Introduction

Cell migration is essential for normal embryonic development, wound healing, and immunity but can be devastating in tumor invasion and metastasis. Netrins are secreted, laminin-related proteins that direct cell and axon migration during neural development (reviewed by [1]). Netrin-1 and netrin receptors DCC, the DCC paralogue neogenin, and UNC5 proteins, are also expressed in many adult tissues [2–9], but their function in mature tissues is poorly understood. Netrin-1 is widely expressed by glioblastoma cells [4]. Reducition of netrin-1 expression is reduced in many cancers, including most high-grade gliomas [12] [13] and loss of DCC correlates with the development of highly invasive glioblastoma multiformae [13]. Furthermore, ectopic expression of ddc in transformed epithelial cells reduced tumorigenicity [14] [15], and expression of DCC antisense RNA in transformed fibroblasts resulted in an increased growth rate, anchorage independence, and tumorigenicity when the cells were transplanted into nude mice [16]. No increased incidence of tumor formation has been detected in conventional DCC knockout mice [17], however, conclusions drawn from this study were complicated by the possibility that tumors may not have had time to develop due to the early post-natal lethality of DCC knockouts. UNC5 homologue netrin receptors signal chemorepulsion, and co-expression of DCC often facilitates UNC5 function (reviewed by [1]). Four UNC5s, UNC5A-D, are expressed in mammals. Altered expression of UNC5A, B, C, and D has been detected in various cancers and tumor cell lines [6,18][19].

Here we investigated the possibility that netrins and netrin receptors influence tumor cell migration. Using human glioblastoma cell lines, we provide evidence that DCC is required for cell motility that promote focal adhesion formation. These findings suggest that disruption of netrin signalling may disable a mechanism that normally restrains inappropriate cell migration.

Abstract

Deregulation of mechanisms that control cell motility plays a key role in tumor progression by promoting tumor cell dissemination. Secreted netrins and their receptors, Deleted in Colorectal Cancer (DCC), neogenin, and the UNC5 homologues, regulate cell and axon migration, cell adhesion, and tissue morphogenesis. Netrin and netrin receptor expression have previously been shown to be disrupted in invasive tumors, including glioblastoma. We determined that the human glioblastoma cell lines U87, U343, and U373 all express neogenin, UNC5 homologues, and netrin-1 or netrin-3, but only U87 cells express DCC. Using transfilter migration assays, we demonstrate DCC-dependent chemotactic migration of U87 cells up a gradient of netrin-1. In contrast, U343 and U373 cells, which do not express DCC, were neither attracted nor repelled. Ectopic expression of DCC by U343 and U373 cells resulted in these cells becoming competent to respond to a gradient of netrin-1 as a chemotaxiant, and also slowed their rate of spontaneous migration. Here, in addition to netrins’ well-characterized chemotropic activity, we demonstrate an autocrine function for netrin-1 and netrin-3 in U87 and U373 cells that slows migration. We provide evidence that netrins promote the maturation of focal complexes, structures associated with cell movement, into focal adhesions. Consistent with this, netrin, DCC, and UNC5 homologues were associated with focal adhesions, but not focal complexes. Disrupting netrin or DCC function did not alter cell proliferation or survival. Our findings provide evidence that DCC can slow cell migration, and that neogenin and UNC5 homologues are not sufficient to substitute for DCC function in these cells. Furthermore, we identify a role for netrins as autocrine inhibitors of cell motility that promote focal adhesion formation. These findings suggest that disruption of netrin signalling may disable a mechanism that normally restrains inappropriate cell migration.

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chemoattraction to netrin-1 and slows the rate of spontaneous cell migration. Our findings support a role for netrins as autocrine inhibitors of cell motility that regulate focal adhesions (FA).

**Results**

**Glioblastoma cells express netrin and netrin receptors**

To determine if netrins regulate glioblastoma cell migration, we first characterized netrin and netrin receptor expression in human astrocytoma cell lines U87, U343, and U373, and in cultures of astrocytes isolated from newborn rat cortex (Fig. 1A). Western blot analysis using an antibody that binds netrin-1 and netrin-3 [20] detected a ~75 kDa band corresponding to full-length netrin in conditioned medium collected from all cells tested. The DCC monoclonal antibody detected a ~185 kDa band corresponding to DCC in astrocyte and U87 cell lysates. In contrast, DCC was not detected in lysates of U343 or U373 cells. The DCC homologue neogenin was expressed by astrocytes and was detected in all glioblastoma cell lysates.

RT-PCR (Fig. 1B) revealed *dcc* expression by U87 cells but not U343 or U373 cells, and *neogenin* and *unc5* expression by all three cell types. U87 and U343 cells express *unc5b* and *c*, and U373 cells express *unc5b*, *c*, and *d*. Netrin-1 expression was detected in U343 and U373 cells, and netrin-3 expression in U87 cells. Netrin-1 and netrin-3 are essentially functionally equivalent: both bind DCC and UNC5 proteins and evoke chemoattractant or chemorepellent responses from responsive cells [21].

We then sought to determine if netrin might exert an autocrine influence on cell migration. We first assessed the relative motility of the three cell lines using a transfilter chemotaxis assay as described [22]. Briefly, cells were cultured on the upper surface of a porous membrane (Fig. 1G) and allowed to migrate across. Following migration, cells remaining on the upper surface of the membrane were scraped off, and the cells that migrated to the underside were fixed, stained, and counted. While this assay is often employed to assess the migration of cells in response to a putative attractant or repellant cue, here we used it in the absence of added factors to compare the relative rates of spontaneous migration of the three glioblastoma lines. U343 and U373 cells, lacking DCC, migrated significantly faster than DCC-expressing U87 cells (Fig. 1C). Notably, the U343 cells, which were originally derived from a grade IV glioblastoma multiforme [23], migrated significantly faster than either the U87 or U373 cells, both of which were originally derived from less aggressive grade III astrocytomas [24].

**Autocrine netrin inhibits U87 cell motility**

We hypothesized that DCC and netrin expressed by U87 cells might exert a kinetic influence on the rate of cell movement, independent of netrin’s influence on directional migration. We therefore tested the effect of disrupting DCC and netrin function on the spontaneous rate of U87 cell migration. The rate of spontaneous migration was not affected by addition of a DCC function-blocking antibody (DCCFB). In order to disrupt autocrine netrin function, netrin function-blocking antibody (NetFB) was added to both the top and bottom compartments of the migration chamber. This resulted in an approximately 10-fold increase in spontaneous migration across the filter relative to the number of...
cells migrating in either medium alone (Control) or in the presence of a control IgG (Fig. 1D).

**Autocrine netrin-1 inhibits migration of U373, but not U343, glioblastoma cells**

Netrin's capacity to inhibit U87 cell motility in a DCC-independent manner led us to determine if a similar mechanism was active in U343 or U373 cells, which do not express DCC. The addition of netrin function-blocking antibody to both the top and bottom compartments of the transfilter assay significantly increased the rate of U373 migration (Fig. 1F), indicating that endogenous netrin-1 inhibits the rate of U373 migration.

Unlike U87 and U373 cells, blocking netrin function did not alter the rate of U343 cell migration (Fig. 1E). Although U343 cells express neogenin and UNC5 homologue netrin receptors, the absence of an increase in the rate of migration may be the result of mechanisms that restrain inappropriate cell motility being more severely disrupted in these cells.

**Netrin-1 is a chemotropic attractant for U87 glioblastoma cells**

Transfer filter migration assays were then used to determine if DCC-expressing U87 cells can respond to a gradient of netrin-1 as a chemoattractant. Addition of 100 ng/ml netrin-1 to the bottom compartment of the migration chamber (NB) produced a significant increase in the number of U87 cells that migrated across the membrane relative to control (medium alone: Fig. 2A, 16 hr assay; Fig. 2B, 48 hr assay). In contrast, when netrin-1 was added to both the top and bottom compartments (NTB), migration was not significantly different from control. This indicates that U87 cells respond to a gradient of netrin-1 as a chemotropic attractant. When challenged with a gradient of netrin-1 whilst in the presence of the DCC-FB antibody in the top and bottom wells (NB DCC-FB), U87 cell migration was not significantly different from control, indicating that the tropic response of U87 cells to netrin-1 requires DCC. Neither U343 nor U373 cells, which do not express DCC, altered their migration in response to a gradient of netrin-1 (Fig. 2C), despite expressing neogenin and UNC5 netrin receptors. These findings suggest that although these receptors may be sufficient to mediate autocrine inhibition of migration (Fig. 1F), they are insufficient for these cells to generate a chemotropic response to a gradient of netrin-1 (Fig. 2C).

**Chemoattractant response of DCC-expressing U343 and U373 cells to a gradient of netrin-1**

To further investigate the contribution of DCC to the regulation of cell motility, we reintroduced the *dcc* gene back into U343 and U373 cells by transfection with a cDNA encoding a DCC-GFP chimera (pDCC-GFP, described by [25]). The proportion of cells expressing DCC-GFP was increased by passaging the cells with Geneticin selection, such that the vast majority of cells seeded in the migration assays expressed DCC. Expression of DCC by U343 and U373 cells was confirmed by western blot (Fig. 1A). Unlike the...
parental U343 and U373 cell lines, DCC-GFP-expressing U343D and U373D cells migrated up a gradient of netrin-1 (Fig. 2D, E), indicating that ectopic expression of DCC now rendered these cells competent to generate a chemotropic response to netrin-1. Like DCC-expressing U87 cells, the gain-of-function migration towards netrin-1 exhibited characteristics of chemotropic attraction, as the cells only responded to a gradient. Uniform presentation of exogenous netrin-1 resulted in migration that was not significantly different from control. The DCCFB antibody blocked the chemoattractant response of U343D and U373D cells, indicating that DCC is required for chemoattraction to netrin-1.

Consistent with the slow migration of DCC-expressing U87 cells, the number of DCC-transfected U343 and U373 cells that migrated under control conditions was substantially reduced relative to that of the parental cells (Fig. 2D, E). These findings suggest that DCC expression decreases the motility of these cells; however, application of the DCC function-blocking antibody (DCCFB) did not increase the rate of migration, as was also found for the U87 cells (Fig. 1D). In contrast, DCCFB completely blocked the chemoattractant migratory response of the U87 cells, and the DCC-transfected U343 and U373 cells to a gradient of netrin-1. These findings are consistent with DCC activating a mechanism that slows non-directional cell migration; however, this mechanism can be differentiated from DCC-dependent chemotraction due to its insensitivity to DCCFB.

Chemoattraction to netrin-1 is converted to repulsion by laminin-1

Laminin-1 exerts a neuromodulatory influence that converts the response of *Xenopus* retinal ganglion cell growth cones to netrin-1 from attraction to repulsion [26]. We therefore investigated the possibility that laminin-1 might influence the migratory response of U87 cells to a gradient of netrin-1 (Fig. 3). When U87 cells were challenged with an ascending gradient of laminin-1 (LB), the number of cells that migrated across the membrane increased. In the presence of a uniform concentration of laminin (LTB), U87 migration was not significantly different from control, indicating that a gradient of laminin-1, like netrin-1, is a chemoattractant for these cells. Interestingly, the combination of an ascending gradient of netrin-1 and a uniform concentration of laminin-1 (LTB NB) dramatically reduced the number of U87 cells that migrated across the membrane to the extent that it was significantly less than control, suggesting that laminin-1 converted the response to netrin-1 from attraction to repulsion. Consistent with this, confronting cells with a descending netrin-1 gradient in the presence of a uniform concentration of laminin-1 (LTB NT) resulted in an increase in migration relative to control. Importantly, this finding provides strong evidence that laminin-1 does not influence the response to a gradient of netrin-1 by arresting cell motility. When the cells were simultaneously exposed to uniform concentrations of netrin-1 and laminin-1, (LTB NTB), fewer cells migrated across

![Figure 3. U87 attraction to netrin is converted to repulsion by laminin-1.](https://www.plosone.org/doi/10.1371/journal.pone.0025408.g003)

(A) U87 migration in the microchemotaxis assay challenged with an ascending gradient of laminin-1 (LB) increased relative to control (C). A uniform distribution of laminin-1 (LTB) does not increase U87 migration. An ascending gradient of netrin-1 and uniform laminin-1 (LTB NB), or uniform distributions of both netrin-1 and laminin-1 (LTB NTB), results in reduced U87 migration. Challenging cells with a descending gradient of netrin-1 with a uniform distribution of laminin-1 (LTB NT), evoked increased migration relative to control. Addition of DCCFB to both the top and bottom compartments in the presence of a uniform distribution of laminin-1 and an ascending gradient of netrin-1 (LTB NB DCCFB) or of uniform distributions of both netrin-1 and laminin-1 (LTB NTB DCCFB) blocked the decrease in migration observed. (B) Schematic depicting migratory responses of U87 cells in (A). Migration assayed after 48 hrs. * p<0.05 vs. control.

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the membrane, indicating that the combined action of netrin-1 and laminin-1 exerts a non-directional effect that inhibits U87 cell motility. These results are consistent with laminin-1 switching netrin-1 from an attractant to a repellent for U87 cells, as previously described for the axons of *Xenopus* retinal ganglion cells [26]. Addition of DCCFB antibody in the presence of a uniform concentration of laminin-1 and either an increasing gradient (LTB NB DCCFB) or uniform concentration (LTB NTB DCCFB) of netrin-1, did not significantly affect migration compared to control, indicating that the laminin-induced repellent response to netrin-1 requires DCC. This is consistent with a requirement for DCC in chemorepellent responses to netrin-1, documented in many cell types [27] [28], including glial precursor cells [29–31][22][32].

Netrin-1 and DCC do not affect U87, U343, and U373 cell proliferation or survival

DCC and UNC5 homologues have been proposed to function as dependence receptors, activating apoptosis in the absence of netrin-1 [33]. This raises the possibility that the effects described above may be due to an influence on cell survival and not motility. Thus, we examined the consequences of manipulating netrin function on the survival of U87, U343 and U373 cells. No significant change in cell number (Fig. 4A), or activation of caspase-3, an indicator of apoptosis (Fig. 4B), was detected following 16 hrs treatment with exogenous netrin-1, laminin-1, or both; nor following disruption of netrin or DCC function using blocking antibodies. Further testing, by blocking netrin and DCC function for 48 hrs, again resulted in no detectable increase in caspase-3 activation (Fig. 4C). In contrast, staurosporine, applied as a positive control, activated caspase-3 and caused extensive cell death (Fig. 4B, C). These findings are consistent with previous analyses of glial precursor cells, indicating that netrin-1 and DCC do not regulate oligodendrocyte precursor survival either in *vitro* or *in vivo* [22][34], and they support the conclusion that the results of transfilter assays reflect changes in cell migration and not effects on cell survival or proliferation.

Endogenous netrin promotes the maturation of focal complexes into focal adhesions

Cell migration requires the formation of transient adhesive contacts with the extracellular matrix (ECM). Initial contacts occur at the leading edge of lamellipodia where integrins bind ECM ligands and recruit proteins such as vinculin and paxillin to

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**Figure 4. Neither netrin-1 nor laminin-1 affect the survival or proliferation of U87, U343, or U373 cells.** (A) Cell viability was assessed by labeling F-actin with Alexa 488-conjugated phalloidin, nuclei with Hoechst, and counting the number of cells. Addition of netrin-1, laminin-1, or both did not affect U87 cell viability. Neither 25 μg/ml NetFB nor 10 μg/ml DCCFB affected cell number. The number of U343 or U373 cells did not change following addition of 100 ng/ml netrin-1 or 25 μg/ml NetFB (16 hr assay). (B) To further assess apoptotic cell death under the same conditions analyzed in panel A, cell lysates were analyzed by immunoblot for the active (cleaved) form of caspase-3. In all three cell lines, a 17 kDa caspase-3 band (black arrowhead) was only observed in lysates exposed to staurosporine, a potent inducer of apoptosis. The white arrowhead indicates a nonspecific 15 kDa immunoreactive band. (C) To determine if netrin regulates apoptosis through a ‘dependence’ mechanism, cells were treated with antibodies blocking either DCC or netrin function for 48 hours. As in panel B, only staurosporine treatment promoted cell death. Ponceau S staining demonstrates equal loading. Ctrl C control; Lam L laminin-1; Net N netrin-1; Netb N netrin function-blocking antibody; DCCb D DCC function-blocking antibody; LN laminin-1 and netrin-1; NDb Netrin-1 and DCCb; LNDb Laminin-1 netrin-1 and DCCb; R pre-immune rabbit IgG; St staurosporine.

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form immature adhesive contacts called focal complexes (FC) (reviewed by [35]). The transition from FC to FA is marked by consolidation of the adhesive contact, an increase in size, and the recruitment of additional proteins including zyxin [36].

The effect of disrupting netrin function on adhesive complex formation in glioblastoma cells was investigated by examining the distribution of paxillin, which is present in both FAs and FCs, and zyxin, which is present in FAs but not FCs. The influence of netrin on FC formation was quantified by subtracting the distribution of zyxin from paxillin (Fig. 5C, H, M, R, W). From paxillin immunoreactivity (Fig. 5B, G, L, O, V) to create images representing regions of paxillin, but not zyxin localization (Fig. 5D, I, N, S, X). Using the ‘paxillin minus zyxin’ images, the density of FCs present in each lamellipodium was calculated. Exposure of U87 cells to an isotype control antibody (RbIgG), DCCFb, or netrin-1, resulted in no change relative to control. In contrast, application of structures restrains cell movement.

Which autocrine secretion of netrin promotes the maturation of described here. These data are consistent with a mechanism in sufficient to saturate the inhibitory response in the conditions the relatively high level of netrin protein secreted by the cells is netrin-1 protein did not influence FC or FA density suggests that the rate of cell migration (Fig. 1). That the addition of exogenous netrin-1 protein did not influence FC or FA density suggests that the relatively high level of netrin protein secreted by the cells is sufficient to saturate the inhibitory response in the conditions described here. These data are consistent with a mechanism in which autocrine secretion of netrin promotes the maturation of FCs into FAs, and that the accumulation of these adhesive structures restrains cell movement.

Netrin and netrin receptors are localized to focal adhesions, but not focal complexes

We next investigated the possibility that netrin and netrin receptors might be localized to FAs or FCs and thereby directly influence their maturation. U87, U343, and U373 cells were labeled with antibodies against paxillin and one of DCC, netrin, or UNC5 proteins. U87 cells were also labeled with anti-DCC and anti-zyxin (Fig. 6). In U87 cells netrin (Fig. 6A–C), DCC (Fig. 6M–O) and UNC5 (Fig. 6G–I) immunoreactivity colocalized with large paxillin-positive foci characteristic of FAs (white arrowhead), but not smaller paxillin-positive structures characteristic of FCs (black arrowhead). In U343 and U373 cells that lack DCC expression, netrin (Fig. 6D–F, P–R) and UNC5 (Fig. 6J–L, V–X) immunoreactivity was similarly localized to FAs but not FCs. Consistent with localization to FAs, DCC and zyxin immunoreactivity colocalized in U87 cells (Fig. 6S–U). Colocalization with markers of FAs is consistent with netrins and netrin receptors regulating cell-substrate adhesion and motility.

Discussion

Here we provide evidence that secreted netrins can function as autocrine inhibitors of cell motility. Our findings support the conclusion that DCC is required for cells to migrate directionally in response to a gradient of netrin-1. Ecotropic DCC expression conferred on U343 and U373 cells the capacity to respond to a gradient of netrin-1. DCC expression also slowed the rate of spontaneous migration in these cells, consistent with DCC restructuring cell movement. The glioblastoma-derived cell lines tested express either netrin-1 (U343, U373) or netrin-3 (U87). Disrupting endogenous netrin-1 or netrin-3 function dramatically increased the rate of U87 and U373 cell movement. U87 cells express DCC while U373 cells do not, indicating that in addition to DCC slowing cell migration, netrins must influence the motility of these cells through a DCC-independent mechanism. Unc5 homologue netrin receptors are required for axonal growth cone repulsion and collapse induced by netrin-1, and co-expression of DCC often facilitates UNC5 function (reviewed by [1]). Our findings support the hypothesis that UNC5 proteins, in collaboration with DCC, underlie the netrin-mediated inhibition of motility described here; however the role of UNC5 homologues in these cells remains to be tested directly.

Consistent with increasing the rate of cell motility, disrupting endogenous netrin function increased the number of lamellipodial FCs, immature adhesive contacts that are associated with cell movement. Netrin, DCC, and UNC5 immunoreactivity was colocalized with FA but not FC markers, suggesting that netrin may act at the nascent FA itself to promote the maturation of FCS to FAs.

Netrin, focal adhesions, and cell motility

Netrin-1 signaling through DCC directs the organization of F-actin [25], regulating the activation of RhoGTPases, PAK1, MAPK, FAK, and Src family kinases [25][37–44]. FAK and Src are also activated downstream of UNC5 proteins in response to netrin [45] [46]. FAs are sites of interaction for many proteins [35]. Our evidence indicates that netrin and netrin receptors are localized to FAs. We hypothesize that netrins may contribute to restricting cell movement by promoting FA maturation. Numerous proteins present in FAs have been implicated in signaling downstream of netrin: FAK, Src, the Ena/VASP proteins [47], Rho-family GTPases Cdc42, Rac, and RhoA [25][48][43] and the GEF Trio [49]. FAK is activated by autophosphorylation that creates a binding site for Src-family kinases. Association with FAK initiates a FAK-Src signaling complex. Extensive tyrosine phosphorylation is a key signaling event observed in focal adhesions, as it is thought to create ‘docking’ sites for recruitment of SH2 domain-containing proteins required for further signaling events (reviewed by [50]). FAK and Src regulate the phosphorylation of UNC5B on multiple tyrosine residues upon netrin binding, and that following these phosphorylation events, Src associates directly with UNC5B via its SH2 domain. Interestingly, this is enhanced by, but does not absolutely require, DCC function [46], perhaps reflecting that co-expression of DCC can facilitate UNC5 function (reviewed by [51]). Notably, FAK is required for the maturation of adhesive complexes [32], and, together with Src, is essential for the normal turnover of FAs [reviewed by [50]]. The findings we present here provide a foundation for investigating the role of netrin-1 in the formation of focal adhesions.

An emerging role for netrin in adhesion and tissue morphogenesis

Netrin-1 and netrin-3 are secreted proteins, which raises the question of how they may contribute to anchoring a cell to either the substrate or another cell. The majority of netrin-1 protein in the
Figure 5. Disrupting netrin function increases the number of FCs and reduces the number of FAs in lamellipodial protrusions of U87 and U373, but not U343, cells. (A, F, K, P, U) U87 cells were labeled with antibodies against paxillin (green) and zyxin (red). FCs present in lamellipodia of U87 cells were identified and quantified by subtracting zyxin immunoreactivity (C, H, M, R, W) from paxillin immunoreactivity (B, G, L, Q, V), revealing localization of paxillin without zyxin (D, I, N, S, X). (Z) Density of paxillin+/zyxin- foci. FAs in U87 cell lamellipodia were identified and quantified by generating images of paxillin and zyxin co-localization (E, J, O, T, Y) and determining the density of paxillin+/zyxin+ foci (AC). 25 μg/ml control rabbit IgG (Rb IgG; K–O), 100 ng/ml netrin-1 (P–T) or 10 μg/ml DCCFB (U–Y) resulted in no change in FC or FA density relative to control medium (A–E). 25 μg/ml NetFB (F–J) significantly increased the density of FCs (Z) and decreased FA density (AC). FCs and FAs of U373 cells were similarly affected (AB, AE). FC of FA density was not altered by control antibody, netrin-1, or NetFB in U343 cells (AA and AD). 100x objective, scale bar = 2 μm. * p < 0.05 vs. control.

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CNS is not freely diffusible, but is bound to cell surfaces and extracellular matrix [5][53](reviewed by [51]). DCC binding immobilized netrin-1 mediates cell-substrate adhesion, consistent with a role for netrin mediating cell-matrix interactions [38][48]. Key roles for netrins and netrin receptors have been identified during tissue morphogenesis (reviewed by [54]), including development of the mammary epithelium [55], pancreas [56], lung [57], lymphangiogenesis [58], and during angiogenesis [59] [60]. Furthermore, overexpression of netrin-1 by cells in the intestinal epithelium of mice led to the formation of focal hyperplasias and adenomas [61]. These authors concluded that the phenotype induced was due to netrin-1 reducing cell death; however, our findings raise the possibility that disruption of appropriate cell-cell interactions as a result of netrin-1 overexpression may contribute to the disorganization of normal epithelial structure.

How might secreted netrins influence tumor cell migration in vivo?

Our findings suggest that loss of netrin function may lead to disruption of appropriate cell-cell and cell-matrix interactions. We have provided evidence that in the presence of laminin-1, netrin-1 becomes a repellent for U87 cell migration, and that this requires DCC (Fig. 3). Importantly, the combined action of netrin-1 and laminin-1 may influence glioblastoma cell migration in vivo. Laminin-1 is restricted to basement membranes and capillary walls in developing and mature CNS [62-64]. Although deregulated cell migration makes an important contribution to the dissemination of tumor cells within the brain, metastasis of brain tumor cells outside the CNS is rare. Glioma cells are attracted to endothelial capillaries in vivo [65] and glioblastoma cells migrate in close association with capillary walls as they disseminate within the brain [66]. Laminin-1 may facilitate this as it promotes glioma cell migration [67] [68]. Based on our evidence that laminin-1 biases cells to respond to netrin-1 as a repellent (Fig. 3, see also [26]), the basal lamina may inhibit the migration of glioma cells expressing netrin-1 and DCC. In contrast, in the absence of netrin function, our findings predict that deregulation of this inhibition of migration will lead to laminin-1 in the basal lamina of blood vessels becoming a permissive substrate that promotes tumor cell migration and dissemination to other brain regions. Correlated loss of DCC expression with tumor progression suggests that netrin and DCC may play an important role in tissue maintenance in adulthood. We propose that appropriate cellular organization may be stabilized by autocrine and paracrine actions of netrin. Our findings suggest that loss of effective netrin signaling may disinhibit a mechanism that normally restrains cell migration. In the absence of netrin-mediated inhibition, local cues such as laminin, are predicted to become potent promoters of migration.

Numerous cell types expressing both netrin and netrin receptors in vivo have been described. We provide evidence that autocrine expression of netrin can restrain cell migration, and promote the maturation of focal complexes into focal adhesions. These findings identify a novel netrin function that may contribute to the formation and maintenance of tissue organization, and identify netrin and its receptors as potential therapeutic targets to inhibit tumor cell migration and dispersion.

Materials and Methods

Cells and cell culture

Human glioblastoma cell lines, U87, U343, U373 (ATCC, Rockville, MD) and astrocytes isolated from newborn mouse brain...
were grown as monolayer cultures in DMEM (Invitrogen, Burlington, ON), 10% heat-inactivated fetal bovine serum (FBS, Invitrogen), 1% glutamax-I (Invitrogen) and 1% penicillin/ streptomycin. All procedures using animals were performed in accordance with the Canadian Council on Animal Care guidelines for the use of animals in research, and were approved by the animal care review board of the Montreal Neurological Institute (approval ID # 4330).

**Antibodies, conditioned media, cell lysates, western blotting, and PCR**

Antibodies against the following were used: cleaved caspase-3 (Asp175, mouse, Cell Signaling Technology, Beverly, MA); DCC (DCCm, mouse, C97-449; BD Biosciences Pharmingen, San Jose, CA; DCCm, goat, A-20; Santa Cruz Biotechnology, Santa Cruz, CA; function-blocking, DCCf, mouse, AF5; Calbiochem, La Jolla, CA); netrin-1 and 3 (PN2, rabbit [5]); netrin function-blocking (545 bp) and (527 bp), netrin-3

PCR

were used at 100 ng/ml. Laminin-1 was used at 10 μg/ml (BD Biosciences, Bedford, MA). Netβ and rabbit isoform control IgG (used as a control) were added at a concentration of 25 μg/ml. DCCFβ was added at a concentration of 10 μg/ml. Statistical significance was calculated using Student’s t-test and error bars represent S.E.M.

**Plasmids and transfection**

U343 and U373 cells were transfected using lipofectamine (Invitrogen) with expression constructs encoding either green fluorescent protein (GFP) alone or DCC tagged at its C-terminus with GFP [25]. Seventy-two hrs after transfection, the medium was changed to selection medium containing Geneticin (Invitrogen).

**Confocal image analysis**

10^4 cells were plated per well in chamber slides (Fisher) coated with 20 μg/ml poly-D-lysine (Sigma) at 4°C overnight, washed with Hanks buffered salt solution (Invitrogen) and allowed to dry. Cells were fixed in 4% PFA, 4% sucrose in PBS, permeabilized with 0.25% Triton X-100 in PBS. Blocking was performed in 3% heat-inactivated normal goat serum, 2% BSA, and 0.125% Triton X-100 in PBS. Cells were then incubated with anti-paxillin and anti-zyxin (Fig. 5), anti-paxillin and one of anti-netrin PN2, anti-UNC5, or anti-DCCGT, or anti-zyxin and anti-paxillin (Fig. 5) for 1 hr. Cells were then incubated with HRP-coupled secondary antibodies and immunoreactivity visualized using chemiluminescence (NEN, MA).

**Transfilter chemotaxis assay**

Cells were plated at a density of 4x10^5 cells/ml on polycarbonate transwell culture inserts (6.5 mm diameter with 8 μm pore size, Corning). 100 μl of cell suspension was added to the upper surface of the filter, and the filters placed in the wells of a 24-well plate over 600 μl of medium. DMEM with 0.2% BSA, 100 U/ml penicillin, 100 μg/ml streptomycin, and 2 mM glutamate was the base medium used for all assay conditions. Following migration, cells on the upper side of the filter were scraped off, and the cells attached to the lower side of the filter fixed with 4% paraformaldehyde (400 ml, 4°C). Filters were rinsed with PBS, and cell nuclei stained with Hoechst dye. Four transwells were used per condition. Four images of each filter were captured using a 10 X objective and nuclei counted using Northern Eclipse software (Empix Imaging, TO). Where pooled results are presented, the value ‘percent migration vs. control’ for a given trial represents the number of cells migrated in that condition expressed as a percentage of the mean number of cells migrating in control conditions. Recombinant netrin-1 protein was purified as described [38] and used at a concentration of 100 ng/ml. Laminin-1 was used at 10 μg/ml (BD Biosciences, Bedford, MA). Netβ and rabbit isoform control IgG (used as a control) were added at a concentration of 25 μg/ml. DCCFβ was added at a concentration of 10 μg/ml. Statistical significance was calculated using Student’s t-test and error bars represent S.E.M.

**Analysis of cell number and apoptosis**

To investigate changes in cell survival or proliferation, cells were plated at a density of 30,000 cells per well in 8-well chamber slides

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References

1. Rajasekharan S, Kennedy TE (2009) The netrin protein family. Genome Biol 10: 239.
2. Fearon ER, Cho KR, Nigro JM, Kern SE, Simons JW, et al. (1990) Genetic alterations during colorectal-tumor development. N Engl J Med 319: 525–532.
3. Manitt C, Colicos MA, Thompson KM, Rousselle E, Peterson AC, et al. (2001) Widespread expression of netrin-1 by neurons and oligodendrocytes in the adult mammalian spinal cord. J Neurosci 21: 3911–3922.
4. Thiebaud K, Mazelin L, Pays L, Llambi F, Joly MO, et al. (2003) The netrin-1 receptor UNC5H is a chemorepellent for oligodendrocyte precursor cells in the embryonic spinal ganglia and shows differential binding to netrin receptors. J Neurosci 19: 415–422.
5. Vogelstein B, Fearon ER, Hamilton SR, Kern SE, Preisinger AC, et al. (1988) Genetic alterations in the cell types of colorectal adenomas and carcinomas. N Engl J Med 319: 525–532.
6. Hedgecock EM, Culotti JG, Hall DH (1990) The unc-5, unc-6, and unc-40 genes guide circumferential migrations of pioneer axons and mesodermal cells on the epidermis in C. elegans. Neuron 4: 61–85.
7. Emelm M, Zhou Y, Su MW, Scott IM, Culott JG (1993) Expression of the UNC-5 guidance receptor on the touch neurons of C. elegans steers their axons dorsally. Nature 364: 527–530.
8. Shekarabi M, Kennedy TE (2002) The netrin-1 receptor DCC promotes filopodia formation and cell spreading by activating Cdc42 and Rac1. Mol Cell Neurosci 19: 1–17.
9. Watanabe H, Copeland NG, Gilbert DJ, Jenkins NA, Tessier-Lavigne M (1999) Axon guidance by diffusible chemoattractants: a gradient of netrin protein in the developing mammalian spinal cord. J Neurosci 19: 4593–4597.
10. Rajasekharan S, Baker KA, Horn KE, Jarjour AA, Antel JP, et al. (2009) Netrin-1 is a chemorepellent for oligodendrocyte precursor cells in the embryonic spinal cord. J Neurosci 23: 5735–5744.

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Author Contributions

Conceived and designed the experiments: AAJ MD TLL TEK. Performed the experiments: AAJ MD TLL NM MS. Analyzed the data: AAJ MD TLL NM TEK. Wrote the paper: AAJ TEK.
47. Lebrand C, Dent EW, Strasser GA, Lanier LM, Krause M, et al. (2004) Critical role of Ena/VASP proteins for filopodia formation in neurons and in function downstream of netrin-1. Neuron 42: 37–49.

48. Moore SW, Correia JP, Lai Wing Sun K, Pool M, Fournier AE, et al. (2008) Rho inhibition recruits DCC to the neuronal plasma membrane and enhances axon chemoattraction to netrin 1. Development 135: 2855–2864.

49. Forsthofel DJ, Lielbl EC, Kolodziej PA, Seeger MA (2003) The Abelson tyrosine kinase, the Trio GEF and Enabled interact with the Netrin receptor frizzled in Drosophila. Development 132: 1983–1994.

50. Mitra SK, Hanson DA, Schlapfer DD (2005) Focal adhesion kinase in command and control of cell motility. NatRevMolCellBiol 6: 56–68.

51. Baker KA, Moore SW, Jarjour AA, Kennedy TE (2006) When a diffusible axon guidance cue stops diffusing: roles for netrins in adhesion and morphogenesis. CurrOpinNeurobiol 16: 529–534.

52. Sieg DJ, Hasuck GR, Schlapfer DD (1999) Required role of focal adhesion kinase (FAK) for integrin-stimulated cell migration. JCell Sci 112(Pt 16): 2677–2691.

53. Manitt C, Kennedy TE (2002) Where the rubber meets the road: netrin expression and function in developing and adult nervous systems. ProgBrainRes 137: 425–442.

54. Hinck L (2004) The versatile roles of "axon guidance" cues in tissue morphogenesis. DevCell 7: 793–799.

55. Srinivasan K, Steckland P, Valdes A, Shin GC, Hinck L (2003) Netrin-1/neogenin interaction stabilizes multipotent progenitor cap cells during mammary gland morphogenesis. DevCell 4: 371–382.

56. Yebra M, Montgomery AM, Diaferia GR, Koidu T, Silletti S, et al. (2003) Recognition of the neural chemoattractant Netrin-1 by integrins alphabeta4 and alphabeta1 regulates epithelial cell adhesion and migration. DevCell 5: 695–707.

57. Liu Y, Stein E, Oliver T, Li Y, Brunken WJ, et al. (2004) Novel role for Netrins in regulating epithelial behavior during lung branching morphogenesis. CurrBiol 14: 897–905.

58. Larrieu-Lahargue F, Welm AL, Thomas KR, Li DY (2010) Netrin-4 induces lymphangiogenesis in vivo. Blood 115: 5418–5426.

59. Lu XW, Le Noble F, Yuan L, Jiang QJ, De Lafarge B, et al. (2004) The netrin receptor UNC5B mediates guidance events controlling morphogenesis of the vascular system. Nature 432: 179–186.

60. Park KW, Crouse D, Lee M, Karmik SK, Soremeni LK, et al. (2004) The axonal attractant Netrin-1 is an angiogenic factor. ProcNatlAcadSciUS A 101: 16210–16215.

61. Maselin L, Bernet A, Bonod-Bidaud C, Pays L, Arnaud S, et al. (2004) Netrin-1 controls colorectal tumorigenesis by regulating apoptosis. Nature 431: 80–84.

62. Yip JW, Yip YP (1992) Laminin—developmental expression and role in axonal outgrowth in the peripheral nervous system of the chick. Brain ResDevBrainRes 68: 23–33.

63. Gordon-Weeks PR, Golding JP, Clarke JD, Tonge D (1992) A study of the expression of laminin in the spinal cord of the frog during development and regeneration. ExpPhysiol 77: 681–692.

64. Hunter DD, Limas R, Ard M, Merlie JP, Sanes JR (1992) Expression of s-laminin and laminin in the developing rat central nervous system. JCompNeur 323: 238–251.

65. von Below C, Hayen W, Hartmann A, Mueller-Klieser W, Albolio B, et al. (2001) Endothelial capillaries chemotactically attract tumour cells. JPathol 193: 367–376.

66. Guillamo JS, Liosowski F, Christov C, Le Guerin C, Defer GL, et al. (2001) Migration pathways of human glioblastoma cells xenografted into the immunosuppressed rat brain. JNeurooncol 52: 205–215.

67. Tynnes BB, Haugland HK, Bjerkvig R (1997) Epidermal growth factor and laminin receptors contribute to migratory and invasive properties of gliomas. Invasion Metastasis 17: 270–280.

68. Knott JC, Mahesparan R, Garcia-Cabreza I, Belge TB, Ekdarden K, et al. (1998) Stimulation of extracellular matrix components in the normal brain by invading glioma cells. IntlCancer 75: 864–872.

69. Rashband WS, ImageJ U. S. National Institutes of Health, Bethesda, Maryland, USA, http://imagej.nih.gov/ij/, 1997–2011.