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Neuronal guidance protein Netrin-1 induces differentiation in human embryonal carcinoma cells

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Abstract

Pluripotent cells within embryonal carcinoma (EC) can differentiate *in vivo* or *in vitro* upon treatment with specific agents. Differentiating EC cells express lower levels of stem cell-related genes like 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 beta-III tubulin, glial filament acidic protein (GFAP), Nestin and gamma aminobutyric acid (GABA) 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 the Neogenin receptor and activation of SHP-2, resulting in increased levels of inactive P-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.

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 that 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 has been shown to be associated with differentiation of EC cells towards 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

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of mouse mammary epithelial cells (9). Recently, it has been demonstrated that Netrin-1 can cause a reduction in the expression of Nanog and Cripto-1 and can increase expression of beta-III tubulin in mouse embryonic stem 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 P-SHP-2 and inactive 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 in either McCoy's 5A medium containing 15% fetal bovine serum (FBS) (NTERA/2) or in DMEM medium containing 10% FBS (NCCIT) and cultured at 37°C in a humidified atmosphere of 5% CO₂. Recombinant Netrin-1 protein was purchased from R&D Systems (Minneapolis, MN). 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 cells/plate), serum-starved overnight, then treated with media alone (control) or with 50ng/ml exogenous soluble Netrin-1 for 30 min. For inhibitor studies, the cells were pre-treated for 16 h with either media alone (control) or anti-Neogenin blocking antibody (12) (1μg/ml; SCBT, Santa Cruz, CA) and for 3 h with either media alone (control) or SHP-2 inhibitor (50μM, 8-Hydroxy-7-(6-sulfo-2-naphthylazo)-5-quinolinesulfonic acid disodium salt - Acros Organics, NJ), followed by stimulation with 50 ng/ml exogenous soluble Netrin-1 for 30 min. Protein extraction and Western blot analysis was performed as described previously (11). The following primary antibodies were used: mouse anti-CR-1 (1:500, Rockland, Gilbertsville, PA); rabbit anti-Neogenin (1:1000, SCBT); rabbit anti-beta III-Tubulin (1:1000, Abcam, Cambridge, MA); mouse anti-Nestin (1:1000, R&D Systems); rabbit anti-SHP-2, anti-phospho(P)-SHP-2^{Y542}, anti-P-src^{Y416} and anti-P-src^{Y527} (1:1000, Cell Signaling; Danvers, MA); mouse anti-GFAP (1:1000, Chemicon); mouse anti-src (1:500; Upstate-Millipore, Billerica, MA) and mouse anti-actin (1:20,000, Sigma, St. Louis, MO). Densitometric analysis of Western blot results was performed with the NIH image program (<http://rbs.info.nih.gov/nih-image>).

Quantitative real time PCR (qRT-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 qRT-PCR was performed on Stratagene MX3000P using Brilliant II SYBR Green QPCR master mix (Stratagene, La Jolla, CA). The following primers were used: hGAPDH for-5'-GAAGGTGAAGGTCTGGAGTC-3' and rev-5'-GAAGATGGTGATGGGATTTTC-3'; hCR-1 for-5'-CACGATGTGCGCAAAGAGA-3' and rev-5'-TGACCGTGCCAGCATTTACA-3'; hNanog for-5'-TGAACCTCAGCTACAAACAGGTG-3' and rev-5'-AACTGCATGCAGGACTGCAGAG-3'; hOct3/4 for-5'-CTTGCTGCAGAAAGTGGGTGGAGGAA-3' and rev-5'-CTGCAGTGTGGGTTTCGGGCA-3'; Nestin for-5'-CAGCTGGCGCACCTCAAGATG-3' and rev-5'-

AGGGAAGTTGGGCTCAGGACTGG-3'. Relative quantification of CR-1 mRNA within the samples was performed using the $\Delta\Delta C_t$ method, as previously shown (11).

Immunofluorescence

EC cells were treated for 48–72 hours with either media alone (control) or 50 ng/ml of exogenous soluble Netrin-1. Cells were incubated for 16 hours at 4°C with the following primary antibodies: rabbit anti-GABA (1:1000; Chemicon); rabbit anti-beta III-tubulin (1:1000, Abcam); mouse anti-Nestin (1:1000, R&D Systems) and mouse anti-GFAP (1:1000, Chemicon). 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*-value < 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

Since 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 WB 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 as compared to untreated control EC cells (Figure 1 A). Netrin-1 did not significantly affect the 72 hr growth rates of either NTERA/2 or NCCIT EC cells (Figure 1B) 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 as compared to untreated controls (Figure 1C). Since 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 (Figure 1 D). This is in agreement with similar observations that describe reduced Nanog and Cripto-1 levels in Netrin-1 treated mouse embryonic stem 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 50 ng/ml of exogenous soluble Netrin-1 increased expression of beta-III tubulin, a common marker for early neuronal differentiation, in NTERA/2 (~ 2-fold) and NCCIT (~3.5-fold) (Figure 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 show increased levels of other markers of early neuronal development, such as Nestin (~2-fold) and GFAP (~3.5-fold) (Figure 2B). Immunofluorescent staining confirmed the higher levels of beta-III tubulin

expressed in Netrin-1 treated NTERA/2 EC cells which appeared to assume a more neuronal-like morphology with dendritic or axonal-like cytoplasmic extensions (Figure 2B). Additional immunofluorescent staining showed increased expression of the neurotransmitter GABA in GFAP positive Netrin-1 treated NTERA/2 EC cells as compared to GFAP negative untreated control cells (Figure 2C). 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 demonstrated expression of Neogenin (Figure 3A) 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 WB analysis (data not shown). Previous studies have shown that a number of the 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 ng/ml 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 (Figure 3B). A similar effect was observed in NCCIT EC cells (not shown). Since 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 MAPK in Netrin-1 treated and untreated NTERA/2 or NCCIT EC cells (not shown). Members of the src family of tyrosine kinases are also affected by SHP-2 activity. Since 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 WB analysis to detect basal levels of Fyn and c-src in NTERA/2 and NCCIT in order 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 (Figure 3 C and D). The potential molecular interplay 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 μ g/ml) or with the SHP-2 inhibitor (50 μ M), alone or in combination (Figure 3C 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 like Nestin (Figure 4A) or repress the expression of Nanog, Oct4 and CR-1 (Figure 4B). 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 like 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 utilized in our study especially since 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 demonstrate 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.

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Figure 1. Exogenous soluble Netrin-1 (50ng/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. (* $P < 0.05$).

Figure 2.

(A) Western blot analysis shows increased expression of beta (β)-III tubulin in NTERA/2 and NCCIT EC cells treated for 12 days with exogenous soluble Netrin-1 (50ng/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 Netrin-1 treated NTERA/2 EC.

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. 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 (D).

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 μ g/ml) and with a synthetic SHP-2 inhibitor (50 μ M) 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)