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Short Article

Inhibition of Endothelial Cell Apoptosis by Netrin-1 during Angiogenesis

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SUMMARY

Netrin-1 was recently proposed to play an important role in embryonic and pathological angiogenesis. However, data reported led to the apparently contradictory conclusions that netrin-1 is either a pro- or an antiangiogenic factor. Here, we reconcile these opposing observations by demonstrating that netrin-1 acts as a survival factor for endothelial cells, blocking the proapoptotic effect of the dependence receptor UNC5B and its downstream death signaling effector, the serine/threonine kinase DAPK. The netrin-1 effect on blood vessel development is mimicked by caspase inhibitors in ex vivo assays, and the inhibition of caspase activity, the silencing of the UNC5B receptor, and the silencing of DAPK are each sufficient to rescue the vascular sprouting defects induced by *netrin-1* silencing in zebrafish. Thus, the proapoptotic effect of unbound UNC5B and the survival effect of netrin-1 on endothelial cells finely tune the angiogenic process.

INTRODUCTION

Netrin-1, originally described as an axon guidance molecule, has recently been shown to function in extraneural processes as well, ranging from a role in branched organs morphogenesis to a function in angiogenesis (Cirulli and Yebra, 2007; Liu et al., 2004; Lu et al., 2004; Wilson et al., 2006). Interestingly, conflicting results were reported regarding the role of netrin-1 during angiogenesis. However, two clear genetic results initially appeared to be at odds: whereas Eichmann, Tessier-Lavigne, and colleagues elegantly demonstrated that the genetic inactivation of *UNC5B* in mice is associated with increased angiogenesis, thereby suggesting an antiangiogenic activity of netrin-1, Li and colleagues showed that the inactivation of *netrin-1a*, which encodes a ligand for UNC5B, is associated with a loss of vessels during zebrafish development (Lu et al., 2004; Wilson et al., 2006).

We investigated here the possibility that these two apparently contradictory observations could be explained by the ability of netrin-1 to block apoptosis that would otherwise be induced by its unbound UNC5B receptor, thus promoting survival of endothelial cells during angiogenesis. Indeed, netrin-1 prevents epithelial cell death by acting as a ligand for the dependence receptors DCC and UNC5A–D, which share the functional property that they are active both in the presence and in the absence of their trophic ligands: whereas ligand binding triggers positive signaling, dependence receptors induce a separate and distinct signaling cascade, leading to cell death when disengaged from their trophic ligands (Llambi et al., 2001; Mehlen et al., 1998; Tanikawa et al., 2003; Bredesen et al., 2005; Mehlen and Bredesen, 2004). In this scheme, the loss of *UNC5B*—i.e., loss of proapoptotic signaling—would indeed be associated with an increased number of blood vessels (Lu et al., 2004). On the contrary, loss of *netrin-1*—i.e., increase of proapoptotic signaling—is expected to be associated with the disappearance of vessels (Wilson et al., 2006). Such a dynamic balance between endothelial cell survival and apoptosis could help to maintain blood vessel integrity, which is, in turn essential for vascular homeostasis, both during vascular development and pathological angiogenesis, as previously described (Alavi et al., 2003; Carmeliet, 2005; Duval et al., 2003; Santoro et al., 2007; Winn and Harlan, 2005). Thus, the initially contradictory results of Eichmann et al. and Li et al. are readily resolved by the dependence receptor hypothesis, and indeed provide further in vivo support for this hypothesis.

RESULTS

Netrin-1 Blocks Endothelial Cell Apoptosis Induced by Its Unbound UNC5B Receptor

To determine whether netrin-1 blocks apoptosis induced by its unbound UNCB receptor, we first determined which netrin-1 dependence receptors are expressed in human umbilical vein/artery endothelial cells (HUVECs/HUAECs). As revealed by quantitative RT-PCR, UNC5A and UNC5B are both expressed in HUVECs and HUAECs (Figure 1A), whereas we failed to detect UNC5C, UNC5D, and DCC (data not shown). Netrin-1 was not expressed in these cells, either at the RNA (Figure 1A) or protein level (see Figures S1A and S1B available online). We then investigated whether netrin-1 behaves as a survival factor for endothelial cells by analyzing its ability to block the spontaneous propensity of HUVECs and HUAECs to undergo cell death under serum starvation. Netrin was used at 50 ng/ml in all in vitro

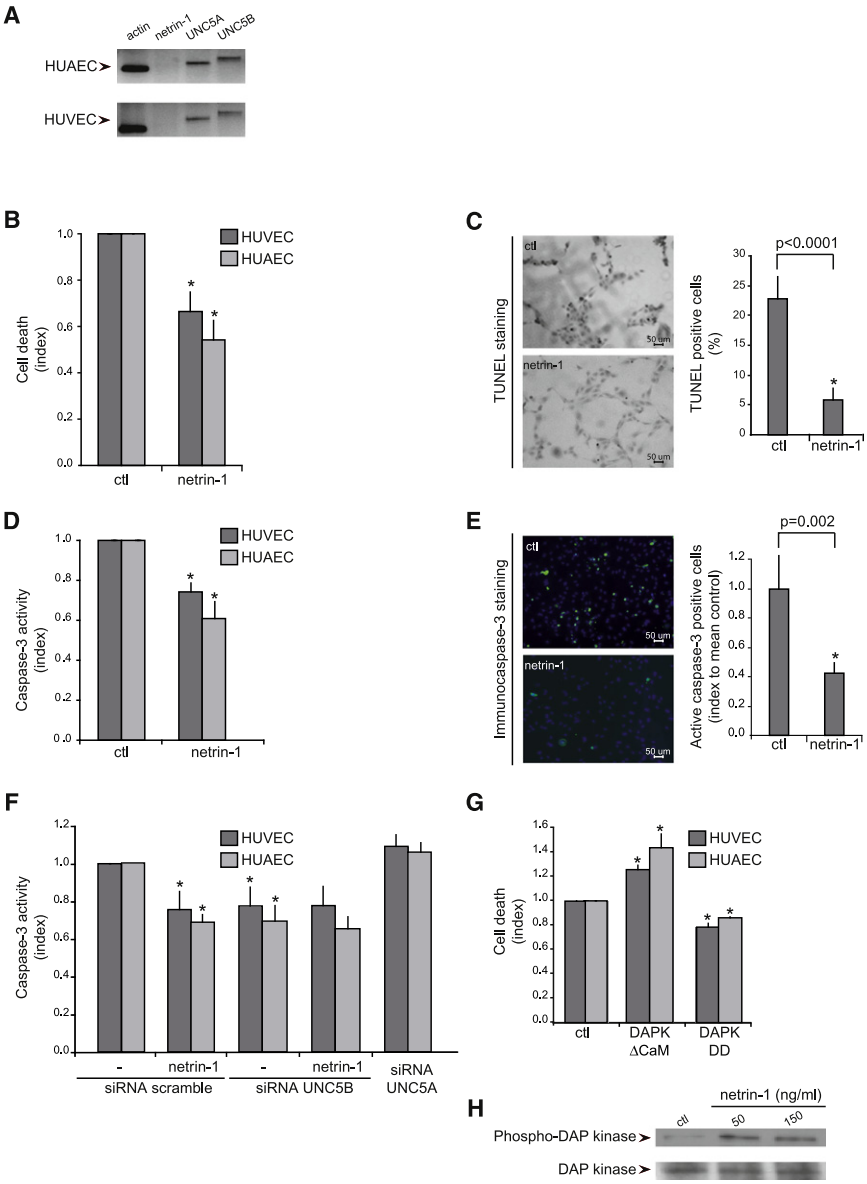


Figure 1. Netrin-1 Prevents Endothelial Cell Apoptosis, Probably via Inhibition of UNC5B-Induced Apoptosis

(A) Human umbilical vein/artery endothelial cells (HUVECs/HUAECs) express UNC5A and UNC5B dependence receptors, but not netrin-1. Quantitative RT-PCRs were performed as described in Experimental Procedures.

(B) Netrin-1 treatment inhibits HUVEC/HUAEC death observed upon serum starvation, as measured by trypan blue exclusion. At least 100 cells per condition were counted. The relative index is shown as the mean ± SEM (n = 3). *, p value < 0.005; **, p value < 0.0001 (Student's t test).

(C) Netrin-1 prevents apoptosis in HUVECs, as measured by TUNEL staining. Quantification of TUNEL-positive cells is mean ± SEM (Student's t test).

(D and E) Netrin-1 treatment reduces caspase-3 activity in HUVECs and HUAECs. Relative index of caspase-3 activity is expressed as the mean ± SEM (n = 3). Immunostaining and the relative index (mean ± SEM) of cleaved caspase-3-positive cells are shown (Student's t test).

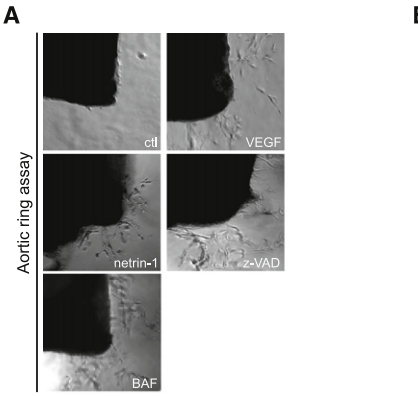
(F) *UNC5B*, but not *UNC5A*, silencing by siRNA is associated with a decrease in caspase-3 activity. Values are means and SEM (n = 3). All p < 0.05 (Student's t test; compared to the level in the control condition).

(G) DAP kinase mediates UNC5B-induced apoptosis in HUVECs and HUAECs. Cell death (Toxilight) in endothelial cells after forced expression of DAP kinase ΔCaM (the constitutively active mutant form of DAP kinase) and of the DAP kinase Death Domain (the dominant-negative form of DAP kinase). Values are means and SEMs. All p < 0.001 (Student's t test; compared to the level in the control condition).

(H) Netrin-1 treatment induces DAP kinase phosphorylation. Anti-phospho-DAP kinase was used to determine the level of enzyme activity.

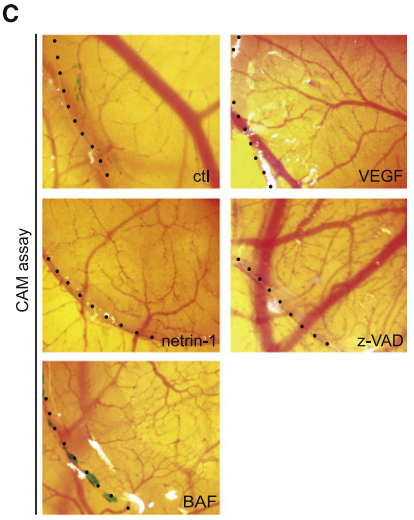
experiments because this dose was previously described to be physiological and functional (Serafini et al., 1994, 1996) (Figure S1C). As shown in Figure 1B, netrin-1 inhibited both HUVEC and HUAEC death (p < 0.005). Similar results were obtained for etoposide-induced cell death (Figure S1D). Netrin-1 prevented endothelial cell apoptosis, as shown by the decreased number of TUNEL-positive cells (Figure 1C) and by the reduced caspase-3 activity (Figures 1D and 1E) (up to 30% for HUVECs and 40% for HUAECs; all p < 0.005). The addition of a recombinant protein corresponding to the extracellular domain of UNC5H2 prevented netrin-1-mediated cell death inhibition, supporting the conclusion that the survival effect is specific for netrin-1 (Figure S1E). Thus, netrin-1 behaves as a survival factor for endothelial cells.

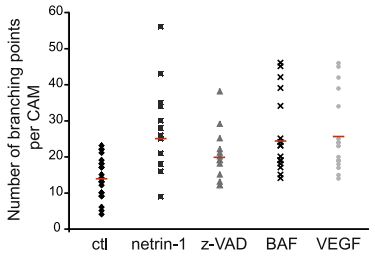
To determine whether UNC5B and/or UNC5A induce apoptosis in endothelial cells, we transiently silenced their expression by using a siRNA strategy (Figure 1F; Figures S1F and S1G). *UNC5B* silencing led to a decreased caspase-3 activity, whereas UNC5A siRNA had no effect on caspase-3 activity (Figure 1F; all p < 0.05). Moreover, the addition of netrin-1 together with *UNC5B* silencing was not associated with an additional survival effect (Figure 1F). Thus, the netrin-1 survival effect on endothelial cells depends on *UNC5B* expression and likely results from an inhibition of apoptosis that would otherwise be triggered by unbound UNC5B. Because UNC5B-induced apoptosis has been shown to be mediated by DAP kinase, we first expressed the constitutively active DAP kinase, DAPK ΔCaM (Shohat et al., 2001), in HUVECs and HUAECs. As shown in Figure 1G, expression of DAPK ΔCaM led to increased cell death (all p < 0.0001). We next forced expression of the dominant-negative mutant of DAP kinase, DAPK DD (Llambi et al., 2005). Similarly to netrin-1 treatment, DAPK DD prevented cell death induction. Moreover, because UNC5B proapoptotic signaling is mediated by the activation of DAP

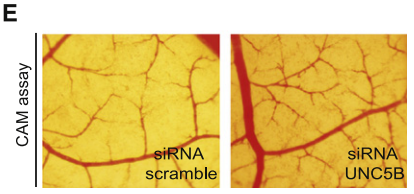


B

	% of aortic rings with vessels length		p-value (χ^2 test)
	<500pi	>500pi	
ctl (n=16)	58.8	41.2	
netrin-1 (n=14)	21.4	78.6	p<0.0001
z-VAD (n=14)	35.7	64.3	p=0.001
BAF (n=8)	25.0	75.0	p<0.0001
VEGF (n=8)	37.5	62.5	p=0.003







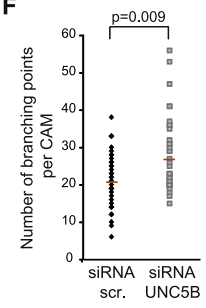


Figure 2. Caspase Inhibitors and *UNC5B* Silencing Mimic Netrin-1's Effect on Neovessel Formation

(A and B) The netrin-1 or caspase inhibitors z-VAD-fmk and BAF promote microvessel formation in an ex vivo murine aortic ring matrigel assay. Aortic rings resected from at least eight different mice are shown per condition. Quantification of microvessel total length (pixels units) was performed as described in Experimental Procedures. All p values are indicated (χ_2 test).

(C and D) Netrin-1 and caspase inhibitors induce neovessel formation in a CAM assay. CAM models were prepared by using 8-day-old chick embryos treated as described in Experimental Procedures ($n \geq 10$). Methylcellulose disks are outlined. De novo angiogenesis is expressed as the number of branching points ($n \geq 13$ fields per condition). Means are indicated by straight lines. *, p value < 0.02; **, p value < 0.0005 (Mann-Whitney U-test).

(E and F) *UNC5B* silencing by siRNA leads to an increase in neovessel formation in a CAM assay. Three siRNA injections were performed between E.5 and E.9, as described in Experimental Procedures. Scramble siRNA was used as a control. De novo angiogenesis is expressed as the number of branching points ($n > 30$ different fields per condition). Means are indicated by straight lines. p value = 0.009 (Mann-Whitney U-test).

kinase through the inhibition of DAP kinase autophosphorylation (Llambi et al., 2005), we next measured the DAP kinase phosphorylation level. As shown in Figure 1H, netrin-1 treatment led to an increase in DAP kinase phosphorylation in HUVECs, presumably by blocking the UNC5B proapoptotic signaling cascade. Together with the observed increase in the number of blood vessels in *UNC5B* mutant embryos (Lu et al., 2004), these in vitro data support the view that netrin-1 is capable of blocking UNC5B-induced apoptosis during angiogenesis.

The Netrin-1 Proangiogenic Effect Is Mimicked by Caspase Inhibition and by Silencing of *UNC5B*

We next challenged the notion that the proangiogenic activity of netrin-1 is related to its ability to promote cell survival, by using ex vivo models of neoangiogenesis. We first used a mouse aortic ring assay and assessed the expression of *UNC5H2*, the murine ortholog of *UNC5B*, in the aortic explants (Figure S2A). Netrin-1 was not found to be expressed in the explants (data not shown). Similarly to what has been observed with VEGF, netrin-1 treatment enhanced capillaries sprouting from matrigel-embedded aortic rings, in comparison to control conditions (Figures 2A and 2B; $p < 0.001$), supporting a proangiogenic activity of netrin-1. Interestingly, caspase inhibitors z-VAD-fmk and BAF also induced neocapillary tube formation, mimicking the effect of netrin-1 (Figures 2A and 2B; $p < 0.002$). Identical results were obtained in the chick-chorioallantoic membrane (CAM) assay (Figures 2C and 2D; Figure S2B for expression analysis). Indeed, we observed a significant increase in neovessel formation in the vicinity of methylcellulose discs soaked either with VEGF, netrin-1, z-VAD-fmk, or BAF (Figures 2C and 2D; $p < 0.02$). Thus, in these ex vivo physiological models, inhibition of apoptosis appears to be sufficient to mimic the effects of netrin-1. Moreover, *UNC5B* silencing by siRNA in CAM (Figure S2C) led to an increase in neovessel ramifications, demonstrating that netrin-1 and its receptor, UNC5B, act in an antagonistic manner during the angiogenic process (Figures 2E and 2F; $p = 0.009$).

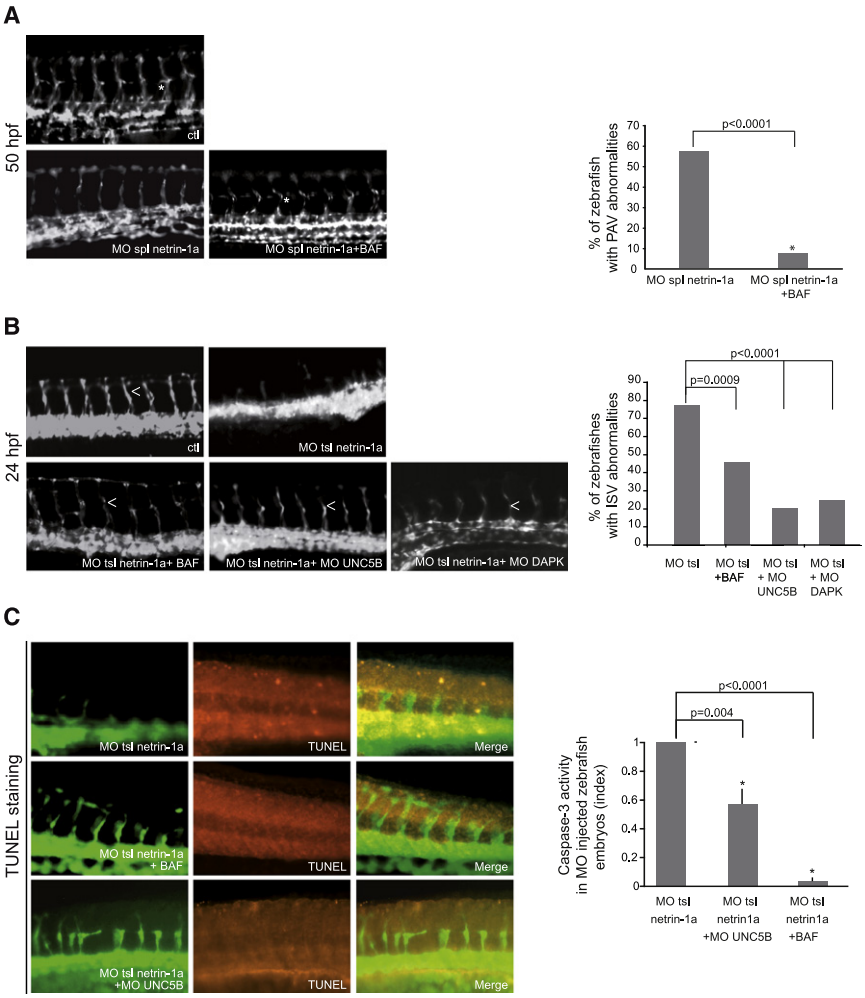


Figure 3. Phenotype of Knockdown *netrin-1a* Zebrafish Embryos Is Rescued by Inhibition of Apoptosis

(A–C) *fli:egfp* zebrafish embryos were injected at the one- to four-cell stage with control (ctl), splice (spl), or translation (tsl)-blocking *netrin-1a*, *UNC5B*, and *DAP kinase* morpholinos, alone or in combination. Phenotypes were analyzed from 24 to 54 hours postfertilization. Representative images of trunk vasculature are shown. Anterior is oriented toward the left. (A and B) The caspase inhibitor BAF restores parachordal vessel formation (asterisks) in (A) zebrafish embryos injected with spl-blocking *netrin-1a* morpholinos and intersegmental vessel formation in (B) zebrafish embryos injected with *netrin-1a* tsl morpholinos. (B) Similar phenotypical rescue was observed by simultaneous injection of *UNC5B* or *DAP kinase* and tsl-blocking *netrin-1a* morpholinos in zebrafish embryos. (A and B) Quantification is presented; p values are indicated (χ_2 test). (C) BAF treatment or injection of *UNC5B*-blocking morpholinos inhibit the increase in apoptosis observed after injection of tsl-blocking *netrin-1a* morpholinos in zebrafish embryos, as shown by TUNEL staining (left panels) or caspase-3 activity (right panel). Caspase-3 activity is expressed as a relative index referred to embryos injected with *netrin-1a* tsl morpholinos solely. Means and SEM are calculated from three independent experiments; p values are indicated (Student t's test).

Netrin-1a Silencing during Zebrafish Development Leads to Vascular Defects that Are Rescued by Caspase Inhibition and by *UNC5B/DAP Kinase* Silencing

To further explore in vivo the cause-and-effect relationship between the proangiogenic and the antiapoptotic activities of netrin-1, we investigated whether antiapoptotic treatment could rescue vessel defects induced by *netrin-1* disruption during zebrafish development. Two *netrin-1* gene orthologs have been characterized in zebrafish, *netrin-1a* and *netrin-1b* (Lauderdale et al., 1997; Strahle et al., 1997). Whereas *netrin-1b* silencing had no obvious effect on vessel development (Figure S3A), both Eichman's group and Li's group have shown, by using different *netrin-1a* morpholinos, that silencing of *netrin-1a* is associated with vessel development defects (Lu et al., 2004; Wilson et al., 2006). *Netrin-1a* silencing with a splice-blocking morpholino (spl-netrin-1a) has been shown to prevent the formation of parachordal vessels (PAVs) (Wilson et al., 2006). Using fli:egfp transgenic zebrafish—a transgenic model targeting the expression of GFP specifically in blood vessels (Lawson and Weinstein, 2002)—we indeed observed at 50–54 hr postfertilization (hpf) that 57.3% of the embryos injected with such spl-netrin-1a morpholinos lacked PAVs (n = 75), whereas the development of intersegmental vessels (ISVs) and dorsal longitudinal anastomotic vessels (DLAVs) was normal (Figure 3A). Treatment of the spl-netrin-1a-injected embryos with the pan-caspase inhibitor BAF successfully reversed this phenotype, since PAV formation occurred in 92.6% of treated embryos (n = 54) compared to 42.7% of untreated ones (Figure 3A; $p < 0.0001$). As described previously (Wilson et al., 2006; Lu et al., 2004), we observed that *netrin-1a* silencing with a translation-blocking morpholino (tsl-netrin-1a, Figure S3B), as well as a third *netrin-1a* blocking morpholino (Figure S3C), led to the disorganization of ISVs, to the absence of the DLAV (Figure 3B), and also to developmental defects. Time-lapse analysis of developing ISVs confirmed the absence or premature arrest of ISVs (Movie S1). Similar defects were previously described in zebrafish embryos knocked down for the small inhibitor of apoptosis, the protein survivin (Ma et al., 2007). Moreover, an increase in caspase-3 activity was observed in *netrin-1a* knockdown embryos (Figure S3D). Treatment of these embryos with BAF was sufficient to rescue ISV formation, since 54.5% of treated embryos displayed at least partial ISVs compared to 23.0% of controls (Figure 3B; $p < 0.001$). In agreement with the dependence receptor notion, simultaneous injection of *UNC5B* morpholinos (Figure S3B) was also able to correct the defects induced by tsl-netrin-1a morpholino injection in 79.6% of the embryos (n = 54) (Figure 3B; $p < 0.0001$). Similar rescue was obtained by coinjecting tsl-netrin-1a morpholinos and morpholinos targeting *dap kinase* (Figure 3B; $p < 0.0001$).

BAF treatment and *UNC5B* morpholino injection both rescued the increase of cell death observed in embryos after *netrin-1a*

silencing, as measured by the TUNEL immunostaining assay and a caspase-3 activity assay (Figure 3C), hence providing further support for the idea that the endothelial cell death inhibited by *netrin-1a* is specifically mediated by UNC5B during vessel development.

DISCUSSION

We have shown here that netrin-1 controls the survival of endothelial cells and promotes angiogenesis, at least in part by blocking apoptosis induced by its unbound UNC5B receptor. Deregulation of this function could explain the modulation of blood vessel formation that we and others have observed (Lu et al., 2004; Wilson et al., 2006). This is in agreement with growing lines of evidence describing the importance of cell death regulation during developmental and pathological angiogenesis (Birdsey et al., 2008; O'Connor et al., 2000; Santoro et al., 2007). Based on the analysis of *netrin-1* and *UNC5B* knockout animal model phenotypes, contradictory conclusions that netrin-1 is either pro- and antiangiogenic were previously reported (Lu et al., 2004; Wilson et al., 2006). Despite additional discrepancies between the various published studies to date (Larrivee et al., 2007; Bouvree et al., 2008; Navankasattusas et al., 2008) that emphasize the complexity of the netrin-1/UNC5B role in angiogenesis (which, in and of themselves, might have resulted from the use of different sources and concentrations of netrin-1 [Yang et al., 2007]), the dependence receptor model proposed here in light of our results indicates that netrin-1 and UNC5B function in an antagonistic manner during angiogenesis and allows for a reconciliation of the apparently opposite genetic observations obtained so far by the Eichmann and Li groups. Even though other effects of netrin-1 on endothelial cells cannot be ruled out—i.e., previously reported netrin-1 effects on in vitro proliferation and migration of endothelial cells (Nguyen and Cai, 2006; Wilson et al., 2006; Park et al., 2004)—these effects probably do not contribute fully to the biological role of netrin-1 during PAV or ISV development, since the inhibition of apoptosis was sufficient to restore adequate vessel development.

Netrin-1 dependence receptors were recently proposed to act as tumor suppressors by inducing apoptosis of epithelial cells that would otherwise develop in settings of trophic ligand unavailability (Grady, 2007; Mehlen and Puisieux, 2006). Loss of the proapoptotic activity of its dependence receptors therefore represents a selective advantage for a tumor cell, as exemplified by the frequent loss of *DCC* and *UNC5C* expression in colorectal cancer (Bernet et al., 2007; Mazelin et al., 2004). Complementarily, gain of netrin-1 expression represents a similar selective advantage for tumor cells, and this phenomenon is indeed observed in some types of aggressive cancers, such as metastatic breast cancers and lung cancer (Delloye-Bourgeois et al., 2009; Fitamant et al., 2008). In light of the results presented here, we propose that this autocrine netrin-1 gain of expression in tumor epithelial cells may have two additive effects. First, as reported, it confers a selective advantage for epithelial tumor cells by inhibiting dependence receptor-induced cell death (Fitamant et al., 2008). Second, it could also potentially favor blood vessel maintenance and/or development, and could consequently promote cancer progression. Therefore, an anticancer approach based on disruption of netrin-1 function, by titration or inhibition of the interaction with its receptors, should eradicate not only tumor epithelial cells but also tumor angiogenic vessels, thus further strengthening the position of netrin-1 as a critical target in cancer.

EXPERIMENTAL PROCEDURES

Endothelial Cell Culture and Transfection

Human umbilical vein and artery cells were cultured according to manufacturer's instructions (Promocell, passages < 8) and were transfected by using the AMAXA Nucleofector system. UNC5A, UNC5B, and scramble siRNAs were designed by Sigma-Proligo. Plasmids encoding mutant DAP kinase (DAPK ΔCam and DAPK DD) were described previously (Llambi et al., 2005).

Cell Death Assays

Cell death assays (Trypan blue, TUNEL immunostaining, caspase-3 activity) were performed as described previously (Llambi et al., 2005; Tauszig-Delamasure et al., 2007; please also see Supplemental Experimental Procedures). Netrin-1 was purchased from Axxora-Apotech. For the netrin-1 titration experiment, rat recombinant UNC5H2-Fc (R&D) was used at 1 μg/ml.

Western Blot Analysis and Immunofluorescence

Protein extracts from endothelial cells were immunoblotted with anti-DAP kinase and anti-phospho-DAP kinase antibodies (Sigma). For immunofluorescence analysis, anti-netrin-1 rat monoclonal antibody (R&D) was used at 1/150 dilution and was revealed by using a specific secondary antibody (Alexa 488 donkey anti-rat, Molecular Probes-Invitrogen).

Quantitative RT-PCR

Total RNA were extracted by using the Nucleospin RNAII kit (Macherey-Nagel), and 1 μg was reverse transcribed by using the iScript cDNA Synthesis kit (BioRad). Real-time quantitative RT-PCR was performed on a LightCycler 2.0 apparatus (Roche) by using the Light Cycler FastStart DNA Master SYBERGreen I kit (Roche). Reaction conditions for all optimal amplification, as well as primer selection, were determined as already described. The sequences of the primers are available upon request.

Aortic Ring Assay

Aortic ring assays were performed as described by Nicosia and Ottinetti (1990). Aortic rings resected from Balb/C mice of 6 to 8 weeks of age were treated or not with netrin-1 (150 ng/ml), z-VAD-fmk (Tebu; 20 nM), BAF (Sigma; 20 nM), or VEGF (Sigma; 13 ng/ml) and photographed with a phase-contrast microscope on day 4. Microvessel outgrowth total length was calculated by using AxioVision Release 4.6 software.

CAM Assay

The chick chorioallantoic membrane (CAM) assay was performed as already described. Netrin-1 (150 ng/ml), z-VAD-fmk (Tebu; 20 nM), BAF (Sigma; 20 nM), VEGF (Sigma), or control vehicles were diluted in 1% autoclaved methylcellulose solution. The methylcellulose disks obtained were placed on a CAM of 8-day-old chick embryos. After 3 days, CAMs were photographed and the angiogenic response was evaluated by measuring the number of secondary ectopic vessel sprouts under the methylcellulose disks by using AxioVision Release 4.6 software. *UNC5B* silencing in CAM was achieved by injection of specific siRNA designed by Sigma-Proligo in CAM blood circulation at E.5, E.7, and E.9. Analysis was performed at E.11.

Morpholino Knockdown of Zebrafish Embryos

A total of 6 ng splice/translation-blocking *netrin-1a*, *UNC5B*, and control morpholinos that were previously designed and characterized were injected alone or in combination in the high yolk of one- to four-cell-stage transgenic fli:egfp zebrafish embryos. A third splice-blocking morpholino specifically targeting *netrin-1a* and a translation-blocking morpholino targeting the zebrafish ortholog of DAP kinase were designed (GeneTools) and injected in embryos at 6 ng and 3 ng, respectively. Sequences of morpholinos are available upon request. Rescue of the phenotype induced by *netrin-1a* silencing was tested by adding 40 nM BAF in the E3 medium 4 hr after morpholino injection. TUNEL labeling of

embryos was achieved as stated in Supplemental Experimental Procedures and revealed by using Cy3-coupled streptavidine (1/400; Jackson Immunoresearch). A caspase-3 activity assay was performed as described above on whole embryos at 24 hr of development. For time-lapse experiments, zebrafish embryos were embedded in a 3% methylcellulose solution with 0.016% tricaine to inhibit movements (Isogai et al., 2003). Transmission videomicroscopic imaging was performed by using a Timelapse Axiovert100M (PLATIM, ENS Lyon).

SUPPLEMENTAL DATA

Supplemental Data include three figures, two movies, and Supplemental Experimental Procedures and can be found with this article online at http://www.cell.com/developmental-cell/supplemental/S1534-5807(09)00079-3.

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REFERENCES

Alavi, A., Hood, J.D., Frausto, R., Stupack, D.G., and Cheresh, D.A. (2003). Role of Raf in vascular protection from distinct apoptotic stimuli. Science *301*, 94–96.

Bernet, A., Mazelin, L., Coissieux, M.M., Gadot, N., Ackerman, S.L., Scoazec, J.Y., and Mehlen, P. (2007). Inactivation of the UNC5C Netrin-1 receptor is associated with tumor progression in colorectal malignancies. Gastroenterology *133*, 1840–1848.

Birdsey, G.M., Dryden, N.H., Amsellem, V., Gebhardt, F., Sahnan, K., Haskard, D.O., Dejana, E., Mason, J.C., and Randi, A.M. (2008). The transcription factor Erg regulates angiogenesis and endothelial apoptosis through VE-cadherin. Blood *111*, 3498–3506.

Bouvree, K., Larrivee, B., Lv, X., Yuan, L., DeLafarge, B., Freitas, C., Mathivet, T., Breant, C., Tessier-Lavigne, M., Bikfalvi, A., et al. (2008). Netrin-1 inhibits sprouting angiogenesis in developing avian embryos. Dev. Biol. *318*, 172–183.

Bredesen, D.E., Mehlen, P., and Rabizadeh, S. (2005). Receptors that mediate cellular dependence. Cell Death Differ. *12*, 1031–1043.

Carmeliet, P. (2005). Angiogenesis in life, disease and medicine. Nature *438*, 932–936.

Cirulli, V., and Yebra, M. (2007). Netrins: beyond the brain. Nat. Rev. Mol. Cell Biol. *8*, 296–306.

Delloye-Bourgeois, C., Brambilla, E., Coissieux, M., Guenebeaud, C., Pedeux, R., Brambilla, C., Mehlen, P., and Bernet, A. (2009). Interference with netrin-1 triggers tumor cell death in non-small cell lung cancer. J. Natl. Cancer Inst. *101*, 237–247.

Duval, H., Harris, M., Li, J., Johnson, N., and Print, C. (2003). New insights into the function and regulation of endothelial cell apoptosis. Angiogenesis *6*, 171–183.

Fitamant, J., Guenebeaud, C., Guix, C., Coissieux, M.M., Puisieux, A., Scoazec, J.Y., Bachelot, T., Bernet, A., and Mehlen, P. (2008). Netrin-1 expression confers a selective advantage for tumor cell survival in metastatic breast cancer. Proc. Natl. Acad. Sci. USA *105*, 4850–4855.

Grady, W.M. (2007). Making the case for DCC and UNC5C as tumor-suppressor genes in the colon. Gastroenterology *133*, 2045–2049.

Isogai, S., Lawson, N.D., Torrealday, S., Horiguchi, M., and Weinstein, B.M. (2003). Angiogenic network formation in the developing vertebrate trunk. Development *130*, 5281–5290.

Larrivee, B., Freitas, C., Trombe, M., Lv, X., Delafarge, B., Yuan, L., Bouvree, K., Breant, C., Del Toro, R., Brechot, N., et al. (2007). Activation of the UNC5B receptor by Netrin-1 inhibits sprouting angiogenesis. Genes Dev. *21*, 2433–2447.

Lauderdale, J.D., Davis, N.M., and Kuwada, J.Y. (1997). Axon tracts correlate with netrin-1a expression in the zebrafish embryo. Mol. Cell. Neurosci. *9*, 293–313.

Lawson, N.D., and Weinstein, B.M. (2002). In vivo imaging of embryonic vascular development using transgenic zebrafish. Dev. Biol. *248*, 307–318.

Liu, Y., Stein, E., Oliver, T., Li, Y., Brunken, W.J., Koch, M., Tessier-Lavigne, M., and Hogan, B.L. (2004). Novel role for Netrins in regulating epithelial behavior during lung branching morphogenesis. Curr. Biol. *14*, 897–905.

Llambi, F., Causeret, F., Bloch-Gallego, E., and Mehlen, P. (2001). Netrin-1 acts as a survival factor via its receptors UNC5H and DCC. EMBO J. *20*, 2715–2722.

Llambi, F., Lourenco, F.C., Gozuacik, D., Guix, C., Pays, L., Del Rio, G., Kimchi, A., and Mehlen, P. (2005). The dependence receptor UNC5H2 mediates apoptosis through DAP-kinase. EMBO J. *24*, 1192–1201.

Lu, X., Le Noble, F., Yuan, L., Jiang, Q., De Lafarge, B., Sugiyama, D., Breant, C., Claes, F., De Smet, F., Thomas, J.L., et al. (2004). The netrin receptor UNC5B mediates guidance events controlling morphogenesis of the vascular system. Nature *432*, 179–186.

Ma, A., Lin, R., Chan, P.K., Leung, J.C., Chan, L.Y., Meng, A., Verfaillie, C.M., Liang, R., and Leung, A.Y. (2007). The role of survivin in angiogenesis during zebrafish embryonic development. BMC Dev. Biol. *7*, 50.

Mazelin, L., Bernet, A., Bonod-Bidaud, C., Pays, L., Arnaud, S., Gespach, C., Bredesen, D.E., Scoazec, J.Y., and Mehlen, P. (2004). Netrin-1 controls colorectal tumorigenesis by regulating apoptosis. Nature *431*, 80–84.

Mehlen, P., and Bredesen, D.E. (2004). The dependence receptor hypothesis. Apoptosis *9*, 37–49.

Mehlen, P., and Puisieux, A. (2006). Metastasis: a question of life or death. Nat. Rev. Cancer *6*, 449–458.

Mehlen, P., Rabizadeh, S., Snipas, S.J., Assa-Munt, N., Salvesen, G.S., and Bredesen, D.E. (1998). The DCC gene product induces apoptosis by a mechanism requiring receptor proteolysis. Nature *395*, 801–804.

Navankasattusas, S., Whitehead, K.J., Suli, A., Sorensen, L.K., Lim, A.H., Zhao, J., Park, K.W., Wythe, J.D., Thomas, K.R., Chien, C.B., et al. (2008). The netrin receptor UNC5B promotes angiogenesis in specific vascular beds. Development *135*, 659–667.

Nguyen, A., and Cai, H. (2006). Netrin-1 induces angiogenesis via a DCC-dependent ERK1/2-eNOS feed-forward mechanism. Proc. Natl. Acad. Sci. USA *103*, 6530–6535.

Nicosia, R.F., and Ottinetti, A. (1990). Growth of microvessels in serum-free matrix culture of rat aorta. A quantitative assay of angiogenesis in vitro. Lab. Invest. *63*, 115–122.

O'Connor, D.S., Schechner, J.S., Adida, C., Mesri, M., Rothermel, A.L., Li, F., Nath, A.K., Pober, J.S., and Altieri, D.C. (2000). Control of apoptosis during angiogenesis by survivin expression in endothelial cells. Am. J. Pathol. *156*, 393–398.

Park, K.W., Crouse, D., Lee, M., Karnik, S.K., Sorensen, L.K., Murphy, K.J., Kuo, C.J., and Li, D.Y. (2004). The axonal attractant Netrin-1 is an angiogenic factor. Proc. Natl. Acad. Sci. USA *101*, 16210–16215.

Santoro, M.M., Samuel, T., Mitchell, T., Reed, J.C., and Stainier, D.Y. (2007). Birc2 (cIap1) regulates endothelial cell integrity and blood vessel homeostasis. Nat. Genet. *39*, 1397–1402.

Serafini, T., Kennedy, T.E., Galko, M.J., Mirzayan, C., Jessell, T.M., and Tessier-Lavigne, M. (1994). The netrins define a family of axon outgrowth-promoting proteins homologous to *C. elegans* UNC-6. Cell *78*, 409–424.

Serafini, T., Colamarino, S.A., Leonardo, E.D., Wang, H., Beddington, R., Skarnes, W.C., and Tessier-Lavigne, M. (1996). Netrin-1 is required for commissural axon guidance in the developing vertebrate nervous system. Cell *87*, 1001–1014.

Shohat, G., Spivak-Kroizman, T., Cohen, O., Bialik, S., Shani, G., Berrisi, H., Eisenstein, M., and Kimchi, A. (2001). The pro-apoptotic function of death-associated protein kinase is controlled by a unique inhibitory autophosphorylation-based mechanism. J. Biol. Chem. *276*, 47460–47467.

Strahle, U., Fischer, N., and Blader, P. (1997). Expression and regulation of a netrin homologue in the zebrafish embryo. Mech. Dev. *62*, 147–160.

Tanikawa, C., Matsuda, K., Fukuda, S., Nakamura, Y., and Arakawa, H. (2003). p53RDL1 regulates p53-dependent apoptosis. Nat. Cell Biol. *5*, 216–223.

Tauszig-Delamasure, S., Yu, L.Y., Cabrera, J.R., Bouzas-Rodriguez, J., Mermet-Bouvier, C., Guix, C., Bordeaux, M.C., Arumae, U., and Mehlen, P. (2007). The TrkC receptor induces apoptosis when the dependence receptor notion meets the neurotrophin paradigm. Proc. Natl. Acad. Sci. USA *104*, 13361–13366.

Wilson, B.D., Li, M., Park, K.W., Suli, A., Sorensen, L.K., Larrieu-Lahargue, F., Urness, L.D., Suh, W., Asai, J., Kock, G.A., et al. (2006). Netrins promote developmental and therapeutic angiogenesis. Science *313*, 640–644.

Winn, R.K., and Harlan, J.M. (2005). The role of endothelial cell apoptosis in inflammatory and immune diseases. J. Thromb. Haemost. *3*, 1815–1824.

Yang, Y., Zou, L., Wang, Y., Xu, K.S., Zhang, J.X., and Zhang, J.H. (2007). Axon guidance cue Netrin-1 has dual function in angiogenesis. Cancer Biol. Ther. *6*, 743–748.