Selective requirements for NRP1 ligands during neurovascular patterning

Joaquim Miguel Vieira, Quenten Schwarz and Christiana Ruhrberg*

Blood vessels and neurons share several types of guidance cues and cell surface receptors to control their behaviour during embryogenesis. The transmembrane protein NRP1 is present on blood vessels and nerves. NRP1 binds two structurally diverse ligands, the semaphorin SEMA3A and the VEGF164 isoform of vascular endothelial growth factor. SEMA3A was originally identified as a repulsive cue for developing axons that acts by signalling through receptor complexes containing NRP1 and plexins. In vitro, SEMA3A also inhibits integrin function and competes with VEGF164 for binding to NRP1 to modulate the migration of endothelial cells. These observations resulted in a widely accepted model of vascular patterning in which the balance of VEGF164 and SEMA3A determines endothelial cell behaviour. However, we now demonstrate that SEMA3A is not required for angiogenesis in the mouse, which instead is controlled by VEGF164. We find that SEMA3A, but not VEGF164, is required for axon patterning of limb nerves, even though the competition between VEGF164 and SEMA3A for NRP1 affects the migration of neuronal progenitor cells in vitro and has been hypothesised to control axon guidance. Moreover, we show that there is no genetic interaction between SEMA3A and VEGF164 during vasculogenesis, angiogenesis or limb axon patterning, suggesting that ligand competition for NRP1 binding cannot explain neurovascular congruence, as previously suggested. We conclude that NRP1 contributes to both neuronal and vascular patterning by preferentially relaying SEMA3A signals in peripheral axons and VEGF164 signals in blood vessels.

KEY WORDS: VEGF, Neuropilin, Semaphorin, Mouse

INTRODUCTION

During embryonic development of all vertebrate species, complex networks of blood vessels and nerves are established to meet metabolic demand and relay information, and these networks communicate to meet physiological demands. Strikingly, the branching of blood vessels throughout the body bears similarities to the branching of axons, and it is therefore not surprising that developing vessels and nerves employ similar cell biological mechanisms to invade and navigate within tissues. For instance, endothelial tip cells and axon growth cones send out filopodia to explore their territory for guidance cues (Gerhardt et al., 2003; Ruhrberg et al., 2002). Many recent studies have explored the idea that endothelial cells and neurons share signalling pathways to control their growth and behaviour. Accordingly, there is now evidence that axon guidance cues control vessel branching and, vice versa, that vascular patterning molecules modify the migration of neurons (reviewed by Eichmann et al., 2005). The cell surface receptor neuropilin 1 (NRP1) has received particular attention because it is expressed both by endothelial cells and many different types of neurons and is essential for neuronal and vascular patterning. NRP1 binds two types of structurally distinct ligands: (1) members of the class 3 semaphorin family and (2) an isoform of vascular endothelial growth factor (VEGF, also known as VEGFA) termed VEGF164 in mice (reviewed by Eichmann et al., 2005). Several distinct NRP1 domains cooperate to mediate ligand binding: the a1 domain in particular is crucial for binding the SEMA domain present in all class 3 semaphorins, and the b1 domain binds VEGF164 (Fig. 1A) (Mamluk et al., 2002; Lee et al., 2003; Gu et al., 2002).

Institute of Ophthalmology, University College London, 11-43 Bath Street, London EC1V 9EL, UK.

*Author for correspondence (e-mail: c.ruhrberg@ucl.ac.uk)

VEGF164 acts in concert with two other major VEGF isoforms termed VEGF120 and VEGF188 (VEGF121 and VEGF189 in humans) to promote vascular development by signalling through several different VEGF receptors (reviewed by Ruhrberg, 2003). VEGF and its main vascular receptor, the transmembrane tyrosine kinase KDR (also known as FLK1 and VEGFR2), are essential for the earliest stages of vascular development in order to promote the differentiation of mesodermal precursors into endothelial cells and their condensation into vessel networks in a process termed vasculogenesis. Importantly, each one of the three major VEGF isoforms is able to direct this process and is therefore able to support embryonic development (Carmeliet et al., 1999; Ruhrberg et al., 2002; Stalmans et al., 2002). This is most likely to be due to the fact that each isoform can bind KDR to control endothelial cell differentiation, proliferation and migration (reviewed by Ruhrberg, 2003).

By contrast, the VEGF isoforms play different roles during later stages of vascular development. Based on a differential affinity for heparan sulphate proteoglycans in the extracellular matrix, the isoforms cooperate to establish chemoattractive gradients around VEGF-secreting cells and attract sprouts from pre-existing blood vessels in a process termed angiogenesis (Park et al., 1993; Ruhrberg et al., 2002). In this fashion, VEGF isoform expression supports the formation of microvessel networks with optimal density and connectivity. In addition, VEGF164 signalling through NRP1 on endothelial cells is thought to contribute to vascular growth by potentiating KDR signalling (Soker et al., 1998). Consistent with this idea, endothelial cells express NRP1 (Kitsukawa et al., 1995), and loss of NRP1 specifically from endothelial cells impairs brain vascularisation (Gu et al., 2003).

Although NRP1 is essential for vascular development, it was first identified as an adhesion protein in the central nervous system (CNS) and then as a receptor for class 3 semaphorins, a family of secreted guidance cues for embryonic nerves (Raper, 2000). Loss of the archetypical class 3 semaphorin SEMA3A causes axon
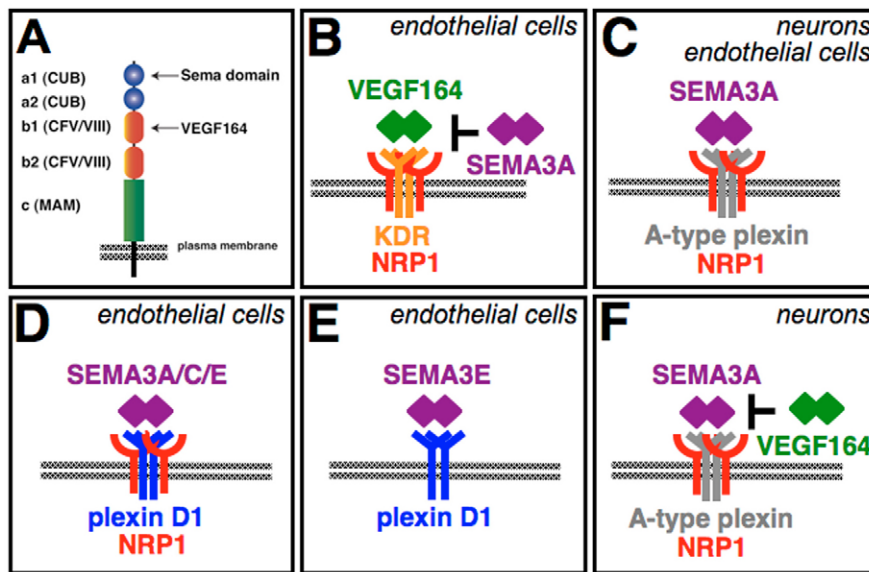
Fig. 1. Working models for the role of class 3 semaphorins in mouse vascular development. (**A**) NRP1 contains several distinct structural domains that cooperate to mediate binding of class 3 semaphorins and VEGF164; the a1 domain is crucial for binding the SEMA domain, the b1 domain for VEGF164 binding. (**B**) Based on tissue culture models, it has been suggested that SEMA3A modulates VEGF signalling by competing with VEGF164 for binding to NRP1. (**C**) SEMA3A may signal directly through complexes containing NRP1 and A-type plexins in endothelial cells, as observed in neurons. SEMA3E and possibly other class 3 semaphorins may influence vascular development by signalling through plexin D1-NRP1 complexes (**D**) and/or through plexin D1 in a mechanism that does not require NRP1 (**E**). (**F**) VEGF164 has been implicated as a modifier of neuronal growth and axon guidance based on its ability to compete with SEMA3A for NRP1 binding in a neuronal progenitor cell line in vitro.

defasciculation and excessive branching of several types of nerves in the developing mouse (Taniguchi et al., 1997). These defects are phenocopied in mice lacking NRP1 and in mice carrying a mutation in the a1 domain of NRP1 that is essential for binding to class 3 semaphorins (Gu et al., 2003; Kitsukawa et al., 1997). Together, semaphorins and neuropilins also play an essential role in heart development. Loss of semaphorin signalling through NRP1 or NRP2 does not compromise heart development; however, when the binding of semaphorins to NRP1 is abolished in a NRP2-null background, septation of the heart outflow tract into an arterial and venous compartment is impaired (Gu et al., 2003). Similar defects are found in mice lacking the neuropilin-ligand SEMA3C and have been attributed to impaired guidance of cardiac neural crest cells into the outflow tract (Feiner et al., 2001).

The observation that SEMA3A competes with VEGF164 for binding to NRP1, so as to inhibit the migration of cultured endothelial cells, made SEMA3A a candidate modifier of vascular development (Miao et al., 1999). Consistent with this idea, SEMA3A has been reported to affect vascular development in lower vertebrates. In zebrafish, one of two Sema3a forms, termed Sema3ab, curbs intersegmental vessel branching, and the implantation of SEMA3A-coated beads disrupts blood vessels in the developing quail limb (Bates et al., 2003; Torres-Vazquez et al., 2004). Serini and co-workers described defective head vessel remodelling, defective intersomitic vessel branching and disruption of anterior cardinal vein formation in mouse embryos lacking SEMA3A (Serini et al., 2003). Loss of SEMA3A was previously shown to cause hypertrophy of the right ventricle and dilation of the right atrium (Behar et al., 1996), but whether these defects occur downstream of impaired lung function (Ito et al., 2000) or impaired vascular growth is not known.

Several different models have been put forward to explain the role of class 3 semaphorins and NRP1 in vascular development (Fig. 1B-F). First, it has been suggested that SEMA3A modulates VEGF signalling by competing with VEGF164 for binding to NRP1 (Miao et al., 1999) (Fig. 1B). Second, SEMA3A might signal directly through complexes containing NRP1 and A-type plexins in endothelial cells, as in neurons (Fig. 1C) (Serini and Bussolino, 2004; Tamagnone and Giordano, 2006). Third, class 3 semaphorins may influence cardiovascular development by signalling through

plexin D1-neuropilin complexes rather than plexin A-neuropilin complexes (Fig. 1D) (Gitler et al., 2004; Torres-Vazquez et al., 2004); importantly, at least one class 3 semaphorin, SEMA3E, can signal through plexin D1 in a mechanism that does not require NRP1 (Gu et al., 2005) (Fig. 1E).

Not only has SEMA3A been implicated as a modifier of VEGF164-mediated endothelial cell growth, but the reverse situation has also been described for neuron culture models, in which the balance of VEGF164 and SEMA3A controls the migration and apoptosis of neuronal progenitor cells (Bagnard et al., 2001). Moreover, VEGF164 has been hypothesised to act as an axonal guidance cue by binding to NRP1 in competition with SEMA3A (Carmeliet, 2003) (Fig. 1F). However, evidence that VEGF acts as an axonal patterning factor in vivo is thus far lacking. By contrast, VEGF164 contributes to the control of neuronal migration, as it is essential for the cell body migration of facial branchiomotor neurons in the mouse hindbrain (Schwarz et al., 2004).

To address which of the above models best describes the role of NRP1-binding semaphorins during vascular development, and to examine whether VEGF164 cooperates with SEMA3A to control axon patterning, we have compared the genetic requirements for these NRP1 ligands during axonal and vascular patterning in the mouse. Unexpectedly, we found no evidence for a competitive relationship between SEMA3A and VEGF164 during endothelial or neuronal growth; rather, NRP1-based signalling pathways display a previously unappreciated context-dependent specificity for either semaphorins or VEGF.

MATERIALS AND METHODS
Animals
To obtain mouse embryos of defined gestational ages, mice were mated in the evening, and the morning of vaginal plug formation was counted as 0.5 days post-coitum (dpc). To stage-match embryos within a litter, or between litters from different matings, we compared somite numbers (9.5 and 10.5 dpc) or facial and limb development (12.5-14.5 dpc). C57Bl/6 mice carrying the *Sema3a*-null allele (Taniguchi et al., 1997) were backcrossed into the CD1 background (Charles River Laboratories, UK) for five or more generations. Heterozygous CD1 mice carrying the *Sema3a*-null allele were then bred to each other to obtain embryos lacking SEMA3A, and to CD1 mice lacking VEGF164 and other heparin/NRP1-binding VEGF isoforms

(*Vegfa*[120/120] *mice*) (Carmeliet et al., 1999; Ruhrberg et al., 2002). NRP1-null mice and mouse mutants deficient in semaphorin signalling through neuropilins (*Nrp1*[sema–/–] *Nrp2*^{–/–}) have been described previously (Gu et al., 2003; Kitsukawa et al., 1997).

For genotyping, genomic DNA from tissue biopsies was analysed by PCR using the Megamix Blue reaction mix (Helena BioSciences) and oligonucleotide primers specific for the gene-targeted loci.
Sema3a locus: 5′-CTGCAGACGCTGGAGGTCCCTGAG-3′, 5′-CAT-TGTCAGCGCGTCTAGTGAGTGTTGG-3′ and 5′-CTTGTAATGGT-TCTGATAGGTGAGGCATGG-3′;
Vegfa locus: 5′-CAGTCTATTGCCTCCTGACCTTCAGGGTG-3′, 5′-CTTGCGTCCACACCGTCACATTAAGTCAC-3′ and 5′-TTCAGAGCG-GAGAAAGCATTTGTTTGTCCA-3′;
Nrp1 locus: 5′-CGTGATATTGCTGAAGAGCTTGGC-3′, 5′-CAAT-GACACTGACCAGGCTTATCATC-3′ and 5′-GATTTTTATGGTCCCG-CCACATTTGTC-3′;
Nrp2 locus: 5′-CAGTGACAACGTCGAGCACAG-3′, 5′-TCAGGACA-CGAAGTGAGAAGC-3′ and 5′-GCTCAATGTAGCTAAGTGGAGGG-3′;
Nrp1[sema–/–] mutation: 5′-AGGCCAATCAAAGTCCTGAAAGACAGTC-CC-3′ and 5′-AAACCCCCTCAATTGATGTTAACACAGCCC-3′.

Alkaline phosphatase-fusion protein-binding assay

Expression vectors encoding secreted alkaline phosphatase (AP) or the fusion proteins AP-SEMA3A, AP-SEMA3C, AP-SEMA3E, AP-SEMA3F or AP-VEGF165 (kindly provided by Drs J. A. Epstein and J. A. Raper, School of Medicine, University of Pennsylvania, Philadelphia, PA) were transfected into HEK-293T cells using FuGENE6 (Roche). Serial dilutions of medium containing AP-fusion proteins were spotted onto nitrocellulose filters, and relative AP activity was determined by incubation with nitroblue tetrazolium (NBT) and 5-bromo-4-chloro-3-indolyl-phosphate (BCIP) (Roche) in a buffer containing 100 mM Tris pH 9.5, 100 mM NaCl, 5 mM MgCl$_2$. For whole-mount AP assays, freshly dissected 11.5 dpc hindbrain tissue was incubated in PBS containing 0.1% Triton X-100 (PBT) and 10% foetal bovine serum for 30 minutes prior to reaction with an appropriate dilution of conditioned medium containing AP-fusion protein for 2 hours at room temperature. Tissues were then washed three times for 20 minutes each with PBT, fixed with 4% formaldehyde for 1 hour at room temperature, and washed again. Endogenous AP was heat-inactivated at 65°C for 3 hours. Tissue-bound heat-stable recombinant AP activity was detected as an insoluble reaction product after incubation with NBT and BCIP as described above. Images were recorded using a MZ16 microscope (Leica) equipped with a ProgRes C14 digital camera (Jenoptiks, Jena, Germany) and OpenLab 3.5.1 software (Improvision).

Immunohistochemistry

Freshly dissected tissue was fixed for 2 hours in 4% formaldehyde, transferred to PBS, then 50% methanol in PBS, and then methanol for 5 minutes each and stored in methanol until use. For immunohistochemistry, samples were rehydrated and endogenous peroxidase activity quenched with 0.1% hydrogen peroxide in PBS for 30 minutes at room temperature. After washing twice in PBT, samples were incubated for 2 hours in blocking solution (PBT containing 10% normal rabbit or goat serum, depending on the secondary antibody used) and then overnight at 4°C in primary antibody diluted in blocking solution. Blood vessels were detected with rat anti-PECAM (also known as PECAM1 and CD31) (BD Pharmingen) or rat anti-endomucin antibodies (gift of Dr D. Vestweber, University of Muenster, Germany). The heart was visualised with mouse anti-smooth muscle actin antibody (Sigma), nerves with rabbit anti-neurofilament antibodies (Chemicon). Samples were washed five times for 1 hour each in PBT at room temperature and then incubated overnight at 4°C with secondary antibodies in blocking solution. Secondary antibodies used were horseradish peroxidase-conjugated rabbit anti-rat IgG or goat anti-rabbit IgG (Dako), and Alexa488-conjugated goat anti-rat IgG, Alexa543-conjugated goat anti-rabbit IgG or Alexa543-conjugated anti-mouse IgG (Molecular Probes). Samples were washed as described above and fixed for 30 minutes (4% formaldehyde in PBS) or developed with diaminobenzidine and hydrogen peroxide (SigmaFast; Sigma) and then fixed. Alexa-labelled samples were

mounted on glass slides using SlowFade (Molecular Probes) and analysed with a LSM510 laser scanning confocal microscope (Zeiss). Images from horseradish peroxidase-labelled samples were recorded using the MZ16 microscope as described above.

Quantitation of vessel and nerve density

To quantify vessel branching in the subventricular vessel plexus, 12.5 dpc PECAM-stained hindbrains of different genotypes were flat-mounted under glass coverslips and photographed at the same magnification. For each sample, the number of branchpoints was determined in three randomly chosen 0.25 mm^2 regions; error bars represent the standard deviation from the mean. For each mutant genotype, between five and 13 samples from at least three different matings were analysed, with the exception of the *Nrp1*[sema–/–] *Nrp2*^{–/–} double-mutant genotype (*n*=3). To quantify the branching of sensory nerves in the limb, we mounted neurofilament-stained limbs at 13.5 dpc under glass coverslips and counted the number of nerve branchpoints in the area corresponding to the future footplate.

RESULTS

Binding of VEGF164 and class 3 semaphorins to blood vessels and nerves

VEGF164, SEMA3A, SEMA3C, SEMA3E and SEMA3F bind neuropilins with different binding affinities in vitro (Raper, 2000; Soker et al., 1998). To compare the relative affinity of these neuropilin ligands for vessels and axons in a region of the developing embryo in which vessels and axons grow in spatiotemporal proximity, we reacted whole-mount hindbrain preparations with AP-tagged neuropilin ligands. This assay allowed us to monitor ligand binding to the subventricular vessel plexus (Fig. 2A-F) (see also Ruhrberg et al., 2002) and to newly formed axon tracts on the opposing brain surface (Fig. 2G-L) in the same specimen. We found that the NRP1-binding SEMA3A and SEMA3C targeted hindbrain vessels in vivo (Fig. 2B,C). However, binding of VEGF164 and SEMA3E to hindbrain vessels was more robust (compare Fig. 2A,D with B,C), presumably owing to the presence of additional receptors such as KDR and plexin D1, respectively (Gu et al., 2005). Even though binding to vessels was comparatively weak, SEMA3A associated strongly with axon tracts on the dorsolateral hindbrain surface (compare Fig. 2H with J). VEGF164 also bound to axon tracts (Fig. 2G). The NRP2-binding SEMA3F was able to bind to axons, but not to vessels in the developing hindbrain (compare Fig. 2E with K), even though it has inhibitory effects on endothelial cells in vitro and on blood vessels in tumour angiogenesis models (Kessler et al., 2004). Vice versa, SEMA3E binding appeared specific for vessels in the hindbrain at this stage (compare Fig. 2D with J), even though it binds to axon tracts in other brain regions (Steffensky et al., 2006).

SEMA3A is not required for the formation of the large axial vessels in the mouse

Because SEMA3A binds to developing vessels and has been implicated in endothelial cell migration, we asked whether it controls vascular development. We initially examined embryos lacking SEMA3A at 9.5 and 10.5 dpc, a time when the major axial vessels have formed and vascular networks have begun to extend throughout the body. Using two different markers for the developing vasculature, endomucin and PECAM (Albelda et al., 1991; Brachtendorf et al., 2001), we unexpectedly found that the vasculature of null mutants was indistinguishable from that of stage-matched wild-type littermates in both C57Bl/6 and CD1 backgrounds (Fig. 3 and data not shown). Specifically, vascular remodelling appeared normal in the head in 5/5 and 11/11 cases in the C57Bl/6 and CD1 backgrounds, respectively; moreover, a paired dorsal aorta and anterior cardinal vein was present in all cases (compare Fig. 3A with B, and G with H). In the trunk,
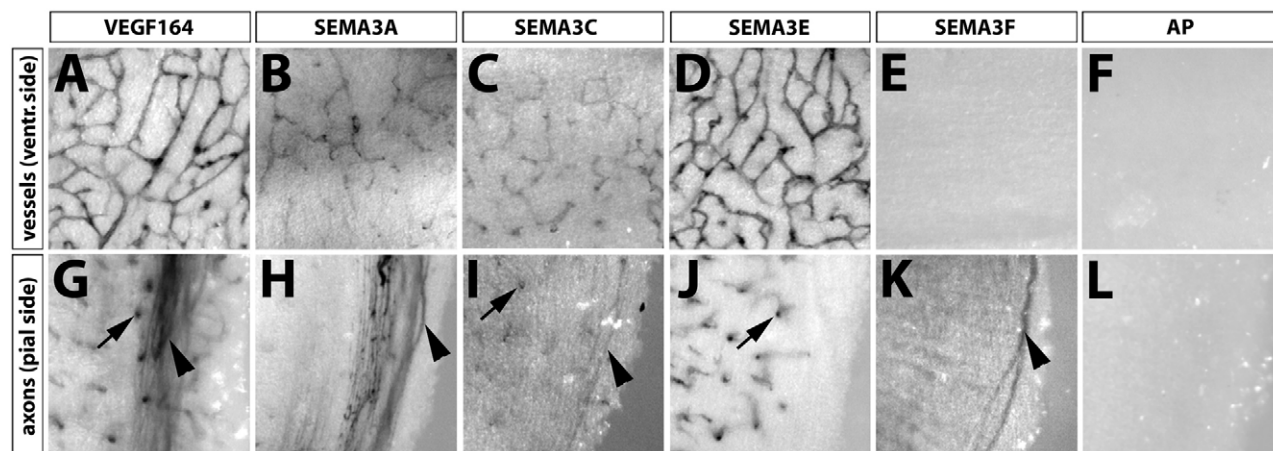
Fig. 2. Distinct binding specificity of different class 3 semaphorins and VEGF164 for blood vessels and nerves. AP-tagged VEGF164 (**A,G**), SEMA3A (**B,H**), SEMA3C (**C,I**), SEMA3E (**D,J**), SEMA3F (**E,K**) and AP alone as a negative control (**F,L**), were reacted with 11.25 dpc mouse hindbrain tissue to examine their binding preference for growing vessels in the subventricular zone (A-F) or axon tracts on the pial brain surface (G-L; arrowheads); binding to vessels entering the brain from the perineural vascular plexus is indicated with arrows. Each panel is 0.25 mm².

intersomitic vessels were present at 9.5 dpc in all cases examined (Fig. 3C,D), and these had branched laterally by 10.5 dpc in 6/6 CD1 mutants examined (Fig. 3E,F). At both developmental stages, the heart and pharyngeal arch arteries appeared grossly normal. Taken together, our results suggest that SEMA3A is not required for the formation of the major axial vessels, vessel branching or vessel remodelling in either the C57Bl/6 or CD1 background at 9.5 dpc. Our findings contrast with those of Serini and co-workers, who recently described defects in all these regions at 9.5 dpc in SEMA3A-null mutants in the CD1 (but not C57Bl/6) background (Serini et al., 2003). To confirm that our mutants had the correct genotype, we examined their nerve patterning in addition to undertaking the usual molecular genotyping. In agreement with previously published observations, we found that loss of SEMA3A caused axon defasciculation in both CD1 and C57Bl/6 backgrounds (compare Fig. 3I with J; see also limb analysis below).

SEMA3A is not required for microvessel branching in the mouse

We extended our analysis of vascular growth in SEMA3A mutants to the developing hindbrain because (1) SEMA3A is expressed in the developing hindbrain (Chilton and Guthrie, 2003; Schwarz et al., 2004); (2) SEMA3A is able to bind hindbrain vessels (Fig. 2); and (3) hindbrain vessels are dramatically affected in NRP1-null mutants (Gerhardt et al., 2004). Again, we found that vessel networks formed normally in the absence of SEMA3A: a normal number of vessels entered the hindbrain from the perivascular plexus (data not shown) and a normal number of branchpoints were present in the vessel plexus that formed in the subventricular zone in 5/5 and 13/13 mutants in the C57Bl6 and CD1 backgrounds, respectively (compare Fig. 4A,D with E,F). Because the branching pattern of microvessels is highly variable between individuals (compare the

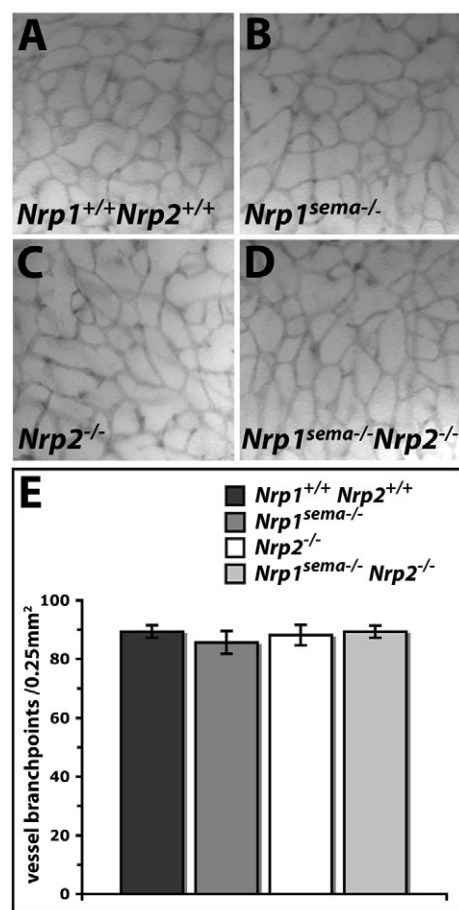**Fig. 3. SEMA3A is not required for vasculogenesis or angiogenesis.** Visualisation of the cardiovasculature in stage-matched littermate mouse embryos expressing (A,C,E,G) or lacking (B,D,F,H) SEMA3A in a CD1 background. (**A,B**) At 9.5 dpc, endomucin-positive vessel networks have extended throughout the embryo and have begun to remodel into the large head vessels. The anterior cardinal vein (arrowhead), dorsal aorta (arrow) and the third and fourth aortic arch arteries are also clearly visible. (**C-F**) Higher magnification of intersomitic vessels (isv) in the trunk region between the forelimb and hindlimb buds at 9.5 (C,D) and 10.5 (E,F) dpc. Vessel branches extend between intersomitic vessels at 10.5 dpc

(wavy arrows), but are rarely seen at 9.5 dpc in this region. (**G-J**) Double labelling of PECAM-positive vessels and neurofilament-positive nerves at 10.5 dpc reveals axon defasciculation of cranial nerves VII, IX and X (compare I and J), but no vessel defects (compare G and H) in embryos lacking SEMA3A. The heart ventricles (v), atria (a) and fourth and sixth aortic arch arteries are clearly visible in embryos lacking SEMA3A. Scale bar: 250 μm in C,D; 500 μm in A,B,E-J.
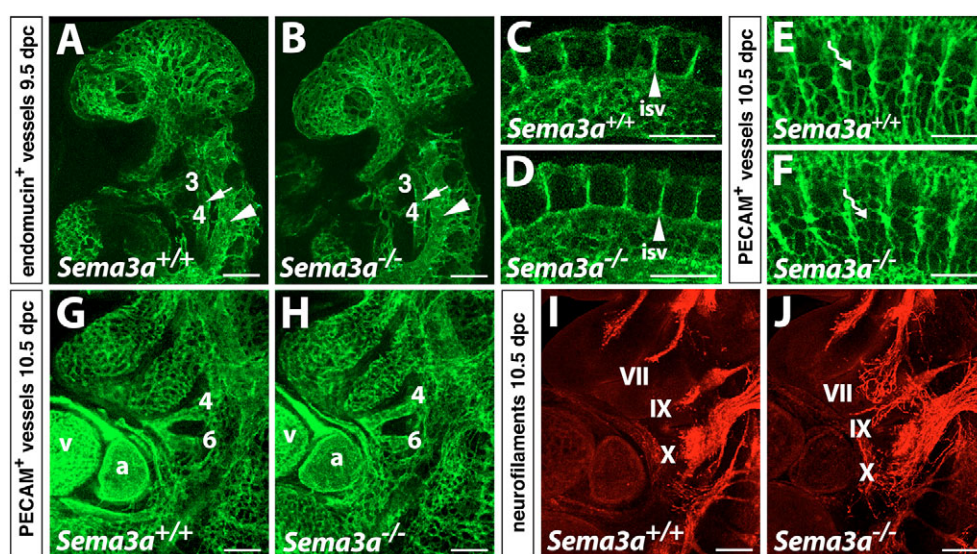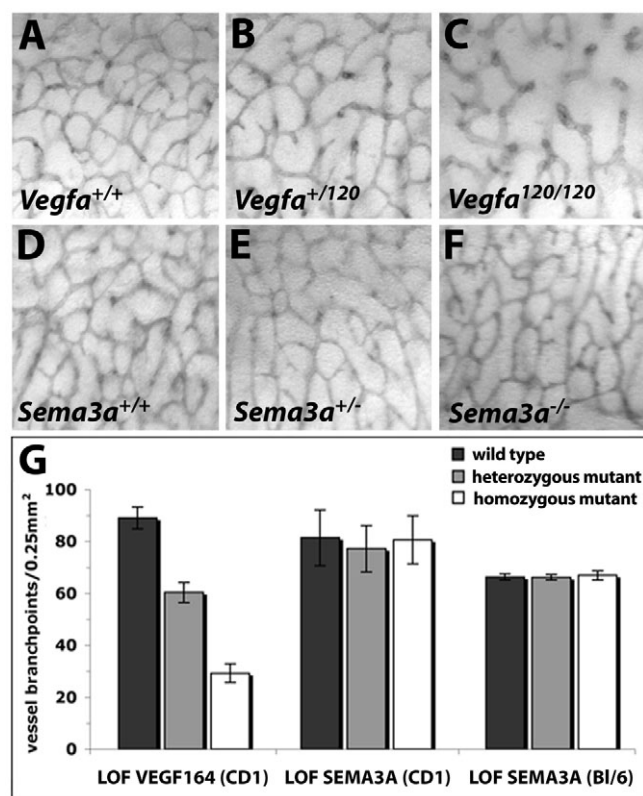
Fig. 4. Loss of heparin/neuropilin-binding VEGF isoforms, but not loss of SEMA3A, impairs brain vascularisation. Visualisation (**A-F**) and quantitation (**G**) of vessel branching in the subventricular zone of 12.5 dpc mouse hindbrains. PECAM-positive vessels are shown in a 0.25 mm^2 area of littermate hindbrains with normal (*Vegfa*$^{+/+}$) or reduced (*Vegfa*$^{+/120}$ and *Vegfa*$^{120/120}$) levels of heparin/neuropilin-binding VEGF isoforms (A-C), or in littermate hindbrains expressing (*Sema3a*$^{+/+}$) or lacking (*Sema3a*$^{+/-}$and *Sema3a*$^{-/-}$) SEMA3A (D-F). (G) Quantitation of vessel branching in hindbrains lacking heparin/neuropilin-binding VEGF isoforms or SEMA3A in a CD1 or C57Bl/6 background. Wild types (*Vegfa*$^{+/+}$ or *Sema3a*$^{+/+}$), dark grey; heterozygous mutants (*Vegfa*$^{+/120}$ or *Sema3a*$^{+/-}$), light grey; homozygous mutants (*Vegfa*$^{120/120}$ or *Sema3a*$^{-/-}$), white. LOF, loss of function.

Fig. 5. Loss of semaphorin signalling through neuropilins does not impair brain vascularisation. Visualisation (**A-D**) and quantitation (**E**) of vessel branching in 12.5 dpc mouse hindbrains. (E) Hindbrains with normal neuropilin function (A) showed a similar amount of vessel branching per 0.25 mm^2 as hindbrains lacking semaphorin signalling through NRP1 (B) or NRP2 (C) or lacking both NRP2 and semaphorin signalling through NRP1 (D).

wild-type patterns in Fig. 4A and D), but their density is conserved for any given tissue at a similar developmental stage, we compared microvessel density in wild-type hindbrains and hindbrains lacking SEMA3A by counting the number of vessel branchpoints per 0.25 mm^2 (Fig. 4G). Whereas the microvessel density was normal in hindbrains lacking SEMA3A, loss of VEGF164 affected microvessel density dramatically in both C57Bl/6 and CD1 backgrounds, as reported previously for outbred Swiss mice (Ruhrberg et al., 2002). Specifically, hindbrains lacking VEGF164 expression from one or both *Vegfa* alleles contained larger vessels with fewer branchpoints compared with wild-type littermates in 13/13 cases (compare Fig. 4A with B,C; see also Fig. 4G). This phenotype is caused by an altered distribution of VEGF in the extracellular matrix when heparin-binding isoforms such as VEGF164 are lost; in addition, VEGF164-signalling through NRP1 is essential for brain vascularisation (Gerhardt et al., 2004; Gu et al., 2003; Kawasaki et al., 1999). We therefore conclude that VEGF, but not SEMA3A, is crucial for microvessel branching in the mouse.

Semaphorin signalling through neuropilins is not required for microvessel branching in the mouse

We next considered whether the lack of a vascular phenotype in SEMA3A-null mutants was due to compensation by other NRP1-binding semaphorins with an ability to bind vessels, such as SEMA3C (see Fig. 2). We examined vessel branching in hindbrains carrying a mutation in the *Nrp1* gene that abolishes binding of the SEMA domain, which is essential for semaphorin signalling through NRP1 (Gu et al., 2003). We found that the loss of semaphorin signalling through NRP1 did not affect vessel branching in 11/11 mutants examined (compare Fig. 5A with B; Fig. 5E). We then considered the possibility that semaphorin signalling through NRP2 might compensate for loss of semaphorin signalling through NRP1 during vascular development, as in heart development (Gu et al., 2003). However, brain vessel branching proceeded normally in mutants lacking NRP2 (5/5 cases; Fig. 5C,E) and in the absence of semaphorin signalling through both NRP1 and NRP2 (3/3 cases; Fig. 5D,E).
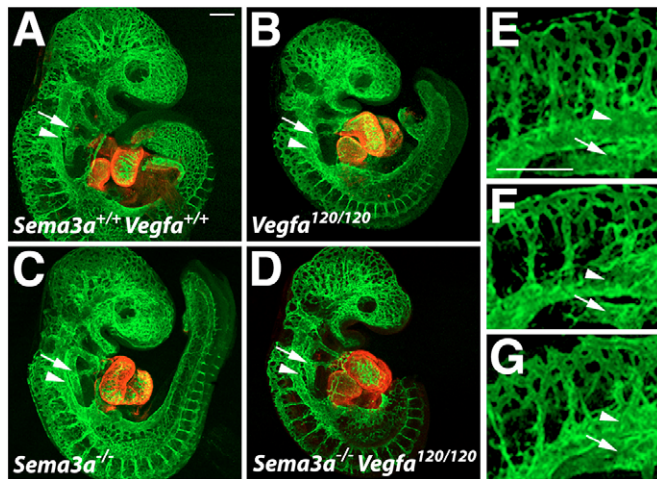
Fig. 6. SEMA3A does not affect vascular patterning in the absence of heparin/neuropilin-binding VEGF isoforms. (**A-D**) The cardiovasculature of 9.5 dpc littermate mouse embryos derived from matings between *Sema3a+/– Vegfa+/120* double-heterozygous mice was labelled with antibodies specific for endomucin (blood vessels, green) and smooth muscle actin (heart, red). The paired dorsal aorta and anterior cardinal vein, as well as the pharyngeal arch arteries and heart, were present in all four genotypes. (**E-G**) Higher magnification of the region containing the dorsal aorta (arrowhead) and anterior cardinal vein (arrow) shows the presence of both these large vessels in littermate wild-type (E), *Vegfa120/120* (F) and *Sema3a–/– Vegfa120/120* (G) embryos. Note that the strong smooth muscle actin staining of the heart necessitated the use of scanning parameters that did not record staining of the dorsal aorta, even though it was immunoreactive. Scale bars: 500 μm.

Similar results were also observed in mutant limbs lacking semaphorin signalling through NRP1 and NRP2, as they were defective in axonal, but not vascular patterning (data not shown). We therefore conclude that semaphorin signalling through neuropilins is not crucial for microvessel patterning in the mouse.

Sema3a and *Vegfa* do not interact genetically during vasculogenesis or angiogenesis in the mouse

It has been reported that VEGF stimulates angioblast migration in frogs and that Sema3aa guides zebrafish angioblasts to the sites where the paired dorsal aorta will form (Cleaver and Krieg, 1998; Shoji et al., 2003). These observations raise the possibility that VEGF and SEMA3A cooperate during the early phase of vessel assembly, with VEGF being an attractive cue for angioblast migration and SEMA3A providing repulsive cues to exclude migrating angioblasts from specific territories. On the other hand, SEMA3A and VEGF164 compete for binding to NRP1 on cultured endothelial cells, and through this competition, SEMA3A may inhibit VEGF164-induced migration (Miao et al., 1999). To address whether VEGF164 and SEMA3A cooperate during vasculogenesis or compete during angiogenesis in the mouse, we examined *Sema3a–/– Vegfa120/120* embryos, lacking both VEGF164 and SEMA3A. Such double-homozygous embryos were recovered from matings of double-heterozygous parents at a frequency predicted by the laws of mendelian inheritance (1/16; *n*=292 embryos from 9.5-14.5 dpc). When we examined litters from double-heterozygote parents, we found that the loss of SEMA3A neither increased nor ameliorated the severity of vascular defects of *Vegfa120/120* embryos
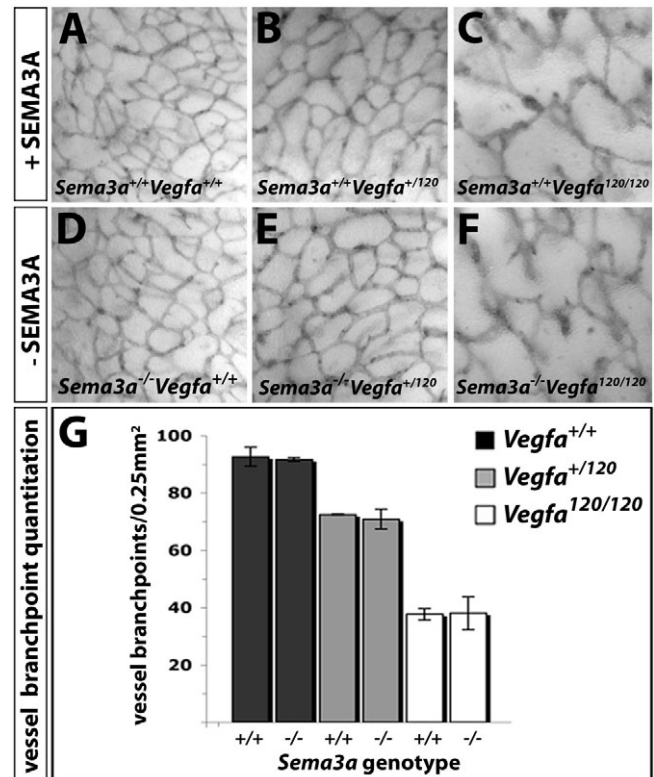

Fig. 7. Loss of SEMA3A does not rescue the vessel defects caused by the loss of heparin/neuropilin-binding VEGF isoforms. Visualisation (**A-F**) and quantitation (**G**) of vessel branching in 12.5 dpc mouse hindbrains derived from *Sema3a+/– Vegfa+/120* matings. (A-F) PECAM-positive vessels in 0.25 mm² areas of hindbrains expressing (*Vegfa+/+*) or lacking (*Vegfa+/120* and *Vegfa120/120*) heparin/neuropilin-binding VEGF differed with respect to the presence (A-C) or absence (D-F) of SEMA3A. (G) Data from hindbrains expressing (*Sema3a+/+*) or lacking SEMA3A (*Sema3a–/–*) were grouped according to their level of VEGF isoform expression. Normal 12.5 dpc hindbrains express more VEGF164 than VEGF120 (*Vegfa+/+*); mutation of one *Vegfa* allele increases VEGF120 at the expense of VEGF164 (*Vegfa+/120*); mutation of both alleles ablates VEGF164 expression (*Vegfa120/120*) (see Ruhrberg et al., 2002).

(Fig. 6). Specifically, embryos expressing VEGF120 at the expense of VEGF164 contained vascular networks with fewer vessel segments and were therefore usually smaller than their wild-type littermates at 9.5 dpc (6/6 cases; compare Fig. 6A with B). This phenotype was not observed in littermates lacking SEMA3A alone (5/5 cases; Fig. 6C; see also Fig. 3). Littermates lacking both SEMA3A and VEGF164 displayed a VEGF120 phenotype (8/8 cases; Fig. 6D). All mutants contained a paired dorsal aorta and paired cardinal veins (Fig. 6E-G), suggesting that the presence of VEGF120 is sufficient to drive angioblast migration and the assembly of the major vessels. We conclude that SEMA3A does not cooperate with VEGF164 to direct vasculogenesis in the mouse.

To address whether SEMA3A is able to influence endothelial cell migration in mutants with reduced VEGF164 levels ('backup role'), we studied vessel branching in the hindbrain. Loss of SEMA3A did not alter the defect caused by loss of the heparin/neuropilin-binding VEGF isoforms (Fig. 7). First, we examined the role of SEMA3A in vessel branching when the level of VEGF120 was higher than that of VEGF164 (10 cases; compare Fig. 7B with E). Then we
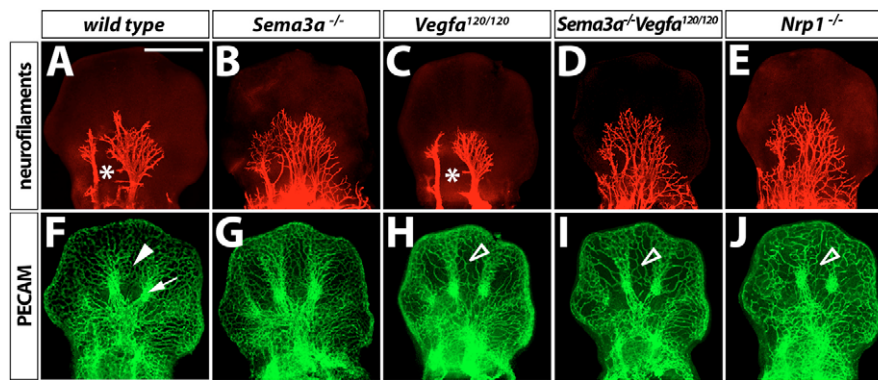
Fig. 8. SEMA3A patterns axons and VEG164 patterns vessel networks in developing mouse limbs. Double label immunohistochemistry for nerves (**A-E**) and vessels (**F-J**) in stage-matched wild-type limbs (*Nrp1*^{+/+}; A,F) and limbs lacking SEMA3A (B,G), VEGF164 (C,H), SEMA3A and VEGF164 (D,I) or NRP1 (E,J) at 12.5 dpc. (A,F) Vessels and nerves grow in close spatiotemporal proximity on the ventral aspect of the forelimb. The two major nerve branches entering the ventral footplate are normally well separated (asterisk). The microvessel network extends throughout the limb, including the areas under which the cartilage anlagen for the digits will form (one such area is indicated with an arrow). Regions of increased vascular density prefigure the sites where major arterial branches supplying the digits will form (one such area is indicated by an arrowhead in F). Note the axon defasciculation in B,D,E; the reduced microvessel branching (arrowheads) and smaller limb size in H-J; and a slight delay in axon extension, but normal axon patterning, in C. Scale bar: 500 μm.

examined the role of SEMA3A in vessel branching when VEGF164 expression was completely abolished (5 cases; compare Fig. 7C with F). In all instances in which VEGF164 levels were lower than VEGF120 levels, the mesh size of vessel networks was reduced independently of SEMA3A, i.e. vessel networks appeared similar in *Vegfa* mutants expressing or lacking SEMA3A (compare Fig. 7A-C with D-F). Moreover, there was no difference in the amount of vessel branching in the presence or absence of SEMA3A (Fig. 7G). The observation that loss of SEMA3A does not affect the phenotype caused by loss of heparin/neuropilin-binding VEGF isoforms during vasculogenesis or angiogenesis suggests that there is no genetic interaction between *Vegfa* and *Sema3a* during vessel development in the mouse.

Functional specialisation of SEMA3A and VEGF164 during neurovascular limb patterning

In developing limbs, axons and vessels grow in close proximity and associate functionally (e.g. Mukouyama et al., 2005), and they express both SEMA3A and VEGF strongly between 11.5 and 13.5 dpc (data not shown) (see also Taniguchi et al., 1997). Developing limbs therefore present a suitable model system to study neuronal and vascular patterning in tandem. We found that the development of limb nerves and limb vessels was compromised in NRP1-null mutants. First, mutants displayed extensive axon defasciculation and axonal overgrowth, as previously described (Kitsukawa et al., 1997) (Fig. 8E and Fig. 10E,A). Second, loss of NRP1 impaired limb vascularisation in 6/6 cases examined (Fig. 8J; compare Fig. 9A with E, and F with J). As expected, limbs lacking SEMA3A displayed axonal defects similar to those of NRP1 mutants (Taniguchi et al., 1997) (Fig. 8B), but they did not show altered microvessel branching in any of seven mutants examined (Fig. 8G; compare Fig. 9A with B, and F with G). Vice versa, loss of VEGF164 impaired limb vascularisation (Fig. 8H and Fig. 9C,H), but did not compromise limb innervation in 16/16 embryos examined (Fig. 8C and Fig. 10). Even though a delay in axon extension was seen in limbs lacking VEGF164 at 12.5 dpc, this was most likely to have been caused by a general delay in development that manifested itself at this time in reduced limb size (Fig. 8C,H). This general developmental delay

occurred in all three genotypes examined, with reduced vascularisation from 9.5 dpc onwards, i.e. in mutants lacking NRP1, VEGF164 or VEGF164 in a SEMA3A-null background (compare Fig. 8F with H-J, and Fig. 6A with B,D). As limbs lacking VEGF164 continued to develop, axonal growth reached normal levels, and at 13.75 dpc nerves on the ventral limb aspect were of similar length in littermate limbs from wild types and mutants lacking VEGF164 in 4/4 embryos examined (compare Fig. 10A with C). Moreover, sensory nerves on the dorsal limb aspect had branched abundantly (compare Fig. 10B with D). The branching pattern of sensory nerves in the skin is not stereotypical, but varies even between different limbs from the same animal. By contrast, the branching frequency appears to be reproducible for any given developmental stage; accordingly, a comparison of the frequency of nerve branching provides a suitable means to identify deficiencies in sensory nerve innervation (see Hafezparast et al., 2003). We therefore determined the number of nerve branchpoints in the dorsal footplate of forelimbs and hindlimbs at 13.75 dpc. We found that sensory nerve branching was increased 2.6-fold in limbs lacking SEMA3A or NRP1, but was not compromised in limbs lacking VEGF164 (Fig. 10G).

To address whether the competition between VEGF164 and SEMA3A for NRP1 binding previously observed in vitro contributes to neurovascular development, we compared the vascular limb phenotype of mouse mutants lacking VEGF164 in the presence or absence of SEMA3A, and the axonal phenotype of SEMA3A mutants in the presence or absence of VEGF164, by examining compound mutants lacking both NRP1 ligands (Figs 8, 9). We found that the absence of SEMA3A neither rescued nor worsened the vessel defect of limbs lacking VEGF164 in 4/4 embryos examined (compare Fig. 9C with D, and H with I). Vice versa, the loss of VEGF164 did not alter the axon branching defect caused by loss of SEMA3A (compare Fig. 8B with D). These observations imply that VEGF164/NRP1 signalling specifically controls vascular development, whereas SEMA3A/NRP1 signalling specifically controls axonal patterning in the limb. Consistent with the idea of an exclusive functional specialisation of both NRP1 ligands during neurovascular patterning in the developing limb, only mutants lacking both VEGF164 and
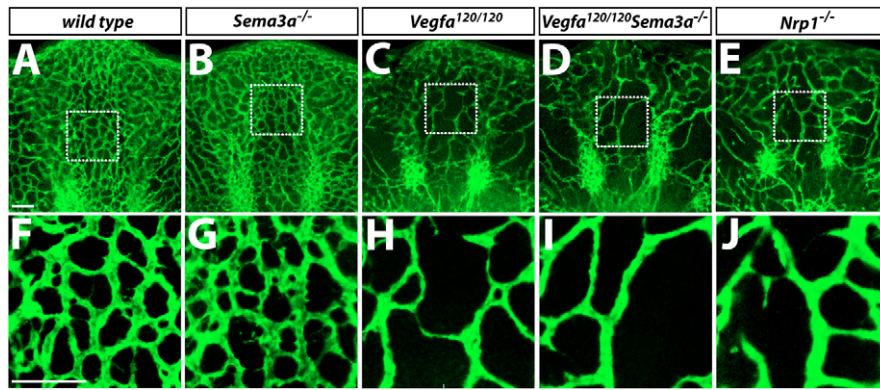
Fig. 9. Loss of VEGF164 but not SEMA3A impairs microvessel branching in the mouse limb. Forelimb vasculature at 12.5 dpc in stage-matched wild-type limbs (*Sema3a*$^{+/+}$; **A,F**), limbs lacking SEMA3A (**B,G**), VEGF164 (**C,H**), SEMA3A and VEGF164 (**D,I**) or NRP1 (**E,J**). Microvessel networks appear normal in the absence of SEMA3A, whereas loss of VEGF164 or NRP1 impairs vessel branching. Vascular defects in limbs lacking both SEMA3A and VEGF164 are similar to those in limbs lacking only VEGF164. Scale bars: 100 μm.

SEMA3A displayed defects in both vascular and axonal limb patterning, similar to those seen in NRP1 mutants (compare Fig. 8D,I with E,J, and Fig. 9D,I with E,J).

DISCUSSION
Semaphorins in vascular development
SEMA3A is able to influence the migratory behaviour of endothelial cells in tissue culture models (Miao et al., 1999; Serini and Bussolino, 2004). However, our analysis of SEMA3A-null mice demonstrates that SEMA3A is not required for vascular growth or patterning in vivo. Specifically, we find that loss of SEMA3A does not impair the formation of the major axial vessels, vessel branching or vessel remodelling in either of two genetic backgrounds examined, the inbred C57Bl/6 or the outbred CD1 strain (Figs 3-8).

Our findings contrast with those of Serini and co-workers, who described dramatic vascular defects at 9.5 dpc in SEMA3A-null mutants in the CD1 background (Serini et al., 2003). The discrepancy between our results and theirs was surprising, given that Serini and co-workers have analysed mice carrying the same gene-targeting event that we examined (Taniguchi et al., 1997). The lack of a vascular phenotype in our analysis of SEMA3A-null mutants was particularly clear in the developing brain, where the quantitative analysis of vessel branching confirmed that a similar number of microvessels was present in wild-type and mutant littermates. Importantly, the number of vessel branches was more variable in the CD1 as compared with the C57Bl/6 background, as expected from an outbred genetic background (e.g. Chia et al., 2005), but variability was similar for all genotypes analysed (compare error bars for both backgrounds in Fig. 4G).

The developmental variability in the CD1 background was particularly obvious at younger gestational ages; for example, we found that the somite number of wild-type embryos within a CD1 litter at 9.5 dpc varied from 19 to 24 (see Fig. S1 in the supplementary material). We therefore always compared embryos that were age-matched (i.e. of the same gestational age) as well as stage-matched (for example, by comparing somite number at 9.5 dpc). The greater variability of vessel branching, and even embryo size within a litter, might have contributed to the conclusion of Serini and co-workers that vascular patterning is abnormal in *Sema3a*-null mutants in the outbred CD1 strain.

In addition, a less robust method of sample analysis might have contributed to the conclusion of Serini and co-workers that formation of the anterior cardinal vein was impaired. To detect the anterior cardinal vein at 9.5 dpc, Serini and co-workers performed whole-mount immunohistochemistry with anti-PECAM and horseradish peroxidase-conjugated secondary antibodies and recorded the image using a conventional light microscope (Serini et al., 2003). Using this method to examine six littermate wild-type embryos at 9.5 dpc, we detected the anterior cardinal vein in 3/6 embryos on the left or right side only (see Fig. S2 in the supplementary material); in 2/6 cases, the anterior cardinal vein was not detectable on any side, and in 1/6 cases it was visible on both sides of the embryo (not shown). Subsequent dissection revealed that a paired cardinal vein had been present in all these cases. When we used antibodies to PECAM or endomucin and recorded images by laser scanning confocal microscopy, we were able to identify the anterior cardinal vein on both embryonic sides in 40/40 whole-mount-stained CD1 embryos at 9.5 dpc without the need to section the samples (compare Fig. S1 with Fig. S2 in the supplementary material; see also Figs 3 and 6). We therefore conclude that laser scanning confocal microscopy is superior to light microscopy for detecting the anterior cardinal vein in whole-mount samples at 9.5 dpc. We also found that endomucin was a superior marker to PECAM for the detection of the vasculature at 9.5 dpc, when blood vessels are relatively more immature than at later developmental stages: whereas blood vessels stained weakly with anti-PECAM antibodies at 9.5 dpc (see Fig. S2 in the supplementary material), blood vessels stained robustly with the same antibodies at 10.5 and 12.5 dpc (see Figs 3 and 4).

Lastly, we cannot exclude the possibility that unknown genetic modifiers have been introduced in either our or their laboratory during backcrossing of SEMA3A C57/Bl6 mice into the CD1 background. For this reason, we extended our analysis to an independently derived line of CD1 SEMA3A mice, but again found none of the previously described vascular defects (J.M.V. and C.R., unpublished).

In a complementary approach, we found that semaphorin signalling through neuropilins was not required for microvessel patterning in the brain and limb (Fig. 5 and data not shown). Our results therefore agree with those of Gu and co-workers, who did not observe vessel defects in mice lacking semaphorin signalling through neuropilins at earlier developmental stages (Gu et al., 2005).
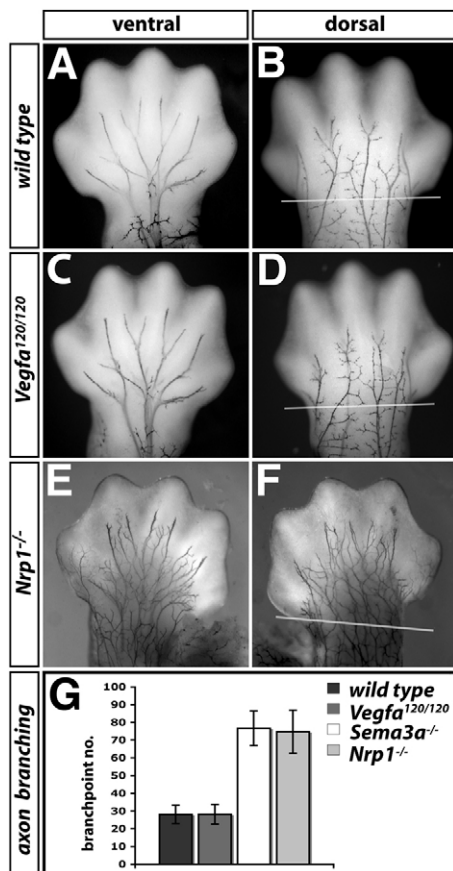
Fig. 10. Loss of SEMA3A but not VEG164 affects sensory nerve branching in the mouse limb. (**A-F**) Visualisation of limb nerves at 13.5 dpc in wild-type forelimbs (*Vegfa⁺/⁺*; A,B) and forelimbs lacking VEGF164 (*Vegfa¹²⁰/¹²⁰*; C,D) or NRP1 (*Nrp1⁻/⁻*; E,F). Ventral (A,C,E) and dorsal (B,D,F) aspects of the forelimb are shown. Note that the delay in axon extension observed at 12.5 dpc (Fig. 8) is no longer apparent at 13.5 dpc. (**G**) Quantitation of sensory nerve branching in the dorsal footplate (i.e. above the grey line in B,D,F) of wild-type limbs (combined data for *Vegfa⁺/⁺*, *Sema3a⁺/⁺* and *Nrp1⁺/⁺*; *n*=17) and limbs lacking VEGF164 (*n*=14), SEMA3A (*n*=5) or NRP1 (*n*=6).

In further support of the idea that semaphorin signalling through neuropilins is not required for the patterning of vascular endothelium, plexin A2, A3 and A4 are all dispensable for vascular development (Q.S.,C.R., K. Waimey, H. J. Cheng and H. Fujisawa, unpublished), even though they are essential co-receptors for semaphorin signalling through neuropilins in neurons (reviewed by Fujisawa, 2004). Moreover, mice lacking both SEMA3A (which binds NRP1) and SEMA3C (which binds NRP1 and NRP2) show normal brain vascularisation (Q.S., J.M.V., C.R. and K. Sabelko, unpublished).

Although semaphorin signalling through neuropilins is not important for microvessel patterning, SEMA3E can signal in a neuropilin-independent mechanism through plexin D1 to pattern intersomitic vessels (Gu et al., 2005). Consistent with the idea that SEMA3E/plexin D1 rather than SEMA3A/NRP1 signalling is important for microvascular development, downregulation of the NRP1 ligand Sema3ab in zebrafish results in only weak vascular defects, but the absence of plexin D1 impairs vascular development dramatically (Torres-Vazquez et al., 2004). Moreover, SEMA3E was the only class 3 semaphorin examined that bound exclusively to

vessels in the hindbrain (Fig. 2), and loss of plexin D1 results in ectopic branching in the hindbrain at 12.5 dpc (J.M.V., C.R., F. A. High, Y. Zhang and J. A. Epstein, unpublished). We therefore conclude that the previously favoured working models detailed in Fig. 1B-D should be abandoned in favour of the more recent plexin D1-based model presented in Fig. 1E to describe the role of semaphorin signalling in endothelial cell growth in the mouse. We cannot exclude, however, that the models described in Fig. 1B-D play a role in the vascular endothelium of other organisms or in cardiovascular tissues other than vascular endothelium in the mouse; accordingly, semaphorin signalling through NRP1 may pattern non-endothelial cell types that contribute to the developing mouse heart, such as cardiac neural crest cells (Gu et al., 2003).

VEGF and axon guidance

Based on the finding that SEMA3A and VEGF164 compete for NRP1 binding in vitro, it has been hypothesised that VEGF164 controls axon guidance, either directly or in competition with SEMA3A (Carmeliet, 2003). However, we have previously shown that SEMA3A, but not VEGF164, controls the behaviour of cranial nerve axons (Schwarz et al., 2004) (see also Fig. 3). We now extend this finding to include limb nerves (Figs 8 and 10). Moreover, we show that the competitive model of VEGF164 and SEMA3A described in Fig. 1F is unlikely to be relevant for axonal patterning (Figs 8 and 10). Although our observations suggest that VEGF164 does not play a general role in axon guidance of NRP1-expressing nerves in the peripheral nervous system, we cannot exclude the possibility that VEGF164 plays a role in axon guidance within the CNS, as it is able to bind to axon tracts in the hindbrain (Fig. 2G). It might be informative to identify NRP1-expressing CNS neurons whose axons are not normally guided by SEMA3A, so as to study their functional requirement for VEGF164, as there appears to exist a mutually exclusive use of either NRP1 ligand. The concept of preferential use of either VEGF164 or SEMA3A by different cell types is not only evident during axonal and vascular patterning in the developing limb and branchial arches, but also in the case of the facial branchiomotor neurons, which use SEMA3A for axon guidance and VEGF164 to position their cell bodies (Schwarz et al., 2004).

Cooperation, not competition, of NRP1 ligands during neurovascular development

Neurovascular co-patterning, as exemplified by the innervation of major arteries and the presence of the vasa nervosum, has recently become a topic of intense interest. NRP1 and its ligands have been considered plausible candidates to coordinate the association of vessels and nerves. Based on experiments with chick limbs carrying SEMA3A implants, it has been proposed that vessels and nerves are both repelled by SEMA3A signals emanating from the prechondrogenic mesenchyme in a mechanism requiring NRP1, and that this mechanism contributes to the emergence of neurovascular congruence (Bates et al., 2003). Although our observations are consistent with a role for NRP1 in controlling the vascularisation of areas surrounding the cartilage anlagen for the digits, we did not identify any vascular defects in SEMA3A mutants in these regions at 12.5 dpc (Figs 8 and 9). Rather, vascular development in the limb appeared to be controlled by VEGF. Moreover, the reduced vessel branching in limbs lacking VEGF164 did not obviously affect nerve patterning (Fig. 10). Taken together, these findings imply that the competition between SEMA3A and VEGF164 for NRP1 binding does not provide the basis for the striking neurovascular congruence seen in the mouse limb at later developmental stages. However, we would like to point out that slight vascular overgrowth occurs in

areas of excess nerve growth after 13.5 dpc in SEMA3A mutants (data not shown), which is likely to be caused by VEGF secretion from ectopic limb nerves (Mukouyama et al., 2005).

Context-dependent ligand selectivity of NRP1-based signalling pathways

The observation that SEMA3A and VEGF164 control mutually exclusive patterning events suggests that NRP1-based signalling pathways display context-dependent ligand selectivity. Such selectivity may be achieved by different means. For example, it is conceivable that NRP1 binds VEGF164 and SEMA3A with different specificity in the presence of different co-factors on the cell surface. However, the hindbrain model suggests that VEGF164 and SEMA3A both bind axons and endothelial cells (Fig. 2). Alternatively, VEGF164 and SEMA3A might bind NRP1 on vessels and neurons independently of specific co-factors on the cell surface, but induce distinct conformational changes in NRP1 that selectively activate VEGFR2 or A-type plexins, respectively, or activate different downstream signalling pathways, because specific co-receptors such as VEGFR2 or A-type plexins are not present in one or the other cell type. As we have not been able to detect A-type plexins in brain endothelial cells or VEGFR2 in embryonic spinal motor/sensory neurons in situ (J.M.V., Q.S. and C.R, unpublished), we favour the latter possibility. Importantly, the specificity of these NRP1-based signalling pathways may be lost under conventional cell culture conditions, in pathological angiogenesis or in cancer, perhaps because of abnormal co-factor expression profiles. Future work should therefore aim to identify the factors that control the selective usage of different NRP1 ligands during neuronal and vascular development.

Supplementary material
Supplementary material for this article is available at
http://dev.biologists.org/cgi/content/full/134/10/1833/DC1

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