Activation of the UNC5B receptor by Netrin-1 inhibits sprouting angiogenesis

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Netrins are secreted molecules with roles in axonal growth and angiogenesis. The Netrin receptor UNC5B is required during embryonic development for vascular patterning, suggesting that it may also contribute to postnatal and pathological angiogenesis. Here we show that unc5b is down-regulated in quiescent adult vasculature, but re-expressed during sprouting angiogenesis in matrigel and tumor implants. Stimulation of UNC5B-expressing neovessels with an agonist (Netrin-1) inhibits sprouting angiogenesis. Genetic loss of function of unc5b reduces Netrin-1-mediated angiogenesis inhibition. Expression of UNC5B full-length receptor also triggers endothelial cell repulsion in response to Netrin-1 in vitro, whereas a truncated UNC5B lacking the intracellular signaling domain fails to induce repulsion. These data show that UNC5B activation inhibits angiogenesis, thus identifying UNC5B as a potential anti-angiogenic target.

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mental processes including angiogenesis (Cirulli and Yebra 2007). Netrins may also act as bifunctional cues during angiogenesis. Wilson et al. (2006) have shown promi-
togenic and promigratory effects with Netrin-1 and Netrin-4 in vitro and proangiogenic effects for both Net-
trins in post-ischemic revascularization processes via unidentified receptor[s]. In contrast, we have previously
observed repulsive actions of Netrin-1 via UNC5B in de-
velopmental angiogenesis (Lu et al. 2004). We here ex-
amine for the first time expression of UNC5B and its
function during postnatal and pathological angiogenesis,
and show that activation of UNC5B with Netrin-1 as an
agonist inhibits angiogenic sprouting and neovascular-
ization.

Results

Unc5b expression during postnatal and pathological angiogenesis

To investigate possible functions of UNC5B during post-
natal and/or pathological angiogenesis, we first exam-
ined its expression at different stages after birth, using
heterozygous unc5b lacZ-plap knock-in mice bred onto
a CD1 background (Lu et al. 2004). Whole-mount X-gal
or alkaline phosphatase (AP) staining allows detecting
both reporter genes inserted into the unc5b locus (Fig. 1;
Supplementary Fig. 1). Staining of brains, hearts, guts,
and skeletal muscle isolated from adult mice (2–8 mo)
showed no vascular staining in any of these organs (data
not shown), indicating that unc5b expression is down-
regulated in the quiescent vasculature. To determine
when unc5b down-regulation occurred, we examined
retinal development, which is a temporally well-charac-
terized, active sprouting angiogenesis process that starts
at postnatal day 0 (P0) (Gariano and Gardner 2005).
Unc5b reporter gene expression was seen in arteries, cap-
illaries, and endothelial tip cells of retinal vessels during
the period of active angiogenesis (P0 until P8–P9)
(Supplementary Fig. 1) and became progressively down-
regulated once angiogenesis of these vessels ceased. At
P12, expression was observed in arteries, but extin-
guished in most capillaries (Fig. 1A–D). After P21, re-
porter staining in retinal vessels became undetectable
(data not shown), and quantitative PCR (qPCR) analysis
confirmed very low unc5b expression levels in adult reti-
nal endothelial cells (Supplementary Fig. 1). Unc5b ex-
pression in tumor arteries (a), but not veins (v), and
expression in hair follicles (arrowheads) and a smooth
muscle cell (*). [M–P] PC3 tumor cells im-
planted for 7 d into a heterozygous unc5b-
lacZ-plap mouse. Whole-mount AP [M]
collagen-IV [N] double staining of a thick section of a tumor nodule. Note unc5b expression in tumor arteries (a), but not veins (v), and in sprouting capillaries (arrowheads). [O,P] Higher magnification of a sprout (yellow arrowhead in M and N) expressing the unc5b lac-Z reporter [O] and double-stained with Collagen-IV [P]. Bars: A,B,E,F, 225 µm; C,D,L–N, 110 µm; G,H, 45 µm; I, 1500 µm; J,K, 2000 µm; O,P, 25 µm.
expression thus appears restricted to vessels undergoing active angiogenesis and becomes down-regulated once these vessels reach quiescence.

To test if induction of neovascularization was accompanied by re-expression of unc5b, we subjected mice to oxygen-induced ischemic retinopathy (OIR) [Smith et al. 1994]. Briefly, mice were exposed to hyperoxia between P7 and P12, followed by return to room air until P17. The return to room air is perceived as a hypoxic insult, leading to VEGF up-regulation and reactivation of angiogenic sprouting. Unc5b was robustly re-expressed in arteries, in arterio–venous shunts, and in sprouting capillaries at P17 [Fig. 1E–H].

To extend these observations, we injected matrigel loaded with the proangiogenic factor bFGF into heterozygous unc5b lacZ-plap knock-in mice to induce sprouting and invasion of subcutaneous vessels into the plug [Kleinman and Martin 2005]. Whole-mount X-gal staining of adult skin showed weak unc5b expression in arteries, while neovessels invading the plugs up-regulated unc5b transcription 2 wk post-injection. Re-expression was observed in arteries, but not in veins, and in sprouting vessels [Fig. 1I–K]. Double staining with the pan-vascular marker PECAM-1 showed that re-expression occurred in endothelial cells and, to a lesser extent, in smooth muscle cells [Fig. 1L]. Results obtained in the unc5b lacZ-plap reporter mice were confirmed in C57/Bl6 mice using in situ hybridization with a probe recognizing unc5b [Supplementary Fig. 2].

To determine if unc5b expression was also up-regulated during tumor angiogenesis, PC3 prostate cancer cells were implanted subcutaneously into unc5b lacZ-plap knock-in mice, harvested after 1 wk and analyzed for unc5b AP reporter expression following by labeling with an anti-Collagen-IV antibody that recognizes vascular basement membranes [Baluk et al. 2003]. No signs of immune reaction could be detected at this time point, presumably due to low HLA surface expression [Bennettskaya et al. 2004]. Robust unc5b expression was observed in vessels invading the tumors, most prominently in arteries, but not veins, and in sprouting capillary endothelium [Fig. 1M–P]. Similar results were obtained using implantation of tumor cell lines in nude mice by in situ hybridization with an unc5b-specific probe [Supplementary Fig. 1].

Finally, we tested for unc5b re-expression following femoral artery ligation in C57/Bl6 and heterozygous unc5b lacZ-plap reporter mice. Paraffin sections of gastrocnemius muscle from either control or ligated limbs 7 and 14 d after femoral artery ligation were prepared. Lectin staining showed numerous enlarged vessels in the ischemic muscle, indicating that ligation had induced a vascular response [Supplementary Fig. 1]. While in situ hybridization failed to detect unc5b up-regulation, X-gal staining revealed some positive cells in the mesenchyme of the ischemic area. Both stainings failed to detect unc5b up-regulation in endothelium of the ischemic vessels [Supplementary Fig. 1]. Thus, during this form of neovascularization, which may not require extensive sprouting, endothelial unc5b expression is not induced.

Taken together, these data show that unc5b expression is dynamically regulated during postnatal life and correlated with sprouting angiogenesis in both physiological and pathological processes.

**Inhibition of matrigel neovascularization by Netrin-1 requires UNC5B**

As unc5b expression accompanied bFGF-induced matrigel neovascularization [Fig. 1I–L; Supplementary Fig. 2], we examined the effects of UNC5B activation by Netrin-1 in this model. Matrigel plugs loaded with saline [control], recombinant Netrin-1 [300 ng/mL or 1 µg/mL], bFGF (300 ng/mL), or bFGF in combination with Netrin-1 were injected into C57/Bl6 or heterozygous unc5b LacZ-plap mice and analyzed after 14 d. Angiogenesis in the C57/Bl6 matrigel plugs was assayed by measurement of hemoglobin content, VEGF Receptor-2 [VEGFR-2] protein concentration, assessment of vessel morphology after perfusion of fluorescently labeled IsolectinB4, and PECAM-1 staining of sections prepared from matrigel plugs [Fig. 2A–D; Supplementary Fig. 2]. Neovascularization in heterozygous unc5b lacZ-plap mice was assayed by whole-mount X-gal staining and counting of the number of X-gal-positive blood vessels on serial paraffin sections prepared from matrigel plugs [Fig. 2E–J]. Plugs containing saline or Netrin-1 alone exhibited no significant invasion of unc5b-expressing blood vessels [Fig. 2A,B,E,H]. Thus, Netrin-1 showed no angiogenic activity in this assay. As expected, bFGF robustly stimulated neovascularization and invasion of unc5b-expressing vessels. In comparison with bFGF alone, vascularization of plugs containing bFGF and Netrin-1 was significantly reduced [Fig. 2A–J; Supplementary Fig. 2]. Higher magnifications of X-gal whole-mount stainings showed numerous unc5b-positive sprouts at the vascular front protruding into the matrigel in bFGF-containing plugs, while protrusion of sprouts was reduced in plugs containing both bFGF and Netrin-1 [Fig. 2J].

Expression of all known Netrin receptors was examined in bFGF-treated matrigel plugs by RT–PCR. Only unc5b and, at lower levels, unc5a and neogenin could be amplified [Supplementary Fig. 2], suggesting that inhibition of bFGF-induced neovascularization by Netrin-1 could be at least partly mediated by the UNC5B receptor. To test this hypothesis directly, we used adult homozygous unc5b-deficient mice. Our previous analysis of an unc5b knock-out mouse had shown that the homozygous mutants die in midgestation [Lu et al. 2004]; however, mating of a large number (>100 females) of heterozygous mutants die in midgestation (Lu et al. 2004). Howev-
Tip cell filopodial retraction in response to Netrin-1 requires UNC5B

To understand how Netrin-1 could inhibit vessel sprouting, we used an ex vivo endothelial sprouting assay. Dorsal aorta explants from wild-type, unc5b+/− and unc5b−/− mice were cultured in semisolid collagen gels. After 48 h, the explants developed sprouts that extended peripherally and were spearheaded by filopodial-extending cells. Whole-mount staining of unc5b+/− and unc5b−/− explants using X-gal showed that sprouting cells expressed unc5b [Fig. 3A,B]. Paraffin sections of explants double-labeled with X-gal and PECAM-1 showed lumenized PECAM-1/X-gal-positive vessels close to the center of the explants [Fig. 3C]. Peripheral X-gal-positive filopodia-extending cells also stained with PECAM-1 [Fig. 3D], indicating that they were endothelial cells.

We then subjected individual endothelial tip cells to gradients of recombinant Netrin-1. The tip cell response was recorded by time-lapse videomicroscopy over 2 h and filopodial length was measured at the beginning and the end of the experiment [Fig. 3E–I]. Injection of Netrin-1 induced filopodial retraction in unc5b+/− and unc5b−/− tip cells [Fig. 3E–I; Supplementary Movie 1]. In contrast, unc5b+/− tip cells showed filopodial movement, but no significant filopodial retraction in response to Netrin-1 [Fig. 3E,H,I; Supplementary Movie 2]. Importantly, the absence of UNC5B did not result in a conversion of the repulsive response to an attractive one, i.e., an increase in filopodial length. Thus, Netrin-1 induces filo-
UNC5B activation inhibits neovascularization

in endothelial cells in vitro. Porcine aortic endothelial cells [PAECs] were stably transfected with a construct encoding full-length (FL) rat UNC5B fused to a C-terminal GFP-tag [UNC5B FL] or with a construct containing a deletion of the entire UNC5B cytoplasmic domain fused to GFP [UNC5B ΔCD]. Immunoblot analysis from PAEC lysates using anti-GFP and anti-UNC5B antibodies showed a single band of the expected size in each cell line, with expression levels of the UNC5B ΔCD clone being higher than for UNC5B FL [Supplementary Fig. 3].

To test for Netrin-1 effects on sprouting of UNC5B-expressing cells, UNC5B FL, ΔCD, or parental PAECs were seeded onto microcarrier beads and embedded in fibrin gels with or without recombinant Netrin-1. Sprouting of UNC5B ΔCD cells or parental PAECs was similar in the presence or absence of Netrin-1, whereas sprouting of UNC5B FL cells was significantly reduced in the presence of Netrin-1 [Fig. 4A–E]. Thus, expression of UNC5B was sufficient to mediate inhibition of endothelial sprouting in response to Netrin-1 in vitro, and sprouting inhibition required the presence of the UNC5B intracellular signaling domain.

To test for possible effects of Netrin-1 on PAEC proliferation, parental, UNC5B FL, and UNC5B ΔCD cells were cultured in low serum and stimulated with either fetal bovine serum [FBS] or different concentrations of recombinant Netrin-1. While robust proliferation was observed in response to serum, no proliferation was induced in the presence of Netrin-1 [Supplementary Fig. 3]. The effects of Netrin-1 on UNC5B-expressing endothelial cells thus involve sprouting rather than proliferation. In contrast to previous reports [Wilson et al. 2006], recombinant Netrin-1 also had no significant effects on HUVEC or HUAEC proliferation, while VEGF robustly stimulated proliferation of both cell types [Supplementary Fig. 3].

To examine if Netrin-1 induced changes in UNC5B receptor localization, subconfluent UNC5B FL and ΔCD PAECs in two-dimensional cultures were treated with recombinant Netrin-1 and observed by fluorescence microscopy. Control or BSA-treated UNC5B FL and ΔCD PAECs showed membrane fluorescence that was particularly strong at cell–cell junctions [Fig. 4F,I]. By 15 min after addition of Netrin-1 at 100 or 500 ng/mL, UNC5B FL PAECs showed membrane fluorescence that was particularly strong at cell–cell junctions [Fig. 4F,I]. By 15 min after addition of Netrin-1 at 100 or 500 ng/mL, UNC5B FL PAECs showed membrane fluorescence that was particularly strong at cell–cell junctions [Fig. 4F,I]. By 15 min after addition of Netrin-1 at 100 or 500 ng/mL, UNC5B FL PAECs showed membrane fluorescence that was particularly strong at cell–cell junctions [Fig. 4F,I]. By 15 min after addition of Netrin-1 at 100 or 500 ng/mL, UNC5B FL PAECs showed membrane fluorescence that was particularly strong at cell–cell junctions [Fig. 4F,I]. By 15 min after addition of Netrin-1 at 100 or 500 ng/mL, UNC5B FL PAECs showed membrane fluorescence that was particularly strong at cell–cell junctions [Fig. 4F,I]. By 15 min after addition of Netrin-1 at 100 or 500 ng/mL, UNC5B FL PAECs showed membrane fluorescence that was particularly strong at cell–cell junctions [Fig. 4F,I].

Figure 3. Unc5b-expressing endothelial tip cells retract filopodia in response to Netrin-1 aortic ring assays. (A,B) Sprouts growing from heterozygous unc5b lacZ-plap aortic rings into collagen gels stain with X-gal. B is a higher magnification of boxed area in A. (C,D) X-gal/PECAM-1 double staining of sections prepared from explants showing lumenized vessels close to the explant [C] and double-staining of tip cells [D]. [*] Tip cell nucleus. (E–I) Tip cell response to gradients of recombinant Netrin-1 recorded by time-lapse videomicroscopy. (E) Quantification of filopodial length at the beginning [t = 0] and the end of the time-lapse movies [t = 120 min]. [F–I] Responses of individual tip cells from unc5b+/− [G, I] and unc5b−/− [H, I] explants to gradients of recombinant Netrin-1 (gradient source indicated by black arrows in F and H). Still images from time-lapse movies at the indicated time points. White arrowheads point to filopodia. Filopodial length is indicated by black lines. Note filopodial retraction in G, but not in I. Total number of tip cells analyzed: six +/+, 17 +/−, and 16 −/−. Error bars, SEM; (*) P < 0.05; (*** P < 0.001, Mann-Whitney U-test. Bars: A, 170 µm; B, 85 µm; C,D, 20 µm; F–I, 45 µm.

UNC5B signaling mediates endothelial repulsion in response to Netrin-1

We asked if signaling from the UNC5B intracellular domain was required to transduce the response to Netrin-1...
Netrin-1 expression in tumor cells repels UNC5B-expressing endothelial cells

To determine if Netrin-1/UNC5B signaling could have a role in tumor angiogenesis, we first examined Netrin-1 expression in tumor cells. We screened 20 different human tumor cell lines of diverse origin for expression of netrins and their receptors by semiquantitative RT–PCR (Fig. 5). Most cell lines expressed netrin-4, but showed no or little expression of netrin-1 or netrin-3. Netrin receptors were also heterogeneously expressed by the tumor cell lines: neogenin was detected in most cell lines, ddc was detected in only one cell line, and expression of the unc5 receptors varied greatly between different lines (Fig. 5).

The low or undetectable levels of the UNC5B ligands netrin-1 and netrin-3 [Leonardo et al. 1997; Wang et al. 1999] were consistent with the absence of inhibitors and thus invasive sprouting of blood vessels into tumors derived from these cells. To test this idea, we examined effects of tumoral Netrin-1 overexpression on the behavior of UNC5B-expressing endothelial cells. Three cell lines were selected for expressing Netrin-1: Miapaca pancreatic cancer cells and Mel2a melanoma cells, which did not express any endogenous nettrins, and PC3 prostate carcinoma cells, which expressed netrin-4. We overexpressed human Netrin-1 in these cells using retroviral gene transfer. Western blot analysis with anti-His antibody recognizing an N-terminal tag [data not shown] or anti-mouse Netrin-1 antibodies, which recognize human Netrin-1, showed that Netrin-1-transduced cells secreted Netrin-1 protein into the medium [Supplementary Fig. 4]. The levels of Netrin-1 in cell supernatants were confirmed by solid phase receptor assay with plates coated with an UNC5B-Fc chimera (Miapaca: 148 ± 51 ng/mL; Mel2a: 98 ± 20 ng/mL; PC3: 19 ± 9 ng/mL) and were consistent with the immunoblotting results. Semi-quantitative RT–PCR showed that except for netrin-1, the expression of other netrins and their receptors was similar between Netrin-1-transduced and empty-vector-transduced or parental cells (Fig. 5; data not shown). Proliferation, apoptosis, and clone-forming ability in soft agar of empty vector- and Netrin-1-transduced cells were comparable [Supplementary Fig. 4]. Thus, retroviral Netrin-1 overexpression had no obvious effect on tumor cell behavior in vitro.

We then tested the response of UNC5B-expressing endothelial cells to Netrin-1 secreted from transduced tumor cells in vitro. Control or Netrin-1-expressing tumor cells were added to UNC5B FL or ΔCD PAECs and the PAEC response was recorded over a period of 24 h. Immunostaining of cocultures with a Netrin-1 antibody showed that Netrin-1-expressing, but not control-infected tumor cells exhibited Netrin-1 immunofluorescence (Supplementary Fig. 5). Netrin-1 immunoreactivity was also observed at the surface of UNC5B FL- and ΔCD-expressing PAECs adjacent to Netrin-1-producing
tumor cells, presumably indicating ligand binding to receptor. Brightest Netrin-1 immunofluorescence was seen on cytoplasmic GFP-positive extensions formed by UNC5B FL cells next to Netrin-1-producing tumor cells (Supplementary Fig. 3). Videomicroscopy showed that by 15 min after addition of Netrin-1-expressing tumor cells, UNC5B FL PAECs adjacent to the added tumor cells aggregated GFP-tagged receptor and retracted their cytoskeleton (data not shown). After 6 and 24 h, Netrin-1-producing tumor cells were spatially segregated from UNC5B FL cells, consistent with a repulsive effect of the tumor cells on endothelial cells expressing UNC5B (Fig. 6D–F). UNC5B-GFP aggregation or retraction of UNC5B FL PAECs was not observed with control-infected tumor cells, and the two cell populations were mixed after 24 h (Fig. 6A–C). Tumor cells expressing Netrin-1 did not induce retraction of UNC5B ΔCD-expressing PAECs, and the two cell populations were again mixed after 24 h (Fig. 6G–I). Quantification of the overlap between tumor cells and PAECs showed a significant reduction in cocultures of UNC5B FL PAECs with Netrin-1-secreting tumor cells (Fig. 6). Thus, Netrin-1 secreted by all three transduced tumor cell lines triggers endothelial cell repulsion after binding to UNC5B.

**Netrin-1 reduces sprouting of unc5b-positive vessels and delays tumor angiogenesis**

The effects of Netrin-1 overexpression on tumor angiogenesis were tested by subcutaneous xenografts of transduced Mel2a, Miapaca, and PC3 tumor cells into nude mice. Tumors were harvested at different time points post-implantation (n = 3 per group per time point) and examined for vascularization using unc5b in situ hybridization and PECAM-1 staining. In control tumors, unc5b expression was prominent in sprouting vessels (Fig. 7B,H; Supplementary Fig. 5). Unc5b expression was also observed around Netrin-1-overexpressing tumors (Fig. 7D, Supplementary Fig. 5). However, these vessels remained localized at the tumor periphery, avoiding Netrin-1-overexpressing tumor areas. Quantification showed a significant reduction of the number of unc5b-positive vessels as well as overall vessel number in Netrin-1-overexpressing compared with control tumors 1 and 2 wk post-implantation (Fig. 7E,K). At later stages post-implantation, the number of unc5b-expressing vessels remained significantly lower in Netrin-1-overexpressing compared with control tumors at all time points and in all tumors analyzed (Fig. 7E,K; Supplementary Fig. 5).
However, blood vessel density in Netrin-1-overexpressing tumors progressively increased and reached levels comparable with control-infected tumors. Thus, compared with controls, Netrin-1 overexpression delayed tumor vascularization, and this delay was correlated with the inhibition of sprouting of \textit{unc5b}-expressing vessels during the initial stages of neovascularization.

Consequently, overexpression of Netrin-1 had mainly inhibitory effects on tumor growth: It greatly inhibited the growth of Mel2a tumors and delayed the growth of PC3 tumors (Fig. 7F, Supplementary Fig. 5). While Netrin-1 overexpression did not significantly affect the growth of Miaapaca tumors (Fig. 7L), histological analysis of these tumors at 6 wk post-implantation showed necrotic tumor areas and a reduction of overall tumor angiogenesis (Fig. 7K, Supplementary Fig. 6). Phospho-histoneH3 staining to label proliferating tumor and endothelial cells in all tumor types at 6 wk post-implantation showed no difference in cell proliferation between Netrin-1-overexpressing tumors and controls (data not shown). CD45 staining revealed no difference in leukocyte invasion (data not shown). The effects of Netrin-1 overexpression on tumor angiogenesis and growth are thus likely a consequence of the initial inhibition of sprouting of \textit{unc5b}-expressing vessels.

We finally tested if UNC5B mediated the inhibitory effects of Netrin-1 overexpression on tumor angiogenesis. Heterozygous and homozygous \textit{unc5b lacZ-plap} knock-in mice received on each flank subcutaneous xenografts of PC3 tumor cells transduced with either control-vector or Netrin-1. Whole-mount collagen-IV staining of PC3 tumors was performed after 1 wk. Low magnification images of tumors implanted in heterozygous mice showed a significant reduction of the vascularized area in the Netrin-1-transduced compared with control tumors (Fig. 8A,B,D). Higher magnification images revealed active sprouting of skin vessels into control vector tumors (Fig. 8E,F) that was inhibited in Netrin-1-expressing tumors, resulting in a blunted appearance of vessels sprouts very similar to that observed in the ma-
UNC5B activation inhibits neovascularization

Figure 7. Netrin-1 overexpression reduces sprouting of unc5b-expressing vessels and delays tumor angiogenesis. [A,B] Mel2a control tumor 4 wk post-implantation. [B] Representative section hybridized with an antisense riboprobe against unc5b showing expression on sprouting vessels invading the tumor (blue, arrowheads). (A) Netrin-1 immunostaining (red) of the same section. Note absence of immunoreactivity in control tumor tissue. Red staining is due to background immunofluorescence of erythrocytes contained in blood vessels. [C,D] Mel2a Netrin-1-overexpressing tumor 3 wk post-implantation. Note that unc5b-positive vessels (blue, arrowheads) avoid Netrin-1-positive tumor areas. [E] Quantification of PECAM-1-positive and unc5b-positive blood vessels per surface area of the tumor in control and Netrin-1-overexpressing Mel2a tumors. [F] Growth curve of Mel2a tumor xenografts implanted in the dorsa of NMRI nu/nu mice. n = 10 mice per group. Data in E and K represent the quantification of two or three tumors per group and three to five sections per tumor. Error bars: SEM; (*) P < 0.05, Mann-Whitney U-test. Bars: A–D, 70 µm; G–J, 125 µm.

trigel experiments [Fig. 8G,H]. Comparison of control vector and Netrin-1-transduced PC3 tumors implanted into homozygous unc5b mutant mice showed no significant difference in vascularized area between Netrin-1 and control-transduced tumor cells [Fig. 8C,D], and the sprouting inhibition induced by Netrin-1 was silenced [Fig. 8I,J]. Thus, Netrin-1 inhibits tumor angiogenic sprouting via UNC5B.

Discussion
We found that expression of the unc5b receptor during postnatal and pathological angiogenesis is restricted to vessels undergoing active angiogenic sprouting. While adult, quiescent vessels were unc5b negative, re-expression was observed during sprouting angiogenesis induced by OIR and matrigel or tumor implantation. Re-expression of unc5b mRNA in neovessels recapitulates the embryonic expression pattern of this receptor [Lu et al. 2004], with high levels seen in arteries and capillary sprouts and lower levels in veins. Interestingly, neovascularization following femoral artery ligation did not lead to endothelial unc5b re-expression, suggesting that this form of angiogenesis, which may not require extensive sprouting, can occur in the absence of UNC5B and that UNC5B may be preferentially required during sprouting angiogenesis. Unc5b transcription is thus part of the active angiogenesis process, although the precise molecular mechanisms regulating its transcription remain to be determined.

Sprouting angiogenesis was reduced after activation of the UNC5B receptor by its ligand Netrin-1. Live imaging in aortic ring assays demonstrated that unc5b-expressing capillary sprouts respond to Netrin-1 stimulation by retraction. Sprouts derived from unc5b−/− mice lose response to Netrin-1. No conversion of repulsion to attraction was observed in the absence of UNC5B in these experiments. Similarly, Netrin-1 alone showed no pro-angiogenic activity in matrigel plugs but potently inhibited bFGF-induced neovessel sprouting. Examination of Netrin receptor mRNA levels in matrigel plugs showed the presence of unc5b mRNA, but the absence of ddc [Nguyen and Cai 2006] and very low levels of neogenin. The presence of repulsive, but not attractive, receptors is consistent with the observed inhibitory Netrin-1 effect. Moreover, Netrin-1-induced inhibition of neovascularization was reduced in unc5b−/− mice, indicating a requirement for UNC5B. Taken together, these data suggest that activation of UNC5B during sprouting angiogenesis triggers repulsive responses of these vessels.

GENES & DEVELOPMENT 2441
Determining the localization of the endogenous UNC5B ligand(s) during postnatal and adult life will establish the physiological relevance of UNC5B signaling during sprouting angiogenesis. This signaling pathway could be part of a negative feedback mechanism to ensure appropriate vessel patterning and to prevent excessive vessel sprouting. A similar negative feedback mechanism involves the endothelial Notch ligand Delta-like 4 (DLL4) (Hellstrom et al. 2007; Lobov et al. 2007; Suchting et al. 2007). DLL4, expressed on endothelial tip cells, binds to Notch receptors on adjacent stalk cells, preventing these cells from also forming sprouts by reducing their response to VEGF. Notch and UNC5B signaling may thus both contribute to angiogenic homeostasis. Alterations in unc5b expression in dll4 mouse mutants (Suchting et al. 2007) suggest a link of these pathways that remains to be fully investigated.

UNC5B could mediate repulsive signaling in response to Netrin-1 directly or, alternatively, be part of a signaling complex involving one or more coreceptor(s). We tested these possibilities using PAECs. Native PAECs fail to respond to Netrin-1 in sprouting and proliferation assays. When transfected with UNC5B FL, but not ΔCD constructs, these cells respond to recombinant Netrin-1 by reduced sprouting and repulsion. Coculture of Netrin-1-producing tumor cells with UNC5B FL, but not ΔCD cells, led to segregation of the two populations over time, clearly indicating a repulsive effect of Netrin-1 mediated by UNC5B signaling. As the extracellular domain of UNC5B can bind Netrin-1 [Hong et al. 1999; data not shown], these results indicate that UNC5B is sufficient to directly mediate Netrin-1 responses in PAECs. Deletion of the cytoplasmic domain of UNC5B abolished the repulsive response to Netrin-1, indicating that this domain is required to trigger downstream signaling events that remain to be fully elucidated (Round and Stein 2007).

Expression of the UNC5B ligands netrin-1 and netrin-3 in 20 different human tumor cell lines was low, while many of these cell lines expressed the related netrin-4, which, however, does not bind UNC5B (see above and Wilson et al. 2006). There are, as yet, few studies of netrin expression in human cancer, however, two studies describe down-regulation of netrin-1 in prostate tumors (Latil et al. 2003) and in a subset of brain tumors and neuroblastomas [Meyerhardt et al. 1999] compared with normal healthy prostate or brain tissue. These data are consistent with the idea that invasive sprouting of blood vessels into tumors is facilitated in the absence of UNC5B ligands, although a more comprehensive investigation of human tumors is required. They are also consistent with our finding that Netrin-1 overexpression in tumor cells led to inhibition of sprouting of unc5b-expressing neovessels and delayed tumor angiogenesis. Inhibition of neovascularization was most pronounced during the initial stages of tumor invasion and likely mediated by UNC5B, as Netrin-1-overexpressing and control tumors implanted into unc5b-deficient mice exhibited similar vascularization. Inhibition of vascularization in Netrin-1-overexpressing tumors up to 2 wk

Figure 8. Netrin-1-induced inhibition of tumor vessel sprouting requires UNC5B. (A–C) Whole-mount Collagen-IV staining of thick sections from PC3 control [A] and Netrin-1-overexpressing [B, C] tumors implanted into unc5b heterozygous [A, B] and homozygous [C] mutant mice. The tumor nodule forming beneath the skin is outlined [dashed, white]. Vessels invading the tumor are outlined [dashed, white]. Vascular area within the tumors is reduced by Netrin-1 in unc5b heterozygous [B] compared with control [A], but not homozygous mutant [C], mice. (D) Computer-assisted quantification of vascular area in tumor nodules. n = 3 tumors per group, four thick sections per tumor (corresponding to the entire tumor) were photographed and quantified. (E–J) Higher magnifications of vessels sprouting from the skin into the tumor area. [E, G, I] Whole mounts were double-stained with Collagen-IV and X-gal. In the overlays shown in F, H, and J, Collagen-IV staining appears as purple color on the dark-blue X-gal staining. Note double-positive vessels (arrowheads) sprouting into control PC3 tumors [F], blunted appearance of vessel sprouts in Netrin-1-overexpressing tumors implanted into unc5b homozygous mutant mice [H], and restored sprouting when Netrin-1-overexpressing tumors are implanted into unc5b homozygous mutant mice [J]. Error bars: SEM. *P < 0.05, Mann-Whitney U-test. Bars: A–C, 800 μm; E–J, 130 μm.
post-implantation led to significant tumor hypoxia [data not shown], presumably inducing high levels of VEGF expression, which could explain why at later stages post-implantation tumor vessel density between Netrin-1-overexpressing and control-transduced tumors reached comparable levels. As only their sprouting was inhibited in the presence of Netrin-1, tumor vessels may switch to other types of growth such as circumferential growth or intussusception (Augustin 2001).

Our results clearly show that UNC5B functions as a receptor for Netrin-1 in vivo, confirming and extending previous studies [Leonardo et al. 1997; Hong et al. 1999, Lu et al. 2004]. It remains to be determined if netrin-1 represents the [only] relevant in vivo ligand for UNC5B in mice. In zebrafish embryos, MO-mediated knockdown of unc5b or netrin-1a led to increased filopodial extensions and aberrant vessel branching of intersegmental vessels [ISV] [Lu et al. 2004]. We have repeated knockdowns using the splice-blocking MO reported by Wilson et al. [2006]. We find that we can fully recapitulate our previous result, i.e., aberrant ISV branching from the dorsal aorta [Supplementary Fig. 7; Supplementary Movie 5]. Our data in zebrafish are thus consistent with netrin-1a as a negative regulator of vessel branching. However, the results reported here do not exclude a possible proangiogenic role of Netrin-1. Nonendothelial cells in the ischemic area expressing unc5b [and perhaps other Netrin receptors] could respond to Netrin-1 and perhaps contribute to ischemic revascularization. In addition, we have not observed endothelial unc5b expression following femoral artery ligation, and stimulation of UNC5B-negative endothelial cells by Netrin-1 could elicit proangiogenic responses. The present study provides multiple lines of evidence indicating that repulsive responses following Netrin-1 stimulation are consistently observed during neovascularization processes where unc5b is expressed, including tumor angiogenic sprouting. Development of UNC5B-selective agonists may be considered as potential therapeutic tools in antiangiogenic strategies.

Materials and methods

Animals

CD1 unc5b LacZ-plap mice were described previously [Lu et al. 2004] and genotyped with the following modifications: primers unc5b wild-type forward, 5'-TGTTGAGAAATTTGATGTGCAGCC-3', and reverse, 5'-GAGGAGAGACCAACCGATGTGAC-3'; mutant forward, 5'-TGTGAGTCACACTGCTCTGCT-3', and reverse, 5'-ATGAGCTGGATGCAGGTGCAGC-3', using 6 pmol of each in a 25-µL PCR reaction [95°C/25 sec, 62°C/30 sec, 72°C/55 sec] × 35 cycles. C57Bl/6 mice were obtained from Charles River Laboratories. NMRI nu/nu mice were obtained from Elevage Janvier and housed in sterile conditions.

Immunohistochemistry and in situ hybridization

For X-gal or AP whole-mount immunostaining, tissues were dissected, washed in PBS, fixed for 10 min at room temperature in 4% PAF, rinsed in PBS, and incubated at 37°C with X-gal staining solution [Lu et al. 2004] or at 65°C for 1 h and subsequently at 37°C in AP developing buffer as described [Leighton et al. 2001]. Whole-mount immunohistochemistry of retinas was done as described [Suchting et al. 2007], using biotinylated IsoldestinB2 (Vector). Images were taken using a Leica TCS SP2 confocal microscope or an Olympus fluorescence microscope. For immunostaining of paraffin or cryostat sections, the following antibodies were used: anti-PECAM-1 [BD Pharmingen], anti-Netrin-1 [R&D], anti-CD45 [Biolegend], and anti-Phospho-His-tone-H3 [Abcam], followed by Alexa 488- or Alexa 555-conjugated secondary antibodies [Molecular Probes], HRP-conjugated secondary antibodies, or tyramide signal amplification using a TSA kit [PerkinElmer]. Nuclei were stained by incubating sections in PBS containing 1 µg/mL 4'-6-Diamidino-2-phenylindole [DAPI, Sigma] for 5 min. For histological analysis of tumors, sections were stained with hematoxylin and eosin (H&E).

For in situ hybridization on paraffin sections [Lu et al. 2004], proteinase K treatment [6 µg/mL] was done for 7 min at 37°C and a 1-kb fragment of rat unc5b [nucleotides 200–1271] was used as a riboprobe. Images were taken using an Olympus microscope.

PCR

For RT–PCR, total cellular RNA was extracted from tumor cells or matrigel plugs using RNAble [Eurobiol] according to the manufacturer’s instructions. Three micrograms of purified total RNA were used as a template to generate first strand cDNA using Moloney murine leukemia virus reverse transcriptase [Invitrogen]. PCR was performed using the following primer pairs: human A2b, 5'-CTATGTCCTACCCGAGGAGC-3' and 5'-ACATGCCCAGGGGAAATTAAAT-3'; human DCC, 5'-AACACTCTGCTAGGACACCC-3' and 5'-CTCCTTAACTGACTGCTGGTTAC-3'; human Unc5A, 5'-CCAGGTCCATGGCCTACACTC-3' and 5'-ATCTGGACGGCATAGTCC-3'; human Unc5B, 5'-AGCTGTCCCTTAATGGCTGGT-3' and 5'-AAAGGCTGTTACATAAGGCC-3'; human Unc5C, 5'-ATTCTGGCCGTCGTGATCTC-3' and 5'-ACAAACCCGGCTGACCT-3'; human Unc5D, 5'-GGTCCCAGCATCATCCTCAGT-3' and 5'-TCCTTAACTGACTGCTGGTTAC-3'. Amplification was carried out with an iCycler (Bio-Rad) [95°C/2 min], 95°C/25 sec, 57°C/5 sec, 68°C/5 min] × 35 cycles. C57Bl6 mice were obtained from Elevage Janvier and housed in sterile conditions.

UNC5B activation inhibits neovascularization
Larriveé et al.

For qPCR, retinal endothelial cells were isolated from P5 and P60 CD1 mice as described (Suchting et al. 2007), with the following modifications: Cells were incubated for 20 min at 4°C with rat anti-mouse-PECAM-1 (BD Pharmingen) [5 μg], and endothelial cells were separated with sheep anti-rat IgG magnetic beads (Dynabeads). QPCR was performed in an iCycler Real-Time PCR detection system (Bio-Rad) using SYBR Green PCR master mix (Qiagen) and the thermocycler conditions recommended by the manufacturer. PCRs were performed in triplicate using 1 μL of cDNA in a total volume of 25 μL. Each sample was analyzed for β-actin to normalize for RNA input and PECAM-1 to normalize for endothelial cell gene expression. Primers were as follows: mouse unc5b, 5'-GATGTCCTATGGTTGATT TGG-3' and 5'-CATAGGAAAGCTTCTGGTG-3'; mouse Pecam-1, 5'-CAGGACGTAGAAGCTG-3' and 5'-TC TAACCTGGCTGGAGA-3'; mouse β-actin, 5'-TGTATAC CAACCTGGGACGACA-3' and 5'-GGGGTGTTGAAGGTCT GAA-3'. Melting curve analysis showed a single sharp peak with the expected Tm for all samples.

Matrigel plug experiments

Ice-cold matrigel (BD) was mixed with PBS, 0.2% BSA, bFGF (R&D), and/or mouse Netrin-1 (R&D) and kept on ice. Mice were anesthetized with ketamine/Rompun, and 500 μL of matrigel was injected subcutaneously at the base of the tail. After 2 wk, mice were killed by cervical dislocation and homogenized in OCT (Tissue-Tek), and frozen. Forty-micron sections (0.5% glutaraldehyde. Matrigel plugs were removed, embedded in OCT (Tissue-Tek), and frozen. Forty-micron sections were imaged by time-lapse videomicroscopy for 2 h under an inverted microscope (Leica). Filopodial length of individual tips was measured using Metamorph software.

Constructs

The FL rat unc5b cDNA in pMT21 vector was recloned in pCDNA3,1A(-) vector (Invitrogen) in-frame with the myc/his tag with the following primers to add restriction sites and mutate the stop codon: 5'-GCTCTAGAGGCGCCCGGACGG CGCCGGG-3' and 5'-CGGAATTCTGGAATCCGGATCTAG CGTGATGGC-3'. The UNC5B construct corresponding to the 424 first amino acids and without most of the cytoplasmic part (ΔCD) was obtained by restriction digest of the FL construct by XbaI and HindIII. Both FL and ΔCD constructs were inserted in pEGFP-N1 vector (Clontech) in-frame with the GFP sequence.

A FL human netrin-1 cDNA was cloned by RT–PCR using oligo-dT(23)VN-primed human embryo polyA-RNA (Clontech; 100 ng) and RNA SuperScript III (Clontech; 200 U) followed by PCR amplification of 1/10 of the RT reaction [95°C/2 min, 95°C/18 sec, 68°C/8 min] × 35, 72°C/10°C] using Pfu DNA polymerase [Promega; 1 U] with 10 pmol of the following primers: forward, 5'-TCTAGATGGGCGAGCTGATCGGC GCAGCTG-3'; reverse, 5'-GGTACCGCCTTCTTCTCAG TGCCCTTCTTCTC-3'. PCR product was gel-purified, adapted to a pCRII-TOP cloning vector (Invitrogen) after 20 min treatment at 72°C with T4 DNA ligase, and sequenced. XbaI-KpnI digestion of this vector was cloned into pcDNA3.1myc-his. H-netrin-1 with the myc and his tags was then cloned in the retroviral vector MSCVneo (Clontech).

PAE cell assays

UNC5B FL or ΔCD PAECs were seeded on eight-well slides (BD) coated with fibronectin at a concentration of 10,000 cells per well or in fibronectin-coated two-well Lab-tek chambered coverglasses (Nunc) [80,000 cells per well] for time-lapse videomicroscopy. The next day, PAECs were incubated with recombinant mouse Netrin-1 (R&D) [0, 10, 100, 500 ng/mL] for 30 min (10 experiments with duplicates for each Netrin-1 concentration) or with recombinant mouse Netrin-1 [300 ng/mL] for time-lapse videomicroscopy.

For sprouting assays, gelatin-coated microcarrier beads (Cy-todex 3, Sigma) were incubated with PAECs. When the cells reached confluence on the beads, equal numbers of PAEC-coated beads were embedded in fibrin gels in 96-well plates. For preparation of fibrin gels, bovine fibrinogen (2.5 mg/mL Sigma) was dissolved in Dulbecco’s modified eagle medium (DMEM)/10% FBS. Aprotinin (0.05 mg/mL Sigma) was added, and the solution was filtered through a 0.22-μm pore-size filter. In some cases, fibrinogen solution was supplemented with mouse Netrin-1 [300 ng/mL]. Following transfer of the fibrinogen solution to 96-well plates, PAEC-coated beads were added at a density of 50 beads per well, and clotting was induced by the addition of thrombin [1.2 U/mL] (Sigma). Following 60 min of incubation, DMEM/10% FBS, either alone or containing 300 ng/mL Netrin-1, was added to the wells. After 24 h, the number of capillary-like tubes formed was quantitated using Metamorph software.
For coculture experiments, transfected PAECs [20,000 cells per well] were plated onto eight-well slides in complete DMEM. The next day, vector control or Netrin-1-expressing tumor cells were stained with PKH26 dye (Sigma) according to the manufacturer’s instructions, and 20,000 cells per well were added to the wells previously seeded with PAECs. Two hours to 24 h later, slides were washed in PBS, fixed in 4% PAF for 5 min, mounted in polyvinyl alcohol mounting medium, and visualized by confocal microscopy.

Cells and retroviral transduction

Tumor cells, PT67 retroviral packaging cells (Clontech), and PAECs were cultured in DEMEM supplemented with 10% FBS (Gibco-Invitrogen) and penicillin-streptomycin (Gibco-Invitrogen). PAECs were transfected with UNCS5 FL or ΔCD constructs using Fugene-6 (Roche) according to the manufacturer’s instructions. Stably transfected clones were selected using G418 [1.2 mg/mL] for 14 d. For each construct, one clone that exhibited strong uniform fluorescence at the surface of all cells was selected for further analysis and expanded. For generation of amphotropic packaged virus, PT67 packaging cells were transfected with MSCPneo-Netrin-1 using Fugene-6. Cell selection was done with 500 µg/mL G418 48 h after transfection. Drug-resistant cells were then expanded and subcultured. For retroviral infection of tumor cells, supernatant was harvested from stably transfected PT67 cells, filtered, and used for repeated infections of tumor cells in the presence of 8 µg/mL polybrene (Sigma). After 48 h, retrovirally infected cells were selected using 700 µg/mL G418. For all experiments, cells transduced with the empty vector MSCPneo were used as controls. Numerous aliquots were frozen, and the same batch of infected cells was used for all experiments.

Determination of the level of hNetrin-1 expression in transduced tumor cells

Tumor cells were grown for 48 h in serum-free DMEM, lysed in buffer containing 1% NP-40, 0.5% sodium deoxycholate, and 0.1% sodium dodecyl sulfate (SDS), with addition of fresh protease inhibitor cocktail (Sigma). Supernatant [7 mL] was concentrated using an Amicon Ultra Centrifugal Device (Millipore). Twenty microliters of 500-µL concentrated supernatant were separated by SDS-PAGE, transferred to PVDF membranes (Millipore), and developed by enhanced chemiluminescence (PerkinElmer). For solid phase receptor assay, concentrated supernatant was incubated on 96-well plates precoated with collagen-1 (BD) in EBM (Clonetics) containing 0.3% FBS. After 24 h, cells were stimulated with recombinant mouse Netrin-1 [R&D] or human VEGF 165 [R&D] every day for 3 d. Twenty-four hours after the third stimulation, cell quantity was measured with CellTiter-Glo (Promega) and luminescence was analyzed with a Fusion plate reader [Packard].

PAECs were seeded on a 96-well plate at 5000 cells per well in DMEM/0.3% FBS and analyzed as above.

Soft agar colony-forming assay

One-thousand-five-hundred cells from each tumor cell line were placed onto six-well plates in DMEM containing 0.3% FBS. After 24 h, cells were stimulated with recombinant mouse Netrin-1 (R&D) or human VEGF 165 (R&D) every day for 3 d. Twenty-four hours after the third stimulation, cell quantity was measured with CellTiter-Glo (Promega) and luminescence was analyzed with a Fusion plate reader [Packard].

Tumor xenografts

Tumor cells [5 × 10⁶ per mouse] were harvested, mixed with an equal volume of Matrigel, and injected in the right dorsa of 4- to 6-wk-old nude mice (NMRI nu/nu; 10 mice per group). The experiment was repeated with a second group of five mice per transduced cell line, with similar results. Tumor size was determined by caliper measurements and tumor volume was calculated by a rational ellipse formula [length × width × depth × 0.5236]. Mice were sacrificed after the tumor grew to <500 mm³. Tumors were excised, weighed, and placed onto six-well plates in DMEM containing 0.3% FBS. After 24 h, cells were stimulated with recombinant mouse Netrin-1 (R&D) or human VEGF 165 (R&D) every day for 3 d. Twenty-four hours after the third stimulation, cell quantity was measured with CellTiter-Glo (Promega) and luminescence was analyzed with a Fusion plate reader [Packard].

Statistical analysis

A two-tailed Mann-Whitney U-test was used for quantification of aortic ring assays, tumor growth, blood vessel counts of matrigel plugs, and measurements of cocultures. All countings were done by two independent observers blinded to the experimental conditions. Dunnett’s test was used for the VEGFR-2 concentrations. A Kruskal-Wallis test was used for hemoglobin concentrations. A Kruskal-Wallis test was used for the VEGFR-2 concentrations. A Kruskal-Wallis test was used for the VEGFR-2 concentrations. P < 0.05 was considered statistically significant.

Zebrafish analysis

Tg(fli1:EGFP)⁺² zebrafish (Lawson and Weinstein 2002) were maintained under standard laboratory conditions. The follow-
ing morpholino oligonucleotide targeting netrin-1a was purchased from GeneTools LLC: 5'-ATGATGGAATTACCCGAC ACATTCGT-3' [Wilson et al. 2006]. Different doses [7.2, 9, and 10.8 ng] of morpholino were injected into single- to four-cell stage zebrafish embryos, using procedures described previously [Stalmans et al. 2003]. Between 30 and 60 injected embryos were analyzed per experiment from 20 h post-fertilization (hpf) to 72 hpf to identify alterations in sprouting of intersegmental vessels and in the formation of the parachordal vessel of the trunk region. Each experiment was repeated at least three times. The penetrance of the phenotype was scored by counting the affected embryos. Images of Tg(fli1:EGFP)y1 embryos were acquired with a Zeiss CLSM510 META NLO camera installed on an inverted AxioVert 200M microscope [Zeiss] equipped with a 1.5-W pulsed Ti:Sa Chameleon laser [Coherent] at minimum laser output.

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Activation of the UNC5B receptor by Netrin-1 inhibits sprouting angiogenesis

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