp1 in cell proliferation was evaluated using MTT assay. MTT assay measures cell proliferation as a function of mitochondrial activity. Mitochondrial dehydrogenases of viable cells cleave the tetrazolium ring in MTT reagent and yield water insoluble purple formazan crystals.

The crystals are dissolved in acidified isopropanol and an increase in absorbance measured spectrophotometrically indicates an increase in cell number (Mossman et al., 1983). MTT reagent was added to MDA-MB-231 breast cancer cell line treated with Nrp1 siRNA or sham-transfected 72 hour post transfection. Cells knockdown for Nrp1 (0.89 ± 0.06) displayed no decrease in cell proliferation in comparison to sham control (0.96 ± 0.13) measured as optical density (OD) at 595 nm (P= 0.19) (Figure 15).
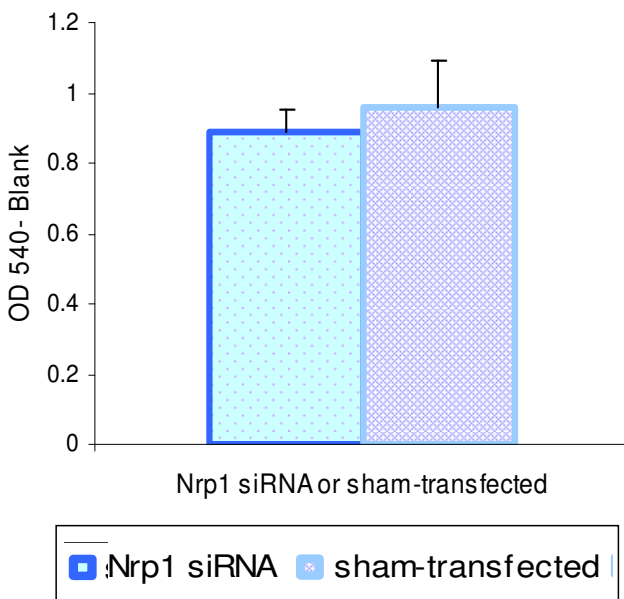
Figure 15 Effect of Nrp1 knockdown on cell proliferation using MTT assay

MDA-MB-231 cells were plated (3 $\times 10^3$ cells/ well) in 96 well plate and treated with Nrp1 siRNA (65nM) or sham-transfected. MTT assay was performed by adding 20% MTT reagent to each well, solubilized in isopropanol and absorbance was detected at 540 nm. There was no significant difference in cell proliferation in the presence or absence of Nrp1 (P=0.19). The above experiment was conducted in three triplicates (or 9 wells) in four independent experiments.

4.4 Nrp1 siRNA treated cells show no changes in apoptosis marker levels

MDA-MB-231 cells treated with Nrp1 siRNA or sham-transfected were induced to undergo apoptosis using etoposide. Etoposide is an anti-neoplastic drug that functions by inhibiting Type II topoisomerases (topo II). During DNA replication, topo II prevents DNA knotting by generating transient breaks in the phosphodiester backbone allowing the DNA molecule to attain a more relaxed topology (Wang, 2002). Etoposide binds to

topo II and prevents DNA re-ligation and hinders replication (Montecucco et al., 2007). Accumulation of DNA breaks leads to cell apoptosis.

Cleavage of Poly (ADP-ribose) polymerase (PARP) is a reliable and established marker for cells undergoing apoptosis (Duriez et al., 1997). PARP normally functions in DNA damage detection and repair (Masson et al., 1995). But when DNA damage is considerably higher than the repair, the cell neutralizes PARP function by cleaving it with caspase (cysteine-aspartic) proteases (Wesierska-Gadek et al., 2004). PARP is one of the earliest proteins targeted during apoptosis and upon cleavage produces the signature 89-kDa fragment.

Treatment with etoposide induced apoptosis as indicated by an increase in cleaved PARP fragment levels (89 kDa) in comparison to the DMSO (solvent for etoposide) negative control (Figure 16, lanes 2, 3 and 4, 5). However knockdown of Nrp1 does not make the cells any more susceptible to etoposide treatment than the sham- transfected control. Same levels of cleaved PARP are observed in Nrp1 siRNA and sham-transfected cells (Figure 16, lanes 3 and 5). Cleaved PARP in the DMSO and untreated controls represents endogenous cell apoptosis (Figure 16, lanes 1, 2 and 4).
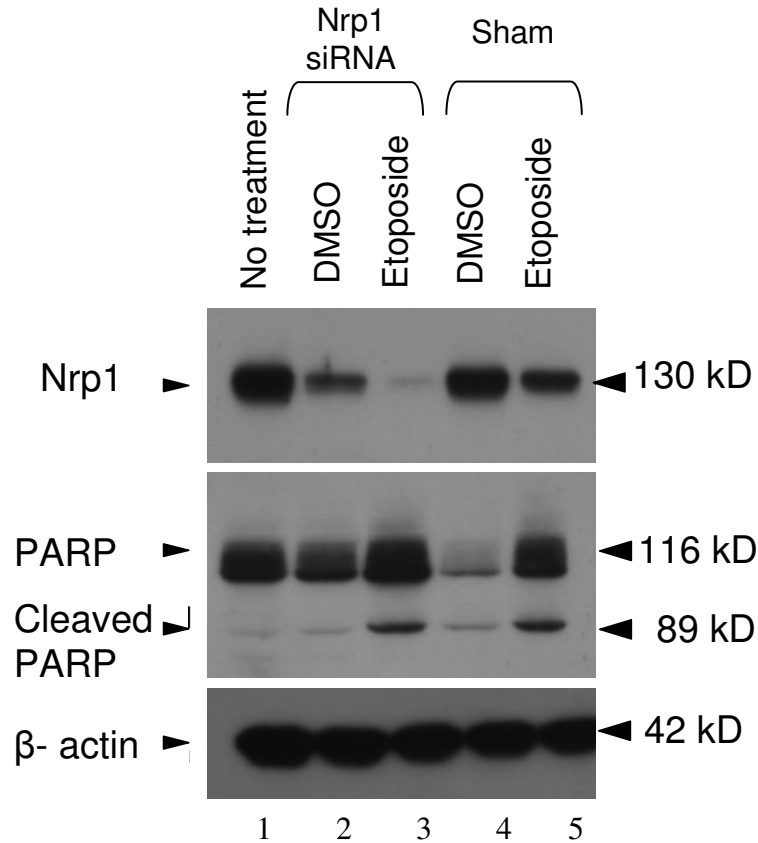
Figure 16 Nrp1 knockdown and changes in cell apoptosis marker (cleaved PARP) levels

MDA-MB-231 cells treated with Nrp1 siRNA (65nM) or sham-transfected were induced to undergo apoptosis using 25µM etoposide or DMSO (etoposide solvent) for 24 hrs. Cell lysates were prepared and probed for total PARP (116kDa), cleaved PARP (89kDa) and Nrp1 (130kDa). Knockdown of Nrp1 (lane 3) did not lead to changes in cleaved PARP levels when compared to the sham-transfected control (lane 5) upon treatment with etoposide. The "no treatment" lane represents MDA-MB-231 cells that were not transfected and not treated with etoposide or DMSO.

4.5 Knockdown of Nrp does not significantly affect cell migration *in vitro*

Nrp1 was first identified as a receptor for semaphorins, which mediate axonal guidance (Kolodkin et al and He et al., 1997). Nrp1 was also shown to potentiate VEGF-mediated endothelial cell chemotaxis (Soker et al., 1998). To determine if Nrp1 also plays a role in tumour cell migration a scratch wound migration assay was conducted. In the scratch wound migration assay the cell monolayer is mechanically disrupted using a pipette tip creating a wound, this stimulates the cells to migrate and heal the wound. Wounds were created in Nrp1 siRNA treated or sham transfected cell monolayers and cell migration was recorded 24 hours post-wound creation (Figure 17a). There was no reduction in cell migration in Nrp1 siRNA treated group (Figure 17b).

Nrp1 is a co-receptor for growth factors (GF) and its effects on tumour cells might not be observed unless the receptor is induced with a GF ligand. Therefore hepatocyte growth factor (HGF), a potent inducer of epithelial cell migration and a reported ligand for Nrp1 was used to induce MDA-MB-231 cell migration (Beviglia et al., 1997, Matsushita et al., 2007 and Hu et al., 2007). As expected, upon addition of HGF, sham-treated cells responded with a higher migration rate than their uninduced counterparts (P=0.048) (Figure 17b). However cells with Nrp1 knockdown also responded equally well to HGF induced migration (P=0.0018) and there was no reduction in cell migration in the absence of Nrp1 (Figure 17b).

HGF interacts with its cognate tyrosine kinase receptor MET on the surface of epithelial cells to activate downstream signalling pathways involved in migration. To investigate if there are any changes at the molecular level in the presence or absence of Nrp1 in the HGF pathway, changes in the phosphorylation of MET was evaluated next (section 4.6).

a.

t_0

t_6

Nrp1 siRNA   Sham-transfected   Nrp1 siRNA +HGF   Sham-transfected +HGF

b.
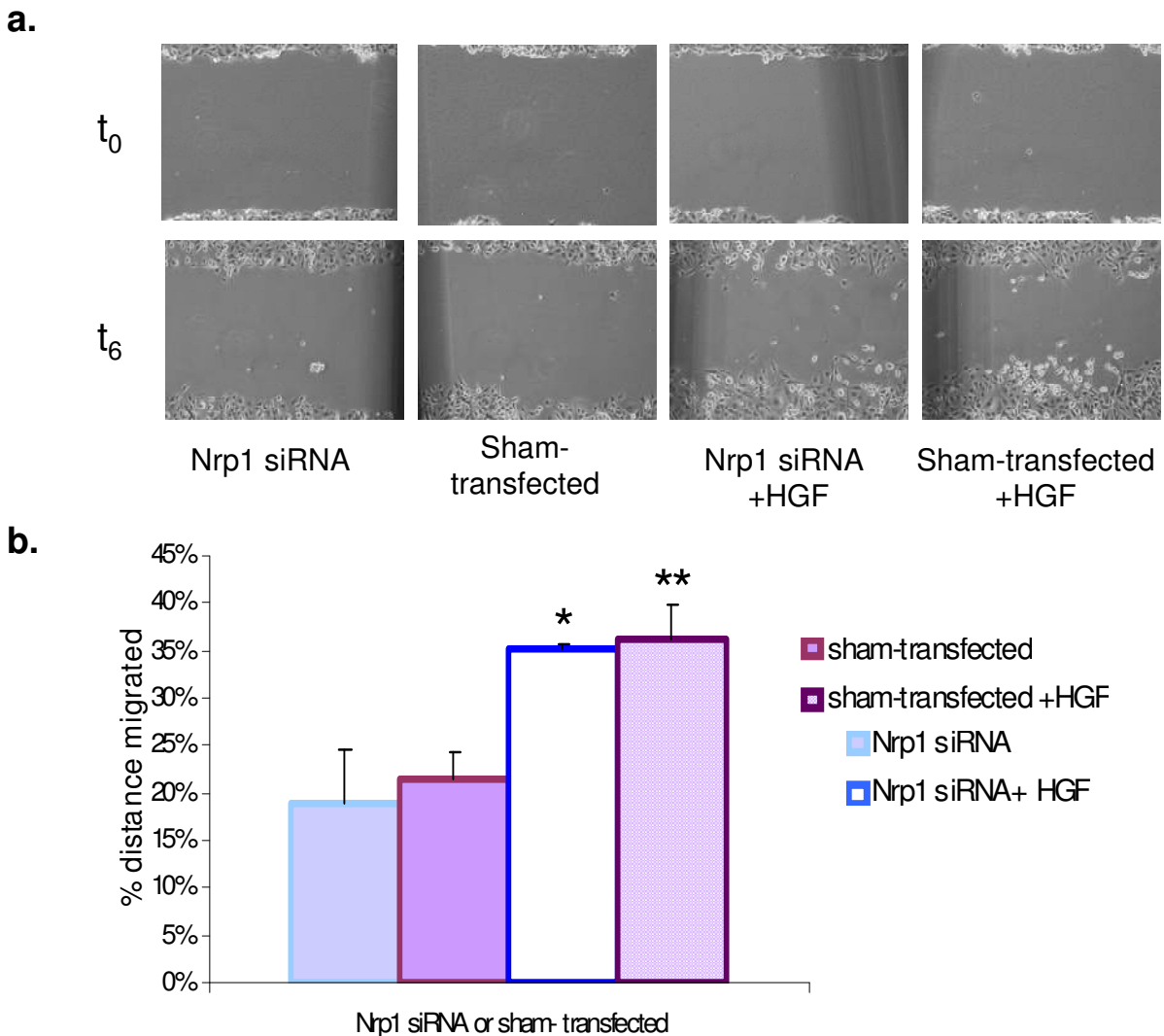
Figure 17 Role of Nrp1 in cell migration independent of HGF and in the presence of HGF

a) Parallel wounds (3 per well) were created in confluent cell monolayers treated with Nrp1 siRNA or sham-transfected. Shown above is a phase-contrast image (10x) of the wound at t_0 and t_6 (6 hours post-wound creation).

b) Percentage distance migrated was calculated using T-Scratch software (Geback et al., 2009). There was no reduction in cell migration in Nrp1 siRNA treated cells (0.18 ± 0.06) in comparison to the sham-transfected control (0.21 ± 0.03). Upon addition of HGF there was significant increase in both sham-transfected (*P=0.0048) and Nrp1 siRNA (**P=0.0018) treated cells when compared to their uninduced counterparts. The above experiment was conducted in triplicates per experiment and repeated in three independent experiments.

4.6 Nrp1 does not potentiate HGF-dependent MET phosphorylation in MDA-MB-231 cell line

Nrp1 siRNA treated cells and the sham- transfected controls were compared for differences in MET receptor tyrosine phosphorylation after stimulation with HGF. Tyr 1349 and Tyr 1356 are two crucial tyrosine residues in the multisubstrate docking site of MET that recruit downstream adapter proteins. The phosphorylation of these two tyrosines is necessary and sufficient for HGF-MET signal transduction (Ponzetto et al., 1994). The minimal HGF concentration required to induce MET phosphorylation at Tyr 1349 in MDA-MB-231 cells was determined to be in the range of 1 to 5 ng/ ml (Figure 18a, lane 4 and 5). In sham transfected cells stimulation with 1 ng/ml HGF led to MET phosphorylation at Tyr 1349 indicated by 131 kDa band (Figure 18b, lane 4). However HGF stimulation of Nrp1 siRNA treated cells did not show any reduction in MET phosphorylation (Figure 18b, lane 2) when compared to the sham-transfected control.

In addition to expressing Nrp1, MDA-MB-231 cells also express Neuropilin-2 (Nrp2) at high levels. Nrp2 is a homologue of Nrp1 and they share many ligands including HGF (Suplice et al., 2008). Therefore Nrp2 in MDA-MB-231 cells might substitute for Nrp1 functions in Nrp1 siRNA treated cells. To address this issue double knockdown of both Nrp1 and Nrp2 was targeted in MDA-MB-231 cell line (section 4.7).
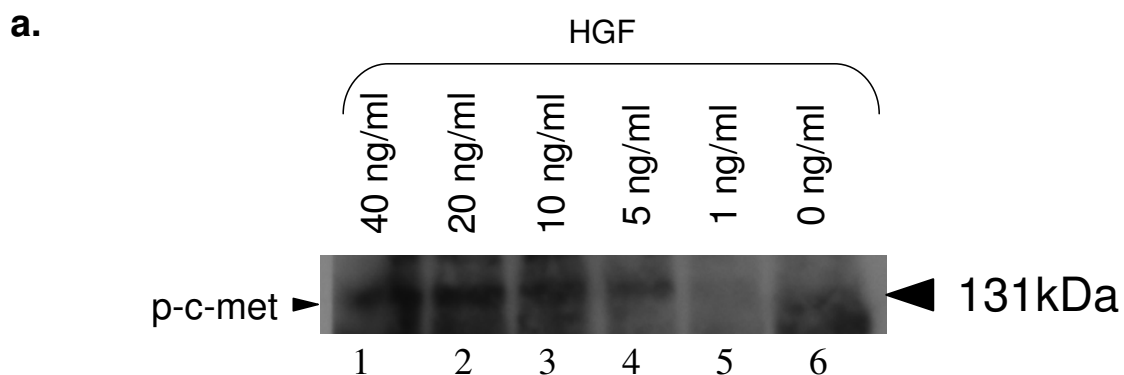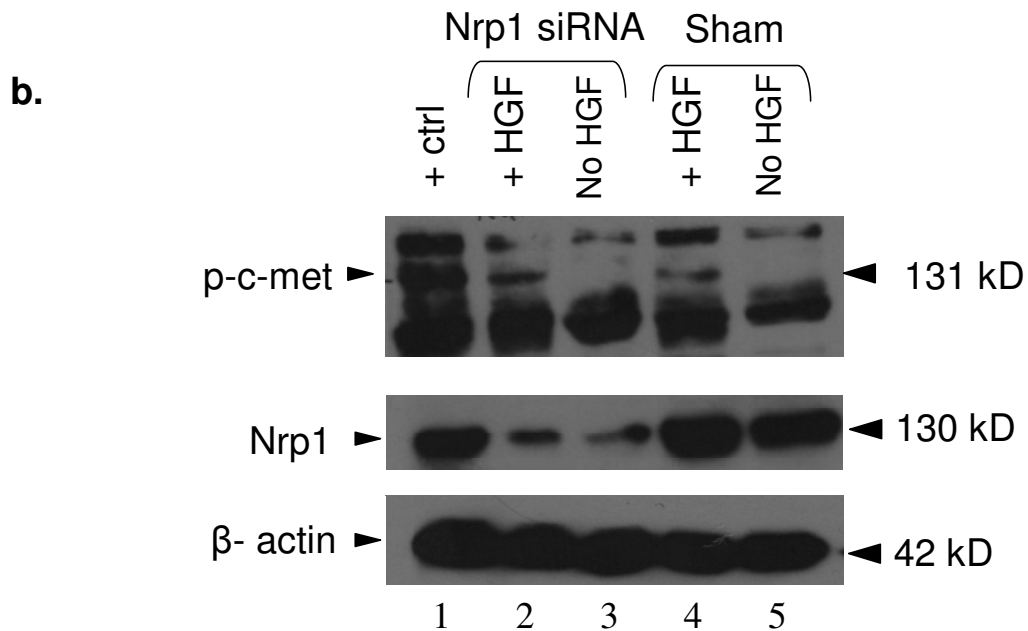
Figure 18 Effect of Nrp1 knockdown and changes in MET phosphorylation after HGF stimulation

a) MDA-MB-231 cells were stimulated with various concentrations of HGF (0 ng/ml to 50 ng/ml). The minimal effective concentration to induce MET phopshorylation was identified to be in the range of 1 to 5 ng/ml.

b) MDA-MB-231 cells knockdown for Nrp1 or sham-transfected were stimulated with 1 ng /ml HGF and MET phosphorylation levels compared. Presence or absence of HGF does not have any effect on MET phosphorylation (lane 2 vs. lane 4). The positive control is cell lysate from MDA-MB-231 cells treated with 50 ng/ ml HGF (lane 1).

4.7 Double knockdown of Nrp1 and Nrp2 in MDA-MB-231 cell line

Neuropilin-2 (Nrp2) can bind and enhance HGF-induced MET activation (Suplice et al., 2008). Therefore it is essential that both Nrp1 and 2 are knockdown before a physiological response is observed in MDA-MB-231 cell line. Consistent with this observation, HUVECs (Human Umbilical Vein Endothelial cells) show a modest 30% reduction in MET phophorylation in Nrp1 siRNA treated cells and 70% reduction when both Nrps are knockdown (Suplice et al., 2008).

MDA-MB-231 cells were transfected with Nrp2 specific siRNA and the knockdown was effective over 144 hours (Figure 19, lane 4) in comparison to the sham-transfected control (Figure 19, lane 5). MDA-MB-231 cells express Nrp2 at levels similar to Nrp1 and therefore equi-molar siRNA concentration of 65nM was used to generate double knockdown (Barr et al., 2005). MDA-MB-231 cells knockdown for both Nrps were stimulated with 1 or 5 ng/ml HGF and MET phosphorylation was evaluated by immunoblotting (see section 1.6 for details). There were no observable changes in MET phosphorylation levels (131 kDa band) in the double knockdown cells (Figure 20, lanes 5 and 6) when compared to the sham transfected control (Figure 20, lanes 1 and 2).

MTT assay was used to evaluate changes in cell proliferation at various time points (48, 96 and 144 hours) after Nrp1 and 2 double knockdown. MDA-MB-231 cells (untreated with transfection reagent or siRNA) displayed the fastest proliferation rate indicated by the steep slope between 48 and 96 hours (Figure 21). The proliferation then reached steady state level as it approached the 144 hour time point. The sham transfected cells and the siRNA treated cells had slower proliferation between 48 and 96 hours post transfection suggesting that oligofectamine itself has an effect on cell proliferation. The single Nrp1 siRNA treated and cells treated with both Nrp1 and Nrp2 have very similar proliferation rate and closely parallel each other at all time points. There is a slight reduction between siRNA treated and sham transfected cells at 96 and 144 hours but not statistically significant.
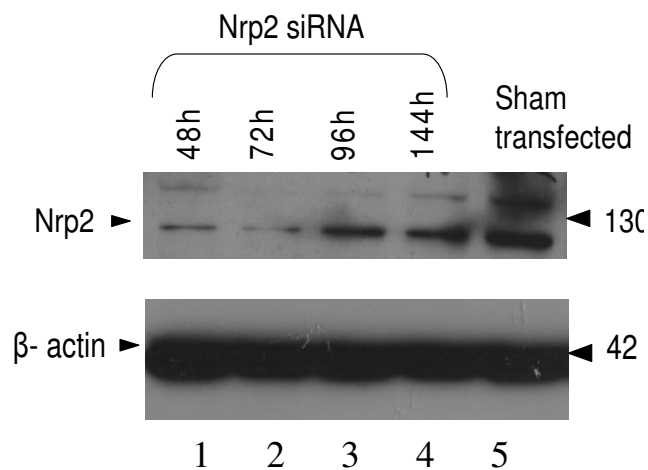
Figure 19 Transient Knockdown of Nrp2 over a time period in MDA-MB-231 cells

MDA-MB-231 cells were transiently transfected with Nrp2 specific siRNA and the efficiency of the knockdown was observed at various time points (48, 72, 96 and 144 hours). The knockdown with Nrp2 siRNA was maintained upto 144 hours (lane 4) in comparison to the sham-transfected control (lane 5). β-actin is used as the loading control.

65
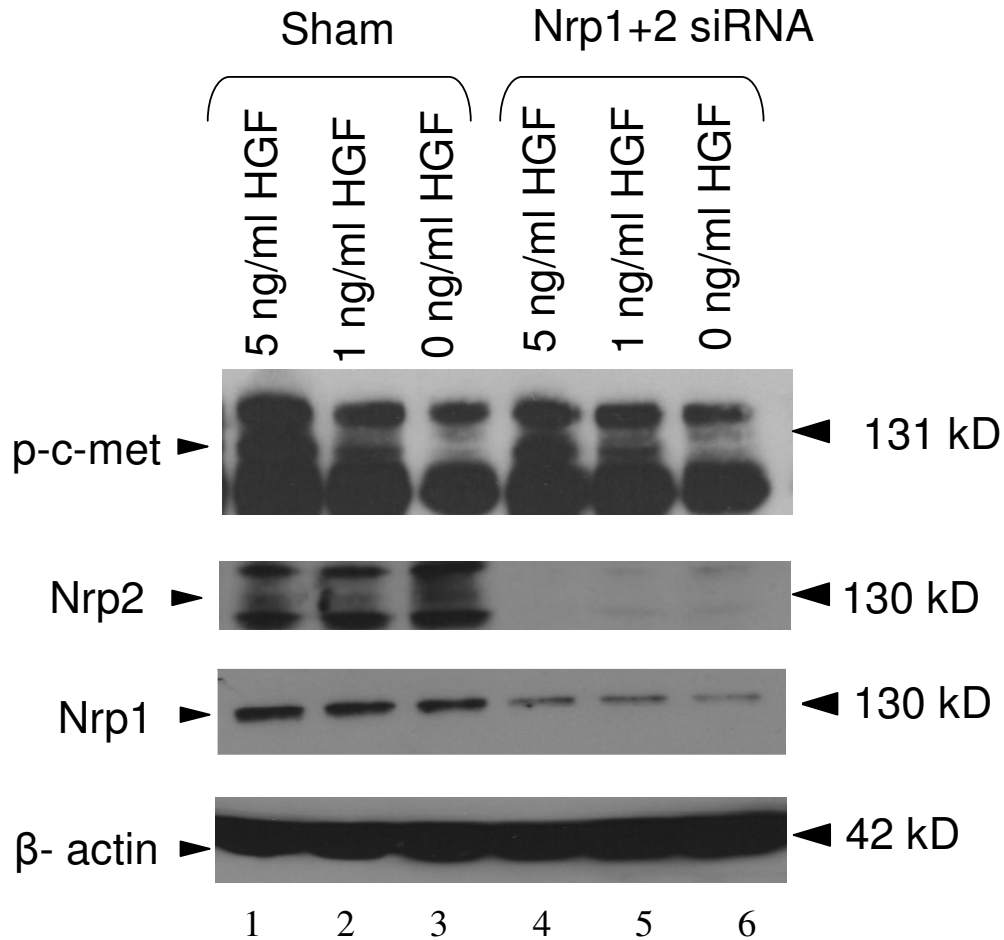
Figure 20 Double knockdown of Nrp1 and 2 in MDA-MB-231 and changes in MET phosphorylation levels

Changes in MET phosphorylation (indicated by 131 kDa band) were evaluated by western blot between Nrp double knockdown and sham transfected cells in MDA-MB-231 cells. Successful double knockdown is indicated by the absence or reduction in Nrp1 and Nrp2 levels in lanes 4, 5, and 6. Induction of MET phosphorylation can be observed by comparing the 0 ng/ml and 1 or 5ng/ml lanes between siRNA treated and sham-transfected groups.
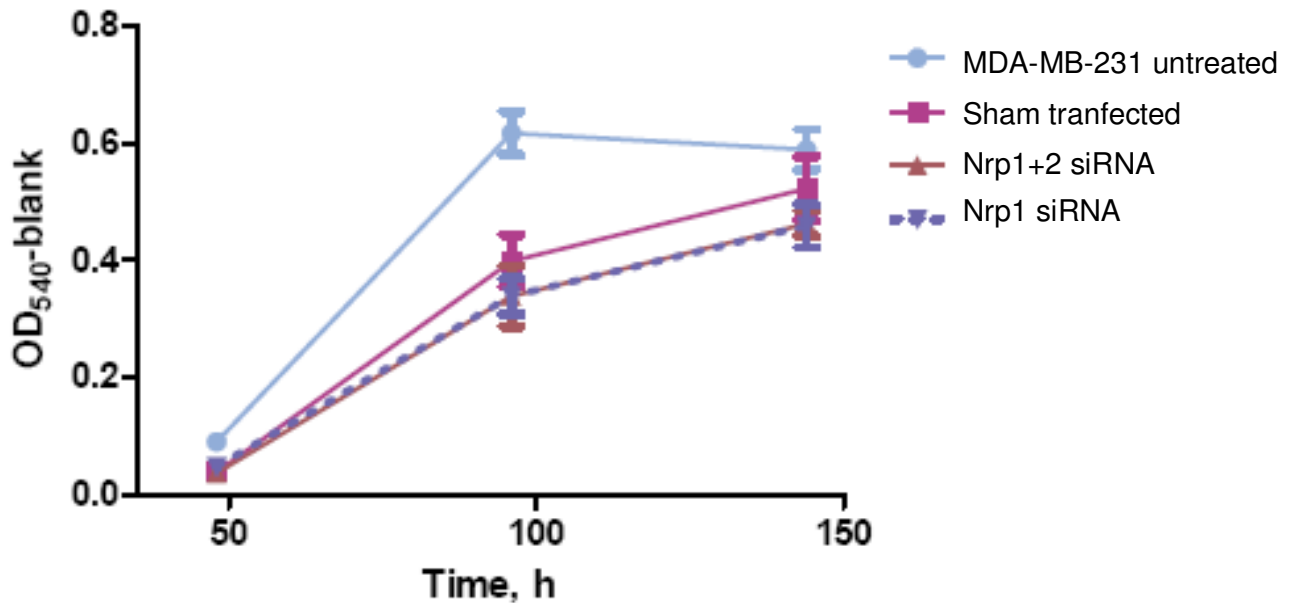
Figure 21 Proliferation of MDA-MB-231 cell line after double knockdown of Nrp1 and 2 in MDA-MB-231

Proliferation of MDA-MB-231 cells was measured by MTT assay at 48, 96 and 144 hours post transfection with Nrp1 and/ 2 siRNA. Differences were measured between untreated MDA-MB-231 cells, sham transfected cells with transfection reagent (oligofectamine) only, Nrp1 siRNA and Nrp1+ 2 siRNA treated cells at each time point. There was a slight reduction in cell proliferation between sham treated and Nrp siRNA treated cells at 96 and 144 hours time intervals, but was not statistically significant.

4.8 Knockdown of Nrp abrogates HGF mediated MET phosphorylation in MCF-7

Nrp did not potentiate HGF dependent MET phosphorylation even after double knockdown of Nrp1 and 2 in MDA-MB-231 (Figure 20). To delineate if the effects observed in MDA-MB-231 are cell line specific, experiments were conducted in MCF-7 cell line. MCF-7 was selected because it has a distinct gene expression profile. MCF-7 is a luminal cell line expressing cytokeratin 8 and 19 and is also Estrogen receptor positive in comparison to the basal MDA-MB-231 and SUM159 cell lines (Curschellas et al., 2004, Charafe-Jauffret et al., 2006). To study the effects of HGF and MET activation, Nrp1 siRNA knockdown was optimized in MCF-7. MCF-7 cell line does not

express Nrp2 and therefore single knockdown was sufficient for these studies (Nasarre et al., 2003; Ellis, 2006).   35nM Nrp1 specific siRNA was used to induce Nrp1 knockdown in MCF-7 cell line (Figure 22a, lane 4). There were no changes in the cell morphology of Nrp1 siRNA treated MCF-7 cells when compared to the sham-transfected control (Figure 22b).

Next, changes in the MET phosphorylation levels after HGF induction were evaluated in the presence or absence of Nrp. Cells were stimulated with 1 ng/ ml HGF and cell lysates were probed for phosphorylated MET by immunoblotting. Addition of HGF stimulated MET phosphorylation in the sham-transfected cells (Figure 23, lane 4) when compared to the uninduced cells from the same group (Figure 23, lane 5). HGF induced MET phosphorylation was greatly abrogated in Nrp1 siRNA treated MCF-7 cells (Figure 23, lane 2) in comparison to the HGF induced sham transfected cells (Figure 23, lane 4). The total MET levels (170 kDa band: MET precursor, 145 kDa band: β- subunit of MET) are unchanged between the two groups (Figure 23, row 2) indicating that the Nrp1 mediated changes are at the phosphorylation level and are HGF-specific.

b.

Sham
transfected

Nrp1 siRNA

4x   10x
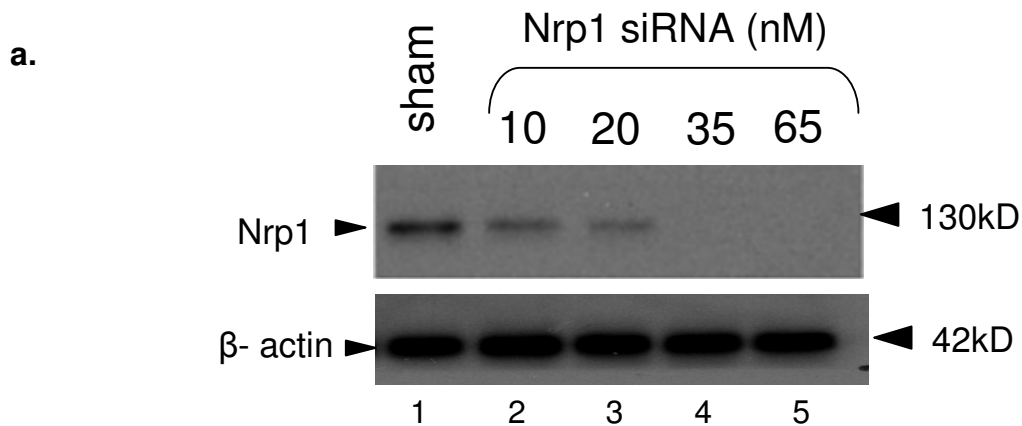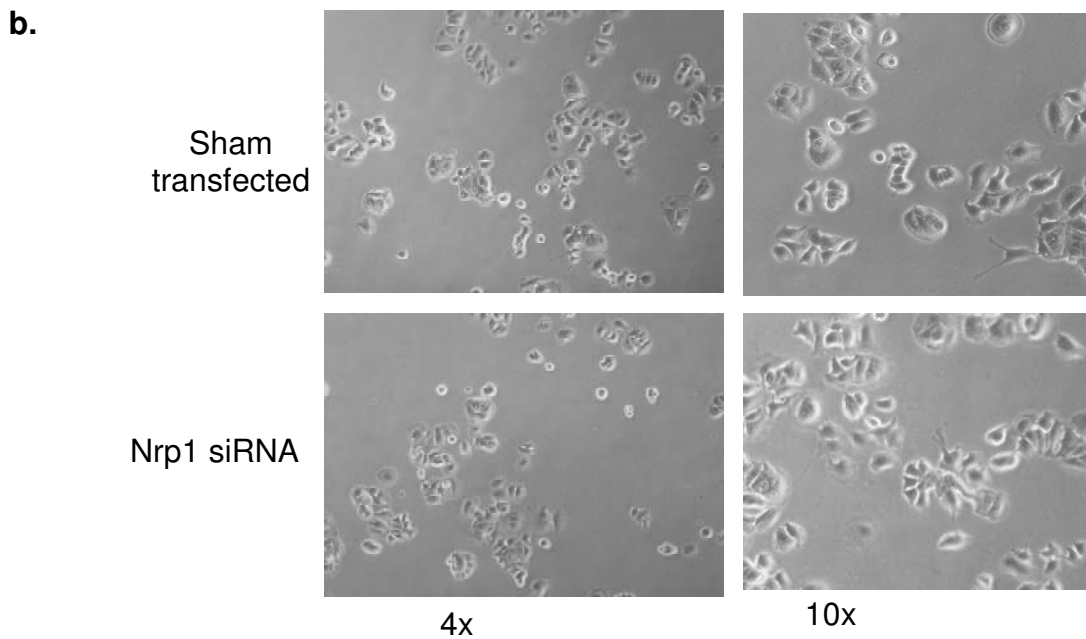
Figure 22 Determination of minimal effective siRNA concentration required for Nrp1 knockdown in MCF-7 cell line

a) MCF-7 cell line was transfected with various concentrations of Nrp1 siRNA (10, 20, 35 and 65nM) and cell lysates were probed for Nrp1 and evaluated for efficiency of the knockdown by western blotting.

b) Photomicrograph of MCF-7 cell line using 4x or 10x magnification on a light microscope. Nrp1 siRNA treated cells did not display any observable morphological changes in comparison to the sham-control.
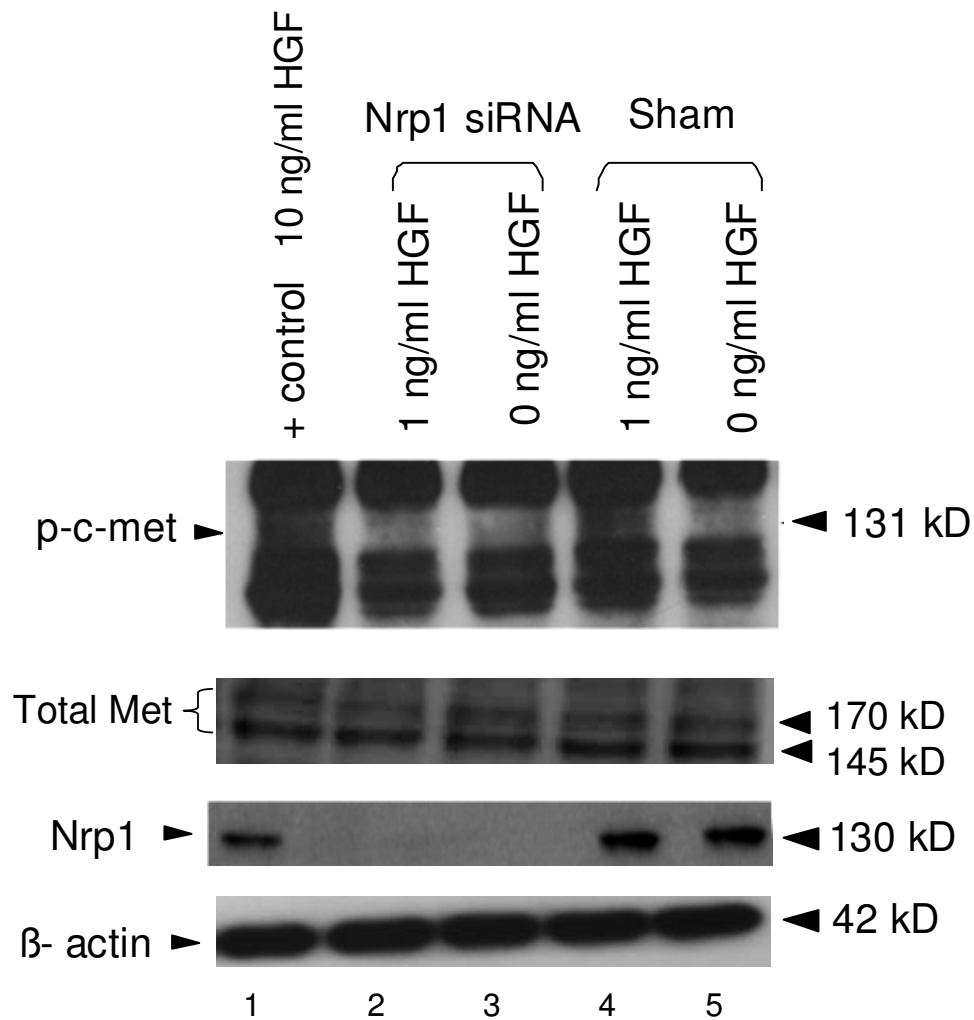
Figure 23 Changes in MET phosphorylation in response to Nrp siRNA knockdown in MCF-7 cell line

Nrp1 siRNA treated or sham transfected cells were uninduced or stimulated with 1ng/ml HGF and whole cell lysates were prepared. Changes in phosphorylated MET (131 kDa) levels were evaluated using western blotting. In addition total MET and Nrp1 levels were evaluated (row 2 and 3 respectively). The positive control for MET phosphorylation is the cell lysate from MCF-7 cells treated with 10ng/ ml HGF (lane 1).

4.9 Nrp1 and 2 regulate primary and secondary mammosphere formation through NF-κB pathway activation

So far the functions of Nrp were evaluated in the bulk tumour population, however if Nrp also plays a role in the Cancer Stem Cell (CSC) subpopulation is unknown. To study the role of Nrp in CSC population a functional assay called the mammosphere assay was used. The mammosphere assay is based on the unique property of stem cells to survive and grow in three-dimensional structures in serum-free suspension (see section 2.6.3). This assay has been successfully used to establish long-term cultures, enriched in stem cells from cell lines and tumour samples (Clayton et al., 2004). MDA-MB-231 cells were treated with Nrp1 and 2 siRNA and changes in the formation of primary and secondary mammospheres were observed. The absence of Nrp significantly reduced both the size and number of primary mammospheres in comparison to the sham control (P<0.05) (Figure 24). The primary mammospheres were passaged once they reached 60 μm in diameter and changes in the formation of the secondary mammosphere were observed (Figure 25). The secondary mammospheres were also significantly reduced in size and number in Nrp1 siRNA treated cells, suggesting important roles of Nrp in CSC biology (P<0.05) (Figure 25).

The canonical NF-κB pathway has recently been identified to play a role in mammary tumorigenesis *in vivo* and shown to regulate tumour stem cell population expansion (Liu et al., 2010). NF-κB activation is also shown to increase the expression of breast tumour CSC markers Nanog and Sox (Liu et al., 2010). To understand if Nrp mediates its functions in mammosphere formation through the NF-κB pathway, the activation of NF-κB was evaluated via an ELISA assay between Nrp knockdown and sham-transfected cells. The NF-κB activation was significantly reduced in Nrp siRNA treated cells in comparison to the sham-transfected control (P<0.01) (Figure 26).

a.

Nrp siRNA treated   Sham transfected

b.

c.
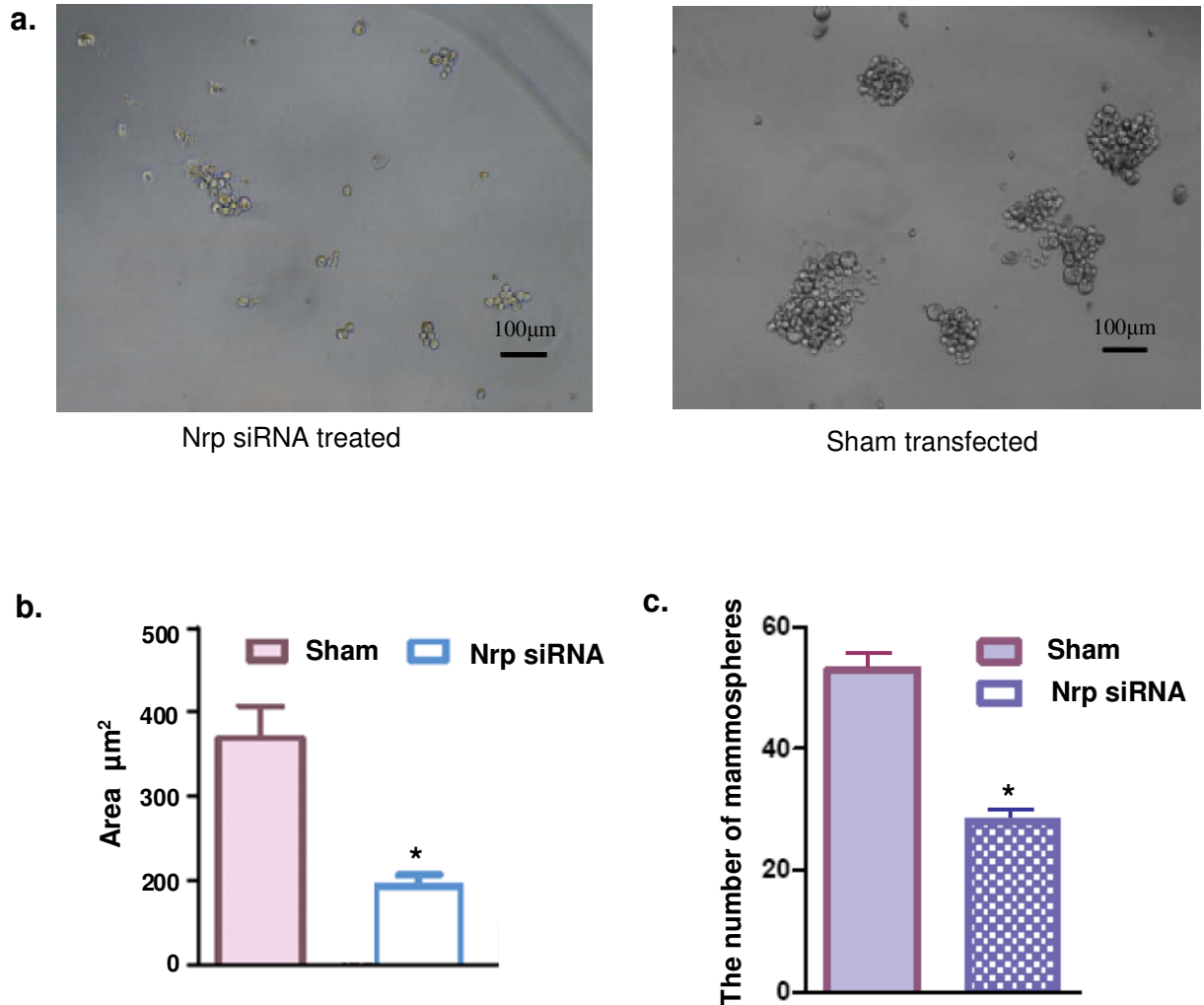
Figure 24 Knockdown of Nrp leads to reduction in size and number of primary mammospheres

a) A photomicrograph of mammospheres at 10x using light microscope (results on day 7). b) Nrp siRNA treatment resulted in reduction in both the size of the mammosphere, measured as the area in micron Sq. (*P<0.05), and c) in number, where all mammospheres larger than 60 µm in size were counted (*P<0.05). Mammosphere assay was conducted in triplicates per group and the results were consistent between two independent experiments.
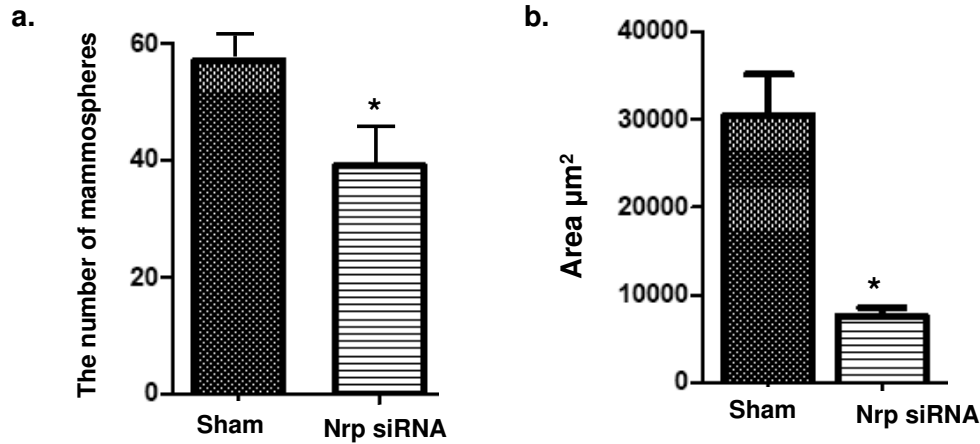
Figure 25 Nrp regulates the number and size of the secondary mammospheres

Secondary mammospheres were generated by dissociating primary mammospheres from Nrp siRNA or sham-transfected groups. Nrp siRNA treated cells formed significantly fewer mammospheres in comparison to the sham-transfected control group (*P<0.05). Also there was a significant reduction in the area (μm^2) of the mammospheres formed between Nrp knockdown and sham-transfected cells (*P<0.05).
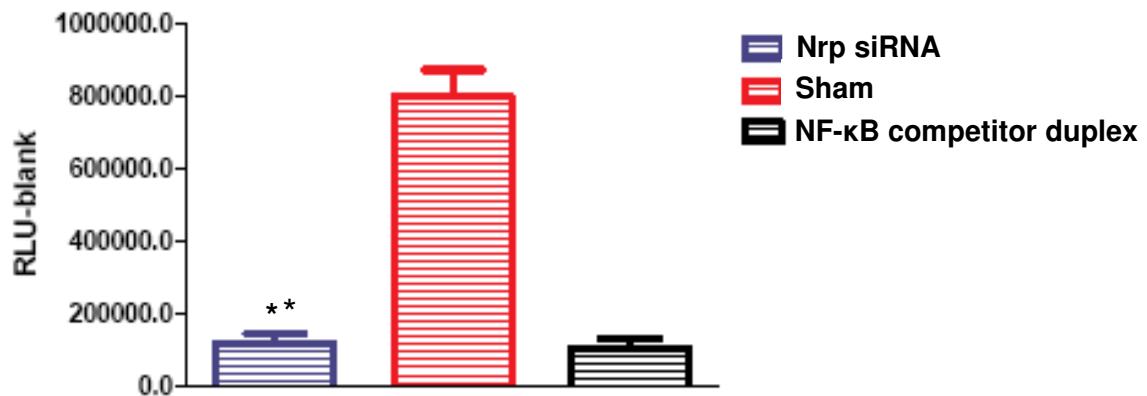
Figure 26 Changes in NF- κB activation in the presence or absence of Nrp

An ELISA assay was used to evaluate changes in NF-κB activation in MDA-MB-231 cells treated with siRNA knockdown (65nM) or sham-transfected. Changes in NF-κB activation are measured in relative chemiluminescence units (RLU). The blank is the RIPA buffer without any protein extract. Nrp knockdown significantly reduces NF-κB pathway activation (**P<0.01) in MDA-MB-231 cell line in comparison to the sham-transfected control. Also shown above is the NF-κB competitor duplex (40pmol) as the negative control.

4.10 Effect of Tranilast on Nrp expression

Tranilast was recently characterized as the drug that can specifically target CSC in human mammary cell lines (Prud'homme et al., 2010). To determine if tranilast has an effect on Nrp, changes in Nrp expression were evaluated using *in vitro* and *in vivo* approaches. *In vitro*, murine 4T1 cell line was treated with 800µmol/L tranilast over 24 and 48 hours (Figure 27a). Tranilast was previously shown to cause cell cycle arrest at the G1 phase and affected the ERK1/2, p38, JNK and TGF-β1 signalling at this dosage (800µmol/L) in 4T1 cell line (Chakrabarti et al., 2009). 4T1 cell line is negative for Nrp2 expression and therefore only changes in Nrp1 expression levels were evaluated (Barr et al., 2005).

BALB/c mice were injected orthotopically with 4T1 cells (5×10^5 cells/ pad) and treated with a therapeutically relevant dose of tranilast (Konneh et al., 1998) or sodium bicarbonate vehicle (Figure 27b). Cell lysates were prepared from cryogenically frozen tumours and changes in Nrp1 expression were evaluated. Tranilast caused reduction in Nrp expression levels in both *in vitro* and *in vivo* settings, in comparison to the corresponding DMSO or sodium bicarbonate vehicle controls (Figure 27). These results (section 4.9 and 4.10) suggest an involvement of Nrp in CSC biology and are discussed in more detail in the next section.
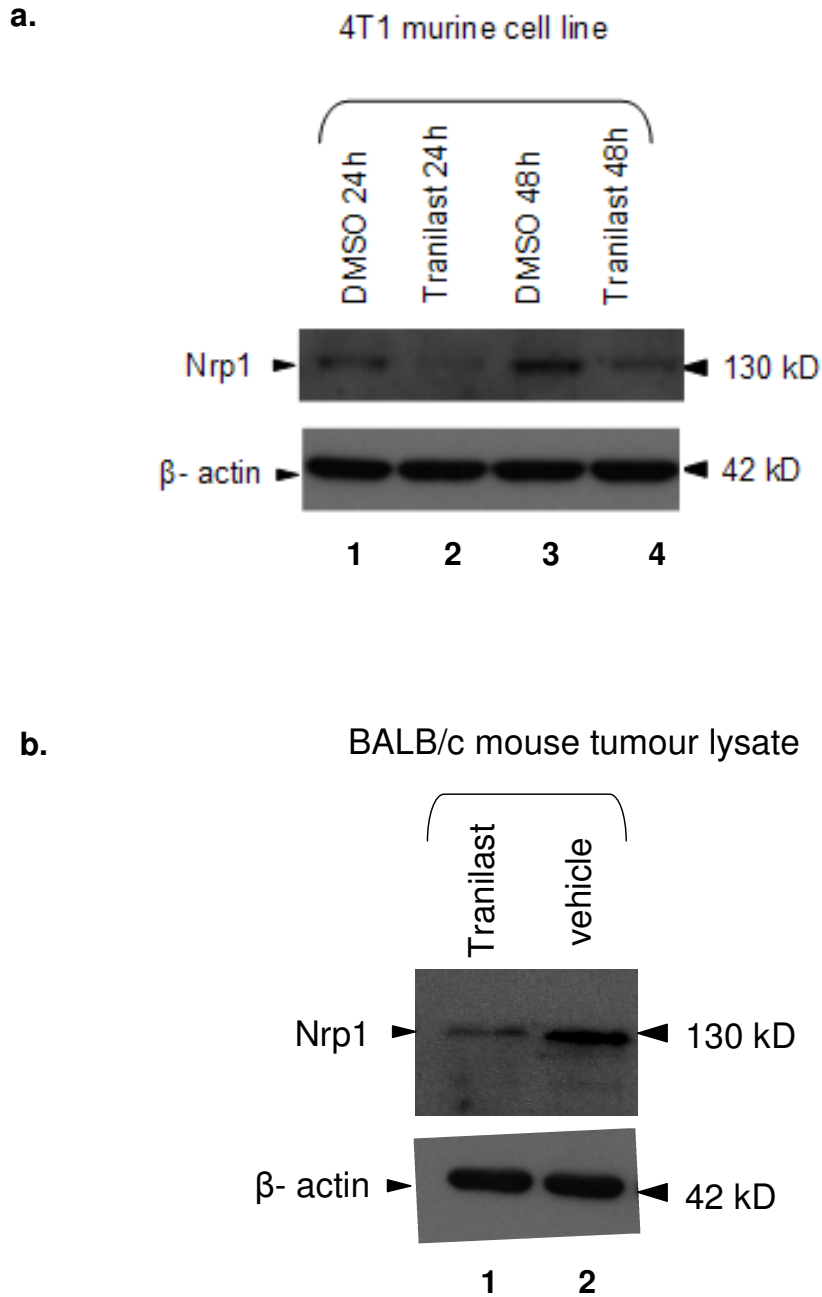
Figure 27 Tranilast reduces Nrp expression both *in vitro* and *in vivo*

a) Treatment of 4T1 murine cell line with 800µmol/l tranilast over a period of 24 or 48 hours resulted in reduction in Nrp1 levels (lanes 2, 4) in comparison to the DMSO vehicle control (lanes 1, 3). b) Tumour lysates form BALB/c mice treated with tranilast (300mg/kg dose) (lane 1) or $NaHCO_3$ control (lane 2) also display qualitatively significant reduction in Nrp1 expression levels.

5 Discussion

The purpose of the current study was to evaluate both growth factor independent and dependent co-receptor function of neuropilin (Nrp) *in vitro* in breast cancer. Nrp1 and Nrp2 are extremely complex surface receptors participating in a diverse range of functions during development, and in the adult organism. They participate in the progression of several human diseases including breast cancer.

The four major findings from these studies are as follows:

a) The role of Nrp in HGF mediated MET phosphorylation and pathway activation is breast cancer cell line dependent.

b) Nrp has no effect on cell proliferation, migration or apoptosis independent of exogenous growth factors in MDA-MB-231 cell line.

c) Nrp plays a role in mammosphere formation through NF-κB pathway activation.

d) The CSC-targeting drug, tranilast, reduces Nrp expression both *in vivo* and *in vitro.*

A.   The role of Nrp in the HGF signalling pathway in breast cancer

HGF/MET pathway activation correlates with breast cancer progression and Nrp is shown to bind HGF, and MET in the signalling complex, in gliomas and pancreatic carcinomas (Jadeszko et al., 2009; Lindemann et al., 2007; Hu et al., 2007; Matsushita et al., 2007). To investigate if Nrp also displays HGF co-receptor functions in breast cancer, changes in MET phosphorylation were evaluated in cell lines with or without knockdown of Nrp.

MDA-MB-231 cells transfected with both Nrp1 and 2 siRNA showed marked reduction in Nrp1/Nrp2 expression, but did not show any changes in MET phosphorylation (Figure 4.7.2), or changes in HGF-mediated cell migration (Figure 4.5). However, in the MCF-7 cell line HGF mediated MET phosphorylation was markedly reduced in the absence of Nrp (Figure 4.8.2). Consistent with these results in MCF-7 cell line, Nrp was shown to

potentiate the HGF pathway and promote glioma progression (Hu et al., 2007). In pancreatic cancer, Nrp was shown to associate with MET and enhance HGF-mediated cell invasion (Matsushita et al., 2007). Recently, Nrp1 was shown to induce MET activation and was positively associated with prostate cancer progression and bone metastasis (Zhan et al., 2010). There are three possible explanations for the absence of an effect in MDA-MB-231 cell line after Nrp knockdown.

1. MDA-MB-231 cell line endogenously expresses high levels of the MET receptor and might not require Nrp as a co-receptor for HGF pathway activation.

MDA-MB-231 and MCF-7 cell lines exhibit substantial genomic, transcriptional, and biological heterogeneity amongst themselves. MDA-MB-231 cell line is a triple-negative basal cell line, expressing little or no E-cadherin and displays a mesenchymal, spindle-cell like morphology. MDA-MB-231 cell line is innately endowed with an invasive phenotype, expressing high levels of extracellular matrix degrading proteases independent of any exogenous HGF addition (Trusolino et al., 2000). MDA-MB-231 cell line expresses high levels of the MET precursor and the mature MET protein, 20- fold higher than in MCF-7 cell line (Matteucci et al., 2009). One of the functions of a co-receptor is to optimize ligand presentation and binding under low ligand and/ receptor concentration (Mythreye and Blobe, 2009). In the presence of high concentrations of MET, MDA-MB-231 cells might not require Nrp for its HGF-dependent co-receptor functions.

2. MDA-MB-231 cell line expresses an alternate MET co-receptor, CD44v6

MDA-MB-231 expresses an isoform of CD44, CD44v6, which has been shown to act as a coreceptor for the receptor tyrosine kinase MET (Orian-Rousseau et al., 2002; Afify et al., 2009). CD44v6 variant is named after exon 6 that is included in its gene transcript, the exon 6 is excluded from other CD44 splice variants. Addition of CD44v6- specific antibodies or CD44v6 specific siRNA completely blocks MET phosphorylation and signal transduction through the HGF pathway (Matzke et al., 2005). The extracellular region of CD44v6 is required for MET activation and its cytoplasmic domain for recruiting ERM proteins (Ezrin-Radixin-Moesin) proteins that bridge the cytoskeleton to the membrane (Orian-Rousseau et al., 2007). Therefore CD44v6 can substitute for Nrp

coreceptor functions in MDA-MB-231 cell line and mediates successful MET phosphorylation in the absence of Nrp. MCF-7 expresses low levels of CD44 and does not express the CD44v6 isoform (Fichtner et al., 1997). Therefore the CD44v6 variant cannot replace HGF co-receptor function of Nrp in MCF-7 cell line and results in reduction of MET phosphorylation upon Nrp knockdown (Figure 28).
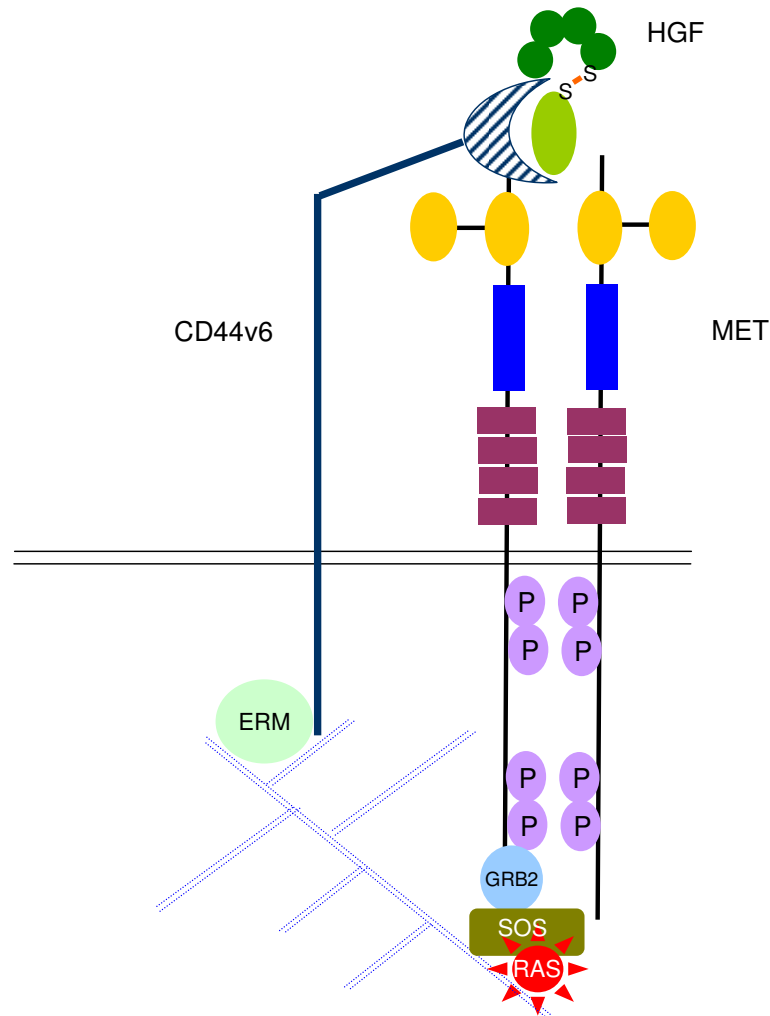
Figure 28 CD44v6, HGF and MET signalling complex
CD44v6 functions as a coreceptor in a ternary complex with HGF and MET, allowing MET phosphorylation. Signalling pathways triggering the activation of ezrin radixin moiesin (ERM) proteins (ezrin) via protein kinase C (PKC) (not shown). Activated ezrin links the cytoskeleton to CD44v6 and leads to Ras activation by its guanidine nucleotide exchange factor Son of sevenless (GEF SOS). Figure adapted from Orian-Rousseau and Ponta, 2008.

3. MDA-MB-231 cell line contains constitutively activated MET receptor in a ligand-independent manner

The nuclei of MDA-MB-231 cells are shown to contain Met cytosolic fragments (CTFs). These CTFs are constitutively activated due to phosphorylation on key tyrosine residues (Tyr 1349, Tyr 1234/ 35) independent of HGF exposure (Previdi et al., 2010; Mettucci et al., 2009). Immunoprecipitation with antibody specific for the cytoplasmic domain of MET, identified CTF as a 60 kDa band, identical to the C-terminal portion of the Met β-chain. We focused on the changes in the full length MET receptor phosphorylation (131 kDa; Figure 3.7.2) after Nrp knockdown and therefore the lower MW CTFs were missed out in our study. There was no immunoreactivity to MET antibodies in MCF-7 nuclear extracts, suggesting the absence of CTFs in this cell line.

A number of receptor tyrosine kinases in addition to MET such as ErbB4 (EGF receptor family member), Notch, and APP, are cleaved within or near the transmembrane domain, by a process called regulated intramembrane proteolysis (Brown et al., 2000). This cleavage releases the cytoplasmic domain, which can translocate to the nucleus and regulate gene expression (Pozner-Moulis et al., 2006). The MET CTFs when fused with Gal4 DNA binding domain transactivated Gal4 Luciferase activity in MDA-MB-231 cell line identifying them as regulators of gene transcription (Mettucci et al., 2009).

Nrp is shown to physically interact with the MET receptor on the tumour cell surface demonstrated by immunoprecipitation experiments (Hu et al., 2007). They also interact with each other in signalling endosomes as part of the HGF, MET, Nrp complex (Matsushita et al., 2007). It is unknown if Nrp can also interact with the CTFs and localize to the nucleus, hampering Nrp co-receptor functions on the cell surface. Further studies are required to investigate the effect of nuclear CTFs on the co-receptor functions of Nrp.

MDA-MB-231 cell line has developed mechanisms such as increased expression of the MET receptor, HGF independent MET pathway activation and expression of CTFs to overcome the need for HGF-dependent co-receptor functions of Nrp. However Nrp is essential for its VEGF and TGF-β1 co-receptor functions in the same cell line (Glinka et

al., 2010; Lee et al., 2007; Barr et al., 2008). This suggests that the functions of Nrp are not only cell type dependent but also sub-cellular context dependent.

B. Nrp has no effect on cell proliferation, migration or apoptosis independent of exogenous growth factors in MDA-MB-231 cell line

Nrp is a co-receptor for several growth factor ligands including HGF, VEGF and TGF-β1. However, it is unknown if Nrp also mediates growth factor independent functions in pathogenesis. Functional assays were conducted in MDA-MB-231 cell line without a growth factor ligand and changes in cell proliferation, migration and apoptosis were evaluated after Nrp1 siRNA treatment.

Nrp1 had no effect on MDA-MB-231 cell line proliferation in MTT assay (Figure 15). In addition, double knockdown of both Nrp1 and Nrp2 did not produce a significant reduction in MDA-MB-231 cell proliferation (Figure 21). Studies conducted *in vitro* in PANC-1 pancreatic cancer cell line showed no changes in cell proliferation after Nrp1 knockdown (Parikh et al., 2004). Colorectal cancer cells (HCT-116) stably transfected with short hairpin RNA (shRNAs) against Nrp2 or control shRNAs also did not display any changes in cell proliferation by MTT assay (Gray et al., 2008). Acute leukemic cell line HEL, treated with Nrp1 siRNA or sham transfected, did not result in significant changes in cell proliferation. However after VEGF treatment the sham transfected leukemic cells increased their proliferation significantly higher than the Nrp1 siRNA treated group in MTT assay (Lu et al., 2007). These studies suggest Nrp1 might not have an effect in tumour cell proliferation independent of growth factor addition.

Nrp plays a role in both endothelial tip cell guidance and directs the axon growth cone during embryogenesis. In scratch wound migration assay, Nrp1 siRNA treated cells did not display a significant difference in their migration rate, in comparison to the control (Figure 17). FBS (Fetal Bovine Serum) contains a rich content of growth factors and therefore was excluded from this assay to prevent activation of GF dependent functions of Nrp. In the aggressive lung cancer cell line, A549, no change in cell migration was observed after double knockdown of Nrp1 and Nrp2 when no FBS was added (Jia et al.,

2010). However, addition of FBS or VEGF led to an observable increase in cell migration in Nrp positive control cells in comparison to the Nrp siRNA treated cells. Both VEGF and serum-dependent migration was inhibited in the presence of EG3287, an antagonist of the b1 domain (Jia et al., 2006). This suggests that the b1 domain of Nrp is involved in GF dependent migration in tumour cells.

The role of Nrp in etoposide- induced cell apoptosis was also evaluated. Knockdown of Nrp did not show a significant difference in PARP cleavage in comparison to the sham-transfected control in the absence of growth factors (Figure 16). *In vitro* studies in human colon adenocarcinoma have identified a role for Nrp1 in regulating apoptosis; however this function of Nrp1 is highly VEGF dependent (Ochiumi et al., 2006). It has been suggested that Nrp allows evasion of apoptosis of the tumour cells *in vivo* because of its ability to promote angiogenesis on which the tumour sustains. A peptide corresponding to the binding site of $VEGF_{165}$ on Nrp induced apoptosis in breast cancer cells (Barr et al., 2005). Nrp1 is shown to promote VEGF- dependent anti-apoptotic effects in many other cell types, including endothelial cells, arthritic synoviocytes and squamous cell lung carcinomas (Kong et al., 2010; Wang et al., 2007; Barr et al., 2007). It is not surprising that the effects of Nrp in proliferation, migration and apoptosis are growth factor dependent because Nrp is a growth factor co-receptor. Binding and responding to GF is an inherent characteristic of Nrp as a GF co-receptor.

C. Nrp plays a role in mammosphere formation through NF-κB pathway activation

Cancer Stem Cells (CSC) is identified as the tumour subpopulation resistant to conventional cancer therapies and also the cause for cancer relapse. Therefore targeting the CSC population that feeds and drives the rest of tumour might lead to effective therapies and better patient prognosis. The role of Nrp in CSC was evaluated using an *in vitro* operational approach by mammosphere assay in MDA-MB-231 cell line. Double knockdown of Nrp1 and Nrp2 in MDA-MB-231 cell line displayed significant reduction (P<0.05) in both primary and secondary mammosphere formation. Both the

number of mammospheres and their size was greatly reduced in the absence of Nrp (Figure 24 and Figure 25). The mammosphere size reflects progenitor cell proliferation, whereas the mammosphere number reflects the stem cell self-renewal capacity. This data suggests that Nrp may function as an intrinsic factor required for the maintenance of the CSC pool and for CSC self renewal activity.

NF-κB pathway is essential for mammary CSC maintenance and survival, therefore changes in this pathway were evaluated next (Zhou et al., 2007; Liu et al., 2010). The activation of NF-κB pathway was greatly abrogated in the absence of Nrp in MDA-MB-231 mammospheres ($P<0.01$) (Figure 26). Nrp is a cell surface coreceptor for several GFs and NF- κB is a cytosolic transcription factor responsive to stimulation by GF (Dreesen et al., 2007). This suggests that NF-κB pathway may be the downstream target of the Nrp receptor in CSC.

However the identity of the GF that stimulates Nrp-dependent activation of NF-κB in mammospheres is unknown. A possibility is fibroblast growth factor (FGF) that was exogenously added as a necessary component to the mammosphere media (Dontu et al. 2003; Cell n Tech, Personal communication). FGF is an essential stemness supporting growth factor (Gotoh, 2009). Interestingly FGF is shown to bind Nrp and can also activate NF- κB (West et al., 2005; Lungu et al., 2008). In breast cancer cells, FGF triggers nuclear translocation of NF-κB p65 subunit, and enhances degradation of cytoplasmic IκBα, which leads to increase in matrix metalloproteinase production (Lungu et al., 2008).

Other possibilities include TGF-β1 that is secreted endogenously by MDA-MB-231 (Guerrero et al., 2010). TGF-β1 is a well established ligand for Nrp and a key player in breast cancer progression (Glinka et al., 2010; Baxley and Serra, 2010). MDA-MB-231 cell line is shown to secrete five times more TGF-β1 when compared to the less invasive MCF-7 cell line. TGF-β1 is also a major inducer of epithelial-to-mesenchymal transition (EMT). Human mammary epithelial cells treated with TGF-β1 underwent EMT and formed 40-fold more mammospheres than the untreated control cells (Mani et al., 2009). These cells also display $CD44^{high}$/ $CD24^{low}$ configuration and possessed

multilineage potential suggesting a link between TGF-β1 driven EMT and cancer stem cell phenotype (Blick et al., 2010; Singh et al., 2010).

To evaluate if TGF-β1 is responsible for Nrp mediated NF- κB activation, endogenously secreted TGF-β1 must be blocked with anti-TGF-β1 antibody and changes in mammosphere number and size should be observed. A reduction in mammosphere formation in the sham-transfected MDA-MB-231 cells after treatment with TGF-β1 antibody would identify TGF-β1 as the GF responsible for Nrp dependent NF-κB activation.

It is interesting to note that the NF-κB responsive elements are genes encoding growth factors, their receptors or other signalling components (Gilmore, 2006). Activated NF-κB p65 was shown to bind the promoter region of Smad7 of TGF-β1 pathway and inhibit its expression (Nagarajan et al., 2000). Smad 7 is an antagonist of TGF-β1 signalling, and prevents binding of Smad2/3 to the activated TGF-βRI (Figure 6) (Hayashhi et al., 1997). This suggests that Nrp dependent autocrine loop may exist between NF-κB activation and TGF-β1 signalling allowing crosstalk between two pathways critical to CSC biology.

D. Tranilast reduces Nrp expression both *in vivo* and *in vitro.*

Tranilast was recently shown to target CSCs in human mammary cell lines by binding to the aryl hydrocarbon receptor (AHR) and prevented metastasis *in-vivo* in orthotopic γ-SCID mice model (Prud'homme et al., 2010). However the downstream molecular targets of tranilast are unknown. Based on the results from mammosphere and NF-κB activation assays, Nrp appears to be important for CSC biology (Figure 24-26). To determine if tranilast has any effect on Nrp, changes in Nrp expression were evaluated both *in vitro* and *in vivo* in a murine model. The expression of Nrp1 was reduced in 4T1 cell line treated with tranilast (Figure 27a). The *in vitro* results were consistent with the *in vivo* results, where syngeneic BALB/c mice treated with tranilast resulted in reduced Nrp1 expression (Figure 27b).

However this data is not sufficient to conclude that Nrp1 is a direct target of tranilast. A better approach might be to identify if the AHR transcription factor can bind the promoter elements upstream of the NRP1 gene, after activation by tranilast. AHR exists as a latent complex and translocates to the nucleus upon ligand binding. In the nucleus AHR forms a transcriptional complex with ARNT (aromatic hydrocarbon nuclear translocator), binds ligand specific promoter elements and regulates their expression (Denison and Nagy, 2003). Electrophoretic mobility gel shift analysis can be used to confirm the presence of an AHR binding site in the promoter region of Nrp1. Also activation of Nrp promoter can be detected by using a luciferase reporter assay in the presence of tranilast.

As mentioned before tranilast attenuates TGF-β1 pathway and inhibits its downstream functions (section 2.6.5) (chakrabarti et al., 2008). In addition, latency-associated binding protein (LTBP) was identified as a novel target for AHR (Gomez-Duran et al., 2009). LTBP is bound to the TGF-β1 propeptide and enhances TG-Fβ1 secretion and is essential for targeting TGF-β1 to specific locations in the extracellular matrix (Hyytiäinen et al., 2004). Reduction in LTBP levels was shown to reduce expression of TGF-β1 activators, such as thrombospondin-1 (TSP-1), plasmin and elastase, although the mechanism is unknown (Gomez-Duran et al., 2006). Interestingly, Nrp is also as an activator of TGF-β1 and its expression levels are reduced after tranilast treatment (Glinka et al., 2010; Glinka and Prud'homme, 2008). Further studies are required to understand if Nrp1 expression is reduced directly by AHR binding to its promoter element or indirectly in response to LTBP downregulation by AHR.

Interestingly, AHR is shown to interact with the NF-κB pathway. Specifically AHR and the NF-κB p65/Rel subunits are shown to physical associate with each other. The binding of AHR to NF-κB p65 forms an inactive complex, and leads to functional antagonism of the NF-κB pathway (Tian et al., 1999) The AHR dependent CSC-targeting role of tranilast might be mediated through reduction in Nrp expression leading to inactivation of the NF- κB pathway and attenuation of TGF-β1 activation.

Based on the results from this study and evidence from literature a model can be proposed to understand the interplay between Nrp, TGF- β1 activation and signalling, tranilast and its receptor AHR, and the NF-κB pathway (Figure 28).
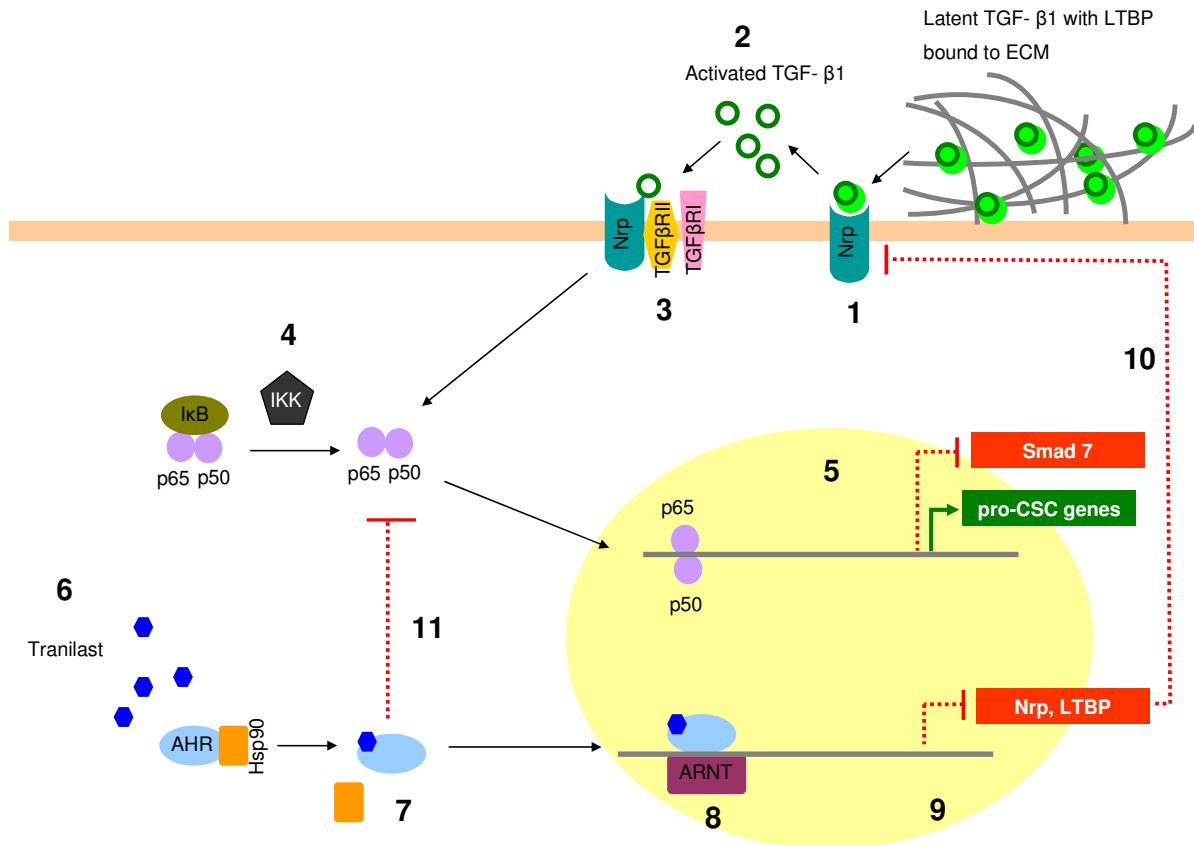
Figure 29 Proposed model for interactions between Nrp, TGF-β signalling, NF-κB, and tranilast

1) Latent TGF-β1 bound to ECM via LTBP is activated by Nrp. 2) The activated TGF-β1 binds to Nrp and its canonical receptor TGFβRII, which recruits TGFβRI for Smad signalling (not shown) 4) and also activates the NF- κB pathway, where IKK phosphorylates (not shown) the IκB to release the p65/p50 heterodimer. 5) The activated p65/p50 NF-κB transcription factor activates genes important for CSC biology. In addition the p65/p50 dimer inhibits Smad7 by binding its promoter, to prevent the Smad7 negative feed back loop activation in TGF-β1 signalling. 6) Tranilast diffuses through the plasma membrane and binds the cytoplasmic AHR. 7) AHR bound to Hsp60 is in an inactive state and is released by tranilast. 8) AHR translocates into the nucleus to bind ARNT and represses the LTBP and Nrp promoter. 9) Repression of LTBP affects the latent TGF- β1 activating function of Nrp. AHR= Aryl hydrocarbon, Hsp90=heat shock protein 90, ARNT= aromatic hydrocarbon nuclear translocator, LTBP= Latent TGF-β binding protein, ECM=extracellular matrix, IκB=Inhibitor of Kappa b, IKK= IκB kinase

6 Future Directions

As an extension to the results obtained from this study, the following experiments are suggested. These studies will help obtain a clearer picture of Nrp's function in breast cancer biology. The first two studies are directed towards Nrp function in the HGF/ MET signalling pathway and rest are concerning the CSC function of Nrp in breast cancer.

1. Identify changes in pathways downstream of HGF/ MET signalling in MCF-7

Knockdown of Nrp1 in MCF-7 abrogated HGF mediated phosphorylation of MET, suggesting Nrp1 dependent functions in this cell line. Next, changes in the phosphorylation of downstream effectors in the HGF/ MET pathway such as MAPK/ ERK and Akt phosphorylation, after Nrp1 knockdown, must be evaluated. A change in the phosphorylation levels of the downstream effectors is a strong indicator of changes at the functional level. For instance changes in the MAPK/ ERK axis would indicate changes in cell scattering and proliferation. Changes in the phosphorylation of Akt would indicate changes in the survival and motility axis. However functional assays such as MTT proliferation, wound migration and apoptosis assay should be conducted to confirm and augment the signalling studies.

2. Overexpression model of Nrp

Gain of function studies by over expression of full length Nrp1 in MDA-MB-453 cell line is currently being optimized. MDA-MB-453 cell line is negative for Nrp expression and non-tumorigenic *in vivo.* Therefore it is an excellent model to test if overexpression of Nrp causes the cell line to acquire a tumorigenic and metastatic phenotype (Gruber and Pauli, 1999; ATCC catalog # HTB131).

3. Differentiation potential of the mammospheres generated from Nrp positive cells

As mentioned before, one of the essential properties of the CSCs relevant to tumour relapse is their multi-lineage differentiation potential. To evaluate multi-lineage potential

of mammospheres, they must be induced to undergo differentiation by growing them in the presence of serum (Grimshaw et al., 2007). Immunohistochemistry analysis can then be performed to test the presence of lineage specific markers such as Cytokeratin 18/8 (CK 18/8) for luminal epithelial lineage and CK14/5 for myoepithelial lineage.

4. Evaluate the self renewal, differentiation and tumorigenic potential of Nrp knockdown cells *in vivo*

It is important to test if the *in vitro* self renewal potential of the Nrp positive cells displayed by the mammosphere formation assay is translated *in vivo*. For *in vivo* studies, shRNA (short hairpin RNA) can be used to generate a stable MDA-MB-231 transfectant lacking Nrp1. Eventually a double knockout for Nrp1 and Nrp2 will be generated. Empty vector can serve as the control for comparison. CSC will be enriched by seeding Nrp positive and negative cells in mammospheres and transplanted *in-vivo* in mice to observe tumorigenicity and metastasis to secondary sites. Immunohistochemistry of the tumour will reveal the multi-lineage differentiation potential of the cells which initiated the tumour. In addition, to evaluate the self renewal capacity, CSCs from the xenograft will be selected in mammospheres and re-implanted in serial transplantation experiments.

5. Identification of the GF that induces Nrp dependent NF-κB activation in mammospheres

As mentioned before, either FGF or TGF-β1, are involved in Nrp dependent NF-κB activation. The involvement of either can be tested by adding FGF or TGF-β1 blocking antibodies to the mammosphere media. Decrease in the mammosphere formation of Nrp positive control cells in the presence of antibodies for these GFs would identify the GF involved. In addition, cell lysates will be prepared from mammospheres and an ELISA will be used to evaluate changes in the NF-κB pathway activation.

6. Test if tranilast inhibits Nrp1 CSC promoting functions

Tranilast was shown to reduce Nrp1 expression and is characterized as a CSC targeting drug. Tranilast can be added to Nrp siRNA treated and sham-transfected cells and changes in NF-κB activation and mammosphere formation will be evaluated.

The above experiments will assist in shaping the role of Nrp as a GF co-receptor in CSC renewal and maintenance. These studies will also identify the GF involved in Nrp dependent NF-κB activation and classify Nrp as a potential molecular target of tranilast.

7 Conclusion

The significance of GF coreceptors and GF signalling related components in cancer biology and therapy is highlighted very well in a recent paper by Ciavarella et al. (2010). Targeted-therapies in cancer include small molecule inhibitors and monoclonal antibodies, which work by inactivating GF, GF receptors or pathway components. Three currently used targeted therapies for the treatment of breast cancer are Herceptin (Trastuzumab), Tykerb (Lapatinib) and Avastin (bevacizumab) which function by targeting HER2/ ERBB2 and VEGF. It is also interesting to note that the production of growth and angiogenic factors by CSC population was identified as crucial for their tumorigenic and metastatic potential (Levina et al., 2009). The Nrp family is identified to play important roles in mediating efficient and effective signalling in VEGF, TGF-β, FGF and PDGF pathways in several cancer cell lines. In the present study, Nrp1 was shown to play a crucial role in HGF/MET signalling in MCF-7 cell line. The Nrp1 siRNA treated MCF-7 cell line failed to undergo phosphorylation at a key tyrosine residue, essential for downstream adaptor recruitment. Further functional assays and changes in the downstream pathway phosphorylation are to be evaluated to define the exact role of Nrp in the HGF/ MET pathway.

In the current study, a novel function of Nrp in CSC self-renewal and proliferation was identified. Knockdown of Nrp in MDA-MB-231 resulted in significant reduction in mammosphere size and number. Nrp was shown to mediate its functions in the CSCs

by activation of the NF-κB pathway. However the identity of the GF responsible for Nrp-dependent NF-κB pathway activation is presently unknown. It is not surprising to note that Nrp has both GF coreceptor and pro-CSC functions, because several other GF coreceptors including CD44 and syndecan are CSC promoters as well (Smalley and Ashworth, 2003; Alexander et al., 2000; Fillmore and Kuperwasser, 2007; Baumann and Krause, 2010).

Interestingly, tranilast, an AHR agonist, known to specifically target CSC in human mammary cell lines was shown to reduce Nrp expression. Several CSC targeting drugs, such as, Parthenolide (PTL), function by inhibiting the NF-κB activation (Guzman et al., 2005). Further experiments are required to test if tranilast can also inhibit NF-κB activation and if these inhibitory functions are mediated through Nrp inhibition.

Apart from its NF-κB mediated CSC functions, Nrp may also play a role in CSC biology through the VEGF pathway. Vascular recruitment for the maintenance and support of the CSC niche in solid tumours is of immense importance. Anti-vascular drugs employed against brain tumour targeted the CSC population (Ghotra et al, 2009). Nrp is a VEGF coreceptor, therefore targeting Nrp may result in the disruption of both CSC niche and the vasculature interaction with the tumour.

In conclusion, Nrp functions as a growth factor coreceptor in several signalling pathways implicated in cancer progression and metastasis. Nrp might to be an intrinsic factor required for the self-renewal activity and maintenance of CSCs. Targeting Nrp may address current issues in breast cancer biology such as cancer relapse and metastasis and improve patient prognosis. Therefore, Nrp appears to be a novel and promising therapeutic target in the treatment of breast cancer.

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