Neuropilins: Role in Signalling, Angiogenesis and Disease[2]

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Abstract

Neuropilins (NRPs) are co-receptors for class 3 semaphorins, and for members of the Vascular Endothelial Growth Factor (VEGF) family of angiogenic cytokines. Genetic analysis of the role of NRPs in mice shows that NRP1 is essential for embryonic neuronal path-finding and cardiovascular development, mediated via semaphorins and VEGF respectively, while NRP2 has a more restricted role in neuronal patterning and lymphangiogenesis. NRPs are thought to mediate functional responses, most importantly cell migration, as a result of complex formation with other receptors, such as plexins in the case of semaphorins and the VEGF receptor, VEGFR2, resulting in enhanced signalling via some intracellular pathways. Recent findings indicate that NRPs may have important biological roles in other physiological and disease-related processes. In particular, NRPs are highly expressed in diverse tumour cell lines and human neoplasms and have been implicated in several biological processes regulating tumour growth *in vivo*, suggesting that NRP1 may be a future therapeutic target in cancer.

Introduction

Neuropilin-1 (NRP1) was discovered as the antigen recognised by the A5 monoclonal antibody, which localised in the tadpoles of *Xenopus laevis* to the neuropile, a superficial layer comprising a dense network of glial cells, synapses, axons, dendrites and neurons [1-3]. The subsequent generation of NRP1 mutants in mice and zebrafish established an essential role of this molecule in embryonic development of the vertebrate nervous and cardiovascular systems [4-7]. A key feature of NRP1 and the structurally related molecule, NRP2, is that they function as receptors for two unrelated types of secreted polypeptide ligand: class 3 semaphorins, a family of molecules with key roles in axonal guidance, and several members of the Vascular Endothelial Growth Factor (VEGF) family of angiogenic cytokines. In both cases NRP1 is important for regulating cell migration, but causes chemorepulsion in response to semaphorins, and chemotaxis in response to VEGF-A. However, despite the wealth of data regarding the developmental functions of NRPs, the cellular functions of NRPs are not entirely clear, and much uncertainty remains regarding the mechanisms through which NRPs mediate the biological effects of their ligands. Moreover, recent findings in diverse physiological and pathophysiological settings have greatly expanded the repertoire of biological processes in which NRPs potentially play an important role [8-10]. This chapter will review NRP biology with a focus on recent advances in understanding the role of NRPs in angiogenesis, in cell signalling and in disease.

Neuropilin Structure

NRP1 and NRP2 are transmembrane glycoproteins of up to 923 and 926 amino acids, respectively, sharing a similar domain structure and an overall amino acid homology of 44% [10,11]. NRPs comprise large extracellular regions containing two CUB (a1/a2) domains, sharing homology with the complement binding factors C1s/C1r, sea urchin fibropellins (called Uegf) and Bone Morphogenetic Protein 1 (BMP1), tandem Factor V/VIII homology (b1/b2) domains with homology to the C-terminal (C1/C2) domains of blood coagulation factors V and VIII [2], and a MAM (c) domain, a 170 amino acid region, also found in the extracellular domains of functionally diverse proteins, including meprin (a cell surface glycoprotein), A5 antigen, and receptor tyrosine protein phosphatase μ (MAM). NRPs have a single transmembrane domain and small cytoplasmic domains of 44 amino acids in NRP1 and 43 in NRP2 (Fig. 1). The carboxy-terminal three amino acids, SEA, present in NRP1 and the NRP2a isoform, form a consensus PDZ domain binding motif, which mediates association with a PDZ domain protein called neuropilin interacting protein-1 (NIP1), synectin or RGS-GAIP-interacting protein (GIPC) [12].

An important role of the extracellular region is ligand binding. The a1/a2 domains are essential for binding of semaphorins, while the b1 domain is required for VEGF-A_{165} binding with the b2 domain also necessary for optimal VEGF-A_{165} binding, and the b1/b2 domains are also important for semaphorin binding. The crystal structure of the NRP1 b1 domain displays a similarity to the three-dimensional structure of the FV/VIII C2 domain [13]. Crystal structures of the NRP1 b1 domain bound either to the peptide ligand, Tuftsin [14], to antibodies directed against either the VEGF or Sema3A binding domains [15], or to small molecule ligand mimetics [16], have identified the VEGF binding pocket and the key residues required for VEGF binding. The c or MAM domain has homology to other MAM domains present in diverse proteins, that are thought to mediate homophilic protein-protein associations important for homodimerisation or oligomerisation, and have also been implicated in regulating protein

stability. The MAM domain is thought to play a role in NRP-1 oligomerisation [17,18], though the function of this region of NRP1 is still poorly understood.

NRP1 is a glycoprotein though the degree of glycosylation appears to vary between different cell types. In several tumour cell lines and in cultured vascular smooth muscle cells (VSMC) NRP1 also occurs as a high molecular weight glycosylated species of >250 kDa in addition to the major species of ~130 kDa corresponding to the full-length protein , and this modified form appears to be expressed at a lower level in cultured endothelial cells [19-21]. NRP1 is modified by addition of an O-linked heparan sulphate and/or chondroitin sulphate glycosaminoglycan (GAG) moiety preferentially to serine 612 in the linker region between the b2 and MAM domains (Fig. 1). In contrast, there is no evidence for a high molecular weight GAG modified form of NRP2. NRP1 and NRP2 are also modified by asparagine(N)-linked glycosylation as indicated by the effect of tunicamycin inhibition of N-glycosylation [22]. CS-GAG modification is reported to enhance both VEGF binding to neuropilin and cell survival, and to down-regulate VEGFR2 expression levels in vascular smooth muscle cells [19]. Further studies are needed to fully determine how glycosylation alters the ligand binding and other functional properties of NRPs.

Neuropilin Ligands

NRPs have the ability to bind with high affinity two structurally unrelated classes of ligands with distinct biological functions, the class 3 semaphorins and several members of the VEGF family. These ligand interactions are summarised in Fig. 2.

Semaphorins

The class 3 semaphorins are secreted proteins with essential roles in the axonal guidance and homing of sensory neurons, most of which require NRP1 or 2 as obligate co-receptors [23-25]. The major semaphorin ligand for NRP1 is Sema3A (also termed collapsin-1), which induces growth cone collapse in subsets of sensory and sympathetic neurons, including the dorsal root ganglia (DRG), and is essential for neurogenesis [26]. NRP1 also binds other secreted class 3 semaphorins with lower affinity, though the biological roles of these interactions are less clear [10]. The best characterized semaphorin ligand for NRP2 is Sema 3F, and Semas 3B, 3C and 3G, but not Sema3A, also bind NRP2 [10].

VEGFs

Vascular endothelial growth factor (VEGF or VEGF-A), also referred to in earlier literature as vascular permeability factor (VPF), is essential for both early formation of primordial blood vessels and endothelial cell differentiation from haematopoietic precursor cells (vasculogenesis) and for the sprouting of new vessels from pre-existing ones (angiogenesis) during development and in the adult [27]. In mammals, the VEGF family includes 5 members

encoded by different genes: VEGFs-A, -B, -C, -D and placental growth factor (PlGF). In addition, VEGF-E is encoded by the *orf* virus, and VEGF-F is found in snake venom [28-31].

Alternative splicing generates multiple VEGF-A isoforms with distinct but partly overlapping receptor affinities and specificities. VEGF-A occurs in at least six different isoforms: $VEGF_{121}$, $VEGF_{145,}$ $VEGF_{165,}$ $VEGF_{165b,}$ $VEGF_{189}$ and $VEGF_{206}$ [31,32]. $VEGF_{121}$ lacks exons 6 (coding for a sequence rich in basic amino acids involved in heparin binding) and 7, and has therefore been thought unable to bind either NRPs or heparin [33]. The most studied and what is generally thought to be the most active and abundant isoform, $VEGF_{165}$, contains exon 7 but lacks exon 6, and is able to bind to both NRPs 1 and 2 [34]. $VEGF_{145}$ lacks exon 7 and binds only to NRP2 [35]. The NRP1 b1 domain is essential for $VEGF_{165}$ binding, and the b2 domain is additionally required for optimal binding, while the a1/a2 domains are largely dispensible for VEGF-A binding [36]. $VEGF_{165}$ binds to NRPs with high affinity through its carboxy-terminal domain encoded by exons 7 and 8. A bicyclic peptide corresponding to the C-terminal 28 amino acid residues of $VEGF\text{-}A_{165}$ encoded by the C-terminal portion of exon 7 and all of exon 8 selectively inhibits VEGF binding to NRP1 [37], and a peptide corresponding to exon 8 of $VEGF\text{-}A_{165}$ and a naturally-occurring peptide with homology to exon 8, Tuftsin, also bind to NRP1 [14,38]. Antibodies specifically blocking $VEGF_{165}$ binding to NRP1 also inhibited endothelial cell migration induced by $VEGF_{121}$ [39]. $VEGF_{121}$, lacking exon 7 but containing exon 8, binds NRP1 *in vitro* in surface plasmon resonance assays, but is unable to promote complex formation between NRP1 and VEGFR2 [39]. These findings are consistent with a model in which initial VEGF contacts with NRP1 are mediated via an interaction between the extreme carboxy-terminal residues encoded by exon 8 and the b1 domain (Fig. 3); binding and NRP1/VEGFR2 complex formation is either stabilised by further interactions between NRP1 and exon 7-encoded residues and/or the exon 7 domain functions as a bridge between NRP1 and VEGFR2. The larger isoforms, $VEGF_{189}$ and $VEGF_{206}$, bind heparin, are not readily diffusible, and are thought to remain sequestered by the extracellular matrix, but theoretically are able to bind NRP1. A novel isoform, $VEGF\text{-}A_{165}b$, has been reported, with a distinct carboxy-terminus, RSLTRKD, encoded by an alternative exon 8 and is unable to stimulate endothelial cell proliferation, angiogenesis or other biological activities [40]. Possibly, the weaker biological activity of $VEGF\text{-}A_{165}b$ is due to its inability to bind NRP1 and thereby promote productive signalling complexes with VEGFR2 [37]. Other factors also influence VEGF binding to NRP1. The affinity of VEGF binding to NRP1, at least *in vitro*, is enhanced by heparin, and by increasing the density of NRP1 [41]. As mentioned above, CS-GAG modification of NRP1 also enhances VEGF binding [19].

VEGF-B and VEGF-E have been shown to bind to NRP1 [42,43], while the VEGFR3 ligands, VEGF-C and VEGF-D, interact with NRP1 and NRP2 [44,45]. PlGF-2 also binds to NRP1 [46]. There is some evidence that other growth factors may bind to NRP1. Thus, FGF-2 binds to NRP1 *in vitro* [47], PDGF-BB has been co-immunoprecipited with NRP1 in VSMC [48], NRP1 associates with PDGFRα in VSMC and in mesenchymal stem cells [21,49], and colocalises with PDGFRα and enhances PDGF-BB binding affinity in hepatic stellate cells [50]. However, definitive evidence for high affinity functional binding of extracellular factors other than VEGFs and semaphorins to NRP1 is so far lacking [21,51], and this question requires further investigation.

NRP genomic organisation and isoforms

The NRP1 gene spans over 120 kb and is composed of 17 exons [52]. The NRP2 gene is > 112 kb, also comprising 17 exons [52]. Human NRP1 and NRP2 genes map to chromosomal positions 10p12 and 2q34, respectively [53]. The strong similarities in the exon-intron organisation of the NRP1 and NRP2 genes (Fig. 1), their exon and intron sizes, and positions of many of the splices sites suggests that they may have originated from a gene duplication event.

Alternative splicing results in the generation of several isoforms of NRP1. One membrane-associated NRP1 isoform has been identified, called NRP1(Δexon16), which lacks the 51 nucleotides corresponding to exon 16 [52,54]. NRP1(Δexon16) does not differ from the common full-length NRP1 in its binding to $VEGF_{165}$, dimerisation with VEGFR2 or regulation of $VEGF_{165}$ signalling [54]. Four NRP1 mRNA isoforms have so far been reported, all predicted to encode soluble proteins containing the a1/a2 and b1/b2 domains but lacking the MAM (c), transmembrane and cytoplasmic domains (Fig.1). These isoforms vary in size from 551 to 704 amino acid residues due to alternative splicing. However, only two soluble NRP1 (sNRP1) isoforms, s_{12}NRP1 (NRP1 isoform b) and s_{IV}NRP1 (NRP1 isoform c), have so far unambiguously been shown to be expressed in protein form. Soluble isoform s_{III}NRP1 contains a1/a2 and b1/b2 domains missing 48 aa at the C terminus of the b2 domain, but contains 13 extra amino acids resulting from a shift in the reading frame of exon 12 [55]. Isoforms s_{III}NRP1 (551 aa) and s_{IV}NRP1 (609 aa) occur in both normal and cancerous human tissues and both bind to $VEGF_{165}$ and Sema3A [55]. Soluble NRP1s may act as decoys, competitively binding and sequestering ligands such as $VEGF_{165}$ and Sema3A, and therefore negatively regulating functions mediated by these cytokines. In support of such a role, sNRP1 inhibits tumour cell growth *in vivo* and triggers tumour cell apoptosis, mimicking the effect of $VEGF_{165}$ withdrawal [56]. However, the effects of sNRP1s may be more complex than suggested by a straightforward decoy role. Thus, whereas an sNRP1 monomer sequesters $VEGF_{165}$ and inhibits its activity, sNRP1 dimers appear to deliver $VEGF_{165}$ to endothelial cell VEGFR2, thereby promoting angiogenesis [57].

Membrane-bound NRP2 exists in two major isoforms, NRP2a which shares 44% overall homology at the amino acid level with NRP1, and NRP2b which is identical to NRP2a in its extra-cytoplasmic domain, but exhibits only 11% homology with NRP2a in its transmembrane and cytoplasmic regions (see Fig. 1). In the mouse, a total of four NRP2a isoforms are generated by alternative splicing resulting in insertion of 0, 5, 17 and 22 (17+5) amino acids after residue 809, situated between the MAM and the transmembrane domain [11]. In the human, two forms of NRP2a have been cloned, $NRP2a_{(17)}$ and $NRP2a_{(22)}$, homologous to the corresponding mouse isoforms. The $NRP2a_{(22)}$ isoform (931 amino acids) results from the insertion of the five amino acids GENFK within the 17 amino acid insertion of $NRP2a_{(17)}$ (926 amino acids; Fig. 1). The $NRP2a_{(0)}$ and $NRP2a_{(5)}$ isoforms have not so far been found in human tissues. These isoforms do not appear to differ in their ligand binding properties, but insertion of residues between the MAM and transmembrane domains might potentially NRP2's ability to form complexes with VEGFR2 or to homodimerise.

NRP2b displays little homology with NRP2a after residue 808 [52] with a distinct cytoplasmic domain lacking the carboxy-terminal PDZ domain recognition sequence, SEA, required for interaction with synectin. Similar to NRP2a, $NRP2b_{(0)}$ and $NRP2b_{(5)}$ isoforms result from

alternative splicing and the insertion of zero or five amino acids after amino acid 808. The marked differences in their cytoplasmic domains suggest that NRP2a and NRP2b isoforms may have divergent functions, a possibility supported by their differential tissue expression. NRP2a and NRP2b are both highly expressed in the brain, but NRP2a is preferentially expressed in the liver, lung, small intestine, kidney and heart, while NRP2b is present in heart and skeletal muscle [52]. A soluble NRP2 isoform is also generated by alternative splicing (see Fig. 1), $_{s9}$NRP2 (1785 bp, 555 aa, 62.5 kDa) consisting of the two a1/a2 domains the b1 domain and a truncated b2 domain followed by the 8 amino acids VGCSWRLPL encoded by intron 9 [52].

Neuropilin Function in Development

Targeted disruption of genes encoding NRPs and their ligands has demonstrated an essential dual role of these molecules in neurogenesis and cardiovascular development (see Table 1). NRP1 null mice die between E10.5 and E14.5, dependent on the genetic background, with a spectrum of cardiovascular and neuronal defects. Both the central nervous system (CNS) and peripheral nervous system (PNS) are severely affected with defects in the homing and fasciculation of spinal, cranial and other sensory neurons [5]. Both small and large vessels of the yolk sacs are disorganised and the capillary network sparse, while aberrant embryonic macrovascular development is characterised by lack of development of the branchial arch, related great vessels and dorsal aorta, transposition of the aortic arch, and insufficient septation of the *truncus arteriosus* [6]. NRP1 over-expression also results in embryonic lethality with excess capillary growth, haemorrhage in the head and neck and a malformed heart, in addition to anarchic sprouting and defasciculation of nerve fibres [58]. Mice with an endothelial-specific NRP1 deletion also exhibit mid-to-late embryonic lethality, a poorly-branched vasculature and multiple defects in the major arteries and defective septation of the major cardiac outflow tract [4]. In contrast, knock-in mice expressing a mutant NRP1 with a 7 amino acid deletion in the a1 domain required for Sema3A binding but retaining the ability to bind VEGF-A, survive to birth, possess no obvious cardiovascular defects, but exhibit aberrant pathfinding of sensory afferent nerves to synapses in the CNS and defasciculation of spinal and cranial nerves, most of the mice dying by P7. Though it has been proposed that Sema3A and VEGF-A interact competitively via NRP1 in cardiovascular development, the analysis of different mouse NRP1 mutants indicates that the major developmental functions of NRP1 reflect tissue-specific actions of either VEGF-A_{165} binding to NRP1 in the endothelium, or Sema3A binding to neuronal NRP1, these two signalling pathways being segregated with relatively little cross-talk between the two types of ligand-NRP1 interaction. Thus, the VEGF-A/NRP1 interaction is thought to be essential for cardiovascular development while the semaphorin/NRP1 signalling axis is essential for formation of cranial and spinal nerve projections, guidance of peripheral projections of bipolar neurons of the vestibular ganglion and the central projections of a subset of axons of cutaneous sensory neurons, and for basal cortical neuron dendrite development [4].

The importance of semaphorin-neuropilin interactions in axonal homing is demonstrated by the close similarities of the neuronal pathfinding defects in NRP1 and Sema3A mutant mice [4,5,59], and in mice lacking Sema3F and NRP2 [60,61,62-64]. The guidance of motor axons from the spinal cord during vertebrate limb bud development serves as a striking illustration of how the expression patterns of NRPs and their specific semaphorin ligands exquisitely choreograph the homing of specific subsets of axons. As they leave the spinal cord, both lateral and medial axons of the lateral motor column (LMCl and LMCm, respectively) express NRP1,

while NRP2 is restricted to the LMCm; chemorepulsive Sema3A is expressed throughout the limb bud whereas Sema3F is localised to the dorsal limb bud. Targeted disruption of either NRP1 or Sema3A causes premature and disorganized invasion of the limb bud by motor axons, and misprojection of both LMCl and LMCm axons. Deficiency of either Sema3F or NRP2 results in selective misprojection of LMCm axons, usually expressing NRP1 and NRP2, to the dorsal limb, while LMCl axons normally expressing only NRP1 track normally to the dorsal limb [62,64]. NRPs do not mediate all functions of their Class 3 semaphorin ligands, however. For example, the findings that loss of either Sema3E or plexin D1 causes a similar disorganisation of the intersomitic vasculature [65-67], while genetic ablation of NRP1 has no such effect, are explained by generation of repulsive cues to endothelial cells as a result of Sema3E binding directly to Plexin D1 independently of NRP1. Interestingly, Sema 6D, which does not bind NRPs, utilizes a complex between a VEGFR2 co-receptor and plexin A1 to mediate signalling essential for cardiac development [68].

While these studies indicate that the cardiovascular and neuronal guidance functions of NRP1 result mainly from spatially distinct and divergent functions of NRP1 expressed in the vasculature or neurons, some degree of co-operation between Sema3A and VEGF binding in cardiovascular development is supported by the finding that knock-in NRP1 mice deficient in Sema3A binding and also null for NRP2 (see Table 1), mice in which VEGF is only able to bind to NRP1, exhibit cardiovascular defects similar to those in endothelial-specific NRP1 null mice [4]. Furthermore, another class III semaphorin, Sema3C, is required for septation of the cardiac outflow tract and thus partially phenocopies the endothelial-specific NRP1 knockout, suggesting that an interaction between NRP1 and Sema3C in the endothelium may be functionally important.

In contrast to NRP1 mutant mice, NRP2 null mice survive to adulthood with no obvious cardiovascular abnormalities, but exhibit a severe reduction of small lymphatic vessels [69] and capillaries, as well as abnormal guidance and fasciculation of cranial and spinal nerves [60,61]. Doubly deficient $NRP1^{-/-}/NRP2^{-/-}$ mice exhibit earlier embryonic mortality than the NRP1 knock out (E8 versus E12-13.5) and have a more severe vascular phenotype resembling the $VEGF\text{-}A_{165}$ and VEGFR2 (KDR) knockouts [70], marked by large avascular areas in the yolk sacs, and head and trunk regions, and a lack of connections between blood vessel sprouts.

An essential role of NRP1 in vascular development has also been demonstrated in other vertebrate species. In the zebra fish embryo, NRP1a is expressed in the neural tube; NRP1b is expressed in the nose and the cranial neural crest cell; NRP2a in the telencephalon and anterior pituitary; and NRP2b in the telencephalon, thalamus, hypothalamus, and epiphysis [71,72]. Morpholino NRP1 knock down in the developing zebrafish [7] produced a severe vascular phenotype characterized by a loss, or anarchic sprouting, of new capillaries from pre-existing intersomitic vessels, but had no effect on the formation of the major axial vessel, suggesting that, in zebrafish, NRP1 is not implicated in vasculogenesis [7]. Defects were also observed in the developing zebra fish nervous system after NRP1 knockdown, characterized by aberrant migration and branching of motor neurons [73,74].

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

the primary vascular plexus before blood has started to flow [75]. In 26-somite embryos, which have a functioning vasculature, NRP1 and NRP2 are differentially expressed in arteries and veins, while the NRP2 ligand, Sema3F, was bound to NRP2-expressing cells [75,76]. Similar to the expression patterns in the chicken embryo, NRP1 is preferentially expressed in the dorsal aorta of zebra fish embryos, and NRP2 transcripts are localised to the posterior cardinal vein [77]. It is unclear at present whether differential NRP expression is crucial for the early embryonic segregation of arterial and venous cells in mammalian embryos, though the relatively mild phenotype in NRP2-deficient mice (Table 1), suggests that NRP2 expression may be less important in this respect.

Receptors and Signalling Mechanisms

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

Plexins

NRP1 mediates the chemorepulsive effects of Sema3A, but does so by acting as the ligand-binding module of a complex or holoreceptor between NRP1 and specific members of a family of transmembrane receptors called plexins [78-80]. NRPs 1 and 2 form complexes with several plexins independently of the presence of semaphorins, including plexins A1, A2, A3 and B1 [79-81]. The human plexin gene family comprises nine members divided into four subfamilies: four plexin As (1 to 4), three plexin Bs (1 to 3), plexin C1 and plexin D1 [36]. Plexins are large transmembrane receptors with extracellular regions comprising a Sema domain (homologous to sema domains in Semaphorins), two or three cysteine-rich regions called Met-related sequences (MRS) domains also found in the MET oncogene family, and three or four immunoglobulin-like domains shared by plexins and transcription factors (IPT), a transmembrane domain, and an intracellular region containing two conserved regions, the functions of which are not yet clearly defined, but show some homology to ras GTPase-activating protein (GAP) domains, and which, in the case of Plexin B1, has been shown to act as a GAP for R-Ras [82].

Sema3A contains both a NRP binding site, and potential sites of interaction with Plexins. Antipenko *et al* have proposed a model based on the crystal structure of the Sema3A Sema domain and mutagenic analysis, in which Sema3A binding results in a 2:2:2 complex between Sema3A, plexin A1 and NRP1, and involving relief of autoinhibition of plexin A1 [83]. This

has been proposed to cause activation of the plexin intracellular domain and the transduction of signals essential for chemorepulsion. Deletion of the cytoplasmic domain of NRP1 did not impair sema3A signalling [18], indicating that neuropilins may function solely as the binding entity of the complex while plexins mediate signalling.

The cytoplasmic domain of plexins is responsible for downstream signalling induced by semaphorins and resulting in the collapse of neurons [78-80]. Monomeric GTPases of the Rho family are thought to play a key role in regulating actin-based motility in neuronal cells [83], and have been implicated in semaphorin-mediated growth cone collapse [84,85]. Recruitment of the small GTPase Rnd to the cytoplasmic Plexin A1 domain triggers cytoskeletal collapse, but this effect is antagonized by RhoD which is also able to bind to Plexin A1 but blocks repulsion of sympathetic neurons [86]. Sema3A-induced cytoskeletal collapse in Cos-7 cells was dependent on co-transfection of Plexin A1 and NRP1, required activation of Rac, but not Rho, and involved a direct interaction between Rac and the plexin A1 cytoplasmic domain [87-89]. If and how NRP1 regulates Plexin-dependent signalling through small GTPases is unclear.

L1 CAM

The immunoglobulin superfamily cell adhesion molecule, L1, is also a potential partner for NRP1 in mediating chemorepulsive Sema3A signals. L1-deficient mice are smaller, less sensitive to touch and pain, and exhibit lack of hind limb coordination compared to littermate controls, and display a striking reduction in the size of the corticospinal tract and in the association of Schwann cells with axons, [90]. That at least part of the phenotype in these mice results from defective axonal guidance orchestrated by Sema3A, is suggested by the inability of Sema3A to repel L1-deficient cortical axons [91], and the formation of stable complexes of L1 and NRP1 mediated by interactions between their extracellular domains [92]. NRP2, which is not required for Sema3A signalling, was unable to associate with L1-CAM [92]. Several L1 mutations located in the NRP1 binding region are associated with some human neurological disorders, including X-linked hydrocephalus and MASA syndrome (Mental retardation, Aphasia, Shuffling gait, Adduced thumbs). Interestingly, one such mutation, L120V, also disrupts L1 association with NRP1, suggesting a causal role of defective L1/NRP1 signalling in human neurological disease [92]. Soluble L1 extracellular domain is able to convert repulsive Sema3A signals into chemoattraction by binding in *trans* to NRP1 and this conversion is mediated by nitric oxide-dependent activation of guanylate cyclase and consequent cyclic GMP synthesis [92], reminiscent of the role of asymmetric guanylate cyclase distribution in determining chemoattractant properties of Sema3A in cortical apical dendrites [93]. The effects of L1 deficiency do not phenocopy the neural defects of Sema3A-deficient mice or NRP1$^{sema3A-/-}$ mice expressing mutant NRP1 unable to bind Sema3A, indicating that L1 regulates Sema3A signalling via NRP1 in a more restricted set of neurons. It is also unclear yet whether the L1/NRP1 complex is either distinct from, or linked with, the plexin/NRP1 holoreceptor.

VEGF signalling

In the endothelium, NRP1 is thought to function primarily as a co-receptor for the VEGF receptor tyrosine kinase (RTK), VEGFR2 (KDR in humans, Flk-1 in mice). NRP1 enhances VEGF binding to VEGFR2, VEGFR2 phosphorylation, and VEGF-induced signalling and migration [94-97], and NRP1 and VEGFR2 associate to form a heterocomplex or 'holoreceptor' [33,37,95-99]. NRP1 is not required for VEGFR2 activation, and it is unclear whether NRP1 complexation with VEGFR2 increases the intrinsic affinity of VEGFR2/KDR for VEGF-A [33, 95, 100]. However, siRNA-mediated NRP1 knockdown resulted in a 50% inhibition in VEGFR2 phosphorylation at Tyr 1175 [101,102], a residue required for VEGFR2-mediated activation of phospholipase C-γ/ERK, cell proliferation and normal embryonic development. It is also important to emphasise that NRP1-dependent VEGF signalling also requires active VEGFR2 [101]. NRP1/VEGFR2 complexation is enhanced by VEGF binding [94,96,102], though some studies have reported constitutive VEGF-A-independent complex formation [95,100]. VEGF-induced NRP1 association with VEGFR2 is likely to result from the dual binding of the cysteine knot motif of VEGF-A_{165} in the core VEGF homology region to VEGFR2, and of the exon 7/8 encoded carboxy-terminal moiety of VEGF-A_{165} to the b1 domain of NRP1 bridging between the receptors to form a tripartite complex. Deletion of the NRP1 carboxy-terminal PDZ domain binding motif decreased NRP1/VEGFR2 co-immunoprepcipitation [103], suggesting that intracellular interactions are also important for heterocomplex formation, though it is unclear whether this involves a direct physical interaction between the NRP1 cytosolic domain and VEGFR2.

Some essential functions of VEGF, including cell survival and proliferation, appear to be largely independent of NRP1. This is illustrated by the findings that activation of the phospholipase C-γ/ERK and Akt pathways, which play major roles in proliferation and survival functions of VEGF, are largely unaffected in the absence of NRP1 or when VEGF binding to NRP1 is selectively inhibited [37,94,101-104,]. Moreover, VEGF-A_{165} and the VEGF-E-NZ2 subtype, which both bind VEGFR2 and NRP1, induced similar increases in PLC-γ tyrosine phosphorylation and activation of ERK and Akt as $VEGF_{121}$, which is unable to promote VEGR2/NRP1 complexation, but exhibited greater angiogenic activity and stimulated more p38 kinase activation than VEGF-A_{121} [105]. However, a study of VEGF-A signalling in mouse endothelial cells expressing NRP1 lacking its cytoplasmic domain indicates that NRP1 is important for VEGF-A ERK signalling [106], an effect which is consistent with the reduction in VEGFR2 Y1175 phosphorylation caused by NRP1 knockdown [101,102]. Recent studies also indicate an important role for NRP1 in mediating VEGF-induced tyrosine phosphorylation of p130Cas, an adapter protein with a key role in regulating actin filament dynamics and cell migration [20,21,101,102,107]. P130Cas associates with several other signalling molecules involved in regulating cell migration and actin cytoskeleton organisation, including the related non-receptor tyrosine kinases, Focal adhesion kinase (FAK) and Pyk2. Interestingly, though VEGF stimulation of p130Cas tyrosine phosphorylation was mediated via Pyk2 [101], NRP1 knockdown did not affect FAK tyrosine phosphorylation at its major autophosphorylation site, Y397. However, both NRP1 knockdown and expression of a NRP1 Y297A mutant deficient in VEGF binding, blocked VEGF-induced FAK tyrosine phosphorylation at Y407 [102], a site that is regulated by Pyk2 [108]. These findings (summarised in Fig. 3) suggest that NRP1 selectively mediates VEGFR2 signalling via a Pyk2/p130Cas pathway important for cell migration. Mice deficient in p130Cas die *in utero* primarily from cardiovascular defects, and exhibit impaired actin filament assembly; p130Cas also appears to be expressed predominantly in the cardiovascular system at the time in embryonic development that lethality occurs in

p130Cas-null mice [109]. These findings are suggestive of a link between NRP1 and p130Cas signalling which may play an important role in developmental angiogenesis.

Recent studies have highlighted the role of NRP1 in endocytosis and intracellular trafficking. VEGF-A_{165} induced clathrin-dependent NRP1 endocytosis, whereas semaphorin 3C (sema3C) stimulated endocytosis via a distinct pathway sensitive to the cholesterol-depleting compound methyl β-cyclodextrin, which is thought to selectively disrupt lipid rafts [110]. NRP1 trafficking in response to both VEGF-A_{165} and Sema 3C was significantly slower in endothelial cells lacking synectin, and synectin$^{-/-}$ endothelial cells also lacked a chemorepellent response to Sema3C, suggesting that Semaphorin-induced NRP trafficking is essential for relay of chemorepulsive cues. Other work suggests that NRP1 endocytosis may also be important for VEGF signalling in arteriogenesis (discussed below).

In contrast to the well-defined signalling role of VEGFR2 in VEGF angiogenic activity, the function of VEGFR1 has not been fully characterised. The VEGFR1 knock out is lethal [111], but a normal phenotype is observed in transgenic mice with Flt-1 lacking the intracellular domain [112]. These findings indicate that VEGFR1 plays a decoy function by sequestering VEGF and therefore limiting its availability for binding to VEGFR2. NRP1 has been found to bind *in vitro* to the VEGFR1/Flt-1 extracellular immunoglobulin-like domains 3 and 4 [41], and it is possible that such a complex may compete for VEGF binding to VEGFR2/NRP1 heterodimers.

Recent findings indicate that VEGF-C interacts with NRP2 in a mainly heparin-independent manner, whereas VEGF-D binding to NRP2 is heparin-dependent [45]. Moreover, VEGFR3 and NRP2 co-localised and co-internalised following stimulation by VEGF-C and -D [45]. The formation of complexes between NRP2 and VEGFR2 or VEGFR3 resulted in a lowering of the activation threshold of VEGFR2 and an enhancement of cell survival and migration induced by VEGF-A and the VEGFR3 ligand, VEGF-C [113]. VEGFR3 and VEGF-C are both strongly implicated in lymphatic vascular development [114], and complex formation between VEGFR3 and NRP2 may help to explain the involvement of NRP2 in lymphangiogenesis as suggested by the phenotype of NRP2 knock out mice [69; Table 1]. Co-immunoprecipitation of cross-linked ^{125}I-VEGF also revealed the existence of a NRP2/VEGFR1 complex [115], but the biological relevance of such a complex is not clear.

Role of the NRP cytosolic domain

While there is strong evidence that NRP1 functions primarily as a co-receptor without an independent signalling role, some findings suggest that NRPs have functions that are not dependent on the known NRP ligands and interacting receptors, and further raise the possibility that NRP1 is able to support functional cellular signalling. For example, VEGF elicits biological responses in some cell types that are NRP-positive but express little or no KDR, such as VSMC [116] and some cancer cells (see below). Antibodies that selectively block VEGF binding to NRP1 have synergistic effects on cencer growth in combination with function-blocking antibodies directed against VEGF [37,94,101], suggestive that NRP1 has VEGF-independent roles at least in tumour vascularisation. The cytoplasmic domains of NRP1 and NRP2 contain a carboxy-terminal consensus PDZ binding motif that associated with the PDZ

protein, synectin or GIPC1 (RGS-GAIP-interacting protein-1), in a yeast two-hybrid screen [12]. A functional role for NRP1 association with synectin in angiogenesis is supported by the finding that expression of NRP1 lacking the C-terminal SEA motif disrupted vessel formation in zebrafish, and that knockdown of either synectin or NRP1 in zebra fish produced similar vascular phenotypes [117]. Furthermore, synectin was found to associate with NRP1 in human endothelial cells, and synectin knockdown inhibited NRP1-mediated endothelial migration [117]. In contrast, the C-terminal PDZ-binding domain does not appear to be important for Sema3A-mediated neuronal pathfinding functions of NRPs [18].

Synectin/GIPC was originally found to associate with one of the regulators of G protein signalling (RGS) proteins, called RGS19 (also known as GAIP) [118]. The GIPC/RGS19 complex is anchored to the cell membrane, localises to clathrin-coated vesicles and has been implicated in endocytosis and intracellular membrane trafficking [119]. Synectin/GIPC binds to G protein coupled receptors and modulates their signalling [119], and can also interact with up to twenty other proteins, including the proteoglycan, syndecan-4 [120], integrin $\alpha5$ and $\alpha6$ subunits [121], the transmembrane semaphorin, M-SemaF [122], and rho GEF (rho guanine exchange factor or syx1) [123]. These findings suggest that synectin has the potential to participate in multimeric protein complexes or scaffolds able to link surface receptors and integrins with intracellular signalling networks. *In vivo* studies show the importance of synectin in the development of a functional vascular system in both the zebrafish and the mouse [124]. Synectin deficient mice are viable, but the mice are smaller than littermate controls, and have a significantly reduced number of small arteries, resulting in impaired vascular functions. Arterial cells from synectin$^{-/-}$ mice exhibited reduced angiogenic and endothelial responses in cell culture models, and aberrant cellular distribution of rac1 [124]. The mainly microvascular arterial defects in synectin knockout mice do not phenocopy the embryonic lethality or aberrant macrovascular development of either global or endothelial-specific NRP1 deficiency, indicating that not all developmental functions of NRP1 are dependent on synectin association. However, it is possible that other related molecules, such as GIPC2 or GIPC3, may compensate for loss of synectin [125].

A mutant mouse line with a knock-in of NRP1 lacking the cytoplasmic domain (NRP1^{Cyto}) has recently been reported. NRP1^{Cyto} mice are viable, fertile, appear phenotypically normal, and also exhibit normal developmental vasculogenesis and angiogenesis. However, they display a restricted phenotype characterised by an abnormally high incidence of crossing of arteries and veins in the retina similar to the increased arteriovenous crossings observed in the human eye disease, branch retinal vein occlusion [126]. These mice also displayed a significant decrease in the number of smaller arteries and arterioles in the kidney, heart and hindlimb vasculatures at P7 and in adults compared to control animals, differences that were concomitant with small reductions in whole body, kidney and heart size [106]. These phenotypic effects of deletion of the NRP1 cytoplasmic domain have similarities to those of synectin deficiency described above, lending support to the concept that a role of NRP1 in developmental and adult arteriolar growth and maturation could be mediated specifically via interaction of the cytolplasmic domain of NRP1 with synectin. Lanahan et al further showed that lack of the NRP1 cytoplasmic domain results in delayed VEGFR2 trafficking, one consequence of which is impaired VEGFR2 phosphorylation at Y1175, a site critical for mediating VEGF-A-induced ERK activation [106]. These findings suggest that the NRP1 cytoplasmic domain may be crucial for normal endocytosis of VEGFR2, and that NRP1-dependent VEGFR2 trafficking plays a key role in intracellular signalling important for arteriolar morphogenesis in development and post-natal organ growth.

The role of NRP1 in endothelial cell function may also be mediated by interactions with integrins. An association between NRP1 and the cytoplasmic domain of the β3 integrin subunit negatively regulates angiogenesis by limiting availability of NRP1 for complexation with VEGFR2 [127]. An interaction between NRP1 and the major fibronectin receptor, integrin α5β1, is reported to mediate α5β1 endocytosis and regulate endothelial cell adhesion to fibronectin [128]. The NRP1 cytosolic domain was required for α5β1 endocytosis possibly due to interaction between the SEA motif and synectin, but did not appear to be necessary for association between NRP1 and α5β1. NRP1 association with β1 integrin is also implicated in pancreatic cancer cell growth, survival and invasion [129].

NRP regulation of cell migration

The most characteristic biological function mediated by NRPs in neuronal cells is chemorepulsion. NRP1 is essential for mediating Sema3A stimulation of growth cone collapse and axon repulsion in DRG neuronal cultures [26,130], while NRP2 is responsible for Sema3F-induced repulsion of superior cervical ganglia [11,131]. Sema3A and 3F have also been reported to promote chemorepulsion in porcine aortic endothelial (PAE) cells expressing, respectively, NRP1 and NRP2 [132,133], and in human endothelial cells [134-136], though other investigators failed to find an inhibitory effect of Sema3A on endothelial cell migration [94]. Sema3A also appears to be required for the correct orientation and chemoattraction of cortical apical dendrites [93]. This chemoattractant role of Sema3A is mediated at least in part via NRP1, as judged by the effects of function-blocking NRP1 antibody. The conversion of the Sema3A/NRP1 axis from chemorepulsion to chemoattraction is determined by the distribution of soluble guanylate cyclase: guanylate cyclase is localised to the dendrite, and inhibition of either guanylate cyclase or protein kinase G disrupted dendrite outgrowth in response to Sema3A [93,137].

Much evidence points to a role of NRP1 in directed endothelial cell migration or chemotaxis. Co-expression of NRP1 and VEGFR2 in PAE cells enhances VEGF binding and chemotaxis [33], and NRP1 mediates endothelial cell attachment to extracellular matrix [138]. VEGF-A_{165} also stimulates morphogenetic responses in renal epithelial cells, including sheet migration and tubulogenesis, through a mechanism dependent on VEGFR2 and which is also blocked by either neutralizing antibody to NRP1 or by the chemorepulsive NRP1 ligand, Sema3A [139]. Antibodies which specifically block VEGF binding to NRP1, inhibited the migratory response to VEGF, *in vitro* endothelial cell sprouting, and neovascularisation *in vivo* [94]. Interestingly, the same study reported that blocking NRP1 antibodies prevent pericyte recruitment to new vessels in mouse neonatal retinal vascularisation and tumour vascularisation models, suggesting a role of endothelial NRP1 in the maturation and stabilisation of developing vessels. The cellular mechanism underlying such a role of NRP1 is unclear, but could occur via one or both of two mechanisms, one in which endothelial NRP1 is important for adhesion or chemoattraction of pericytes to developing vessels, and/or a role for NRP1 expressed in pericytes or VSMC in adhesion and migration of these cells [94]. NRP1 is highly expressed in

human aortic and coronary artery smooth muscle cells (HCASMC), both as a core protein of 130 kDa and as a high molecular weight species modified by *O*-linked glycosylation [19-21]. Inhibition of NRP1 function significantly decreases the migratory response of VSMCs to PDGF-BB and PDGF-BB signalling via p130Cas, similar to findings in endothelial cells [21]. In support of a functional role of NRP1 in VSMC *in vivo*, apart from neurons, epicardium and endothelium, NRP1 is reported to be most strongly expressed in late mouse embryos in the VSMC of large vessels [140]. Proteoglycans play important roles in VSMC *in vivo*, and it is possible that the CS-GAG modification of NRP1 in VSMC may play a role in migratory functions of this molecule in developing or remodelling arteries. However, there remains a lack of information regarding expression of NRP1 in arterial VSMC *in vivo* either during development or in adult vessels, and determination of a biological role of NRP1 in VSMC will await analysis of appropriate animal models. Interestingly, NRP2 is expressed in mouse bladder and gastrointestinal smooth muscle in vivo, and increased bladder smooth muscle cell contractility was reported in NRP2-deficient mice [141], indicating a novel functional role of NRP2 in visceral (non-vascular) smooth muscle cell function *in vivo*.

Analysis of vascularisation in mutant NRP1 mice indicate that this molecule plays a key role in the guidance of specialized endothelial tip cells in newly sprouting vessels [27]. Tip cells are highly polarised cells with abundant filopodia at the migrating edge, studded with VEGRF2, and are responsive to gradients of VEGF-A. NRP1-deficient mice exhibit defective endothelial cell migration whereas endothelial proliferation appears unaffected in these mice [142,143]. NRP1 expression is also downregulated by the Notch signalling pathway, which plays a key role in suppressing the tip cell phenotype [144]. These findings do not necessarily indicate that NRP1 is essential for endothelial cell migration *per se*, but rather that NRP1 and its ligand VEGF specify the trajectories of migrating cells, similar to its pathfinding and homing role in neuronal patterning.

Other cell functions

Several studies indicating a role for NRP1 in cell adhesion to the extracellular matrix, are suggestive that such a role of NRP1 may be independent of known ligand interactions [102,138,145]. Shimuzu *et al*. found that recombinant NRP1 proteins supported adhesion of a variety of cell lines, including L cells, HEK293T, COS-7, HeLa, p19, KB and NIH3T3, and identified specific regions in the NRP1 b1 and b2 domains for adhesion, but showed that neither Sema3A nor VEGF-A_{165} interfered with this activity [145]. Furthermore, siRNA-mediated NRP1 knockdown disrupted endothelial cell adhesion to fibronectin, laminin or gelatin, while silencing of VEGFR2 had little effect, suggesting that an NRP1-mediated adhesive function is independent of VEGF-A signalling through VEGFR2 [138]. Blocking NRP1 antibodies appeared to have no effect on adhesion of endothelial cells to fibronectin [94], consistent with the independence of any adhesive function from VEGF binding.

NRP1 is also implicated in regulating endothelial permeability. Sema3A induced vascular permeability in vivo, an effect that was inhibited in adult mice with a conditional deletion of endothelial NRP1, or in wild-type mice treated with a blocking antibody directed against NRP1 [146]. Transendothelial resistance of PAE expressing VEGFR2/KDR and NRP1 was decreased in response to $VEGF_{165}$ treatment compared to cells expressing either receptor alone, and

inhibition of NRP1 in human pulomanry artery endothelial cells decreased $VEGF_{165}$-induced increased permeability [147]

Neuropilin Functions in Disease and Adult Tissues

Cancer

NRP1 and NRP2 are expressed by a wide variety of human tumour cell lines and diverse human neoplasms [8,10,33], and are implicated in mediating effects of VEGF and Semaphorins on the proliferation, survival and migration of cancer cells [148-151]. Over-expression of NRP1 in Dunning rat prostate AT2.1 carcinoma cells increased tumour growth *in vivo* [150], while NRP1 knockdown using siRNA inhibited breast carcinoma cell migration [152], and a peptide targeted to the VEGF binding site of NRP1 induced breast tumour cell apoptosis [153]. Furthermore, NRP1 plays a role in mediating or potentiating PDGF-BB and HGF/Scatter factor signalling through their receptor tyrosine kinases in both glioma and pancreatic cancer cell lines, regulating tumour progression, migration and invasion [20,101,154,155]. NRP1 is expressed in patient specimens from lung, breast, prostate, pancreatic and colon carcinomas, but not in corresponding normal epithelial tissues [22,156-161]. NRP1 has also been found in several other tumours including melanoma [162], astrocytoma [163] and neuroblastoma [164]. NRP2 expression was reported in lung cancer [156,157], neuroblastoma [164], pancreatic cancer [165], osteosarcoma [166] and bladder cancer [167]. It has been suggested that NRP1 is more prevalent in carcinomas (mainly of epithelial origin), whereas NRP2 may be more frequently expressed in non-carcinoma neoplasms such as melanomas, leukaemias and neuroblastomas [10,168,169]. However, there appears no sharp distinction between the types of neoplasms expressing NRPs 1 and 2, and often they are co-expressed in tumour cells. Furthermore, different cell lines derived from the same tumour types, such as glioma [170], may exhibit divergent patterns of NRP1 and NRP2 expression.

Clinical studies suggest that NRP1 plays a role in tumour growth and disease progression [8,10]. Overexpression of NRPl has been demonstrated to be associated positively with the metastatic potential, advanced stage and clinical grade of prostate carcinoma [159]. NRP1 upregulation in gastrointestinal carcinomas appears to correlate with invasive behavior and metastatic potential [171]. Co-expression of NRP1 and NRP2 also increased in the progression from dysplasia to microinvasive lung carcinoma, and correlated significantly with tumour progression and poor prognosis in patients with non-small cell lung carcinoma [156]. NRP1 also appears to be preferentially expressed in metastatic cells, and is found, for example in the metastatic breast cancer MDA-MB-231 and melanoma MDA-MB-435 cell lines but not in the nonmetastatic cell line MDA-MB-453 or some nonmetastatic tumours [33,148].

While most studies have indicated a pro-tumourigenic role of NRPs, some reports suggest that NRP1 plays a more complex role in some tumour types. Thus, NRP1 over-expression in Panc-1 cells was found to reduce tumour volume and incidence [172], whereas the same group showed that FG pancreatic carcinoma cells expressing NRP1 have increased resistance to anoikis and cytotoxic drugs [173]. Furthermore, some findings suggest that not all effects of VEGF in

tumour cells may be dependent on NRP1. Lee *et al* found that survival effects of VEGF in the breast carcinoma MDA-MB-231 and MCF-7 cell lines were mediated by internally expressed VEGFR1/Flt1, and were unaffected by NRP1 knockdown [174].

There is also some evidence pointing to potentially differential or antagonistic roles of NRP1 and NRP2 in tumour cell regulation. Sema3F, the best-characterised ligand for NRP2, induces a poorly vascularised non-metastatic phenotype in melanoma xenografts in mice [133] and, suggestively, Sema3F and a second NRP2 ligand, Sema3B, are both localised to the 3p21.3 chromosomal region, which is commonly deleted in human lung cancers [175,176]. These findings suggest that Sema ligands for NRP2 are potential tumour suppressors. Confirmation of such an anti-tumourigenic role of NRP2 awaits further work.

Recent studies provide direct evidence that NRP1 contributes to tumour cell growth and tumour neovascularisation *in vivo*. A peptide that inhibits VEGF binding to NRP1 has been reported to inhibit angiogenesis and growth of tumour xenografts [177]. An antibody targeted to the b1 domain that specifically blocks VEGF-A binding to NRP1 causes a range of effects in endothelial cell cultures including inhibition of VEGFR2 complex formation, VEGF-induced migration, and vascular sprouting, reduces angiogenesis in a neonatal retinal neovascularisation model, and inhibits tumour growth and tumour vascularisation in mouse xenograft models. Blocking NRP1 antibody alone had a relatively small effect on tumour growth, but produced a strong additive effect when used in combination with the blocking VEGF antibody, bevacizumab or Avastin [94], now approved for clinical use in patients with late stage colorectal carcinoma. Interestingly, Pan *et al* reported that the anti-tumour activity of blocking NRP1 antibodies was not dependent on NRP1 expression in the tumour cell line used in the xenograft model. Furthermore, this study also found no evidence of direct effects of NRP1 antibodies on tumour cell proliferation, suggesting that their anti-tumourigenic effects were mainly due to inhibition or destabilisation of the tumour vasculature. High expression of NRP1 is reported to correlate with poor overall survival in patients with Medulloblastoma, the commonest pediatric malignant brain tumour [178]. Furthermore, inhibition of either NRP1 and the NRP1 ligand PlGF in mice results in medulloblastoma regression, decreased metastasis, and increased overall survival. Interestingly this study reports that PlGF derived from tumour stromal cells acts through NRP1 and independently of VEGFR1 to promote tumor cell survival.

Another approach to targeting NRP1 therapeutically in cancer is to use small molecule antagonists of the ligand interaction with the NRP1 extracellular domain. A peptidomimetic of the exon 8-encoded region of $VEGF_{165}$, EG00229, was designed on the basis of structure activity relationship analysis of the bicyclic peptide, EG3287, corresponding to the C-terminal 28 $VEGF_{165}$ amino acid residues. EG3287 and EG00229 inhibited VEGF-induced VEGFR2 phosphorylation and endothelial cell migration and enhanced the in vitro cytotoxic effect of paclitaxel and 5-fluorouracil in cancer cells [16,151]. Small molecule inhibitors of NRP1 have potential as novel anti-cancer therapeutics.

The concept that the role of NRP1 in tumour growth is exclusively or even largely confined to its contribution to tumour vascularisation or to VEGF signalling in endothelial cells is being challenged by recent work which provides new insights into the role of NRP1 in cancer. One such development highlights the role of NRP1 in the cancer stem cell niche. Human CD133+ glioma stem-like cells (GSC) express VEGFR2 and NRP1 and together these receptors mediate the autocrine survival and growth of these cells dependent on GSC-derived VEGF-A [179]. It has been proposed that such self-renewal of GSC may explain to modest effects of anti-VEGF therapy (bevacizumab) in clinical studies of Glioblastoma multiforme and to resistance to

bevacizumab after an initial response. A VEGF-VEGFR2-NRP1 signalling axis has also been implicated in generating a perivascular niche for proliferation of cancer stem cells in squamous cell skin tumours [180]. NRP1 was expressed in cancer stem cells from skin papillomas induced by chemical carcinogenesis in mice, and mice with a conditional NRP1 deletion specifically in the epidermis did not develop skin papillomas induced by 25 weeks of phorbol ester treatment compared with skin papillomas in 100% of control mice. In this model VEGF plays a dual role, stimulating angiogenesis via VEGFR2 expressed on endothelial cells, and promoting CSC proliferation via NRP1 expressed on CSC.

Another challenge to the paradigm of NRP1 acting in cancer primarily as a mediator of VEGF-driven tumour vascularisation has come from studies of regulatory T cells (T regs), a subset of the T cell repertoire that plays an important role in negatively regulating the anti-tumour immune response. Several studies have found that NRP1 is highly expressed on T regs positive for Foxp3 and CD4 in mice [181,182]. Furthermore, mice with a T cell-specific deletion of NRP1 display impaired melanoma growth accompanied by a marked reduction in tumour-infiltrating Foxp3+ T regs and a concomitant augmentation in activity of effector CD8+ T cells [183]. This study also reported that tumour-derived VEGF together with NRP1 expressed in T-cells are important for chemotactic recruitment of Foxp3+ T regs to tumours. These findings suggest a novel role for NRP1 in T regs in suppressing the anti-tumour immune response mediated effector T cells. While aspects of the mechanism underlying this role of NRP1 remain to be clarified, it appears that NRP1 does not directly mediate the suppressive effect of Foxp3+ T regs on effector T cells, but functions mainly in the recruitment of T regs to the tumour microenvironment. It is at present unclear whether NRP1 is involved in infiltration of T regs into human tumours, and this should be an important goal of future research in this area.

Immune system

NRP1 was found to be expressed in naïve T-cells and immature dendritic cells (antigen presenting cells), cell types that interact during the primary immune response in the secondary lymphoid organs [184], and are essential for triggering the proliferation and differentiation of mature T cells that will later interact again with antigen presenting cells to mediate antigen elimination [12]. NRP1 expressed on naïve T cells also mediated their clustering with NRP1-expressing Cos-7 cells, and the stimulation of resting T cell proliferation by dendritic cells was reduced by around 50% by blocking NRP1 antibodies [184]. T cell activation was also reduced by Sema3A [185,186]. NRP1 distribution in T cells was polarised, and NRP1 co-localised with the T cell marker, CD3, at the interface between dendritic cells and naïve T-cells [184]. While the biological relevance of these findings for the immune response *in vivo* is unclear, they are consistent with the formation of homophilic NRP1 interactions between dendritic cells and immature T cells, which contribute to an early step in formation of the immunological synapse, essential for T cell maturation [184]. As mentioned above, among T cell lineages, at least in mice, NRP1 is predominantly expressed in Foxp3+ CD4+ T regs, and it has been shown that NRP1 increases the longevity of interactions between T regs and immature dendritic cells, enhancing antigen sensitivity [182]. It has been proposed that in the absence of inflammatory stimuli, this may confer an advantage of T regs over naïve helper T cells, resulting in enhanced suppression of the immune response. Recent findings show that NRP1 is expressed in mice at high levels on natural (n) T reg cells that arise in the Thymus, but is present at a low level in

induced or adaptive T regs (iTregs) generated in peripheral tissues through induction of Foxp3 [187,188]. However, Weiss *et al* reported that in some highly inflammatory settings, such as the spinal cords of mice with spontaneous autoimmune encephalomyelitis (EAE) and the lungs of mice with chronic asthma, iT regs can express NRP1 [188]. NRPs 1 and 2 have also been detected on human basophils, $VEGF_{165}$ stimulated basophil chemotaxis [189].

Wound healing

There is evidence that NRP1 is upregulated in response to tissue injury and may be involved in regeneration and repair [10]. In *Xenopus* when the optic nerve was crushed and allowed to regenerate, the level of NRP1 increased and remained elevated for weeks before finally declining after healing [3]. NRP1 was also found to be strongly expressed in the neovasculature during wound angiogenesis in a murine model of dermal wound healing, while blocking anti-NRP1 antibody reduced wound vascularisation [190]. Optic nerve injury in a rat model resulted in cell invasion at the site of injury by microglia, oligodendrocytes, and astrocytes associated with induction of semaphorin 3A and NRP1 [191]. NRP1 may protect neuronal cells against damage resulting from stress or injury. Thus, IFNγ activation of microglia, a cell type that becomes activated following neuronal injury, caused upregulation of NRP1 and plexin A1 and was associated with induction of microglial apoptosis by Sema3A, suggesting that a NRP1-mediated pathway may protect neurons against damage caused by activated microglia [192]. Expression of NRPs, VEGFs and Class III semaphorins are also upregulated by cerebral ischemic injury in different animal models [193-201]. For example, in a mouse model of cerebral ischemia, NRP1 mRNA expression increased rapidly and remained elevated for at least a month after the ischaemic event [193]. Interestingly, NRP1 was not only localised to the ischemic neurons but also to the endothelial cells of the brain vessels. These findings are suggestive of a dual role for NRP1 in the response to cerebral injury, in the promotion of both neuronal growth and cerebral angiogenesis.

Liver Cirrhosis

NRP1 upregulation was observed in activated Hepatic stellate cells (HSCs) in two rat models of liver fibrosis, and was detected in human biopsies of liver cirrhosis caused by both hepatitis C and steatohepatitis [50]. Analysis of PDGF/PDGFRβ– and TGFβ-mediated signalling in HSC showed that NRP1 knockdown inhibited PDGF-BB-induced chemotaxis, and enhanced Rac1 activity via an association with the c-Abl tyrosine kinase, while NRP-1 overexpression increased cell migration and TGF-beta-dependent collagen production [50]. The role of NRP1 in PDGFRβ-mediated chemotaxis appears to result from enhanced receptor affinity for ^{125}I-PDGF-BB . Furthermore blockade of NRP1 using a neutralizing antibody reduced recruitment of HSCs, and decreased liver fibrosis in a rat model of liver injury, suggesting a role for NRP-1 as a modulator of pathophysiological liver fibrosis [50].

Other functions

NRP1 expression has been reported in other cell types, including bone marrow-derived progenitor cells [202], platelets [203], and the granulosa and theca cells in the follicles of the bovine ovary [204], though the role of NRP1 in these systems has not yet been defined. A novel role for NRP1 has been identified in mediating cell entry of the Human T-cell lymphotropic virus type 1 (HTLV-1), apparently through viral mimicry of $VEGF_{165}$ [205].

Conclusions and Perspectives

Neuropilins are receptors for Class III Semaphorins and for members of the VEGF family, with essential roles in neuronal patterning and cardiovascular development, respectively. Much evidence points to a key function of NRP1 in the migratory guidance of both axons and endothelial cells, a role that is presumably controlled by gradients of extracellular ligands and the co-expression of signalling receptors, plexins in neuronal cells and VEGFRs in endothelial cells. Though complex formation between NRPs and VEGFR2 or plexins seem to be key to understanding the mechanism of action of NRPs, in endothelial cells and neurons respectively, many other aspects of the molecular basis for NRP regulation of intracellular signalling or cell guidance remain enigmatic. Associations between the NRP carboxy-terminus and the PDZ domain protein, synectin, appears to offer a clue to the mechanism underlying the role of NRP1 in arteriolar morphogenesis, but does not explain the full complexity of NRP1 functions in development revealed by phenotypes of NRP1 deficient mice. Further work delineating the role of NRP protein-protein associations, and the identification of novel protein interactions with the NRP intracellular domain is likely to reveal novel insights into the mechanisms mediating NRP functions.

The different expression patterns and phenotypes of the respective mutant mice indicate that they play distinct roles in the development of both the neuronal and vascular systems, though they also have the ability to interact. While NRP2 is important for guidance of some classes of neuronal axons, the cellular function of NRP2 is less well studied than for NRP1, and it is unclear what its primary functions are in endothelial, lymphatic or cancer cells. In cells co-expressing NRP1 and NRP2, how, if at all, do they interact and what is the impact on VEGFR2 and plexin signalling? Does NRP2 play a modulatory or inhibitory role in NRP1 functions? Furthermore, while the most widely accepted model for NRP function is that NRPs are ligand-binding but non-signalling co-receptors, unable to function independently of their partner receptors in neuronal and endothelial cells, evidence from studies in endothelial, cancer and T-cells, does not readily fit this mechanistic paradigm, and suggests additional modes of NRP1 function. NRP1 is expressed in cell types, such as immature T-cells and VSMC, which are not typical targets for either semaphorins or VEGFs, and many cancer cells may express NRP1 in the absence of co-expression of either VEGFRs or plexins. There is evidence that NRP1 may participate in homophilic adhesive interactions between cells, in, for example, the formation of early contacts – the so-called 'immune synapse' – between naïve T-lymphocytes and immature dendritic cells. The details of these cell-cell interactions, and the precise role NRP1 plays

remain to be elucidated, but they are suggestive that NRP1 plays roles independent of its known complement of ligands and receptor partners, particularly in adhesion.

In addition to their major biological roles in neurogenesis and cardiovascular development, recent evidence has identified novel functions for NRPs in other physiological processes such as T-cell maturation, and in disease. A major recent development has been the emergence of a role for NRPs in cancer. NRPs are expressed on many tumour cells and they appear to be important in several aspects of tumour growth and spread, including cell migration and metastasis, interactions between neoplastic cells and the tumour microenvironment, T cell regulation of anti-tumour immunity and generation of cancer stem cells. Since the role of NRP1 in cancer appears to be distinct from that of VEGF, targeting NRP1 may complement and potentiate the anti-tumour effects of therapies targeted at VEGF, such as bevacizumab. These findings indicate that therapeutic targeting of NRPs may offer a novel approach to the inhibition of tumour growth and metastasis.

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Declaration of competing interests

No compteting interests.

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Table 1. Phenotypes of Neuropilin and Synectin mutant mice.

Key, $NRP1^{Endo-/-}$, endothelial-specific NRP1 knockout; $NRP1^{tg}$, mice globally overexpressing NRP1; $NRP1^{Sema3A-/-}$, mice with a knock-in of a CUB domain deletion unable to bind Sema3A, $NRP1^{Cyto}$, mice with a knock-in of NRP1 lacking the cytoplasmic domain.

Figure Legends

Figure 1. Neuropilin gene organisation and protein structure.

The NRP1 gene comprises 17 exons (black rectangles), encoding a full-length protein of 923 amino acids (aa) consisting of an extracellular region with two tandem CUB (a1 and a2) domains (complement binding factors C1s/C1r, Uegf, Bone morphogenetic protein 1 BMP1), two tandem FV/VIII homology (b1 and b2) domains , a linker region and one MAM (c) domain (meprin, A5 antigen, receptor tyrosine phosphatase μ,); a single transmembrane domain (TM); and a 44 aa cytoplasmic (Cyt) domain containing a C-terminal PDZ (post synaptic density protein (PSD95), Drosophila disc large tumor suppressor (DlgA), and zonula occuldens-1 protein) binding domain motif, with the sequence, SerGluAla (SEA). Locations of binding sites for semaphorins and VEGF, and the putative oligomerisation domain are indicated. Arrowheads indicate: the positions of Ser 612 in NRP1, the major site of O-linked chondroitin sulphate glycosaminoglycan (CS-GAG) modification; insertion at residue 808 in NRP2 of five amino acids GlyGluAspPheLys (GENFK); and addition of 9 amino acids ValGlyCysSerTrpArgLeuProLeu (VGCSWRLPL) following truncation of the b2 domain at residue 548 in soluble NRP2 (sNRP2). Soluble NRP1 (sNRP1) is truncated at the linker region between the second FV/VIII and the MAM domain and ends with the 3 amino acids GlyIsoLys (GIK). Note that not all sNRP1 isoforms contain Ser 612, and are not all modifiable by CS-GAG addition. Percentage amino acid homologies between the domains of full length NRP1 and NRP2a isoforms (a1/a2, b1/b2, MAM, and intracellular domains, respectively, left to right) and between the NRP2a and NRP2b isoforms (extracellular region, and transmembrane domain plus intracellular domain, respectively) are indicated.

Figure 2. Neuropilins, their ligands and co-receptors.

A. Class 3 Semaphorins bind NRP1 and NRP2 with differing, and partially overlapping specificities and affinities. Plexins A1 and A2 are the major signalling receptors for class 3 Semaphorins, and complex with NRPs and Semaphorins to form a holoreceptor essential for mediating effects of Semaphorins on growth cone collapse and axonal guidance in neurogenesis. The plexin cytoplasmic domain contains two blocks of homology shared with GTPase activating proteins. Key: IPT, immunoglobulin-like domains shared by plexins and transcription factors; MRS, Met-related sequences

B. Specific isoforms of several members of the VEGF family bind with high affinity to NRP1 and NRP2, with distinct specificities. VEGF specificities for their receptor tyrosine kinases are also shown. The major VEGF-A signalling receptor, VEGF receptor 2, (the human homologue called kinase insert domain-containing receptor, the mouse homologue, Flk-1; VEGFR2/KDR/Flk-1), forms complexes with NRP1 and is important for optimal VEGFR2 signalling and function in endothelial cells. The VEGF-A_{121} isoform can bind to NRP1 but is unable to promote NRP1/VEGFR2 complexation. The role of NRP1 in VEGF-A signalling is likely to be important for mediating the essential functions of NRP1 in cardiovascular development revealed by mutant mouse studies. NRP1 has also been reported to associate with VEGFR1/Flt1, and NRP2 can complex with the receptor for VEGF-C and VEGF-D, VEGF

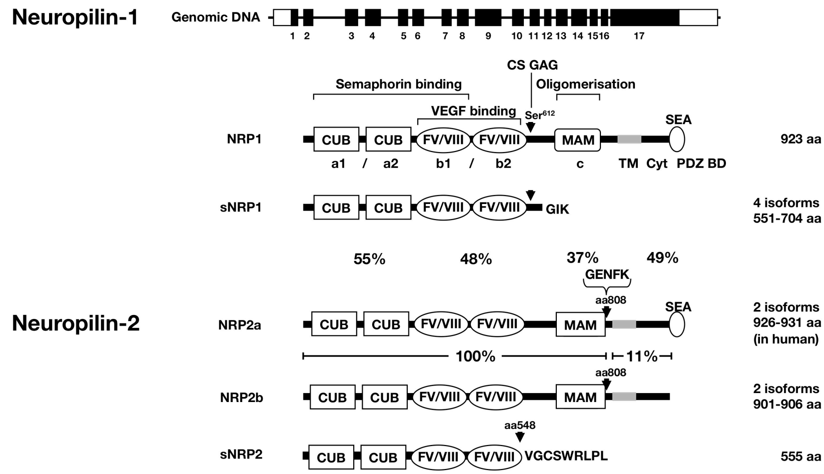
receptor 3, also called fms-related tyrosine kinase receptor 4 (VEGFR3/Flt-4). See text and legend to Fig.1 for key to abbreviations and further details. Key: Ig-like, immunoglobulin-like.

Figure 3. VEGF signalling via NRP1 .

A: VEGF (VEGF-A) is able to signal via VEGFR2 in the absence of NRP1. VEGF binding to VEGFR2 stimulates signalling via phospholipase C-gamma (PLC-γ), leading to activation of Protein kinase C (PKC) and extracellular signal-regulated protein kinases 1 and 2 (ERK1/2), and phosphoinositide 3-kinase (PI3K), mediating activation of the serine-threonine kinase, Akt/PKB (product of the oncogene encoded by the lymphomagenic murine retrovirus, AKT8, and activation of Focal adhesion kinase (FAK) via increased phosphorylation of Y397.

B: VEGF-A_{165} binds to NRP1 and promotes complex formation. The NRP1 carboxy-terminal PDZ domain binding motif associates with the PDZ protein synectin and this is thought to be important for the role of NRP1 in VEGF-A signalling and function mediated via VEGFR2,. NRP1/VEGFR2 complex formation is implicated in signalling via p38 kinase, ERKs1/2, and a Pyk2 pathway leading to p130Cas tyrosine phosphorylation, via mechanism(s) that are still unclear. NRP1-dependent VEGF signalling is particularly important for directed cell migration.

Fig. 1



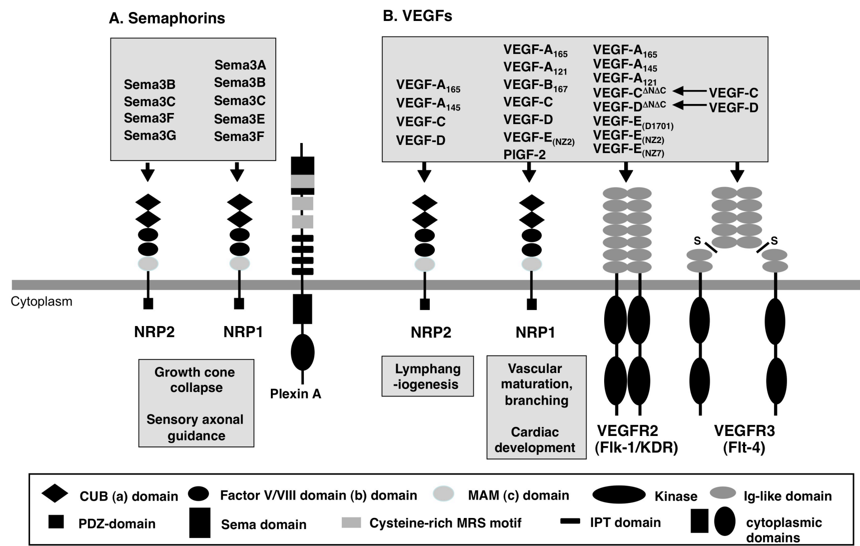


Fig. 2
A. Semaphorins
Sema3B
Sema3C
Sema3F
Sema3G
Sema3A
Sema3B
Sema3C
Sema3E
Sema3F
B. VEGFs
VEGF-A165
VEGF-A145
VEGF-C
VEGF-D
VEGF-A165
VEGF-A121
VEGF-B167
VEGF-C
VEGF-D
VEGF-E(NZ2)
PlGF-2
VEGF-A165
VEGF-A145
VEGF-A121
VEGF-CΔNΔC
VEGF-DΔNΔC
VEGF-E(D1701)
VEGF-E(NZ2)
VEGF-E(NZ7)
VEGF-C
VEGF-D
Cytoplasm
NRP2
NRP1
Plexin A
NRP2
NRP1
VEGFR2
(Flk-1/KDR)
VEGFR3
(Flt-4)
S
S
Growth cone collapse
Sensory axonal guidance
Lymphang -iogenesis
Vascular maturation, branching
Cardiac development
CUB (a) domain
Factor V/VIII domain (b) domain
MAM (c) domain
Kinase
Ig-like domain
PDZ-domain
Sema domain
Cysteine-rich MRS motif
IPT domain
cytoplasmic domains

Fig. 3

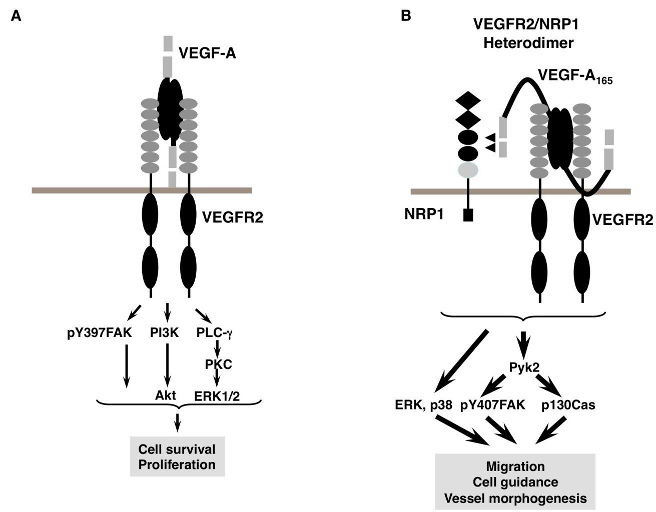


Table 1. Phenotypes of Neuropilin and Synectin Mutant Mice

Genotype	Phenotype	References
NRP1$^{-/-}$	Die between E10 and E13.5 with extensive cardiovascular and neuronal defects. Embryos have impaired endothelial tip cell guidance in the developing CNS. Small and large vessels of the yolk sac are disorganised and its capillary network is sparse. Mice show an agenesis of the brachial arch, related great vessels and dorsal aorta.	Kitsukawa *et al.,* 1997 [5] Gerhardt *et al.,* 2004 [90] Kawasaki *et al.,* 1999 [6]
NRP1$^{Endo-/-}$	Mid to late embryonic lethality, abnormal poorly-branched vasculature, multiple cardiac defects including *truncus arteriosus* and failure of septation of cardiac outflow tract.	Gu *et al.,* 2003 [4]
NRP2$^{-/-}$	Embryos survive to adulthood, but display abnormal guidance and fasciculation of some cranial nerves and fewer small lymphatic vessels and capillaries.	Giger *et al.,* 2000 [65] Chen *et al.,* 2000 [64] Yuan *et al.,* 2002 [63]
NRP1 $^{-/-}$/NRP2 $^{-/-}$	Earlier embryonic mortality (E8) than NRP1 knockout with a phenotype resembling that of VEGF-A_{165} and VEGFR2 (KDR) knockouts.	Takashima *et al.,*2002[66]
NRP1^{tg}	Embryonic lethality with excess capillary growth, haemorrhage in the head and neck. Embryos have a malformed heart with anarchic sprouting and defasciculation of nerve fibres.	Kitsukawa *et al.,* 2000 [62]
NRP1 $^{Sema3A-/-}$	Survive until birth but most die perinatally by P7. Aberrant guidance of sensory afferent nerves to synaptic targets in CNS, defasciculation of spinal and cranial nerves. No obvious cardiovascular defects.	Gu *et al.,* 2003 [4]
NRP1$^{Sema3A-/-}$/NRP2$^{-/-}$	Cardiac defects with persistence of the *truncus arteriosus*, failure of septation of the cardiac outflow tract and atrial enlargement. Similar to NRP1$^{Endo-/-}$ mice.	Gu *et al.,* 2003 [4]
NRP1^{Cyto}	Viable, fertile, macroscopically normal. Increased retinal arteriovenous crossing. Reduction in arteries and arterioles in heart, kidney, skeletal muscle; small reduction in body size.	Fantin et al 2011 []; Lanahan *et al.,* 2013 [4]
Synectin$^{-/-}$	Viable and fertile; 30% smaller with fewer small arteries.	Chittenden *et al.,*2006 [127]