Oncogene (2007) 26, 5615–5625

www.nature.com/onc

ORIGINAL ARTICLE

Opposing roles of netrin-1 and the dependence receptor DCC in cancer cell invasion, tumor growth and metastasis

S Rodrigues[1,2], O De Wever[1,3], E Bruyneel[3], RJ Rooney[4] and C Gespach[1,2]

[1]INSERM, U673, Paris, France; [2]Université Pierre et Marie Curie-Paris 6, Faculté de Médecine, Laboratory of Molecular and Clinical Oncology of Solid Tumors, Paris, France; [3]Ghent University Hospital, Laboratory of Experimental Cancerology, Ghent, Belgium and [4]Genome Explorations Inc., Memphis, TN, USA

Deleted in colon cancer (DCC) and UNC5 function as netrin dependence receptors by inducing apoptosis in the absence of their ligand and accordingly were recently designated as putative conditional tumor suppressors. Herein, we determined whether netrin-1 and its receptors are implicated in cancer cell invasion and tumor progression. Expression of DCC, UNC5 and adenosine A2B-receptors (A2B-Rs) was investigated by reverse transcription polymerase chain reaction in human colon cancer cells. The impact of DCC restitution and netrin-1 was evaluated on collagen type I invasion, tumor growth and metastasis in nude mice, cancer cell survival and gene expression profiling. Flow cytometry, poly(ADP-ribose)-polymerase-1 and caspase-8 activation were used to evaluate the impact of DCC on cell death. Both netrin-1 and A2B-R activation induced the invasive phenotype through the Rho–Rho kinase axis in DCC-deficient human colorectal cancer cells. Restitution of wild-type DCC blocked invasion induced by netrin-1, A2B-R agonist and other agents. Ectopic expression of netrin-1 led to increased growth of human colon tumor xenografts in athymic mice. Conversely, introduction of wt-DCC in kidney MDCKts.src-ggl cells strongly inhibited metastasis in lymph nodes and lungs and increased sensitivity to apoptosis in hypoxia. DNA microarrays revealed that netrin and DCC had common and divergent impacts on gene expression linked to cell cycle, survival, surface signaling and adhesion. Our findings underscore that netrin is a potent invasion and tumor growth-promoting agent and that DCC is a metastasis suppressor gene targeting both proinvasive and survival pathways in a cumulative manner.
Oncogene (2007) **26,** 5615–5625; doi:10.1038/sj.onc.1210347; published online 5 March 2007

Keywords: adenosine A2B receptors; src; apoptosis; caspase-8; hypoxia; cDNA microarrays

Correspondence: Dr C Gespach, INSERM U673, Site Saint-Antoine, 75571 Paris Cedex 12, France.
E-mail: gespach@st-antoine.inserm.fr.
Received 30 August 2006; revised 9 January 2007; accepted 24 January 2007; published online 5 March 2007

Introduction

Netrin-1 belongs to a family of laminin-related secreted proteins expressed in the brain and peripheral tissues (Serafini *et al*., 1994). Netrins act through immunoglobulin-like transmembrane receptors, namely deleted in colon cancer (DCC)/neogenin and UNC5A-D, as well as the G-protein-coupled adenosine A2B-receptor (A2B-R), acting as a DCC co-receptor (Arakawa, 2004). Somatic loss of heterozygosity at chromosome 18q, frequently observed in colon, pancreatic and breast cancers, is often associated with loss and abnormalities of the putative tumor suppressor gene DCC (Fearon *et al*., 1990). The tumor suppressive functions of the dependence receptors DCC and UNC5 are linked to their ability to induce apoptosis when they are not engaged with their ligand netrin (Mehlen and Puisieux, 2006). In gut, netrin-1 exerts a key role on the maintenance, integrity, migration and renewal of the intestinal epithelium by induction of cell survival in proliferating crypt progenitors, where netrin levels are high. On top of villi where netrin levels are low, both DCC and UNC5 exert proapoptotic activities and epithelial cell shedding, because the death receptors DCC are constitutively expressed throughout the crypt–villus axis (Mazelin *et al*., 2004). Consistent with this scenario, inhibition of cell death by forced expression of netrin-1 in DCC-proficient mouse intestinal epithelial cells inhibits apoptosis and leads to the induction of hyperplastic and neoplastic lesions, following disruption of the netrin gradient and netrin expression all along the crypt–villus axis.

Netrin is a chemotropic molecule predominantly implicated in axonal outgrowth and cell migration orientation in the developing nervous system (Tessier-Lavigne and Goodman, 1996). Both DCC and UNC5 mediate the attractive and repulsive effects of netrin-1 on axonal growth and guidance (Hong *et al*., 1999). Implication of the adenosine A2B-R in mediating netrin-1-dependent axon outgrowth has been proposed (Corset *et al*., 2000). Netrin-1 and netrin receptors also control morphogenesis of endothelial cells and vascular smooth muscle cells and are implicated in the reorganization of the cytoskeleton, as well as epithelial cell adhesion and migration in lungs, mammary glands and pancreas (Shekarabi and Kennedy, 2002; Wilson *et al*.,

2006). Netrin dependence receptors integrate multiple signaling pathways including Rac1 and Cdc42 small GTPases, mitogen-activated protein kinase, and src and focal adhesion kinase (Forcet *et al*., 2002; Ren *et al*., 2004). Because these pathways and cellular functions can be activated in cancer and/or participate directly in promoting tumor development, we addressed the question of whether netrin and DCC are implicated in the regulation of cancer cell invasion and metastasis

Results

Proinvasive activity of netrin, adenosine A2B-R and their associated signaling pathways

We first established the *in vitro* relevance of netrin-1 in cellular invasion by assaying the expression of netrin receptors DCC, UNC5 and A2B adenosine receptors by reverse transcription–polymerase chain reaction (RT–PCR) in colon adenoma and adenocarcinoma cell lines PCAA/C1 and HCT8/S11 (Figure 1a, inset). Both cell types were positive for netrin-1, UNC5A-C and adenosine receptors A2A/A2B, but were DCC-deficient. Normal epithelial crypts isolated from the human colon were positive for DCC by identification of the corresponding 538-bp PCR product (not shown). Netrin-1, the high-affinity adenosine A2A/A2B-R agonist NECA, and adenosine each induced an invasive phenotype in HCT8/S11 cells in a dose-dependent fashion, with an EC_{50} of 0.1 nmol/l, 0.3 μmol/l and 7 μmol/l, respectively (Figure 1a). The proinvasive activity of netrin-1 was confirmed in premalignant PCAA/C1 colon adenoma cells and DCC-deficient human colon cancer cells HT-29 (not shown). The A2A-R agonist CGS (0.1–10 μmol/l) was ineffective, suggesting that the proinvasive activity of NECA occurs through A2B-R activation. In accordance, invasion produced by NECA and adenosine was abolished by the A2B-R antagonist enprofylline at low concentrations (10 μmol/l), whereas higher concentrations (50–300 μmol/l) were required to neutralize the response to netrin-1 and the thromboxane-A2 receptor agonist U466 (Figure 1b). It is notable that the EC_{50} of NECA and adenosine on cellular invasion are in good agreement with their interaction on typical human adenosine A2B-R (Linden *et al*., 1999). Consistent with this, we provide evidence that NECA, but not netrin-1 or the A2A-R agonist CGS, increased cAMP generation in HCT8/S11 cells (Figure 1c). This cAMP response was not altered by simultaneous addition of netrin-1 and NECA. In contrast, the combination of netrin-1 and NECA, but not netrin-1 combined with CGS, conferred additive proinvasive activity (Figure 1c, inset), which suggest that netrin-1 and A2B-R operate through distinct but convergent signaling pathways to induce invasion in DCC-deficient HCT8/S11 cells.

At the mechanistic level, A2B-R are coupled to Gαs and Gαq/G$\beta\gamma$ heterotrimeric G-proteins connected with their downstream effectors protein kinase A (PKA) and phospholipase C (PLC), respectively. The PKA inhibitor KT5720 suppressed invasion induced by netrin or

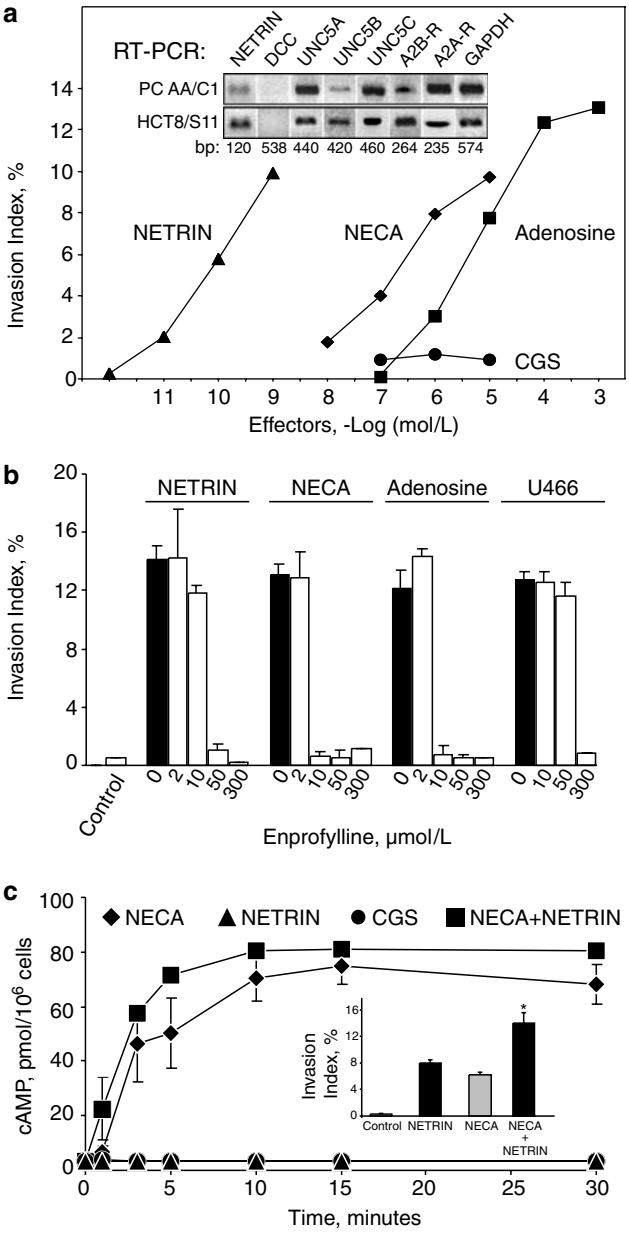


Figure 1 Functional expression of netrin and adenosine A2A/A2B receptors in human colon adenocarcinoma HCT8/S11 cells. (**a**) Dose-effect of netrin-1, NECA, CGS-21680 and adenosine on collagen type I invasion by HCT8/S11 human cancer cells. Inset: Expression of netrin-1 and its receptors DCC, UNC5A-C and adenosine A2A/A2B by RT–PCR in HCT8/S11 cells and premalignant adenomatous PC/AA/C1 cells isolated from a patient with familial adenomatous polyposis coli. Amplicons of netrin-1: 120 bp; DCC: 538 bp; UNC5A: 440 bp; UNC5B: 420 bp; UNC5C: 460 bp; adenosine A2B-R: 264 bp; adenosine A2A-R: 235 bp; GAPDH: 574 bp. (**b**) Dose–effect of the adenosine A2B-R antagonist enprofylline on cellular invasion induced by either netrin-1 (2.5 nmol/l), NECA (10 μmol/l), adenosine (0.1 μmol/l) and the TXA2-R agonist U466 (10 μmol/l). HCT8/S11 cells were preincubated for 2 h with enprofylline (2–300 μmol/l), before the addition of the test agents. (**c**) Kinetics of cAMP generation in HCT8S11 cells exposed to single and combined additions of netrin-1 (2.5 nmol/l), and NECA (10 μmol/l). Comparisons with additions using the A2A-R agonist CGS-21680 (10 μmol/l) or netrin-1 combined with NECA.

NECA, but was ineffective on the adenosine stimulus (Figure 2a). Conversely, stimulation with netrin and NECA was insensitive to U73122, while this PLC inhibitor abrogated the adenosine signals. As Rho GTPases and Rho-kinase ROK are required for the activation of the actin cytoskeleton and epithelial cell movements and invasiveness (Nguyen *et al*., 2005), we next neutralized these small GTPases by function-blocking pharmacological inhibitors and interfering mutants. Inhibition of Rho-GTPases (with C3T exoenzyme) and ROK (with Y27632) obliterated the invasive responses promoted by netrin-1 and NECA, but not adenosine in HCT8/S11 cells (Figure 2a). As expected (Nguyen *et al*., 2005), the adenosine proinvasive pathways linked to Gαq are not interrupted by pharmacological inhibitors and molecular interventions targeting the Rho-ROK axis, including C3T, Y27936, the RhoA antagonist DC-RhoD (G26V-RhoD) and the interfering mutants DN-RhoA (T19N-RhoA) and DN-ROK (DRB/PH). In contrast, DN-Rac1 (T17N-Rac1) and DN-Cdc42 (T17N-Cdc42), as well as the phosphatidylinositol 3-kinase (PI3K) kinase inhibitor wortmannin, abolished the invasive responses promoted by the proinvasive agents netrin-1, NECA and adenosine. Given that stress fiber formation and RhoA are required for cell motility and contraction, we next examined the impact of netrin-1 and NECA on the activation status of RhoA. In agreement with our data in Figure 2a, netrin-1 and NECA caused strong activation of RhoA (fourfold) in HCT8/S11 cells (Figure 2b), which was associated with reorganization of F-actin to parallel organized stress fibers in most of the cell colonies. Cell retraction was associated with the formation of needle-like extensions containing actin filaments, which were occasionally seen along the cell margins, together with the persistence of peripheral actin bundles (Figure 2c). Our data in Figures 1 and 2 support the conclusion that the proinvasive activity determined by the A2B-R is cAMP/PKA-dependent and PLC-independent. In contrast, the physiological cAMP-inducing agent adenosine required the Gαq/PLC pathway as a prerequisite for stimulating invasion via PKA and Rho-ROK-independent mechanisms. Of note, cAMP-dependent/PKA-independent pathways include Epac proteins (exchange protein directly activated by cAMP) that are guanine nucleotide exchange factors for the small GTP-binding proteins Rap1 and Rap2 regulating a vast array of cellular and biological responses, including induction of

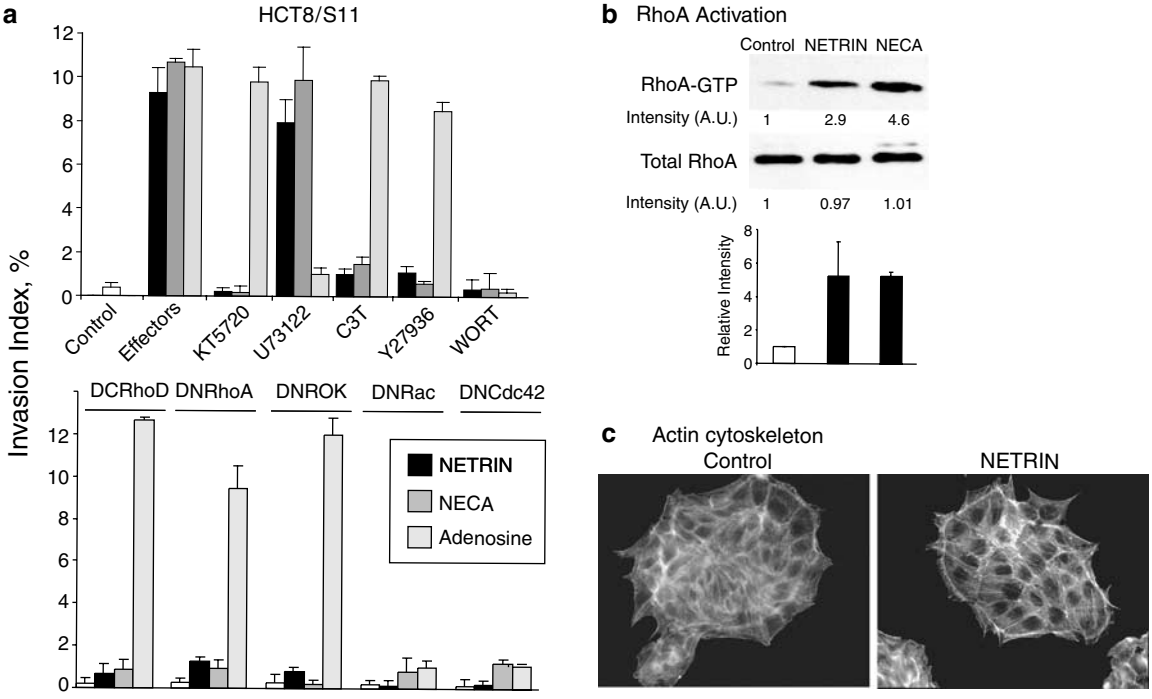


Figure 2 Signaling pathways and activation of the actin cytoskeleton associated with the proinvasive activity of netrin-1, NECA and adenosine in human colon adenocarcinoma HCT8/S11 cells. (**a**) Top: proinvasive activity of netrin-1 (2.5 nmol/l), NECA (10 μmol/l) and adenosine (0.1 mmol/l) in HCT8/S11 cells and its reversion by pharmacological inhibitors targeting PKA (KT5720, 1 μmol/l), PLCβ (U73122, 1 μmol/l), Rho-GTPases (C3T exoenzyme, 3 μg/ml), Rho-kinase ROK (Y27632, 10 μmol/l) and PI3K (wortmannin: WORT, 10 nmol/l). Bottom: stably transfected HCT8/S11 cells expressing the dominant negative mutants DNRhoA, DNRac, DNCdc42, DNROK and the constitutively activated form of the RhoA antagonist RhoD (DCRhoD) were assayed for their invasive capacity in response to netrin-1, NECA and adenosine. (**b**) RhoA activity in cell lysates prepared from HCT8/S11 cells grown in serum-free conditions and treated for 30 min with netrin-1 (2.5 nmol/l) and NECA (10 μmol/l). The activated GTP-bound form of RhoA was assayed using sepharose beads linked to the Rho-binding domain of Rhotekin. Relative intensity of the RhoA-GTP signals (arbitrary units, AU) was quantified using ImageQuant Software, according to total RhoA levels detected by direct immunoblotting. (**c**) F-actin stress fiber formation and cell retractation in HCT8/S11 cells treated for 30 min with netrin-1 (2.5 nmol/l) or vehicle (Control). The cells were stained for F-actin using rhodamine-conjugated phalloidin.

the caudal homeobox gene Cdx-2 involved in colon epithelial cell differentiation and carcinogenesis, integrin-mediated cell adhesion, cancer cell invasion and metastasis (Chen *et al*., 2005; Gao *et al*., 2006). Interestingly, Epac was found upstream PLC and PLD, which are necessary for the translocation and activation of PKC, suggesting that adenosine and this cAMP-GEF mediates a Gαs/cAMP-to-PLC crosstalk (Hucho *et al*., 2005). Taken together, our data suggest that the A2B-R is the key signaling platform for determination of the invasive phenotype induced by adenosine through PLC in human colon cancer cells and that signaling crosstalks between A2B-R and adenosine receptors A1 (Gαi/Gαo/IP3/Ca2 +) and A3 (Gαi/Gαq/IP3) are also potentially involved in this process.

Opposing roles of netrin and DCC ectopic expression on the invasive phenotype

Next, we tested whether the invasive phenotype conferred by external addition of netrin-1 could be extended to HCT8/S11-netrin cells stably transfected by an expression vector encoding netrin-1. As with parental HCT8/S11 cells, control vector transfected HCT8/S11-pcDNA3 cells were noninvasive and external addition of netrin or NECA simulated collagen type I invasion. The invasive phenotype exhibited by the HCT8/S11-netrin cells (Supplementary data 1) was reversed by the inhibitors of the Rho-ROK axis and PI3K (Kotelevets *et al*., 1998; Barbier *et al*., 2001; Nguyen *et al*., 2005). Conversely, restoration of wt-DCC in DCC-deficient HCT8/S11 cells obliterated the proinvasive activity of netrin-1, NECA and other proinvasive agents, including TGFα and trefoil factors. This was associated with a decreased ability of netrin and NECA to increase RhoA-GTP levels in HCT8/S11-wt-DCC cells (twofold), compared to the fourfold stimulation observed in parental cells (not shown). Treatment of HCT8/S11.wt-DCC cells with netrin reversed the invasion blocking activity exerted by DCC on the invasion promoters TGFα and TFFs. Notably, mutation of the caspase-3 cleavage site in DCC (DCC-Stop and D1290N mutants) abrogated the invasion suppressive functions of DCC (Supplementary data 1). Our data demonstrate that the intracytoplasmic part of DCC plays a key role in the invasion suppressor role of DCC. This signaling platform integrates several signaling elements using Src and Fyn family kinases, FAK, phosphatidylinositol transfer protein-α and PLC, as well as the membrane–cytoskeleton linkers ezrin and merlin (Martin *et al*., 2006; Xie *et al*., 2006). Other mechanisms using other DCC intracellular domains and/or extracellular domains (fibronectin repeats) may play additional roles, as shown for the processing of the DCC intracellular domain, nuclear translocation and transcriptional functions (Taniguchi *et al*., 2003).

Convergent and divergent gene expression signatures following ectopic expression of netrin and wt-DCC

cDNA microarray analysis shown in Supplementary data 2 indicated that both netrin-1 and DCC decreased expression of a number of genes associated with cell growth control, cell-cycle regulation and apoptosis, including *p57KIP2*, *BCL3*, *BIRC6*, *NF1*, *NFKBI* zeta and transcription regulatory proteins *EGR1*, *ELF3*, *ETS2*, *FOS*, *H19*, *KLF9*, *KLF10*, *MSX2*, *THRAP2* and *TRIP12*. In addition, DCC, but not netrin-1, decreased the expression of other genes associated with apoptosis (*BCL6*, *BIK*, *BTG1*, *PIM1* and *SIAH1*), cell-cycle regulation (*CYCLIN D2*, *CYCLIN G2*, *CHES1*, *DUSP1*, *FYN*, *GADD45α*, *RAD50*, *Septin 3* and *Septin 6*) and transcription control (*FOXP1*, *HDAC4*, *NR1D1*, *NR2F1* and *NR5A2*), in addition to several genes related to cellular invasion, angiogenesis, neurite morphogenesis (*NEUROPILIN*, *NRCAM*) and cell surface antigens (*CD38*, *CD44*). Parenthetically, considerable attention has already been focused on the involvement of *CD44* and *NEUROPILIN* genes in cancer cell adhesion, proliferation, invasion and metastasis (Hill *et al*., 2006; Nguyen *et al*., 2006). In coherence with our functional data, we observed that wt-DCC downregulate the expression of the *SIAH1* gene (seven in absentia homolog 1) encoding the ubiquitin-conjugating enzyme Siah-1 mediating the degradation of DCC via the proteasome pathways, whereas netrin was found to exert the opposite regulation via ubiquitin–proteasome degradation of DCC (Hu *et al*., 1997; Kim *et al*., 2005). In contrast, netrin-1, but not DCC decreased the expression of a number of genes associated with cytokine/cell surface receptor-mediated signals (*ADORA*, *CHRM1*, *CCL15*, *CYSLTR*, *DP1*, *DUSP6*, *EPOR*, *IFNGR1*, *IFI27*, *IRF8*, *ISGF*, *PRLR*, *TNF15*, *TNFR1B*, *TNFR21*, *TNFR25* and *VEGF*), cell adhesion and the cell matrix or motility (*CLAUDIN 2*, *CLAUDIN 3*, *FN1*, *INTEGRINS α3*, *β4*, *KERATIN 8-like 2*, *MESOTHELIN*, *NEUROLYSIN*, *PROCADHERIN 1* and *TIMP3*). Interestingly, both integrins α3 and β4 are components of the netrin-1-binding sites in cellular signaling (Yebra *et al*., 2003). Restitution of wt-DCC in HCT8/S11 cells increased the expression of a few genes, including alkaline phosphatase, decay accelerating factor (*CD55*), and the melanoma antigen *MAGE A2*. Finally, ectopic expression of netrin-1 (not with DCC) upregulated several genes involved in cellular growth and guidance, including *BDNF*, a brain-derived neurotrophic factor acting through the tyrosine kinase receptors TrkB and the Tiam-Rac cascade (Grider *et al*., 2006; Miyamoto *et al*., 2006), *KIT ligand*, *SOCS2*, a member of the suppressors of cytokine signaling, and several transcription factors (*LEF1*, *NF1/B* and *SATB2*). These convergent and divergent profiles are consistent with the idea that DCC and netrin form a decision point governing cell fate and phenotype.

Differential impact of netrin and wt-DCC on primary tumor growth and cancer cell metastasis

As shown in Figure 3a, both control HCT8/S11-pcDNA3 and wt-DCC cells formed tumors with similar onset and growth curves in athymic mice. In contrast, netrin increased twofold the tumorigenicity of HCT8/S11-netrin cells at day 45 post-injection in nude mice

($P<0.05$, Figure 3a, inset). Similarly, expression of wt-DCC had no impact on the growth of the metastatic kidney MDCKts.src-ggl-luci.wt-DCC cancer xenografts (Figure 3b, left), but decreased by 60% the weight of their corresponding axillary ganglia at day 110 compared to their control counterparts MDCKts.src-ggl-luci (Figure 3b, right). We checked for the persistence of wt-DCC expression in tumors initiated by colon and kidney cancer cell xenografts. The 538-bp amplicon was detected in HCT8/S11.wt-DCC and MDCKts.src-ggl-luci.wt-DCC tumors resected at days 45 and 110 (Figure 3a and b, insets).

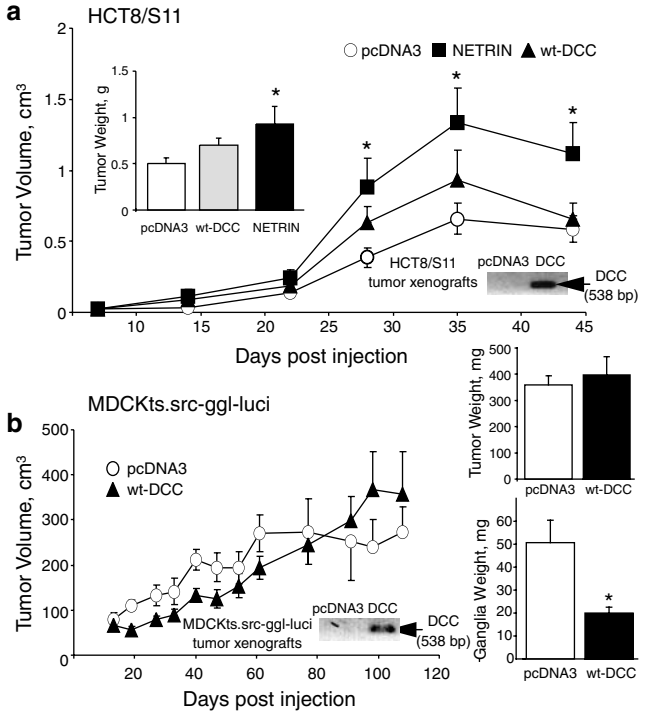


Figure 3 Differential impact of ectopic expression of netrin-1 and wt-DCC on tumor growth and metastasis. (**a**) Control vector-transfected HCT8/S11-pcDNA3 cells, HCT8/S11-Netrin and HCT8/S11.wt-DCC cells, as well as pcDNA3- and wt-DCC-transfected MDCKts.src-ggl-luci cells (**b**), were subcutaneously injected as cancer cell xenografts in nude mice (4 and 10×10^6 cells per mouse, eight or nine mice per group, respectively). Tumor volume was measured once a week and tumor and regional ganglia weight (insets) at end of the experiments. Expression of the exogenous human wt-DCC was confirmed by RT–PCR in both wt-DCC-transfected HCT8/S11.wt-DCC and MDCKts.src-ggl-luci cells (insets in **a** and **b**). Data are means$\pm$s.e.m. and are representative of another experiment. *$P<0.05$, according to the Student *t*-test.

These results suggest that DCC signaling negatively controls cellular dissemination and the metastatic process in MDCKts.src-ggl-luci xenografts. To test this interpretation, we quantified the presence of luciferase-positive cancer cells in primary tumors established from pcDNA3 and wt-DCC-transfected MDCKts.src-ggl-luci cancer cell xenogratfs, as well as in their corresponding axillary lymph nodes and lungs. Table 1 shows that MDCKts.src-ggl-luci cell counts are about three times lower in wt-DCC primary tumor xenografts (16.6×10^6 cells) than their pcDNA3 control counterparts (53.4×10^6 cells, $P<0.002$). Notably, expression of wt-DCC decreased lymph node invasion by cancer cells, because only eight of 25 ganglia were invaded in mice bearing wt-DCC tumor xenografts (0.28×10^6 cells), as opposed to 23 of 28 ganglia that were luciferase-positive in mice injected with control pcDNA3 xenografts (3.5×10^6 cells, $P<0.015$). A similar picture emerged in the corresponding lungs of these animals as five of nine control animals revealed MDCKts.src-ggl-luci-positive cancer cells in lungs (52.3×10^6 cells), whereas luciferase activity was undetectable in lungs from animals bearing the wt-DCC xenografts. Thus, wt-DCC decreased modestly the cancer cell mass in primary tumors (3.2-fold), but reduced 12.5-fold the metastasis to regional lymph nodes and blocked cancer cell dissemination in the lung. To confirm the invasion suppressor role of wt-DDC in MDCKts.src-ggl-luci cells, we confirmed that collagen invasion induced by netrin, NECA, adenosine and TGFα in pcDNA3 cells was drastically inhibited by the ectopic expression of wt-DCC (Supplementary data 3).

Cell death activity of wt-DCC in hypoxic conditions

These data led us to consider the impact of ectopic DCC expression on cell survival and apoptosis in the pcDNA3 and wt-DCC MDCKts.src-ggl-luci variants. Cells were therefore submitted to environmental stress conditions

Table 1 Quantification of luciferase-positive cells in primary tumors, regional lymph nodes and lungs after subcutaneous implantation of pcDNA3- and wt-DCC-transfected MDCKts.src-ggl-luci cells in nude mice

	Luciferase-positive MDCKts.src-ggl cancer cells								
	Primary tumors			*LN*			*Lungs*		
Tumor type	*Total number*	*means$\pm$s.e.m. (10^6 cells)*	*Range (10^6 cells)*	*Positive LN/ number studied*	*Means$\pm$s.e.m. (10^6 cells)*	*Range (10^3 cells)*	*Positive lungs/ number studied*	*Means$\pm$s.e.m. (10^3 cells)*	*Range (10^3 cells)*
pcDNA3	9	53.4$\pm$6.6	(20–82)	23/28	3.5$\pm$1.4	(7–26 900)	5/9	52.3$\pm$22	(3.7–130)
DCCwt	9	16.6$\pm$2.8	(5.1–28)	8/25	0.28$\pm$0.19	(5.5–1670)	0/9	(ND)	

Abbreviations: DCC, deleted colon cancer; LN, lymph nodes. At 110 days post-implantation of the MDCKts.src-ggl-luci xenografts, pcDNA3 and wt-DCC tumors were excised from immunodeficient mice and homogenized for the luciferase assay. Luciferase signals were linearly correlated with pcDNA3 and wt-DCC cell number over a range of 200–150 000 cells. About 1500 luciferase units corresponded to 200 pcDNA3 cells and to 400 wt-DCC cells. Recovery of luciferase-positive cells in tissues was checked after direct injection of pcDNA3 and wt-DCC MDCKts.src-ggl-luci cells in lungs.

induced by serum deprivation and hypoxia, two situations mimicking the tumor microenvironment during cancer progression. In normoxia, there was no significant effect of wt-DCC on apoptosis in either confluent or proliferating cells cultured in the presence or absence of serum (Figure 4a). However, in hypoxic conditions, wt-DCC increased the incidence of apoptosis from 9.3% in pcDNA3 cells to 20.6% in wt-DCC cells ($P<0.003$), a response that was not further increased by serum withdrawal in wt-DCC cells ($P<0.048$). Similarly, addition of netrin (2.5–12.5 nmol/l) to pcDNA3 and wt-DCC cells was unable to reverse the apoptosis induced by DCC and hypoxia in MDCKts.src-ggl-luci cells cultured in the presence or absence of serum ($P<0.016$ and 0.012 compared to pcDNA3 cells). To identify the mechanisms linked to the apoptosis induced by ectopic expression of wt-DCC in hypoxia, we focused on critical effectors and signals of the death pathways, namely cleavage of the poly(ADP-ribose)polymerase-1 (PARP) and caspase-8, two common signatures of the intrinsic and extrinsic apoptosis pathways (Hipfner and Cohen, 2004). In general, the apoptotic process is activated by initiator caspases such as caspase-8, involved in anoikis and cancer cell dissemination, in integrin and in a cellular death receptor-dependent manner (Lahti *et al*., 2006). Activation of the extrinsic pathway was therefore determined by immunoblotting for cleaved caspase-8. As shown in Figure 4b, the 40 kDa cleaved caspase-8 fragment was evidenced in wt-DCC cells exposed to hypoxia in the presence and absence of netrin. Downstream apoptosis events include activation of executioner caspases such as caspase-3, -6 and -7, which target various substrates including PARP. Accordingly, Western blot analysis showed that wt-DCC in hypoxia induced the appearance of the characteristic 85 kDa cleaved active PARP fragment in MDCKts.src-ggl-luci cells cultured in the presence or absence of netrin. These molecular signatures were confirmed by the morphological appearance of apoptotic MDCKts.src-ggl-luci.wt-DCC cells in hypoxia (Figure 4c). Our data suggest that the cell death activity of wt-DCC observed under hypoxic conditions can explain, at least in part, the reduced cancer cell mass in the primary tumors.

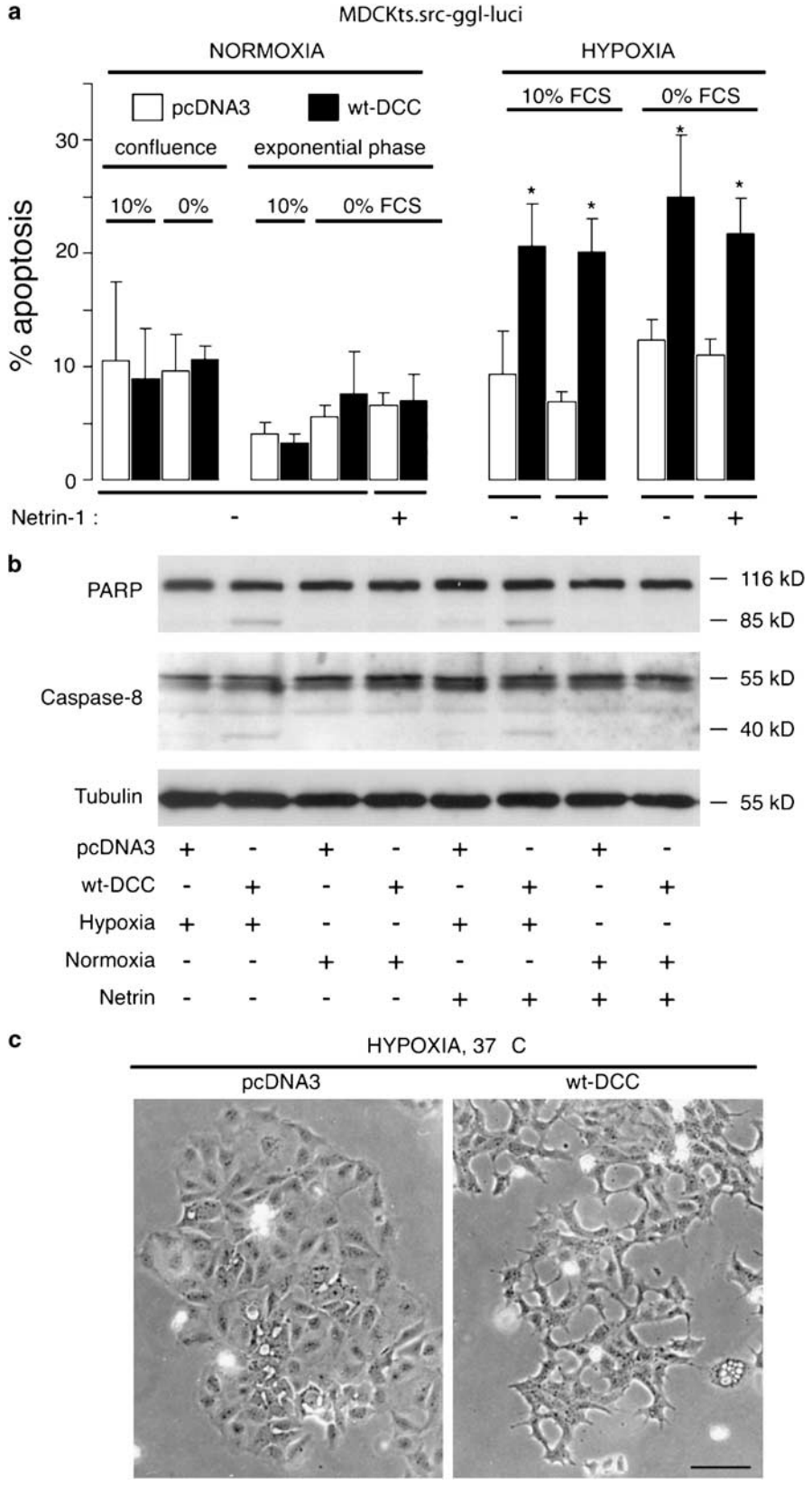
Discussion

DCC has been discovered as a putative tumor suppressor gene in the colon cancer field and was revealed to be a netrin receptor, mediating axonal guidance in the central nervous system. Recent evidence also indicates that both netrin and DCC are playing a major role in the coordinated migration and survival of normal intestinal epithelial cells along the crypt–villus axis, in agreement with the concept that DCC belongs to the dependence receptors family toward their respective ligands (Mazelin *et al*., 2004). A gradient of netrin-1 was observed along crypt–villus axis with high levels of netrin-1 associated with the self-renewal of intestinal progenitor cells and low netrin levels at the mucosal surface (Mazelin *et al*., 2004). Progressive loss of available netrin-1 is associated with increased alterations of cell viability and epithelial cell shedding at the mucosal surface, suggesting that the netrin-1/DCC duo had a significant role in maintaining intestinal homeostasis through epithelial stem cell renewal and differentiation, migration and death. Accordingly, forced expression of netrin-1 in the intestinal epithelium of tg-netrin transgenic animals was associated with a 50% decrease of apoptotic intestinal cells and was also shown to confer a tumor growth advantage in $APC^{+/1638N}$ mice at the adenoma-carcinoma transition. Indeed, Tg-netrin/APC mice showed higher incidence of high-grade adenoma with focal invasion of the muscularis mucosa (Mazelin *et al*., 2004).

The data presented here demonstrated that netrin-1 induces proinvasive and tumor growth-promoting signals in DCC-deficient colon cancer cells and also showed that DCC acts as a tumor suppressor gene by counteracting cancer cell invasion and metastasis. The proinvasive signals initiated by netrin-1 in the present study are concordant with the expression and retention of the UNC5 netrin receptors in premalignant adenoma cells PCAA/C1 and colon cancer cells HCT8/S11, even with the loss of DCC receptors in these models. It is suggested that UNC5 receptors and integrins compensate for the loss of DCC toward migration and cancer invasion induced by netrin-1. This assumption does not exclude the apoptotic potential of UNC5 receptors observed after genetic transfer and certain pathophysiological situations (Mehlen and Puisieux, 2006). Moreover, both external addition and ectopic expression of netrin-1 initiated an invasive phenotype in colon cancer cells, suggesting that both paracrine and autocrine mechanisms are involved with netrin expression in normal and transformed colon mucosa. In agreement, we have identified by RT–PCR the accumulation of the transcripts encoding netrin and its receptors UNC5(A–C) and A2B-R in human colon adenocarcinoma (stages B1, C2 and D) and their corresponding control mucosa,

Figure 4 Molecular and cellular determination of apoptosis in pcDNA3 and wt-DCC-transfected MDCKts.src-ggl-luci cells cultured under normoxic and hypoxic conditions. (**a**) Flow cytometry analysis in confluent and exponentially growing MDCKts.src-ggl-luci.pcDNA3 and-wt-DCC cells. Effect of serum deprivation and overnight hypoxia on the percentage of apoptosis at the sub-G1 fraction in cells incubated for 24 h in the presence (+) or absence (−) of netrin-1 (2.5 and 12.5 nmol/l). *$P<0.05$, according to the Student's *t*-test. (**b**) Expression and cleavage of the apoptosis-related proteins PARP (85 kDa) and caspase-8 (40 kDa) by SDS–PAGE (8 and 12%, respectively) and immunoblot analysis. MDCKts.src-ggl-luci.pcDNA3 and -wt-DCC cells were incubated in normoxic and hypoxic conditions in the presence (+) and absence (−) of netrin-1 (10 nmol/l). The tubulin immunoreactive bands (55 kDa) served as an internal control. (**c**) Representative morphological appearance by phase contrast microscopy of cell shrinkage and apoptotic body formation in hypoxic MDCKts.src-ggl-luci.wt-DCC cells.

whereas loss and downregulation of the DCC transcripts were observed (not shown). Consistent with these findings, 18q loss of heterozygosis is indeed observed at increasing frequency during the adenoma-to-carcinoma conversion and in invasive colorectal cancers in FAP patients (Miyaki *et al*., 1990).



Our data support the notion that the proinvasive activity of adenosine is mediated by the A2B-R through integration of complex signaling networks and cross-talks between adenosine receptors, including coactivation of classical A1, A2(A/B) and A3 receptor subtypes in human colon cancer cells. Recent evidence supports other diverse roles for atypical nonconventional adenosine cell surface receptors or transporters (Tan *et al*., 2006). In coherence with our data, it has been demonstrated that the A2B-R is induced in hypoxia, inflammatory bowel diseases and promotes an angiogenic phenotype, as measured by endothelial tube formation (Kong *et al*., 2006). Thus, we found that the PLC signaling cascade activated by the physiological agonist adenosine is the critical invasion effector acting through Rho/ROK-independent mechanisms and the Ca^{2+}-dependent myosin light chain kinase that is believed to promote the contractility of the actin cytoskeleton, as shown previously in our laboratory (Nguyen *et al*., 2005). Both adenosine and the A2B-R are linked to cAMP pathways and to the recruitment of DCC receptors from an intracellular pool to the cell surface, in a netrin-dependent manner (Bouchard *et al*., 2004). In contrast, we have shown that the proinvasive activity of netrin is determined by PKA, Rho/ROK and PI3K-dependent pathways via a PLC-independent mechanism. This picture is probably more complicated because netrin-1 also interacts and signals with integrins $\alpha6\beta4$ and $\alpha3\beta1$ and the integrin-associated kinase FAK for the activation of cell adhesion and migration (Yebra *et al*., 2003). Integrins are also considered dependence receptors for extracellular matrix components during loss of adhesion-mediated apoptosis, that is, anoikis. Accordingly, primary and adenocarcinoma-derived pancreatic epithelial cells were shown to adhere very efficiently to netrin relative to collagen types I and IV, fibronectin and laminin 1,5 (Yebra *et al*., 2003).

Consistent with the cell death activity of the DCC dependence receptor, few DCC-resistant clones were isolated upon ectopic expression of DCC in HCT8/S11 and MDCKts.src cells. These cell lines escaped from the mechanisms of apoptosis promoted by the DCC tumor suppressor gene. We confirmed the invasion suppressive functions of DCC in metastatic MDCKts.src-ggl.wt-DCC cells showing that DCC remarkably exerts dominant functions over the src oncogene. The src tyrosine kinase is activated at early stages during colon cancer progression and plays major roles in cancer cell survival and invasion (Rivat *et al*., 2003). We found that wt-DCC significantly reduced the number of luciferase-positive MDCKts.src-ggl cancer cells in primary tumors, suggesting that DCC expression alters their viability and survival in the tumor microenvironment. Accordingly, the use of hypoxic conditions revealed that wt-DCC still mediates detrimental functions to cell viability in a netrin-independent manner, as visualized by caspase-8 and PARP cleavage and morphological criteria. Thus, we can assume that the survival mechanisms that arose during the selection of MDCKts.src-ggl.wt-DCC cells are diverted and abolished in hypoxia. Supporting this interpretation, the apoptosis dependence receptor UNC5 harbors a C-terminal domain related to the death domain of the Fas and TNF receptors that are connected with the apoptosis initiator caspase-8 (Mehlen and Puisieux, 2006). In opposition to netrin-1, DCC expression promotes convergent tumor-suppressive functions by inducing increased susceptibility to apoptosis and impairing cellular invasion and metastasis. Thus, our data link the invasion and metastatic potential of cancer cells with their ability to survive in primary tumors, as circulating micro-metastases, and to extravasate in target organs where they must face new modalities for homing, survival and growth. Although DCC reduced by 70% the number of luciferase-positive cancer cells in primary tumors at 110 days post-implantation, this tumor-suppressor gene had no impact on the dimension and weight of the tumor. These paradoxal data can be attributed to large variations in the percentage of stromal content in solid tumors, a situation influenced by complex interactions between cancer cells and their neighboring stromal cells, (myo)fibroblasts, and endothelial and immune-competent cells. Quantitative measurements revealed that stromal content varied from 18 to 64% in colon carcinomas, possibly in conjunction with specific chromosomal aberrations and somatic mutations, associated with loss and gain of functions by tumor suppressor genes and oncogenes (Dr R Fijneman and Professor G Meijer, personal communication). Taken together with the present study, these clinical data may explain part of the individual differences in the observation and interpretation of tumor responses during chemotherapy of primary and metastatic human solid tumors.

Opposing and divergent roles of netrin-1 and DCC in cell survival, invasion, tumor growth and metastasis demonstrated in the present study integrate several pathways involved in cellular transformation. Initial DNA microarray analyses clearly indicate that netrin-1 and DCC have distinct, but overlapping effects on gene expression that are linked to signaling elements involved in cellular growth, survival and cellular adhesion. Netrins and the dependence receptors DCC/neogenin, UNC5 and integrins play a key role in normal development in the brain–gut axis by regulating major functions linked to axonal guidance and survival, pancreatic and mammary gland morphogenesis, intestinal epithelial cell homeostasis and angiogenesis (Hebrok and Reichardt, 2004; Wilson *et al*., 2006). DCC appears to influence a greater number of genes associated with cell cycle and apoptotic pathways, whereas netrin-1 exhibits a greater selective impact on genes associated with cell surface signaling and cell adhesion, motility and the extracellular matrix. Taken together with recent advances in this field showing the developmental role of netrin-1 in neovascularization (Wilson *et al*., 2006) and cancer progression (Mazelin *et al*., 2004), our data suggest that new compounds and strategies targeting the netrin and DCC pathways may bring therapeutic benefit in colon cancer patients.

Materials and methods

Cell lines, DNA constructs and stable transfections

HCT8/S11 and HT-29 were maintained in culture medium containing RPMI-1640 and DMEM, respectively (GIBCO-BRL, Invitrogen, Cergy Pontoise, France), supplemented with antibiotics and 10% fetal calf serum (FCS). HCT8/S11 cells were stably transfected using the lipofectAMINE plus reagent (GIBCO-BRL) and one the following expression vectors (Mehlen *et al*., 1998): pcDNA3.netrin-1 encoding a myc-tagged chicken netrin-1; wild-type pCMV.DCC encoding the full length DCC (wt-DCC); truncated pCMV.DCC-Stop-1291 lacking its intracellular domain under the caspase cleavage site, following the insertion of a stop codon at position 1291; mutated pCMV.DCC-D1290N containing the aspartic acid asparagine substitution at position 1290 of the caspase cleavage site, and the control empty vector pcDNA3. After 48 h, cultures were selected for 2 weeks with 400 μg/ml G418. HCT8/S11-resistant colonies were ring-cloned for analysis of ectopic expression of DCC and netrin-1. Only one clone was positive from 12 G418-resistant colonies tested after transfection with each DCC variant (wt, Stop-1291 and D1290N). HCT8/S11 cells stably transfected by the dominant-negative forms of the RhoA GTPases (DNRhoA, DNRac and DNCdc42), Rho-kinase ROK (DNROK) and the constitutively activated form of the RhoA antagonist RhoD (DCRhoD) have been characterized elsewhere (Nguyen *et al*., 2005). The kidney cancer cell line MDCKts.src established after stable transfection of a thermosensitive variant of v-src and the PCAA/C1 adenomatous colon cell line derived from a patient with familial adenomatous polyposis were maintained in DMEM supplemented with 10–20% FCS. Because v-src transformed MDCK cells were previously described to exhibit a metastatic potential after orthotopic implantation (Kadono *et al*., 1998), we derived a metastatic subline from MDCKts.src cells that consistently and spontaneously metastasize to regional lymph nodes and lungs. For this purpose, *in vivo* selection was carried out by subcutaneous implantation of 10×10^6 MDCKts.src cells in athymic nude mice. After 4 months, axillary lymph nodes were minced and cultured to isolate metastatic variants. After two rounds of *in vivo* selection via subcutaneous injection and regional lymph node invasion in new recipient mice, the resulting metastatic cell line was stably transfected by the PGL3 luciferase reporter vector (Promega, Charbonnières, France). Thirty-six clones were then isolated and propagated using a selectable puromycin gene in pBabe and luciferase activity was measured. Clone 18 associated with high luciferase activity was then isolated and designated MDCKts.src-ggl-luci. This clone 18 was subsequently transfected by the pcDNA3 and the pCMV.wt-DCC vector. Three sets of transfections allowed the selection of 54 individual hygromycin-resistant colonies. Among them, only one clone of MDCKts.src-ggl-luci.wt-DCC cells (clone 13) was positive for wt-DCC expression.

Western blot and immunofluorescence

Cells were homogenized at 4°C in lysis buffer containing phenylmethylsulfonyl fluoride, dithiothreitol, aprotinin, pepstatin A and leupeptin as protease inhibitors. Proteins were resolved in Laemmli buffer, denaturated for 5 min at 95°C and then separated in 6–12% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) gels and transferred to polyvinylidene fluoride membranes (GE Healthcare, Orsay, France). The blots were then probed for 1 h at room temperature with primary monoclonal antibodies directed against the myc epitope to reveal chicken netrin-1 (1:500, BD Biosciences, Erembodegem, Belgium), human DCC (1:100, Oncogene Science, Paris, France) and RhoA (1:100, Santa Cruz Biotechnologies, Heidelberg, Germany). Membranes were reprobed for 1 h with secondary antibody consisting of peroxidase-linked goat anti-mouse IgGs (1:2000, Santa Cruz Biotechnologies) and revealed by enhanced chemiluminescence (GE Healthcare, Orsay, France). For F-actin staining, single HCT8/S11 cells were seeded on coverglasses and grown as colonies of 20–30 cells. Then the cells were cultured for 24 h under serum-free conditions before stimulation with 3 nmol/l netrin-1 for 30 min. After fixation in 3.7% formaldehyde (10 min) and permeabilization for 5 min with 0.2% (v/v) Triton X-100 in buffered Hank's balanced salt solution, cells were incubated with rhodamine-coupled phalloidin (Calbiochem-VWR, Strasbourg, France). Cells were visualized under a Leica DMR fluorescence microscope (Rueil-Malmaisons, France).

RT–PCR and DNA microarrays

RNA was extracted from cells preserved in RNA*later*, using Trizol reagent, following the manufacturer's instructions (Ambion, Huntingdon, UK). Total RNA was reverse-transcribed by Superscript II reverse transcriptase in the presence of 500 ng RNA and hexarandom primers (Invitrogen). Fragments of cDNA corresponding to netrin-1 and DCC, UNC5A, UNC5B, UNC5C and adenosine A2A-B receptors were amplified by 35 cycles of PCR using *Taq* Gold polymerase (Perkin–Elmer, Zaventem, Belgium) and the following primers and annealing temperatures: Netrin-1 (amplimer: 120 pb, 5′TTTCACTACGATCGGCCCTGGCA GC and 5′ATAGAGCTCCATGTTGAATCTGCAGCGC, 62°C); DCC (amplimer: 538 pb, 5′CATCTTCAAGTAACCG and 5′TGGAGTCGGCTGATCTGCA, 56°C); UNC5A (amplimer: 440 pb, 5′GCACAAGCCGGAAGACGTGAG and 5′GCATCGTGGGTGTCATGCAGG, 56°C); UNC5B (amplimer: 420 pb, 5′CAAGGCAGAAAGTACCCTCCCGCT and 5′CAGCACCTCCTTCAGTGCTAC, 56°C); UNC5C (amplimer: 460 pb, 5′GGCAGCAAGACAAGATCTGC and 5′ ATGGGTGGCCTCATAGTTTC, 56°C); A2A-R (amplimer: 235 pb, 5′GCCATCACCATCAGCACCG and 5′GCAT GGGAGTCAGGCCGATG, 56°C); A2B-R (amplimer: 264 pb, 5′GACTTCTACGGCTGCCTCT and 5′ CCATCC-CAGGGTTCTGTGCAGTT, 56°C).

For the DNA microarrays, first and second strand cDNA was synthesized from 15 μg of total RNA using the SuperScript Double-Stranded cDNA Synthesis Kit (Invitrogen, Cergy Pontoise, France) and oligo-dT_{24}-T7 (5′-GGC CAG TGA ATT GTA ATA CGA CTC ACT ATA GGG AGG CGG-3′) primer (Sigma-PrOligo, St Louis, MO, USA) according to the manufacturer's instructions. The purity and concentration of RNA samples were determined from $OD_{260/280}$ readings using a dual-beam UV spectrophotometer and RNA integrity was determined by capillary electrophoresis using the RNA 6000 Nano Lab-on-a-Chip kit and the Bioanalyzer 2100 (Agilent Technologies, Wilmington, DE, USA). cRNA was synthesized and labeled with biotinylated UTP and CTP by *in vitro* transcription using T7 promoter-coupled double-stranded cDNA as a template and the Bioarray HighYield RNA transcript labeling kit (ENZO Diagnostics Inc., Farmingdale, NY, USA). The fragmented cRNA was hybridized for 16 h at 45°C to GeneChip Human Genome U133 Plus 2.0 arrays (Affymetrix, Santa Clara, CA, USA), which cover over 47 000 human transcripts and variants representing approximately 39 000 genes (Affymetrix, Santa Clara, CA, USA). The arrays were then stained with

phycoerythrin-conjugated streptavidin (Invitrogen) and the fluorescence intensities were determined using a GCS 3000 high-resolution confocal laser scanner (Affymetrix). The scanned images were analysed using programs resident in GeneChip Operating System v1.4 (GCOS, Affymetrix). The expression data were analysed by MAS5 statistical algorithms resident in GCOS. The signal intensity for each gene was calculated as the average intensity difference, represented by ($\sum$(PM−MM)/(number of probe pairs)), where PM and MM denote perfect-match and mismatch probes (Lockhart *et al*., 1996). GCOS-generated detection and change calls were used to identify robust expression changes.

Cyclic AMP and RhoA pulldown

HCT8/S11 cells were preincubated for 10 min in KRB buffer containing 10 mmol/l (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) (pH 7.4), 0.1% bovine serum albumin and 1 mmol/l isobutylmethylxanthine as a phosphodiesterase inhibitor. Cyclic AMP content was measured by radioimmunoassay (GE Healthcare). Data are expressed as picomoles of cAMP produced per 10^6 cells. To determine the RhoA activation status, HCT8/S11 cells were serum starved for 24 h before treatment with netrin-1 (2.5 nmol/l) and NECA (10 μmol/l) for 30 min. GTP-bound RhoA was precipitated after 45 min incubation at 4°C with the Rhotekin Rho-binding domain fused to agarose beads (Upstate Biotechnology, Lake Placid, NY, USA). GTP-bound and total RhoA protein levels were detected by immunoblotting, as described (Nguyen *et al*., 2005). The relative intensity of the immunoreactive bands was determined with ImageQuant software (GE Healthcare).

Collagen invasion and tumor xenografts

HCT8/S11 and MDCKts.src-ggl-luci cells were seeded on top of collagen type I gels (Upstate Biotechnology). Cultures were incubated for 24 h at 37°C and then invasive and superficial cells were counted in 12 fields of 0.157 mm^2 using an inverted microscope. The invasion index was the ratio of the number of cells invading the gel over the total number of cells counted in each field. All data are from at least three separate experiments and are means ± s.e.m.

HCT8/S11 and MDCKts.src-ggl-luci cancer cells and their corresponding counterparts (4 and 10×10^6 cells, respectively) were injected subcutaneously into the flanks of nude mice. Tumor volume (V) was calculated using the formula $V = 0.4 \times a \times b^2$, with a being the length and b the width of the tumor. In mice receiving MDCK.ts.src-ggl-luci xenografts, the axillary ganglia and lungs were also removed and processed for the determination of luciferase activity (Promega, Charbonnières, France) and cancer cell number. The statistical significance was assessed by unpaired Student's t-test and $P < 0.05$ was considered statistically significant.

Hypoxia and apoptosis

Where indicated, cancer cell cultures were placed under hypoxic conditions for 24 h at 37°C in a sealed incubator chamber device, as described (Nguyen *et al*., 2005). The impact of hypoxia and DCC on apoptosis was determined by flow cytometry (FACS Caliburn cytofluorometer, Becton Dickinson, Le Pont de Claix, France) after the cells were stained with propidium iodine (50 μg/ml) or activation of PARP and caspase-8 by Western blotting, using their corresponding antibodies (each at 1:1000, BD Biosciences) and tubulin as loading control (1:4000, Sigma).

Reagents, peptides and pharmacological inhibitors

Netrin-1 and TGFα were from R&D Systems Europe Ltd (Oxon, UK). Adenosine, NECA, CGS-21680 (designated as CGS) and enprofylline were from Sigma. Trefoil peptides were a generous gift from Professor BR Westley and Dr L Thim (University of Newcastle, UK and Novo Nordisk, Denmark). C3T and Y27632 were kindly provided by Dr Flatau (INSERM U627) and Yoshitomi Pharmaceutical Industries Ltd (Osaka, Japan).

Abbreviations

A2B-R, adenosine A2B-receptor; DCC, deleted in colon cancer; EGF-R, epidermal growth factor receptor; GCOS, GeneChip Operating System v1.4; GPCR, G-protein-coupled receptors; PARP, poly(ADP-ribose)polymerase-1; PI3K, phosphatidylinositol 3-kinase; PKA, protein kinase A; PLCβ, phospholipase Cβ; ROK, Rho-kinase; RT–PCR, reverse transcription–polymerase chain reaction; TGFα, transforming growth factor α; TFFs, trefoil factors.

Acknowledgements

This work is supported by INSERM, ARC, the Fund for Scientific Research Flanders (Brussels, Belgium) and the Scientific Exchange Program between the Flemish community and France (Grant I.2006/2007.03). We are indebted to Dr Lamarche-Vane and to Dr Mehlen for providing the initial batch of recombinant netrin-1 and the expression vectors encoding netrin-1 and the DCC.

References

Arakawa H. (2004). Netrin-1 and its receptors in tumorigenesis. *Nat Rev Cancer* **4**: 978–987.

Barbier M, Attoub S, Calvez R, Laffargue M, Mareel M, Altruda F *et al*. (2001). Weakening link to colorectal cancer? *Nature* **413**: 796.

Bouchard JF, Moore SW, Tritsch NX, Roux PP, Shekarabi M, Barker PA *et al*. (2004). Protein kinase A activation promotes plasma membrane insertion of DCC from an intracellular pool: a novel mechanism regulating commissural axon extension. *J Neurosci* **24**: 3040–3050.

Chen L, Wang P, Andrade CF, Zhao IY, Dube PE, Brubaker PL *et al*. (2005). PKA independent and cell type specific activation of the expression of caudal homeobox gene Cdx-2 by cyclic AMP. *FEBS J* **272**: 2746–2759.

Corset V, Nguyen-Ba-Charvet KT, Forcet C, Moyse E, Chedotal A, Mehlen P. (2000). Netrin-1-mediated axon outgrowth and cAMP production requires interaction with adenosine A2b receptor. *Nature* **407**: 747–750.

Fearon ER, Cho KR, Nigro JM, Kern SE, Simons JW, Ruppert JM *et al*. (1990). Identification of a chromosome 18q gene that is altered in colorectal cancers. *Science* **247**: 49–56.

Forcet C, Stein E, Pays L, Corset V, Llambi F, Tessier-Lavigne M *et al*. (2002). Netrin-1-mediated axon outgrowth requires deleted in colorectal cancer-dependent MAPK activation. *Nature* **417**: 443–447.

Gao L, Feng Y, Bowers R, Becker-Hapak M, Gardner J, Council L *et al*. (2006). Ras-associated protein-1 regulates extracellular signal-regulated kinase activation and migration

in melanoma cells: two processes important to melanoma tumorigenesis and metastasis. *Cancer Res* **66**: 7880–7888.
Grider JR, Piland BE, Gulick MA, Qiao LY. (2006). Brain-derived neurotrophic factor augments peristalsis by augmenting 5-HT and calcitonin gene-related peptide release. *Gastroenterology* **130**: 771–780.
Hebrok M, Reichardt LF. (2004). Brain meets pancreas: netrin, an axon guidance molecule, controls epithelial cell migration. *Trends Cell Biol* **14**: 153–155.
Hill A, McFarlane S, Mulligan K, Gillespie K, Gillespie H, Draffin JE *et al*. (2006). Cortactin underpins CD44-promoted invasion and adhesion of breast cancer cells to bone marrow endothelial cells. *Oncogene* **25**: 6079–6091.
Hipfner DR, Cohen SM. (2004). Connecting proliferation and apoptosis in development and disease. *Nat Rev Mol Cell Biol* **5**: 805–815.
Hong K, Hinck L, Nishiyama M, Poo MM, Tessier-Lavigne M, Stein E. (1999). A ligand-gated association between cytoplasmic domains of UNC5 and DCC family receptors converts netrin-induced growth cone attraction to repulsion. *Cell* **97**: 927–941.
Hu G, Zhang S, Vidal M, Baer JL, Xu T, Fearon ER. (1997). Mammalian homologs of seven in absentia regulate DCC via the ubiquitin-proteasome pathway. *Genes Dev* **11**: 2701–2714.
Hucho TB, Dina OA, Levine JD. (2005). Epac mediates a cAMP-to-PKC signaling in inflammatory pain: an isolectin B4(+) neuron-specific mechanism. *J Neurosci* **25**: 6119–6126.
Kadono Y, Okada Y, Namiki M, Seiki M, Sato H. (1998). Transformation of epithelial Madin-Darby canine kidney cells with p60(v-src) induces expression of membrane-type 1 matrix metalloproteinase and invasiveness. *Cancer Res* **58**: 2240–2244.
Kim TH, Lee HK, Seo IA, Bae HR, Suh DJ, Wu J *et al*. (2005). Netrin induces down-regulation of its receptor, deleted in colorectal cancer, through the ubiquitin-proteasome pathway in the embryonic cortical neuron. *J Neurochem* **95**: 1–8.
Kong T, Westerman KA, Faigle M, Eltzschig HK, Colgan SP. (2006). HIF-dependent induction of adenosine A2B receptor in hypoxia. *FASEB J* **20**: 2242–2250.
Kotelevets L, Noe V, Bruyneel E, Myssiakine E, Chastre E, Mareel M *et al*. (1998). Inhibition by platelet-activating factor of Src- and hepatocyte growth factor-dependent invasiveness of intestinal and kidney epithelial cells. Phosphatidylinositol 3′-kinase is a critical mediator of tumor invasion. *J Biol Chem* **273**: 14138–14145.
Lahti JM, Teitz T, Stupack DG. (2006). Does integrin-mediated cell death confer tissue tropism in metastasis? *Cancer Res* **66**: 5981–5984.
Linden J, Thai T, Figler H, Jin X, Robeva AS. (1999). Characterization of human A(2B) adenosine receptors: radioligand binding, western blotting, and coupling to G(q) in human embryonic kidney 293 cells and HMC-1 mast cells. *Mol Pharm* **56**: 705–713.
Lockhart DJ, Dong H, Byrne MC, Follettie MT, Gallo MV, Chee MS *et al*. (1996). Expression monitoring by hybridization to high-density oligonucleotide arrays. *Nat Biotechnol* **14**: 1675–1680.
Martin M, Simon-Assmann P, Kedinger M, Martin M, Mangeat P, Real FX *et al*. (2006). DCC regulates cell adhesion in human colon cancer derived HT-29 cells and associates with ezrin. *Eur J Cell Biol* **85**: 769–783.
Mazelin L, Bernet A, Bonod-Bidaud C, Pays L, Arnaud S, Gespach C *et al*. (2004). Netrin-1 controls colorectal tumorigenesis by regulating apoptosis. *Nature* **431**: 80–84.
Mehlen P, Puisieux A. (2006). Metastasis: a question of life or death. *Nat Rev Cancer* **6**: 449–458.
Mehlen P, Rabizadeh S, Snipas SJ, Assa-Munt N, Salvesen GS, Bredesen DE. (1998). The DCC gene product induces apoptosis by a mechanism requiring receptor proteolysis. *Nature* **395**: 801–804.
Miyaki M, Seki M, Okamoto M, Yamanaka A, Maeda Y, Tanaka K *et al*. (1990). Genetic changes and histopathological types in colorectal tumors from patients with familial adenomatous polyposis. *Cancer Res* **50**: 7166–7173.
Miyamoto Y, Yamauchi J, Tanoue A, Wu C, Mobley WC. (2006). Trk binds and tyrosine-phosphorylates Tiam1, leading to activation of Rac1 and induction of changes in cellular morphology. *Proc Natl Acad Sci USA* **103**: 10444–10449.
Nguyen QD, Rodrigues S, Rodrigue C, Rivat C, Bruyneel E, Emami S *et al*. (2006). Inhibition of VEGF-165 and Semaphorin 3A-mediated cellular invasion and tumor growth by the VEGF signaling inhibitor ZD4190 in human colon cancer cells and xenografts. *Mol Cancer Therapeutics* **5**: 2070–2077.
Nguyen QD, De Wever O, Bruyneel E, Hendrix A, Xie WZ, Lombet A *et al*. (2005). Commutators of PAR-1 signaling in cancer cell invasion reveal an essential role of the Rho-ROK axis and tumor microenvironment. *Oncogene* **24**: 8240–8251.
Ren XR, Ming GL, Xie Y, Hong Y, Sun DM, Zhao ZQ *et al*. (2004). Focal adhesion kinase in netrin-1 signaling. *Nat Neurosci* **7**: 1204–1212.
Rivat C, LeFloch N, Sabbah M, Redeuilh G, Bruyneel E, Mareel M *et al*. (2003). Synergistic cooperation between the AP-1 and LEF-1 transcription factors in activation of the matrilysin promoter by the src oncogene: implications in cellular invasion. *FASEB J* **17**: 1721–1723.
Serafini T, Kennedy TE, Galko MJ, Mirzayan C, Jessell TM, Tessier-Lavigne M. (1994). The netrins define a family of axon outgrowth-promoting proteins homologous to *C. elegans* UNC-6. *Cell* **78**: 409–424.
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.
Tan EY, Richard CL, Zhang H, Hoskin DW, Blay J. (2006). Adenosine downregulates DPPIV on HT-29 colon cancer cells by stimulating protein phosphatase(s) and reducing ERK1/2 activity via a novel pathway. *Am J Physiol* **291**: C433–C444.
Taniguchi Y, Kim SH, Sisodia SS. (2003). Presenilin-dependent 'gamma-secretase' processing of deleted in colorectal cancer (DCC). *J Biol Chem* **278**: 30425–30428.
Tessier-Lavigne M, Goodman CS. (1996). The molecular biology of axon guidance. *Science* **274**: 1123–1133.
Wilson BD, Ii M, Park KW, Suli A, Sorensen LK, Larrieu-Lahargue F *et al*. (2006). Netrins promote developmental and therapeutic angiogenesis. *Science* **313**: 640–644.
Xie Y, Hong Y, Ma XY, Ren XR, Ackerman S, Mei L *et al*. (2006). DCC-dependent phospholipase C signaling in netrin-1-induced neurite elongation. *J Biol Chem* **281**: 2605–2611.
Yebra M, Montgomery AM, Diaferia GR, Kaido T, Silletti S, Perez B *et al*. (2003). Recognition of the neural chemoattractant Netrin-1 by integrins alpha6beta4 and alpha3beta1 regulates epithelial cell adhesion and migration. *Dev Cell* **5**: 695–707.

Supplementary Information accompanies the paper on the Oncogene website (http://www.nature.com/onc).