Neuropilin 1 (NRP1) hypomorphism combined with defective VEGF-A binding reveals novel roles for NRP1 in developmental and pathological angiogenesis

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ABSTRACT
Neuropilin 1 (NRP1) is a receptor for class 3 semaphorins and vascular endothelial growth factor (VEGF) A and is essential for cardiovascular development. Biochemical evidence supports a model for NRP1 function in which VEGF binding induces complex formation between NRP1 and VEGFR2 to enhance endothelial VEGF signalling. However, the relevance of VEGF binding to NRP1 for angiogenesis in vivo has not yet been examined. We therefore generated knock-in mice expressing Nrp1 with a mutation of tyrosine (Y) 297 in the VEGF binding pocket of the NRP1 b1 domain, as this residue was previously shown to be important for high affinity VEGF binding and NRP1-VEGFR2 complex formation. Unexpectedly, this targeting strategy also severely reduced NRP1 expression and therefore generated a NRP1 hypomorph. Despite the loss of VEGF binding and attenuated NRP1 expression, homozygous Nrp1Y297A/Y297A mice were born at normal Mendelian ratios, arguing against NRP1 functioning exclusively as a VEGF164 receptor in embryonic angiogenesis. By overcoming the mid-gestation lethality of full Nrp1-null mice, homozygous Nrp1Y297A/Y297A mice revealed essential roles for NRP1 in postnatal angiogenesis and arteriogenesis in the heart and retina, pathological neovascularisation of the retina and angiogenesis-dependent tumour growth.

KEY WORDS: NRP1, VEGF, Angiogenesis, Arteriogenesis, Retina, Hindbrain

INTRODUCTION
NRP1 is a transmembrane receptor for the VEGF165 isoform (VEGF164 in mice) and the neuronal guidance cue SEMA3A, with essential roles in both vascular and neuronal development (reviewed by Pellet-Many et al., 2008; Raimondi and Ruhrberg, 2013). Accordingly, Nrp1-null mice die before birth with severe cardiovascular and neuronal defects (Kitsukawa et al., 1997; Kawasaki et al., 1999). Mice lacking NRP1 in vascular endothelial cells (ECs) also show defective cardiovascular development, whereas mice carrying a mutated extracellular domain that abolishes SEMA3A, not VEGF164, binding show defective nerve, but not blood vessel, patterning (Gu et al., 2003; Vieira et al., 2007). These and other genetic, biochemical and cell biological data support a model in which VEGF165 binding induces complex formation between NRP1 and VEGFR2 (KDR – Mouse Genome Informatics) to enhance VEGFR2 signalling during EC migration in vitro (e.g. Soker et al., 2002; Wang et al., 2003; Evans et al., 2011) and arteriogenesis in vivo (Lanahan et al., 2013).

The extracellular NRP1 a1/a2 and b1/b2 domains are crucial for recognition of SEMA3A, whereas the major VEGF binding site resides in the b1 domain, with some contribution of the b2 domain (Gu et al., 2002). Structural studies of the b1 domain have identified key residues important for ligand interactions in a VEGF165-binding pocket (von Wronski et al., 2006; Vander Kooi et al., 2007; Jarvis et al., 2010). For example, mutation of Y297 or D320 in the b1 domain impairs VEGF165 binding and complex formation between NRP1 and VEGFR2, which reduces VEGF165-stimulated EC migration in vitro (Jarvis et al., 2010; Herzog et al., 2011).

Though NRP1 is essential for developmental angiogenesis, the contribution of the NRP1-VEGF interaction to physiological or pathological angiogenesis is unclear. We therefore generated mice with a Y297A mutation in the b1 VEGF binding site (supplementary material Fig. S1A). Inadvertently, insertion of a Nrp1Y297A/Y297A mutant cDNA reduced mRNA and protein expression of the mutant allele, generating a novel mouse model with NRP1 hypomorphism. Despite combining loss of VEGF binding with reduced NRP1 expression, Nrp1Y297A/Y297A homozygous mice were mostly viable at birth, without the severe and lethal cardiovascular phenotypes of full or endothelial-specific Nrp1-null mutants. This observation implies that NRP1 has important VEGF-independent roles in angiogenesis which most likely synergise with its known role as a VEGFR2 co-receptor. Moreover, the postnatal viability of Nrp1Y297A/Y297A mice enabled the study of newborn and adult mutants, thereby extending genetic evidence for the established function of NRP1 in cardiovascular development to include essential roles in promoting postnatal and pathological angiogenesis.

RESULTS AND DISCUSSION
Reduced NRP1 expression in Nrp1Y297A/Y297A mice

Previous studies demonstrated that the highly conserved Y297 residue in the NRP1 b1 domain (supplementary material Fig. S1A,B) is essential for VEGF165 binding to human NRP1 (Jarvis et al., 2010; Herzog et al., 2011). The Y297A mutation also inhibited high affinity binding of 125I-VEGF165 to murine NRP1 in vitro (supplementary material Fig. S1C). Heterozygous mice expressing a mutated Nrp1Y297A cDNA from exon 2 of the

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endogenous Nrp1 locus were generated (Fig. 1A) and interbred to produce homozygous Nrp1Y297A/Y297A offspring (Fig. 1B-D). Unexpectedly, immunoblotting revealed a severe reduction of NRP1 in Nrp1Y297A/Y297A and a moderate reduction in Nrp1Y297A/+ mice compared with wild-type (WT) littermates (Fig. 1E,F). Immunohistochemistry confirmed reduced NRP1 expression in Nrp1Y297A/Y297A tissues (supplementary material Fig. S2A,B). Reduced NRP1 protein levels correlated with decreased Nrp1 mRNA expression in all Nrp1Y297A/Y297A tissues examined (supplementary material Fig. S2C). Impaired expression of the Nrp1Y297A allele may be due to the disruption of intronic regulatory elements deleted by insertion of the mutant cDNA into the Nrp1 locus. Allele-specific quantitative PCR (qPCR) of heterozygous mice demonstrated significantly reduced expression of the mutant
allele compared with the WT allele in all tissues examined (Fig. 1G). Mice expressing the Nrp1<sup>Y297A</sup> allele are therefore hypomorphic for NRP1 with markedly reduced NRP1 expression as well as reduced VEGF<sub>164</sub> binding and represent a novel model in which to examine NRP1 roles in angiogenesis in vivo.

Increased mortality and impaired growth in postnatal Nrp1<sup>Y297A/Y297A</sup> mice

Genotyping of offspring from crosses between heterozygous mice yielded the expected Mendelian ratio for WT, heterozygous and homozygous mice before birth, and only a small, non-significant reduction in the number of Nrp1<sup>Y297A/Y297A</sup> offspring on postnatal day (P) 0 (supplementary material Table S2), indicating that residual NRP1 expression from the Nrp1<sup>Y297A</sup> allele prevents the mid-gestation lethality of full NRP1 knockouts. By contrast, homozygous mutants were under-represented in litters at P21, and the number of adult Nrp1<sup>Y297A/Y297A</sup> mice (~8 weeks) was ~50% lower than expected (supplementary material Table S2). Follow-up from birth to 6 weeks of age showed increased mortality of Nrp1<sup>Y297A/Y297A</sup> mice predominantly within the first 2 weeks of postnatal life (Fig. 1H). Nrp1<sup>Y297A/Y297A</sup> mice were also significantly smaller than WT littermates at P7 (Fig. 1I) and at all ages examined between 6 and 18 weeks (Fig. 1J,K). Heterozygous mice had normal weights at P7, but showed a small, significant size reduction at 8, 10, 12 and 14 weeks (data not shown). These results indicate that low NRP1 expression combined with inhibition of VEGF<sub>164</sub> binding causes postnatal mortality and reduces body weight in surviving mice.

Defective myocardial vascularisation in postnatal Nrp1<sup>Y297A/Y297A</sup> mice

Post mortem analysis of mutants that had died on P7 revealed blood-filled lungs (Fig. 1L), indicative of congestive heart failure. Increased mortality and reduced body size are also seen in Vegfa<sup>120/120</sup> mutants that die perinatally owing to ischemic cardiomyopathy caused by impaired myocardial vascularisation (Carmeliet et al., 1999). Similarly, P7 Nrp1<sup>Y297A/Y297A</sup> hearts contained fewer coronary arteries and capillaries than control hearts (Fig. 1M-P). Moreover, capillaries in mutants appeared abnormal and were sparse, particularly in the subendocardium, consistent with reports that angiogenesis occurs in an epicardial-to-endocardial gradient (Tomanek, 1996). The similar vascular defects in Nrp1<sup>Y297A/Y297A</sup> and Vegfa<sup>120/120</sup> myocardium, combined with a predominance of NRP-binding VEGF isoforms relative to VEGF<sub>120</sub> in this tissue (Ng et al., 2001), suggest that the perinatal vascularisation of myocardium depends on VEGF binding to NRP1.

Embryonic angiogenesis is mildly impaired in Nrp1<sup>Y297A/Y297A</sup> mutants

We next investigated the effect of the Y297A mutation on VEGF binding to NRP1 and on vascular development in the hindbrain on embryonic days (E) 11.5 and E12.5, when blood vessels sprout and fuse to form the subventricular vascular plexus (SVP) (Fantin et al., 2010). Although Nrp2, Vegfr1 (Flt1 – Mouse Genome Informatics) and Vegfr2 mRNA levels were unaffected, Nrp1 transcripts were reduced by 50% in Nrp1<sup>Y297A/Y297A</sup> compared with WT hindbrains (Fig. 2A). In agreement, immunostaining showed reduced NRP1 protein levels in the mutants, both in axons and vessels (Fig. 2B,C). Alkaline phosphatase (AP)-conjugated VEGF<sub>165</sub> ligand binding to NRP1-positive pial axons was abolished in Nrp1<sup>Y297A/Y297A</sup> mutant, similar to Nrp1-null, hindbrains (Fig. 2D). By contrast, AP-VEGF<sub>165</sub> binding to vessels was unaffected (Fig. 2D), consistent with high VEGFR2 expression by hindbrain ECs, but not axons (Lanahan et al., 2013). AP-SEMA3A binding to NRP1-expressing axons in Nrp1<sup>Y297A/Y297A</sup> mice was unperturbed (Fig. 2E). However, AP-SEMA3A binding to SVP vessels was impaired, even though binding to radial vessels on the pial side was unaffected (Fig. 2E), perhaps due to the differential effect of the Nrp1 mutation on overall NRP1 protein levels in the two brain regions.

E12.5 Nrp1<sup>Y297A/Y297A</sup> hindbrains exhibited a similar number of radial vessels compared with WT littermates (Fig. 2F,G), but significantly reduced vessel branching in the SVP (Fig. 2H,I), as observed for Nrp1-null mice (Gerhardt et al., 2004; Fantin et al., 2013a). Yet, the effect of the Nrp1<sup>Y297A/Y297A</sup> mutation on SVP angiogenesis was less severe than that of complete or endothelial-specific NRP1 loss, which causes a catastrophic failure of neighbouring vessels to interconnect, giving rise to large vascular tufts at vessel termini (Gerhardt et al., 2004; Fantin et al., 2013a). Importantly, the mild vascular defects in Nrp1<sup>Y297A/Y297A</sup> hindbrains do not phenocopy Vegfa<sup>120/120</sup> hindbrains expressing VEGF<sub>120</sub>, but lacking the NRP1-binding VEGF isoforms; specifically, Vegfa<sup>120/120</sup> hindbrains contain fewer pial vessels as well as poorly branched and enlarged vessels throughout the brain, and these defects can be attributed to perturbed VEGF gradients (Ruhberg et al., 2002). As in the hindbrain, vessel density was only mildly affected in head and trunk of E9.5 Nrp1<sup>Y297A/Y297A</sup> embryos, and the formation of the pharyngeal arch arteries and the intersomitic vessels appeared to be unaffected (supplementary material Fig. S3). The mild vascular phenotype of Nrp1<sup>Y297A/Y297A</sup> embryos compared with the severe phenotype of Nrp1-null and Vegfa<sup>120/120</sup> mice argues against a major role for VEGF<sub>164</sub>-signalling through NRP1 in embryonic angiogenesis.

Retinal angiogenesis and arteriogenesis are defective in Nrp1<sup>Y297A/Y297A</sup> mice

The impact of the Y297A mutation on postnatal vascular development was further investigated in the retina, where angiogenesis begins at P0 and is essentially complete by P21 (Fruttiger, 2007). Examination of the P7 retinal vasculature by wholemount immunofluorescence for the vascular marker collagen IV revealed a significant reduction in the extension of the primary vascular plexus towards the retinal margin in Nrp1<sup>Y297A/Y297A</sup> compared with WT retinas (Fig. 3A,B). The number of arteries and veins was also reduced in Nrp1<sup>Y297A/Y297A</sup> retinas (Fig. 3A,C; data not shown), and mutants displayed significantly reduced vessel coverage by vascular smooth muscle cells (VSMCs) positive for smooth muscle α-actin (SMA) (Fig. 3A,D,E). Similar to the hindbrain, the Nrp1<sup>Y297A</sup> mutation greatly reduced NRP1 expression in Nrp1<sup>Y297A/Y297A</sup> compared with WT retinas (Fig. 3F).

By P21, delayed angiogenesis in the primary vascular plexus had largely recovered in homozygous mutants (Fig. 3G), but delayed formation of the deep and intermediate plexi was indicated by reduced vascular density (supplementary material Fig. S4). The number of arteries and their VSMC coverage remained significantly reduced (Fig. 3G-J). Moreover, incomplete vascularisation was observed in parts of the peripheral retinas, where vessels terminated in endothelial tufts covered with SMA-positive VSMCs and NG2 (CSPG4 – Mouse Genome Informatics)-positive pericytes (Fig. 3K). As NRP1 is not essential for endothelial cell proliferation (Jones et al., 2008), but is important for migration (Wang et al., 2003; Pan et al., 2007; Evans et al., 2011), tuft formation is likely to be due to abnormal endothelial cell sprouting. Nrp1<sup>Y297A/Y297A</sup> retinas also contained an increased number of arteriovenous crossings (Fig. 3L,M), similar to mice lacking the NRP1 cytoplasmic domain.
(Fantin et al., 2011) or with haploinsufficient expression of VEGF in the neural lineage (Haigh et al., 2003).

We cannot presently resolve whether low NRP1 expression levels and/or inhibition of VEGF164 binding to NRP1 are primarily responsible for the observed retinal vascular defects in Nrp1Y297A/Y297A mice. However, their similarity to defects in Vegfa120/120 retinas (Stalmans et al., 2002), combined with similar vascular defects in the P7 myocardium (Fig. 1M-P), suggests that NRP1-mediated VEGF-signalling is more important for perinatal than embryonic vascular development.

**Reduced angiogenesis in Nrp1Y297A/Y297A mice with oxygen-induced retinopathy**

The postnatal viability of Nrp1Y297A/Y297A mice allowed us to examine the impact of Nrp1 hypomorphism and defective VEGF binding on pathological angiogenesis. We used a model of oxygen-induced retinopathy, in which sequential exposure of neonatal mice to hypoxia and normoxia causes obliteration of central retinal capillaries, followed by hypoxia-induced VEGF upregulation and abnormal neovascularisation (Smith et al., 1994). Vaso-obliterration was slightly reduced, consistent with reduced vascular dropout after hypoxia or increased revascularisation of avascular areas after return to normoxia, whereas pathological neovascularisation was strongly and significantly decreased in Nrp1Y297A/Y297A compared with WT littermates (Fig. 4A-D). These observations show that NRP1 contributes substantially to ocular neovascularisation.

**Reduced VEGF-induced angiogenesis and tumourigenesis in Nrp1Y297A/Y297A mice**

VEGF stimulation of pathological angiogenesis in Nrp1Y297A/Y297A mice was assessed in the aortic ring assay, in which adult aortic explants undergo angiogenic sprouting upon growth factor

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**Fig. 2. Mild angiogenesis defects in Nrp1Y297A/Y297A hindbrains.** (A-C) VEGF receptor expression in Nrp1Y297A/Y297A hindbrains. qPCR analysis for Nrp1, Nrp2, Vegfr1 and Vegfr2 relative to Actb (A) (n=3; mean ± s.d.; **P<0.01, ns, not significant) and whole-mount immunofluorescence staining for NRP1 and IB4 (B) in E11.5 hindbrains. (C) Single NRP1 channels are shown in greyscale and heat-map (red, blue and green indicate high, low and medium pixel intensity, respectively). Arrowheads indicate axons. (D,E) AP-VEGF165 (D) and AP-SEMA3A (E) binding in E12.5 Nrp1Y297A/Y297A hindbrains; arrowheads indicate axons; asterisks highlight absent ligand binding to axons; open triangles indicate absent SEMA3A vessel binding. (F-I) Whole-mount PECAM immunostaining and quantification of vessels in E12.5 hindbrains, imaged (F) and quantified (G) on the pial side or imaged (H) and quantified (I) on the SVP side; the arrow indicates a vascular tuft; n≥4; mean ± s.d.; ***P<0.001. Scale bar: in C, 100 μm (B-F,H).
stimulation (Baker et al., 2011). VEGF<sub>164</sub> increased vessel sprouting from WT aortic rings, but this response was suppressed by the Nrp1<sup>Y297A</sup> mutation (Fig. 185-187). By contrast, serum stimulated sprouting from WT and mutant aortic rings similarly (Fig. 189-191). We next examined the effect of the Y297A mutation on angiogenesis-dependent growth of syngeneic B16-F1 murine melanomas (Franco et al., 2002). These tumours produce high levels of VEGF, and their growth is blocked by VEGFR2 inhibition, demonstrating dependence on VEGF-driven angiogenesis (Prewett et al., 1999). Whereas tumours in WT mice grew rapidly, tumours were significantly smaller in age- and sex-matched Nrp1<sup>+/Y297A</sup> littermates and essentially absent in homozygotes at two weeks (>90% inhibition in homozygous mutants; Fig. 192-194). Collectively, these genetic data show that NRP1 plays an important role in pathological angiogenesis driven by VEGF. Pan et al. (Pan et al., 2007) demonstrated that function-blocking antibodies targeting VEGF binding to NRPI reduce human non-small cell lung carcinoma xenograft tumour size in mice by 37%. The greater effect of the Y297A mutation compared with blocking antibodies (>90% versus 37%) may reflect use of different tumour models and/or an additional effect of reducing NRPI levels compared with inhibiting only VEGF binding to NRPI, perhaps due to the contribution of ligands other than VEGF to tumour angiogenesis.

Conclusions
The consensus model for the endothelial role of NRPI is that it functions as a receptor for VEGF<sub>165</sub> and complexes with VEGFR2 to generate a holoreceptor that optimally transduces angiogenic signals. However, this model has hitherto been based almost entirely on biochemical and cell culture studies, combined with the analysis of a mouse model that abolishes all endothelial NRP1 functions. The severe reduction in NRP1 expression caused by the Nrp1<sup>Y297A</sup> allele did not permit definitive distinction of VEGF binding from other NRPI functions requiring normal expression. However, the mild embryonic vascular phenotypes combined with the extended viability of Nrp1<sup>Y297A/Y297A</sup> relative to full and endothelial-specific NRP1 knockout mice clearly demonstrates for the first time that VEGF binding to NRPI is not essential for embryonic angiogenesis and, therefore, that NRPI functions in embryonic ECs predominantly in a VEGF-independent role. Because embryonic vascular functions of NRPI do not involve semaphorin binding (Gu et al., 2003; Vieira et al., 2007), future studies will be needed to define the precise mechanism of NRPI function in angiogenesis,
taking into account recent reports of NRP1 as a modulator of PDGF, TGFβ and integrin signalling (Valdembri et al., 2009; Cao et al., 2010; Evans et al., 2011; Pellet-Many et al., 2011; Yaqoob et al., 2012).

MATERIALS AND METHODS

Mice
Animal experiments were conducted in accordance with the Animal Care and Ethics Guidelines of University College London (UCL) and the United Kingdom Home Office Animals (Scientific Procedures) Act of 1986. Site-directed mutagenesis of mouse Y297 was performed as described (Herzog et al., 2011). The start codon and coding region of exon 2 of murine WT Nrp1 were replaced by homologous recombination in 129Sv/Pas embryonic stem cells (ESCs) with murine cDNA containing Nrp1Y297A, targeted ESCs were injected into C57/BL6 blastocysts and resulting male F1 chimeras were bred with C57BL/6 females expressing CRE to generate heterozygous Nrp1+/Y297A mice lacking a neo cassette (Genoway; Lyon, France; Fig. 1). Genotyping and sequencing of genomic or cDNA from tail biopsies were performed with the primers listed in supplementary material Table S1.

Expression studies
mRNA levels were measured by qPCR using cypophilin as a normaliser and quantified with qStandard software (UCL). Protein expression in tissue lysates was determined by immunoblotting using rabbit anti-NRP1 (1:500; stock code 3725, Cell Signaling) and horseradish peroxidase-conjugated secondary antibody (1:10,000; stock code sc-2030, Santa Cruz), or immunostaining with goat anti-NRP1 (1:50; stock code AF566, R&D Systems) and biotinylated secondary antibody (1:200; stock code E0466, Dako).

Assays
Cell culture, VEGF-binding assay, wholemount immunofluorescence, alkaline phosphatase (AP)-fusion protein binding assay, oxygen-induced retinopathy, aortic ring assays and tumour cell injections were all performed and quantified according to published procedures (Franco et al., 2002; Jia et al., 2006; Connor et al., 2009; Herzog et al., 2011; Baker et al., 2011; Fantin et al., 2013b).

Statistics
Results are presented as means ± s.d. or s.e.m., as specified in each figure legend. Statistical significance of differences between samples was determined by a two-tailed Student’s t-test; more than two datasets or grouped datasets were compared, respectively, by one-way or two-way analysis of variance followed by post-hoc tests. Genotype distribution was analysed using the χ² test, survival ratios using the Log-rank (Mantel-Cox) test. Statistical analyses were performed with Prism 5 (GraphPad Software); P<0.05 was considered significant.

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Competing interests
This study was financially assisted in part by Ark Therapeutics Ltd, which has had an interest in developing therapies to inhibit NRP1.

Author contributions
A.F., B.H., C.R. and I.Z designed the research, performed the research, analysed the data and wrote the paper. L.D. performed research. A.P., M.M. and M.Y. performed research and analysed the data.
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Supplementary material
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