Neuronal Guidance Protein Netrin-1 Induces Differentiation in Human Embryonal Carcinoma Cells

Mario Mancino,[1,2] Claudia Esposito,[2] Kazuhide Watanabe,[1] Tadahiro Nagaoka,[1] Monica Gonzales,[1] Caterina Bianco,[1] Nicola Normanno,[2,3] David S. Salomon,[1] and Luigi Strizzi[1]

[1]Mammary Biology and Tumorigenesis Laboratory, National Cancer Institute, Bethesda, Maryland; [2]CROM–Istituto Nazionale Tumori "Pascale" Napoli, Mercogliano (AV), Italy; and [3]Cell Biology and Preclinical Models Unit, Istituto Nazionale Tumori-Fondazione Pascale, Naples, Italy

Abstract

Pluripotent cells within embryonal carcinoma (EC) can differentiate *in vivo* or *in vitro* on treatment with specific agents. Differentiating EC cells express lower levels of stem cell–related genes, such as *Cripto-1*. We show that migration of human EC cells (NTERA/2 and NCCIT) can be reduced following treatment with the guidance molecule Netrin-1. Moreover, Netrin-1 treatment increased the levels of β-III tubulin, glial filament acidic protein, Nestin, and γ-amino-butyric acid and reduced the expressions of Cripto-1, Nanog, and Oct4 in EC cells. These Netrin-1–induced effects in the EC cells were mediated via binding of Netrin-1 to the Neogenin receptor and activation of SHP-2, resulting in increased levels of inactive phosphorylated c-src$^{(Y527)}$. These results suggest that Netrin-1 can induce neuroectodermal-like differentiation of human EC cells by affecting c-src signaling via SHP-2 activation and regulation of Nanog, Oct4, and Cripto-1 expressions. [Cancer Res 2009;69(5):1717–21]

Introduction

Embryonal carcinoma (EC) is a germ-line tumor consisting of cells that can be induced to differentiate with agents, such as retinoic acid (1). The malignant phenotype of EC cells can be reversed when injected into the mouse blastocyst and become incorporated into normally developing embryonic tissues (2). Transcription factors, such as Oct4 and Nanog, which regulate self-renewal and pluripotency of embryonic stem (ES) cells (3), are expressed in EC cells (4, 5), suggesting that these transcription factors could also regulate pluripotency in EC cells. Cripto-1, an epidermal growth factor family member and target gene of Oct4 and Nanog, is expressed in ES cells and during the initial stages of development (6). Cripto-1, also known as teratocarcinoma-derived growth factor-1 since first isolated from human NTERA/2 EC cells (7), can induce cellular transformation *in vitro* and increase tumor incidence *in vivo* (6). Interestingly, repression of Cripto-1 is associated with differentiation of EC cells toward a neuroectodermal lineage (7). However, little information is known about how external factors, such as guidance molecules, may regulate the levels of Nanog and/or Oct4 and result in the differentiation of cancer cells with pluripotent characteristics.

Netrin-1, a secreted guidance molecule, can bind to specific cell surface receptors and regulate the development, functional differentiation, and trafficking of both neuronal and extraneuronal cells (8). Netrin-1 can also regulate Cripto-1–induced cellular motility and allometric outgrowth of mouse mammary epithelial cells (9). Recently, it has been shown that Netrin-1 can cause a reduction in the expression of Nanog and Cripto-1 and can increase expression of β-III tubulin in mouse ES cells, suggesting that Netrin-1 may affect early neuroectodermal differentiation in pluripotent cells (10).

Here, we investigate the effect of Netrin-1 on human EC cells. Exogenous soluble Netrin-1 was able to reduce migration and induce increased levels of markers of early neuroectodermal differentiation in NTERA/2 and NCCIT EC cells. These responses to Netrin-1 were accompanied by increased levels of active phosphorylated SHP-2 (P-SHP-2) and inactive phosphorylated c-src$^{(Y527)}$ [P-c-src$^{(Y527)}$]. Furthermore, Netrin-1–treated EC cells showed reduced levels of Cripto-1, Nanog, and Oct4. These results suggest that Netrin-1 can regulate differentiation in human EC cells.

Materials and Methods

Cell culture, recombinant proteins, migration, and proliferation assays. Human NTERA/2 and NCCIT EC cells were grown either in McCoy's 5A medium containing 15% fetal bovine serum (FBS; NTERA/2) or in DMEM containing 10% FBS (NCCIT) and cultured at 37°C in a humidified atmosphere of 5% CO_2. Recombinant Netrin-1 protein was purchased from R&D Systems. Migration and cell proliferation assays were performed as previously described (11). The experiments were performed in triplicate and repeated twice.

Western blot analysis. The human EC cells were seeded in 60-mm plates (6×10^5 per plate), serum starved overnight, and then treated with medium alone (control) or with 50 ng/mL of exogenous soluble Netrin-1 for 30 min. For inhibitor studies, the cells were pretreated for 16 h with either medium alone (control) or anti-Neogenin blocking antibody (1 μg/mL; Santa Cruz Biotechnology; ref. 12) and for 3 h with either medium alone (control) or SHP-2 inhibitor [50 μmol/L, 8-hydroxy-7-(6-sulfo-2-naphthylazo)-5-quinolinesulfonic acid disodium salt; Acros Organics] followed by stimulation with 50 ng/mL of exogenous soluble Netrin-1 for 30 min. Protein extraction and Western blot analysis were performed as described previously (11). The following primary antibodies were used: mouse anti-Cripto-1 (1:500; Rockland); rabbit anti-Neogenin (1:1,000; Santa Cruz Biotechnology); rabbit anti-β-III tubulin (1:1,000; Abcam); mouse anti-Nestin (1:1,000; R&D Systems); rabbit anti-SHP-2, anti-P-SHP-2$^{(Y542)}$, anti-P-c-src$^{(Y416)}$, and anti-P-c-src$^{(Y527)}$ (1:1,000; Cell Signaling); mouse anti-glial filament acidic protein (GFAP; 1:1,000; Chemicon); mouse anti-src (1:500; Upstate-Millipore); and mouse anti-actin (1:20,000; Sigma). Densitometric analysis of Western blot results was performed with the NIH Image program.[4]

[4] http://rbs.info.nih.gov/nih-image

Note: D.S. Salomon and L. Strizzi contributed equally to this work.

Requests for reprints: Mario Mancino, Mammary Biology and Tumorigenesis Laboratory, National Cancer Institute, 37 Convent Drive, Room 1112, Bethesda, MD 20892. Phone: 301-443-3133; Fax: 301-402-8652; E-mail: mario.mancino@cro-m.eu.

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Quantitative real-time PCR. EC cells were plated at a density of 6×10^5 in 60-mm plates and serum starved for 24 h. The cells were then stimulated with exogenous soluble Netrin-1 at different concentrations for 30 min. Total RNA was isolated using RNeasy Mini kit (Qiagen) and quantitative real-time PCR (qRT-PCR) was performed on Stratagene MX3000P using Brilliant II SYBR Green QPCR master mix (Stratagene). The following primers were used: hGAPDH, 5′-GAAGGTGAAGGTCG-GAGTC-3′ (forward) and 5′-GAAGATGGTGATGGGATTTC-3′ (reverse); hCripto-1, 5′-CACGATGTGCGCAAAGAGA-3′ (forward) and 5′-TGACC-GTGCCAGCATTTACA-3′ (reverse); hNanog, 5′-TGAACCTCAGCTACAAA-CAGGTG-3′ (forward) and 5′-AACTGCATGCAGGACTGCAGAG-3′ (reverse); hOct3/4, 5′-CTTGCTGCAGAAGTGGGTGGAGGAA-3′ (forward) and 5′-CTGCAGTGTGGGTTTCGGGCA-3′ (reverse); and Nestin, 5′-CAGCTGGCG-CACCTCAAGATG-3′ (forward) and 5′-AGGGAAGTTGGGCTCAGGACTGG-3′ (reverse). Relative quantification of Cripto-1 mRNA within the samples was performed using the C_t method, as previously shown (11).

Immunofluorescence. EC cells were treated for 48 to 72 h with either medium alone (control) or 50 ng/mL of exogenous soluble Netrin-1. Cells were incubated for 16 h at 4°C with the following primary antibodies: rabbit anti-γ-aminobutyric acid (GABA; 1:1,000; Chemicon), rabbit anti-β-III tubulin (1:1,000), mouse anti-Nestin (1:1,000), and mouse anti-GFAP (1:1,000). Cells were processed for immunofluorescent staining as previously described (9).

Statistical analysis. The statistical significance of the various groups in the different experiments was calculated with the nonparametric Mann-Whitney *U* test. Statistical tests were two sided and data were considered statistically significant with $P < 0.05$.

Results and Discussion

Netrin-1 reduces migration and expression of Cripto-1, Nanog, and Oct4 in NTERA/2 and NCCIT human EC cells. Because the expression of Netrin-1 in human EC cells is unknown, we determined whether it would be expressed in NTERA/2 or NCCIT EC cells. In our study, we could not detect Netrin-1 protein by Western blot in either NTERA/2 or NCCIT EC cells (data not shown). However, the addition of 50 ng/mL of exogenous soluble Netrin-1 significantly reduced ($P < 0.05$) the migration of NTERA/2 and NCCIT EC cells compared with untreated control EC cells (Fig. 1*A*). Netrin-1 did not significantly affect the 72-hour growth rates of either NTERA/2 or NCCIT EC cells (Fig. 1*B*), suggesting that Netrin-1 did not reduce migration by inhibiting proliferation in the EC cells. A reduction in Cripto-1 levels can lead to lower migratory rates in Cripto-1–expressing cells (9, 11); therefore, we determined whether Netrin-1 treatment could affect Cripto-1 levels in NTERA/2 and NCCIT EC cells. Western blot and qRT-PCR analysis show a significant reduction in Cripto-1 protein and mRNA levels ($P < 0.05$) in Netrin-1–treated NTERA/2 and NCCIT EC cells compared with untreated controls (Fig. 1*C*). Because Cripto-1 is a target gene of the stem cell–related transcription factors Oct4 and Nanog (13), we investigated whether Netrin-1 could affect the expressions of Oct4 and/or Nanog in NTERA/2 and NCCIT EC cells. Exogenous soluble Netrin-1 significantly reduced ($P < 0.05$) Oct4 and Nanog mRNA expression in NTERA/2 and NCCIT cells (Fig. 1*D*). This agrees with similar observations that describe reduced Nanog and Cripto-1 levels in Netrin-1–treated mouse ES cells (10).

NTERA/2 and NCCIT EC cells assume early neuronal-like characteristics when treated with Netrin-1. Exogenous agents, such as retinoic acid, can induce neuronal-like features in EC cells (1). We investigated whether the effect of Netrin-1 on Cripto-1, Nanog, and Oct4 expression might be associated with differentiation of NTERA/2 and NCCIT EC cells. Western blot analysis shows that 12-day treatment of NTERA/2 or NCCIT EC cells with

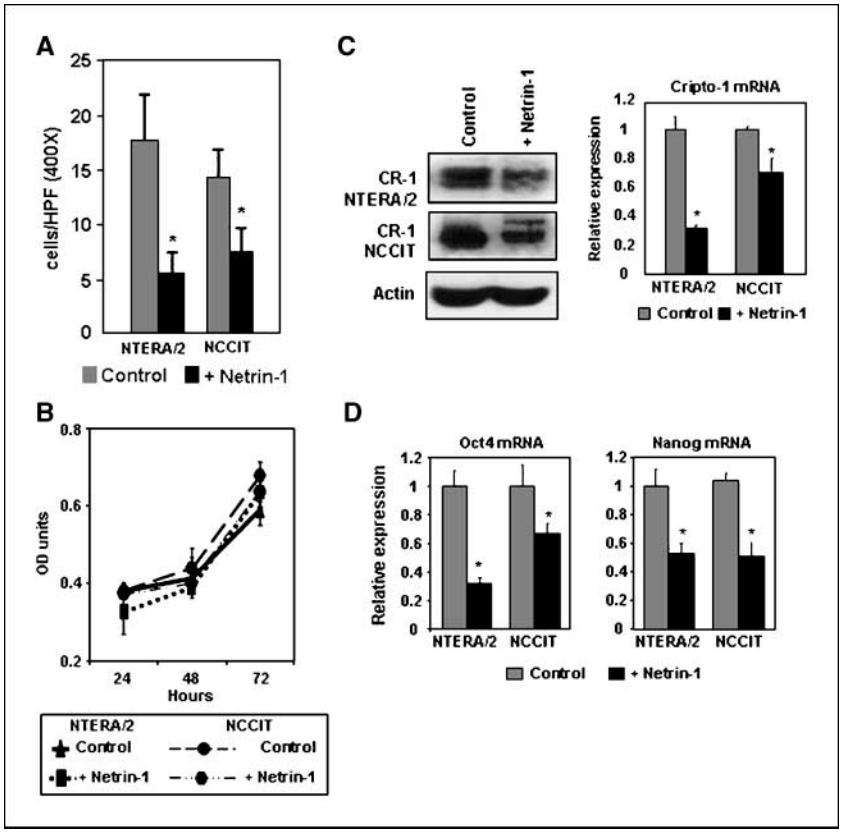

Figure 1. Exogenous soluble Netrin-1 (50 ng/mL) reduced (*, $P < 0.05$) migration of NTERA/2 and NCCIT EC cells (*A*) without affecting proliferation (*B*) and significantly reduced the expressions of (*C*) Cripto-1 (*CR-1*) protein and mRNA and (*D*) Oct4 and Nanog mRNA (*, $P < 0.05$). *HPF,* high-power field; *OD,* absorbance.

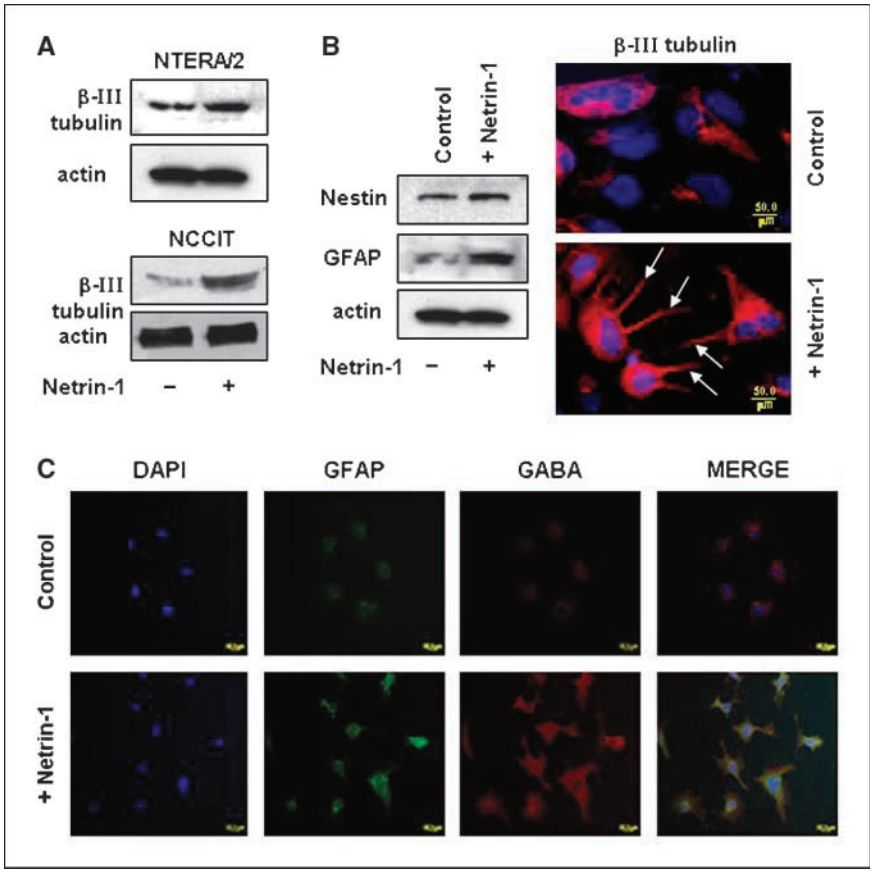

Figure 2. *A,* Western blot analysis shows increased expression of β-III tubulin in NTERA/2 and NCCIT EC cells treated for 12 d with exogenous soluble Netrin-1 (50 ng/mL). *B,* Western blot analysis shows increased expression of GFAP and Nestin in Netrin-1–treated NTERA/2 EC cells. Increased expression of β-III tubulin in Netrin-1–treated NTERA/2 EC cells was also detected by immunofluorescent staining. Of note is the neuronal-like morphology with axonal/dendritic-like cellular extensions (*white arrows*) in Netrin-1–treated NTERA/2 EC cells. *C,* immunofluorescent staining shows increased GFAP and GABA expressions in Netrin-1–treated NTERA/2 EC. *DAPI,* 4′,6-diamidino-2-phenylindole.

50 ng/mL of exogenous soluble Netrin-1 increased expression of β-III tubulin, a common marker for early neuronal differentiation, in NTERA/2 (~2-fold) and NCCIT (~3.5-fold; Fig. 2*A*) EC cells. Thus far, these results show that Netrin-1 can exert comparable effects in both NTERA/2 and NCCIT EC cells. Further analysis of Netrin-1–treated NTERA/2 cells shows increased levels of other markers of early neuronal development, such as Nestin (~2-fold) and GFAP (~3.5-fold; Fig. 2*B*). Immunofluorescent staining confirmed the higher levels of β-III tubulin expressed in Netrin-1–treated NTERA/2 EC cells, which seemed to assume a more neuronal-like morphology with dendritic- or axonal-like cytoplasmic extensions (Fig. 2*B*). Additional immunofluorescent staining showed increased expression of the neurotransmitter GABA in GFAP-positive Netrin-1–treated NTERA/2 EC cells compared with GFAP-negative untreated control cells (Fig. 2*C*). We could not detect mRNA expression of either GATA4 or Brachyury, markers of endoderm and mesoderm differentiation, respectively, in Netrin-1–treated or untreated NTERA/2 or NCCIT EC cells (data not shown), suggesting that Netrin-1 may be more effective in regulating the expression of markers of neuroectodermal differentiation in human EC cells.

Netrin-1 activates SHP-2 and increases levels of inactive c-src in NTERA/2 EC cells. Netrin-1 can bind DCC/Neogenin and UNC5H family of receptors (14). Western blot analysis showed expression of Neogenin (Fig. 3*A*) in NTERA/2 and NCCIT EC cells but not UNC5HA (data not shown), suggesting that Netrin-1 effects observed in NTERA/2 or NCCIT EC cells are most likely mediated via Neogenin. Changes in expression of either Neogenin or UNC5H in Netrin-1–treated and untreated NTERA/2 or NCCIT EC cells were not detected by Western blot analysis (data not shown). Previous studies have shown that several responses that are induced by Netrin-1 can be mediated via recruitment and activation of intracellular signaling molecules, such as the tyrosine phosphatase SHP-2 (15). In fact, treatment of NTERA/2 EC cells with 50 or 100 ng/mL of exogenous soluble Netrin-1 showed approximately 3- and 2.5-fold increase ($P < 0.05$), respectively, in the levels of active P-SHP-2 as determined by Western blot analysis (Fig. 3*B*). A similar effect was observed in NCCIT EC cells (data not shown). Because SHP-2 is known to regulate many intracellular tyrosine kinases in different cell types (16), we determined whether this is also true in Netrin-1–treated EC cells. We could not detect differences in the activation of Akt, focal adhesion kinase (FAK), or mitogen-activated protein kinase in Netrin-1–treated and untreated NTERA/2 or NCCIT EC cells (data not shown). Members of the src family of tyrosine kinases are also affected by SHP-2 activity. Because Fyn and c-src activity have been implicated in Netrin-1 cellular effects (17), it is possible that they might perform a role in the Netrin-1–induced effects in NTERA/2 and NCCIT EC cells. We therefore performed Western blot analysis to detect basal levels of Fyn and c-src in NTERA/2 and NCCIT to determine which of these tyrosine kinases could be affected by Netrin-1–induced SHP-2 activity. We could not detect activated Fyn in Netrin-1–treated or untreated NTERA/2 or NCCIT EC cells (data not shown). However, we did detect an increased ratio between inactive c-src [c-src$^{(Y527)}$] to active c-src [c-src$^{(Y416)}$] in Netrin-1–treated NTERA/2 and NCCIT EC cells (Fig. 3*C* and *D*). The potential molecular interplay

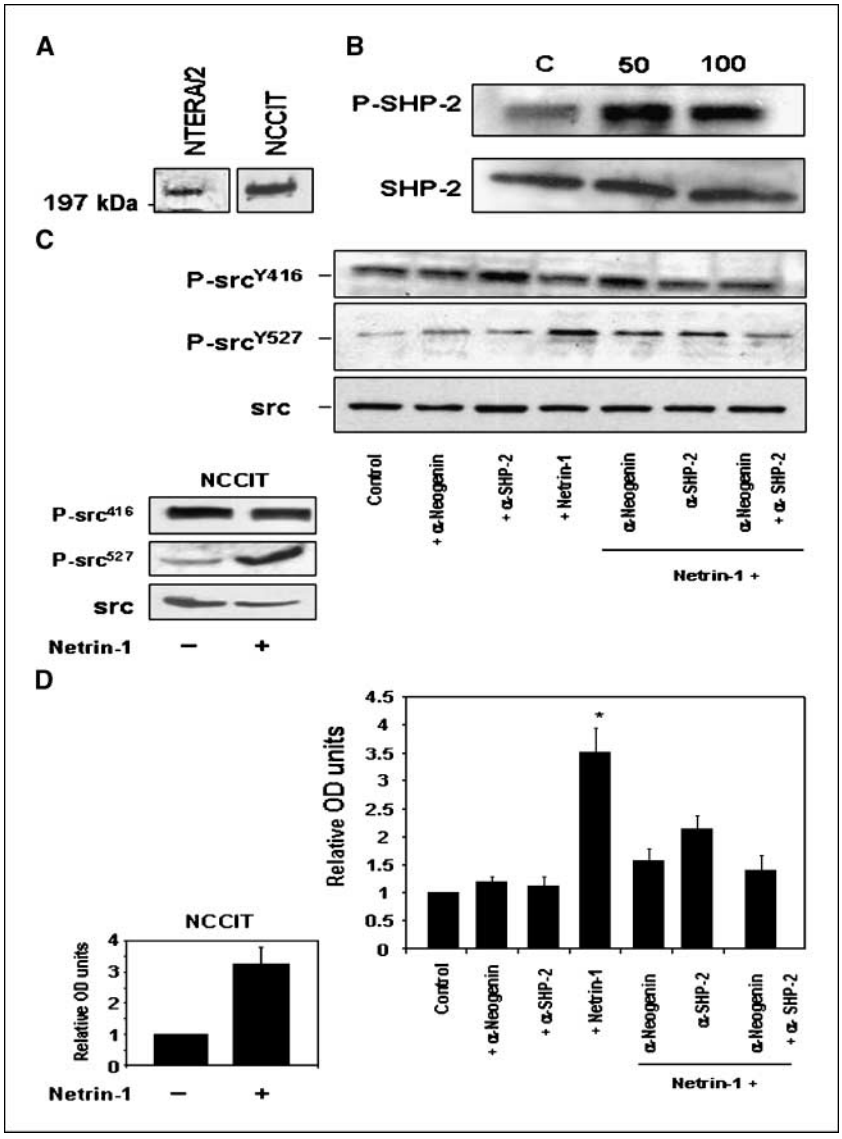

Figure 3. *A,* Western blot analyses show expression of the Netrin-1 receptor Neogenin in NTERA/2 and NCCIT EC cells. *B,* P-SHP-2 expression is increased in NTERA/2 EC cells treated with 50 or 100 ng/mL of soluble Netrin-1. *C,* Western blot analysis shows reduced expression of active c-src$^{(Y416)}$ with concomitant increase in the expression of the inactive c-src$^{(Y527)}$ in Netrin-1–treated NTERA/2 and NCCIT (*inset*) EC cells. *D,* densitometric analysis of the results in *C* shows a significant increase (*, $P < 0.05$) in the ratio between the expression of inactive [P-c-src$^{(Y527)}$] to active [P-c-src$^{(Y416)}$] forms of c-src in NTERA/2 and NCCIT (*inset*) EC cells after treatment with Netrin-1.

between Netrin-1, SHP-2, and c-src was further investigated in NTERA/2 cells. We found that the Netrin-1–induced effect on c-src was significantly reduced when NTERA/2 cells were pretreated with anti-Neogenin blocking antibody (1 μg/mL) or with the SHP-2 inhibitor (50 μmol/L), alone or in combination (Fig. 3*C* and *D*). Furthermore, when NTERA/2 EC cells were pretreated with the anti-Neogenin blocking antibody and with the SHP-2 inhibitor, Netrin-1 could no longer induce the expression of neuroectodermal markers, such as Nestin (Fig. 4*A*), or repress the expression of Nanog, Oct4, and Cripto-1 (Fig. 4*B*). These results suggest that exogenous soluble Netrin-1 may affect human EC cells by signaling via its receptor Neogenin and triggering the activation of SHP-2 resulting in reduced c-src activity.

We show that SHP-2 activation and reduction of active c-src is associated with reduced levels of Nanog, Oct4, and Cripto-1 and acquisition of neuroectodermal features in Netrin-1–treated human EC cells. Previous work has shown that activation of SHP-2 is associated with differentiation of ES cells (18). Although other tyrosine kinases, such as Fyn and c-Yes, can play a role during the maintenance of pluripotency (19), our data suggest that c-src may be more essential in the Netrin-1–induced effects observed in the human EC cells used in our study especially because Fyn, another mediator of Netrin-1 signaling (17), was not detected in the EC cells, nor was active FAK, a downstream component of Netrin-1–induced Fyn activity (20). Taken together, these results show that Netrin-1 can reduce the aggressiveness of EC cells by influencing signaling pathways known to regulate pluripotency and affect the expression of stem cell–related transcription factors and target gene(s). These findings support the rationale for future investigation of the potential use of Netrin-1 for treatment of human EC.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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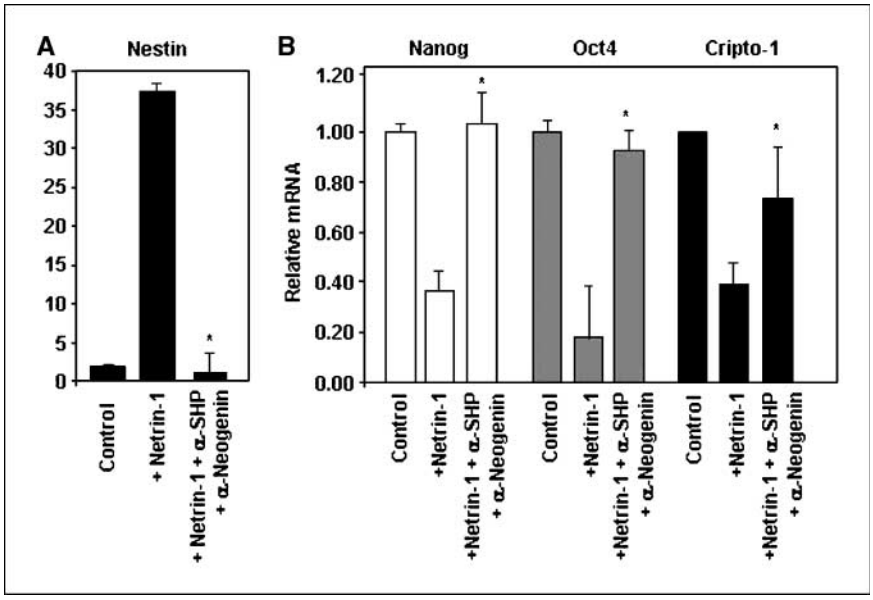

Figure 4. Antagonizing the Netrin-1 effect in NTERA/2 EC cells by treating the cells with a specific anti-Neogenin functional blocking antibody (1 μg/mL) and with a synthetic SHP-2 inhibitor (50 μmol/L) not only significantly inhibited Netrin-1–dependent induction of Nestin (*A*) but also significantly inhibited Netrin-1–dependent reduction of Nanog, Oct4, and Cripto-1 (*B*). *, $P < 0.05$.

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