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Doctoral School of Oncology and Genetics

Cycle XXI

Ph.D. in Oncological Genetics

Neuronal guidance protein Netrin-1 induces neuroectodermal-like differentiation by regulating the expression of stem cell markers Nanog, Oct4 and Cripto-1.

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Thesis suitable for the title of "Doctor Europeus"

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Ph.D. dissertation board

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Orsay, september 13th, 2009

To whom it may concern,

I am sending you my report on the PhD work of
Mario M A N C I N O on the subject

« **Neuronal guidance protein Netrin-1 induces neuroectodermal-like differentiation by regulating the expression of stem cell markers Nanog, Oct4 and Cripto-1**».

This PhD Thesis is divided in five main parts of variable sizes : The first one is a literature review of the main aspects of the thesis, which concerns embryonal carcinoma cells (EC) and three important proteins involved in early development including self-renewal and the maintenance of pluripotency. The second one is the Material and methods part including cell culture, molecular biology and biochemical methods and some classical imaging and statiscal tests. The third part is dedicated to the results, the fourth chapter to the discussion. Expectingly, references (106) are given in the fifth chapter. This PhD thesis contains 51 pages including the front page and the PhD dissertation board.

The candidate seems to know perfectly well the scientific literature surrounding his theme. The literature review is focusing on the main aspects of the thesis. It is concise and bring the main information to introduce the questions of this PhD thesis. The introduction brings together the necessary elements required to understand his

work. However, even self explicit, the candidate could have written the clear aim(s) of his PhD thesis at the end of the introduction. It has to be noted that Mario did not fall in the trap to give a general introduction on early development, embryology, stem cells and all proteins involved in this events. The quality of the figures is adequate.

The materials and methods chapter is clear and seems to be complete. Altogether, this demonstrates that Mario Mancino is perfectly able to manage classical molecular biology, cell biology and biochemical experiments.

In EC cells, exogenous Netrin affects migration but not proliferation. The reduction of migration is at least in part associated with the reduction of Cripto-1. It would have been of interest to verify that Cripto-1 affects effectively the migration of EC cells. Moreover, he shows that the presence of Netrin induces the reduction of Oct4 and Nanog (two upstream regulators of Cripto-1). The treatment of EC cells with Netrin induces a modification of shape of the cells, a modification of its cytoskeleton (reported by the presence of b-III tubulin), the induction of the neurotransmitter GABA. They suggest that Netrin induces preferentially a differentiation towards ectoderm due to the absence of GATA4 and T-Brachyury. In the second part of the work, Mario Mancino shows that Netrin is affecting signalling via its receptor Neogenin and triggering the activation of SHP-2 which could result in the modification of the levels of active c-src. Altogether, these experiments appear to be solid. However, additional experiments showing the consequences at the functional level and determination would have been nice to present.

The discussion chapter is proper but it could have bring more perspectives and eventually more personal thoughts. Moreover, a general abstract could have be presented. Finally, no publication is reported in the PhD thesis.

In conclusion, the quality of the presented work is very good and fulfils the conditions that are required to allow Mr Mario Mancino to take his PhD examination.

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To whom it may concern

Report on the PhD Thesis
of
Mario M A N C I N O

Title

Neuronal guidance protein Netrin-1 induces neuroectodermal-like differentiation by regulating the expression of stem cell markers Nanog, Oct4 and Cripto-1

The present thesis is classically structured as expected and spanning 51 pages. The author starts with an introduction into the main aspects of the work and presents a comprehensive review of the relevant literature. The role of embryonal carcinoma cells and the stem cell markers Nanog, Oct4 and Cripto-1 were described in detail.

In material and methods the applied technologies such as methods of cell culture, biochemistry and molecular biology of the markers and the applied statistics were extensively described.

In the following part results are presented extensively and adequately discussed. Finally in more than 100 references the literature, relevant for this field, is cited.

Mario Mancino has shown that he is familiar with the topic of his thesis and the literature about embryonal carcinoma cells (EC) and the stem cell markers investigated. The informations necessary to understand the aspects of his word were concisely presented and focus to the aspects of his work.
In material and methods, he presented a complete description of the technology applied in cell and molecular biology, which he used in his investigations. The main results found in the present thesis: Netrin reduces migration embryonal carcinoma cells and diminishes the Oct4 and Nanog production. On the other hand, Netrin modificates the cytoskeleton of embryonal carcinoma cells with changes in the shape of the treated cells. Changes tent to a differentiation to ectodermal cells. In further investigations Mario Mancino found that Netrin is altering Neogenin responses and activating SHP-2.

In summary, Mario Mancino found interesting biological and molecular pathways. The study should be continued in order to show the biological consequences of his findings. The methods and results are discussed adequately, tables and figures are clearly structured, however a short abstract is missing.

Final conclusion: I want to recommend the present thesis of Mario Mancino to be accepted and to allow him to appear for his PhD examination, as the volume and quality of his work good and consists of all parts that should be covered in a PhD thesis.

(PD Dr. Wolfgang Marek)

TABLE OF CONTENTS

INTRODUCTION

Testicular germ cell tumors, which include human embryonal carcinoma (EC), teratocarcinoma, and seminoma, are the most common solid tumors of young men (1). Their incidence has increased 2–3-fold in most Western countries over the past 50 years (2). The reasons for this increasing incidence are unclear, although a role for environmental estrogen analogues affecting germ cell development *in utero* has been proposed. Fortunately, these tumors are highly susceptible to chemotherapy, and current treatment regimens, including monitoring of serum tumor marker levels, are very successful (3). Nevertheless, treatment failures occur, and even successful treatment is often accompanied by significant morbidity.

EC cells are probably the single most important malignant component of non-seminomatous germ cell tumors and are thought to give rise to the other cell types of teratomas by differentiation. EC cells can be differentiated with exogenous factors like retinoic acid (4) and the malignant phenotype reversed as they become incorporated into normally developing embryonic tissues after injection into the mouse blastocyst (5).

Transcription factors, such as Oct4 and Nanog are expressed in EC cells (6;7) and function to maintain the self-renewal and pluripotency of embryonic stem cells (ES) (8). ES cells are pluripotent cells derived from the inner cell mass (ICM) of the mammalian blastocyst. They are capable of indefinite self renewing expansion in culture. Depending on culture conditions, these cells can differentiate into a variety of cell types. Oct4, and Nanog are key regulators essential for the formation and/or maintenance of the ICM during mouse preimplantation development and for self-renewal of pluripotent ES cells.

A common target gene of Oct4 and Nanog is Cripto-1, a member of the epidermal growth factor family of proteins expressed in embryonic stem cells and during the initial stages of development (9). Cripto-1, also known as teratocarcinoma derived growth factor-1 since first isolated from human NTERA/2 EC cells(10), can induce cellular transformation *in vitro* and increase tumor incidence *in vivo*(9). Interestingly, repression of Cripto-1 has been shown to be associated with differentiation of EC cells towards a neuroectodermal lineage (10). However, little information is known about how external factors, such as guidance molecules, may regulate the levels of

Nanog and/or Oct4, which could result in the differentiation of cancer cells with pluripotent characteristics.

Netrin-1, a secreted guidance molecule, can bind to specific cell surface receptors, such as Neogenin, and regulate the development, functional differentiation and trafficking of both neuronal and extraneuronal cells (11). Netrin-1 can also regulate Cripto-1-induced cellular motility and allometric outgrowth of mouse mammary epithelial cells (12). 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 the early neuroectodermal differentiation marker beta-III tubulin in mouse embryonic stem cells suggesting that Netrin-1 may affect differentiation of pluripotent cells (13).

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 were accompanied by an increases in the levels of active P-SHP-2 and inactive P-c-src$^{(Y527)}$. Furthermore, Netrin-1 treated EC cells exhibited lower levels of expression of Cripto-1, Nanog and Oct4. These

results suggest the potential for Netrin-1 to induce differentiation of human EC cells.

Embryonal carcinoma: an overview

Teratocarcinomas, a subset of germ cell tumours (GCTs), provide a striking paradigm of the stem cell concept of cancer. They are highly malignant tumours containing a disorganized array of many somatic and extraembryonic cells, together with nests of EC cells. EC cells are the 'pluripotent' stem cells of these cancers, capable of self-renewal as well as differentiation into a very wide range of cell types. The differentiated derivatives of the EC cells are typically non-malignant, so that malignancy, as shown by the ability to regenerate the whole cancer including its differentiated elements, is the property of the EC stem cells. This was demonstrated by the classic experiments of Kleinsmith and Pierce (14) in which they showed that transplantation of a single EC cell to a new host mouse is sufficient to regenerate a new tumour. However, extensive studies during the 1970s also showed a close relationship between EC cells from murine teratocarcinomas and the pluripotent ICM (inner cell mass) cells of the blastocyst stage of early mouse embryos (15). This, together with an understanding of how to culture and characterize EC cells *in vitro*, culminated

in the isolation of ES (embryonic stem) cell lines by explanting ICM cells from mouse embryos in 1981 (Fig 1) (16,17).

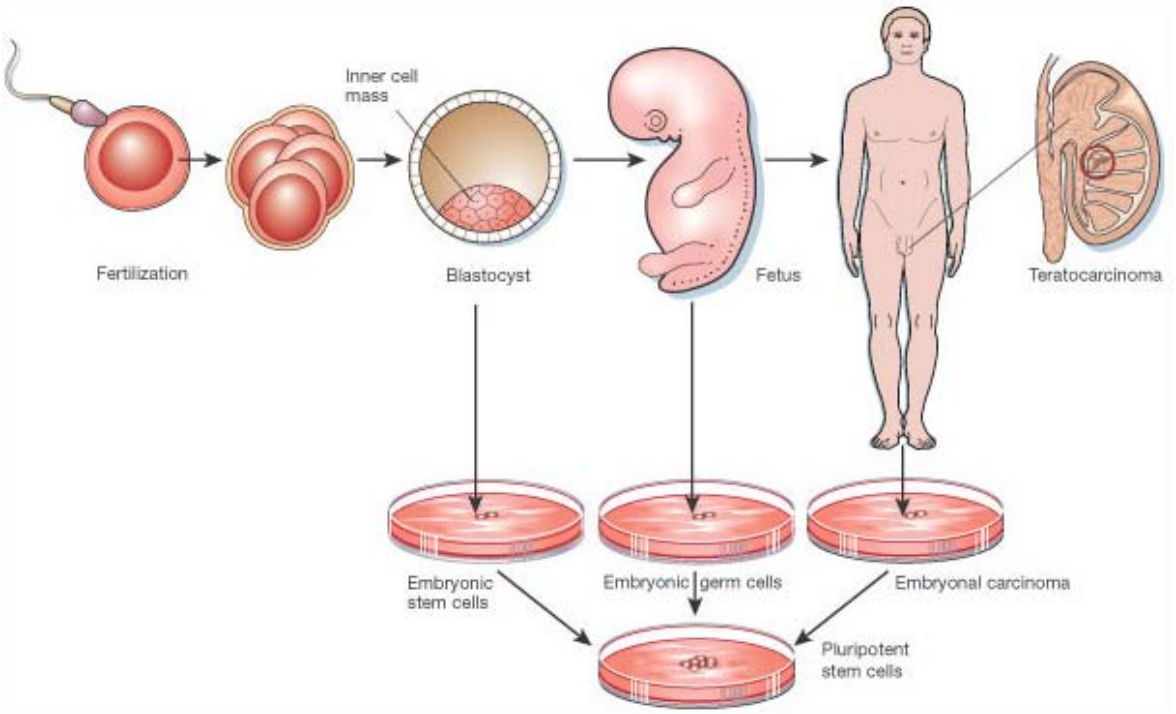

Fig 1 Embryonic stem (ES) cells are derived from the inner cell mass of the pre-implantation embryo. Embryonic germ (EG) cells are derived from primordial germ cells (PGCs) isolated from the embryonic gonad. Embryonal carcinoma (EC) cells are derived from PGCs in the embryonic gonad but usually are detected as components of testicular tumours in the adult. All of the three pluripotent stem cell types are usually derived by culture on layers of mitotically inactive fibroblasts, termed feeder layers.

The pathology of human and mouse GCTs is significantly different, while the properties of human and mouse EC cells also differ from one another. For example, while trophoblastic differentiation from human EC cells is common, it does not normally occur from mouse EC cells (18). Furthermore, they express different patterns of characteristic surface antigens. For example, human EC cells typically express the glycolipid antigens

SSEA3 (stage-specific embryonic antigen-3) and SSEA4, but not SSEA1, the high molecular mass proteoglycan antigens TRA-1-60, TRA-1-81 and GCTM2 and the protein antigens Thy1 and MHC class 1; in contrast, murine EC and ES cells express SSEA1 but not the other markers (19). It is noteworthy, then, that when human ES cells were finally derived by explanting the ICM of human blastocysts, their properties closely paralleled those of human EC cells and were distinct from those of mouse ES cells (20,21). Human ICM cells from blastocysts also express similar patterns of surface antigen expression to both human EC and ES cells, again confirming the relationships between these cell types and emphasizing that the differences from the corresponding mouse cells most probably represent species differences in embryogenesis (22). Nevertheless, like murine EC and ES cells, human EC and ES cells characteristically express the transcription factors OCT4 and Nanog, which are down-regulated upon differentiation (23).

Despite their similarities, teratocarcinoma-derived EC cells present only a caricature of ES cells. In contrast with ES cells, EC cells often only have a limited capacity for differentiation, and many EC cell lines have

completely lost this ability, such cell lines are called ‘nullipotent’. Many, particularly in humans, are also karyotypically abnormal (24). Such differences from ES cells are not particularly surprising when it is appreciated that EC cells have necessarily been selected for tumour growth. While many facets of cell biology might contribute to better survival of tumour cells, a particular feature that is pertinent in the case of pluripotent stem cells is their capacity to choose between the production of daughter stem cells (self-renewal) on the one hand, and differentiation on the other. Since the differentiated derivatives of EC cells have limited competence for extended proliferation and survival, one might infer that pluripotent stem cells like EC cells will be subject to strong selection for mutations that tend to limit differentiation, even a small increase in the probability of self-renewal compared with differentiation could have a significant selective advantage during tumour progression. If indeed the selection of variants with reduced capacity for differentiation is to be expected in the growth of EC cells, similar selection might also be anticipated in ES cells on prolonged passage in culture. Recently, it has been demonstrated that human ES cells in culture commonly acquire additional copies of chromosome 17, particularly its long

arm (17q) and also chromosome 12, particularly its short arm (12p) (25). What is striking about this observation is that the same chromosomal additions are very common in EC cells from human teratocarcinomas (26-28). Furthermore, amplification of mouse chromosome 11, which is syntenic with much of human chromosome 17q, has also been reported in mouse ES cells (29), although mouse chromosome 8 is also commonly amplified too. One inference from these observations is that additional copies of a gene(s) encoded by these chromosomes contribute to enhancing the capacity of both EC and ES cells for self-renewal and to encode key components of the molecular mechanism by which these stem cells choose whether or not to commit to differentiation. To date, the identity of these genes remains unknown.

Oct3/4 and Nanog transcriptor factors in EC cells

The pluripotency of these neoplasms has recently been explained by a close resemblance of their expression profile to that of embryonic inner cell mass cells studied in culture as embryonic stem cells, with high expression of transcription factors associated with pluripotency, such as Nanog and Oct3/4 (Fig 2).

Oct3/4, also known as *otf3* or *pou5f1*, is a member of the POU family of transcription factors, which is expressed in pluripotent mouse and human embryonic stem and germ cells (30– 35). Expression of this gene is down-regulated during differentiation (36). Furthermore, knocking out the *pou5f1* gene in mice causes early lethality because of lack of inner cell mass formation (37) because pou5f1 is critical for self-renewal of embryonic stem cells (38). Interestingly, pou5fl has been linked to the capacity of proper outgrowth of somatic cell clones (39). During human development, expression of *POU5F1* is found at least until the blastocyst stage (40) in which it is involved in gene expression regulation. The protein activates transcription via octamer motifs (ATGCAAAT) located distally or proximally from transcriptional start sites (41). POU5F1 binding sites have been

identified in various genes, including *fibroblast growth factor 4* and the 1.5-kb alternative promoter of the *platelet-derived growth factor* receptor (42). The data indicate that pou5f1/POU5F1 functions as a master switch in differentiation by regulating cells that have, or can develop, pluripotent potential. Moreover has been demonstrated that *POU5F1* transcripts are found in a specific set of human testicular GCT of adolescents and young adults (TGCT): the seminomas and embryonal carcinomas (43). In addition, the precursor lesions of TGCT, known as CIS (44), also express *POU5F1* (43). These lesions are composed of cells that are considered to be the malignant counterpart of an embryonic germ cell, most likely a PGC (44–46). In contrast, no expression was found in the differentiated components of nonseminomas, *i.e.*, teratomas, yolk sac tumors, and choriocarcinomas (43). Indeed, expression of *POU5F1* has been reported in embryonal carcinoma cells lines, and down-regulation of expression is found upon differentiation (33, 42). Nanog also plays a critical role in regulating the cell fate of the pluripotent ICM during embryonic development, maintaining the pluripotent epiblast and preventing differentiation to primitive endoderm (47). It has been identified in human ES cells (48-50), and more recently, also in embryonal

carcinoma (51). A more detailed analysis of Nanog in germ cell tumours demonstrated a pattern of expression essentially identical to that of Oct3/4 (52, 53). A common feature of these genes is their link to pluripotency; they prevent further differentiation of the cell and ensure a ‘stock’ of undifferentiated cells to renew the tissue. Outside the early embryonic development, Nanog and Oct3/4 are only found in immature germ cells. A high expression of these genes is a probable explanation of the ability of EC cells to undergo reprogramming to pluripotent embryonal carcinoma and further differentiation to teratomas, which may contain all types of somatic tissues.

Cripto-1, a target gene of Oct4 and Nanog, expressed in EC cells

Cripto-1, a member of the epidermal growth factor (EGF)-CFC family, implicated in embryogenesis and carcinogenesis, can enhance migration, invasion, branching morphogenesis and EMT of several mammary ephitelial cell lines. Moreover, recently, a meta-analysis of 38 original studies revealed that TDGF-1, which is the gene name of Cripto-1 is highly ranked as a specific marker of ES cells. Seventeen out of 20 lists showed that Cripto-1 is specifically expressed in EC cells (54).

Together with Nanog and Oct4, Cripto-1 has been suggested to play a role in self-renewal and maintenance of pluripotency (8). It has been shown to be a direct target gene in embryonic stem (ES) cells for Nanog and Oct4 suggesting that many of the functions of Nanog and/or Oct4 may be mediated in part through expression of Cripto-1 (54). Cripto-1 functions through at least three different pathways:1) as a co-receptor for the TGF β- related proteins Nodal and growth and differentiation factors 1 and 3 (55-57); 2) as a ligand for glypican-1/ c-Src/MAPK/PI3K-Akt signaling (58) and 3) as an inhibitor for Activin/TGF β signaling (59) (Fig 2). During early mouse embryogenesis, Cripto-1 mRNA expression is found in the embryonic ectoderm following

implantation of the blastocyst. On day 6.5 of gestation, Cripto-1 is detected at increasing levels in the epiblast cells undergoing epithelial to mesenchymal transition (EMT) as they migrate through the nascent primitive streak and in the developing mesoderm cells (60, 61-63). By day 7, Cripto-1 is detected mostly in the truncus arterious of the developing heart. With the exception of the developing heart, little if any expression of Cripto-1 mRNA can be detected in the remainder of the embryo after day 8 (61,64). As previously mentioned, Cripto-1 null mice (Cripto-1-/-) succumb at day 7.5 due to their inability to gastrulate and form appropriate germ layers (65).

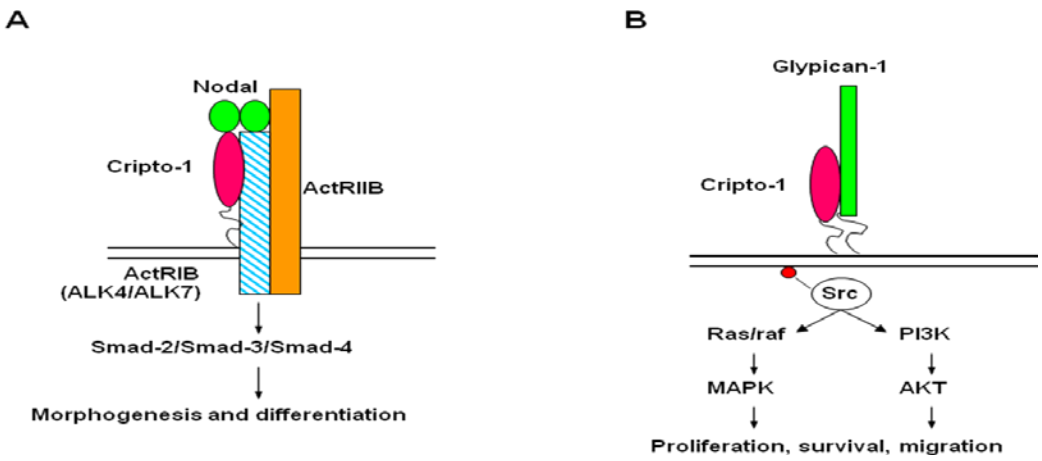

Fig. 2 Schematic Overview of Nodal and Cripto-1 Signaling Pathways. **A**) Canonical Nodal signaling using Cripto-1 as a co-receptor. Nodal can also activate this pathway, albeit less efficiently, independently of Cripto-1. **B**) Cripto-1 Nodal-independent signaling via binding with Glypican-1 and activating src/Ras/raf/PI3K downstream signaling.

Netrin-1: diversity in development

Various studies have identified different chemotropic factors that regulate the direction of cell migration. Most of these have been identified during neuronal development and include proteins such as, Slits, Ephrins, Semaphorins (66), Sonic hedgehog (67), bone morphogenic proteins (68), Wnts (69) and Netrins (70,71). Sequence and functional analysis have shown that Netrins are a conserved family of secreted proteins that have regional homology to laminins and are capable of regulating axonal outgrowth (70,72,73). The direction of Netrin-dependent neuronal outgrowth is determined by the cellular expression of receptors belonging to either the DCC (deleted in colon cancer) or UNC5 families of Netrin-1 receptors (74,75). These single-pass transmembrane receptors contain immunoglobulin domains with DCC containing fibronectin type-3 domains and with UNC5 containing a thrombospondin type-I domain (76). The DCC receptors, which include the structurally similar Neogenin receptor, mediate attraction, whereas repulsion is mediated by a complex of DCC and UNC5 receptor families (Fig 3) (77,78). The highly conserved family of UNC receptors possesses a high level of structural and sequence homology in the ligand binding extracellular domain (79). In humans, UNC5 receptors are composed of UNC5HA, UNC5HB and UNC5HC and correspond to the rodent orthologues UNC5H1, UNC5H2 and UNC5H3, respectively (80).

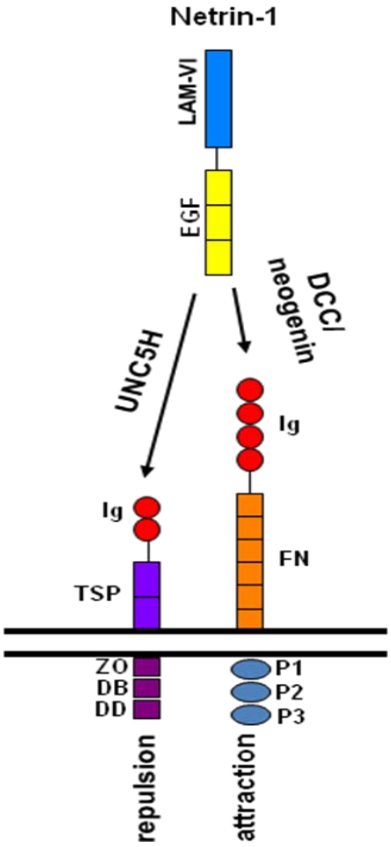

Fig 3 Attractive and repulsive netrin–receptor complexes. The attractive effects of Netrin1 are mediated by homodimers of DCC proteins (DCC and Neogenin in vertebrates). By contrast, UNC5 proteins mediate repulsion, in the presence of DCC (DCC-dependent repulsion),

Recent studies have found functioning Netrin molecules outside the nervous system, in the pancreas, intestine (81,82), lung (83) kidney, heart and vasculature (84-86) where they presumably play a role in the development of these organs by regulating the migration of different types of cells. Regulation of the expression of Netrin-1 and its receptors may play a role in tumorigenesis. In fact, Netrin-1 was shown to be reduced in tumors of the prostate and of the nervous system (87,88). Low levels of somatic mutations of DCC have been identified in cancers of the brain, stomach, pancreas, colorectum and testicle (80) and in a series comparing human colorectal tumors with corresponding normal tissues, of the different UNC5 receptors studied, UNC5A, the orthologue of rodent UNC5H1, showed the highest

percentage of altered expression (89). Netrin-1 and Neogenin have been shown to be involved in maintaining adhesion between cap cells and luminal cells in the mammary gland terminal end buds (90). As Cr-1 is also expressed in the terminal end buds of developing mammary glands (91) and is capable of inducing migration by deregulating cell adhesion and promoting EMT in mammary epithelial cells. Finally, it has been demonstrated that Netrin-1 can cause a reduction in the expression of Nanog and Cripto-1 and can increase expression of the early neuroectodermal differentiation marker beta-III tubulin in mouse embryonic stem cells suggesting that Netrin-1 may affect differentiation of pluripotent cells (13)

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 37ºC in a humidified atmosphere of 5% CO_2. Recombinant Netrin-1 protein was purchased from R&D Systems (Minneapolis, MN). Cell migration was assessed using fibronectin coated transwell. migration assay Kit (Chemicon, Temecula, CA). Briefly, NTERA/2 and NCCIT EC cells were treated with various concentrations of Netrin-1 recombinant protein. After 24 h incubation, cells that migrated through the porous membrane were stained with crystal violet solution (Chemicon). The crystal violet solution was eluted with elution buffer (Chemicon) and absorbance was read at 595 nm. Medium containing 2% FBS was used in the lower chamber as chemoattractant. The experiments were performed in triplicate and repeated twice.

Western blot analysis

The human EC cells were seeded in 60 mm plates ($6x10^5$ 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 (90) (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.

Actively growing cells were harvested from culture, washed in PBS and then lysed in lysis buffer (50 mM Tris-HCl (pH 7.4), 250 mM NaCl, 5 mM EDTA, 50 mM NaF, 0,1% triton-X-100, 0,1mM Na3Vo4) containing Protease Inhibitor Cocktail (Roche, Complete, Mini, EDTA-Free). The lysates were kept on ice for 30 min, vortexed twice, and centrifuged at 4°C for 20min at 14,000 rpm in an Eppendorf microcentrifuge; and clear supernatant recovered. Protein concentration was determined by the Bradford assay. Equal amount of cell extracts (50 Ug) were electrophoresed through 10%-

12% (depending on protein analyzed) acrylamide SDS-denaturing gel, electroblotted onto nitrocellulose membrane and probed with the appropriate primary and secondary antibodies. Blocking and incubation with primary and secondary antibodies were performed in 5% nonfat dry milk and 0.1% Tween20 in TBS. 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

EC cells were plated at a density of $6x10^5$ 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. Cells were washed with PBS and total RNA was isolated using RNeasy mini Kit (Qiagen) according to the manufacturer's protocol. Two μg of total RNA was used for cDNA synthesis using the RETROscript Kit (Ambion, Foster City, CA) following the manufacturer's instructions. Quantitative real-time PCR was performed on Stratagene MX3000P using Brilliant II SYBR Green QPCR master mix (Stratagene, La Jolla, CA). The primers for the analysis of GAPDH, CR-1, Nanog, Oct3/4 and Nestin mRNA expression were selected by using the Primer Express software (PE Biosystems) (Tab.1). Relative quantification of CR-1 mRNA within the samples was performed using the ΔΔCt method (ΔCtsample - ΔCtcalibrator = ΔΔCt; relative quantity = $2^{-\Delta\Delta Ct}$), as suggested by the manufacturer (PE Biosystems).

Tab.1

Primers used for real time-PCR		
	Sense	**Antisense**
hGAPDH	GAA GGT GAA GGT CGG AGT C	GAA GAT GGT GAT GGG ATT TC
hCR-1	CAC GAT GTG CGC AAA GAG A	TGA CCG TGC CAG CAT TTA CA
hNanog	TGA ACC TCA GCT ACA AAC AGG TG	AAC TGC ATG CAG GAC TGC AGA G
hOct3/4	CTT GCT GCA GAA GTG GGT GGA GGA A	CTG CAG TGT GGG TTT CGG GCA
Nestin	CAG CTG CGC ACC TCA AGA TG	AGG GAA GTT GGG CTC AGG ACT GG

Immunofluorescence

Approximately $2x10^4$ EC cells were seeded in Lab-Tek dual chamber slides (Nalge Nunc, Naperville, IL) and treated for 48-72 hours with either media alone (control) or 50 ng/ml of exogenous soluble Netrin-1. Culture medium was removed and cells were washed twice with PBS, fixed with ice-cold 100% methanol for 10 minutes and air-dried. Slides were then washed three times with PBS, blocked for 30 minutes with 5% normal goat serum and incubated for 1 hour 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 again washed three times with PBS and incubated for 30 minutes with secondary antibodies (1:600) (Molecular Probes, Eugene, OR). Slides were finally mounted with Vectashield (Vector Labs, Burlington, CA), a mounting medium containing DAPI for identification of cell nuclei.

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

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 sought to determine 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, however, significantly reduced ($P<0.05$) the migration of NTERA/2 and NCCIT EC cells as compared to untreated control EC cells (Fig 4).

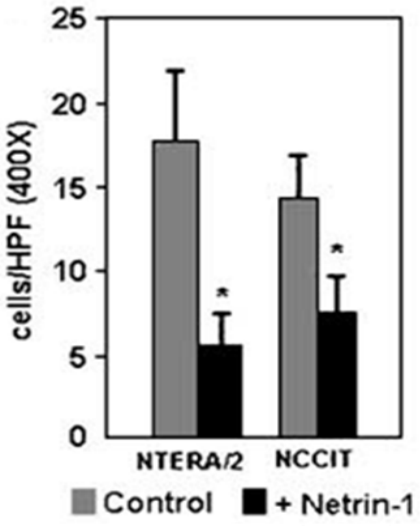

Fig 4 Exogenous soluble Netrin-1 (50 ng/mL) reduced (*, $P < 0.05$) migration of NTERA/2 and NCCIT EC cells. HPF, high-power field.

Exogenous Netrin-1 did not significantly affect the 72 hr growth rates of either NTERA/2 or NCCIT EC cells (Fig 5) arguing against a potential anti-proliferative effect of Netrin-1 that may have reduced the migratory capabilities of the Netrin-1 treated EC cells.

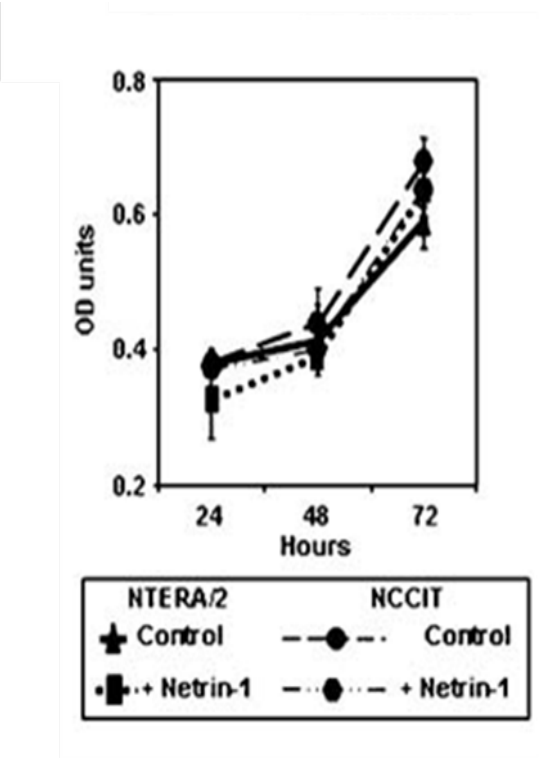

Fig 5 Exogenous soluble Netrin-1 (50 ng/mL) does not affect proliferation of NTERA/2 and NCCIT EC cells. OD, absorbance.

Since a reduction in Cripto-1 levels can lead to lower migratory rates of Cripto-1-expressing cells such as mouse mammary epithelial cells and human EC cells (12,92), we determined whether the reduced migration observed in the Netrin-1 treated NTERA/2 and NCCIT EC cells could be associated with a reduction in Cripto-1 levels. 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 control EC cells (Fig 6 A-B).

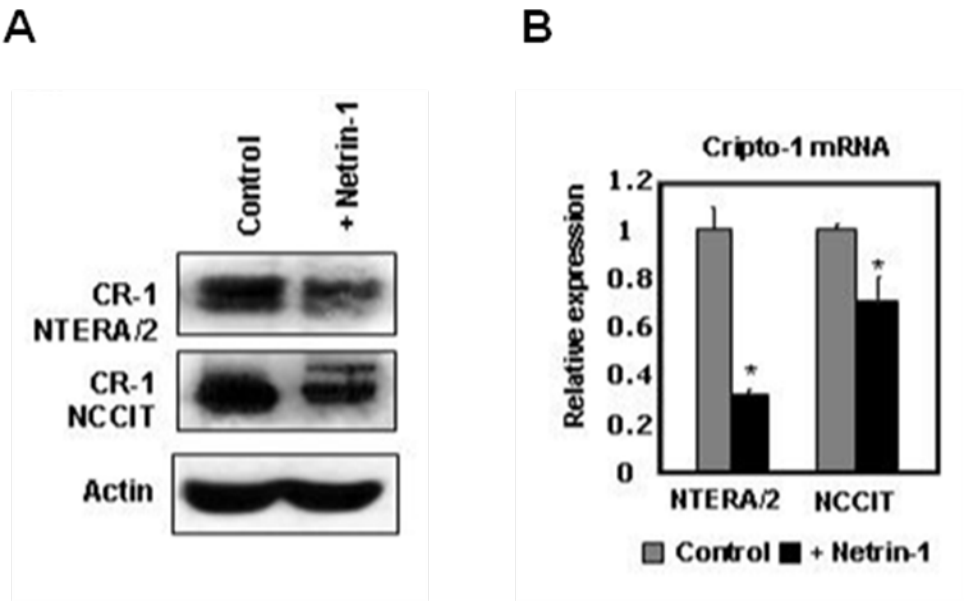

Fig 6 Exogenous soluble Netrin-1 (50 ng/mL) significantly reduced the expressions of Cripto-1 (CR-1) protein (A) and mRNA (B) (*, $P < 0.05$).

Since Cripto-1 is a target gene of the stem cell-related transcription factors Oct4 and Nanog (54), we investigated whether lower Cripto-1 expression is accompanied by reduced Oct4 and/or Nanog expressions in Netrin-1 treated NTERA/2 and NCCIT cells. A significant reduction ($P<0.05$) in the levels of Oct4 and Nanog mRNA was detected in the Netrin-1 treated NTERA/2 and NCCIT cells (Fig 7). This is in agreement with similar observations that describe reduced Nanog and Cripto-1 levels in Netrin-1 treated mouse embryonic stem cells (13).

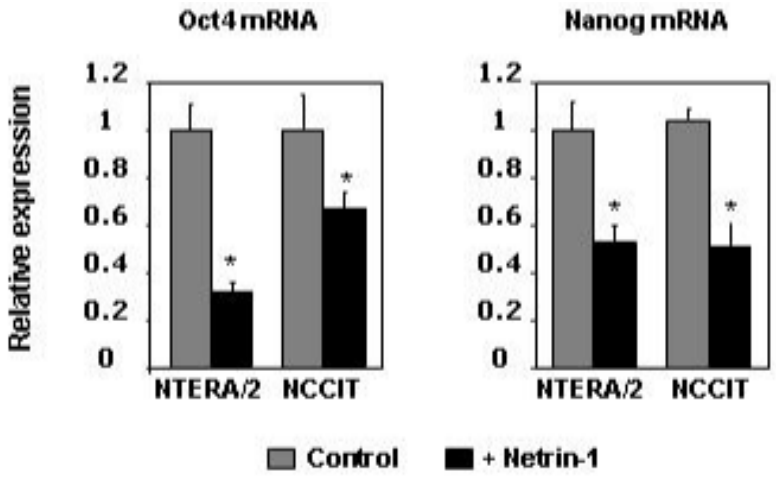

Fig 7 Exogenous soluble Netrin-1 (50 ng/mL) significantly reduced the expressions of Oct4 and Nanog mRNA (*, $P < 0.05$).

NTERA/2 and NCCIT EC cells assume early neuronal-like characteristics when treated with Netrin-1

Previous studies have shown that it is possible to induce neuronal-like features in EC cells following treatment with different agents, such as retinoic acid (4). We investigated whether the effect of Netrin-1 on Cripto-1, Nanog and Oct4 expression might be associated with the differentiation of NTERA/2 and NCCIT EC cells. Western blot analysis of cell lysates from NTERA/2 or NCCIT EC cells treated with 50 ng/ml of exogenous soluble Netrin-1 for 12 days showed increased expression of beta-III tubulin, a common marker for early neuronal differentiation, in NTERA/2 (~ 2-fold) and NCCIT (~3.5-fold) (Fig 8) EC cells.

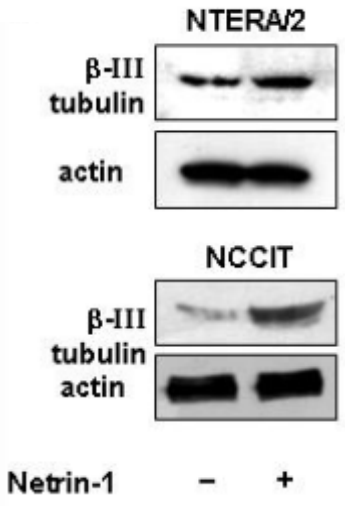

Fig 8 Western blot analysis shows increased expression of beta-III tubulin in NTERA/2 and NCCIT EC cells treated for 12 d with exogenous soluble Netrin-1 (50 ng/mL).

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, as compared to untreated control cells, of other markers of early neuronal development, such as Nestin (~2-fold) and GFAP (~3.5-fold) (Fig 9).

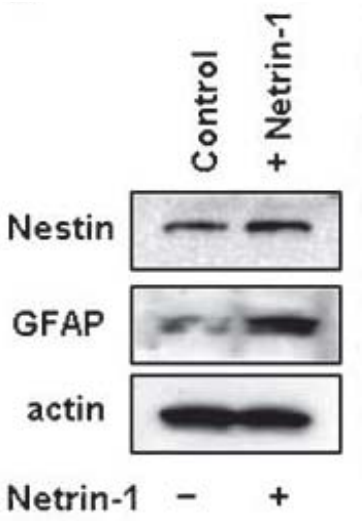

Fig 9 Western blot analysis shows increased expression of GFAP and Nestin in Netrin-1–treated NTERA/2 EC cells.

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 including dendritic or axonal-like cytoplasmic extensions (Fig 10).

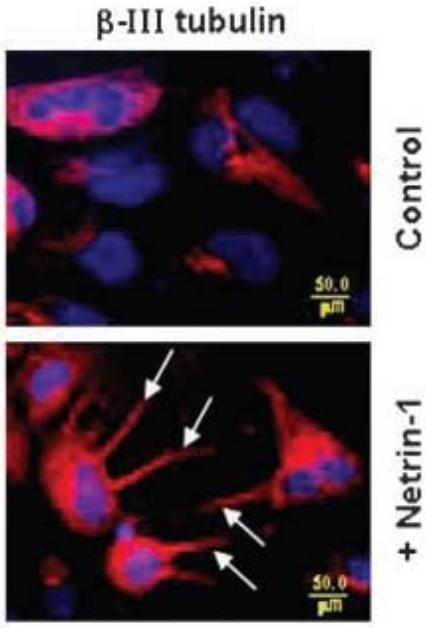

Fig 10 Increased expression of beta III-tubulin in Netrin-1–treated NTERA/2 EC cells was also detected by immunofluorescent staining. Of note is the neuronal-like morphology with axonal/dendritic-like cellular extensions (white arrows) in Netrin-1–treated NTERA/2 EC cells.

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 (Fig 11).

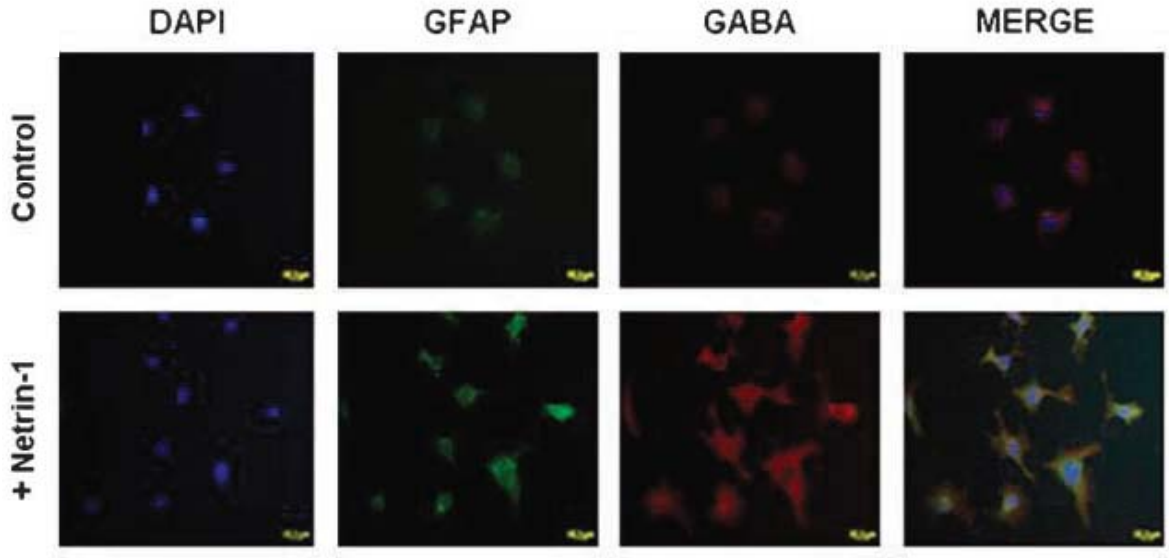

Fig 11 Immunofluorescent staining shows increased GFAP and GABA expressions in Netrin-1–treated NTERA/2 EC. DAPI, 4¶,6-diamidino- 2-phenylindole.

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 that are common to early precursors of neurons or glial cells in human EC cells.

Netrin-1 activates SHP-2 and increases levels of inactive c-src in NTERA/2 EC cells

Netrin-1 generally binds to two types of receptors: the DCC/Neogenin family of receptors and the UNC5H family of receptors (93). Western blot analysis demonstrated expression of Neogenin (Fig 12) in NTERA/2 and NCCIT EC cells but not UNC5HA (data not shown) suggesting that the biological effects of Netrin-1 observed in NTERA/2 or NCCIT EC cells are most likely mediated via Neogenin. We could not detect by Western blot analysis changes in the level of expression of either Neogenin or UNC5H between Netrin-1 treated and untreated NTERA/2 or NCCIT EC cells (data not shown). Previous studies have shown that a number of the responses induced by Netrin-1 can be mediated via recruitment and activation of intracellular signaling molecules such as, the tyrosine phosphatase SHP-2 (94). In fact, treatment of NTERA/2 EC cells with 50 ng/ml or 100 ng/ml of exogenous soluble Netrin-1 showed, on average, an approximate 3- and 2.5-fold increase ($P<0.05$), respectively, in the levels of active P-SHP-2 as

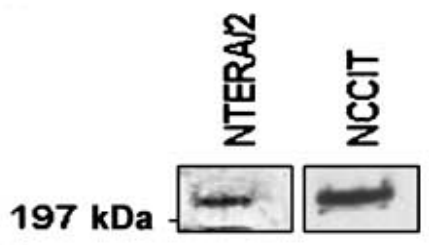

Fig 12 Western blot analyses show expression of the Netrin-1 receptor neogenin in NTERA/2 and NCCIT EC cells.

determined by Western blot and densitometric analysis of bands (Fig 13). A similar effect was observed in NCCIT EC cells.

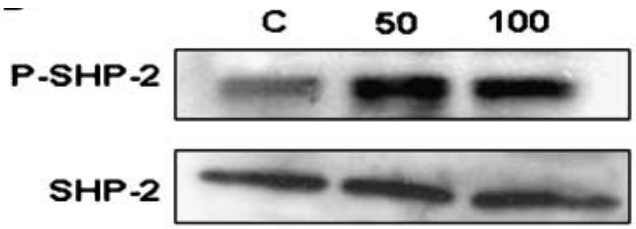

Fig 13 P-SHP-2 expression is increased in NTERA/2 EC cells treated with 50 or 100 ng/mL of soluble Netrin-1.

Since SHP-2 is known to regulate a number of intracellular tyrosine kinases in a variety of cell types (95), we determined whether this is also true in EC cells before and after Netrin-1 treatment. 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 (96) it is possible that these tyrosine kinases might perform a role in the Netrin-1 induced effects observed in the 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 in NETRA/2 and NCCIT EC cells. We could not detect activated Fyn in Netrin-1 treated or untreated NTERA/2 or NCCIT EC cells (data not shown). However we did find that Netrin-1 treatment resulted in an increased ratio between inactive c-src (c-src^{Y527}) to active c-src (c-src^{Y416}) in NTERA/2 and NCCIT EC cells (Fig 14 A-B). The potential molecular interplay between Netrin-1, SHP-2 and c-src was further investigated in NTERA/2 cells.

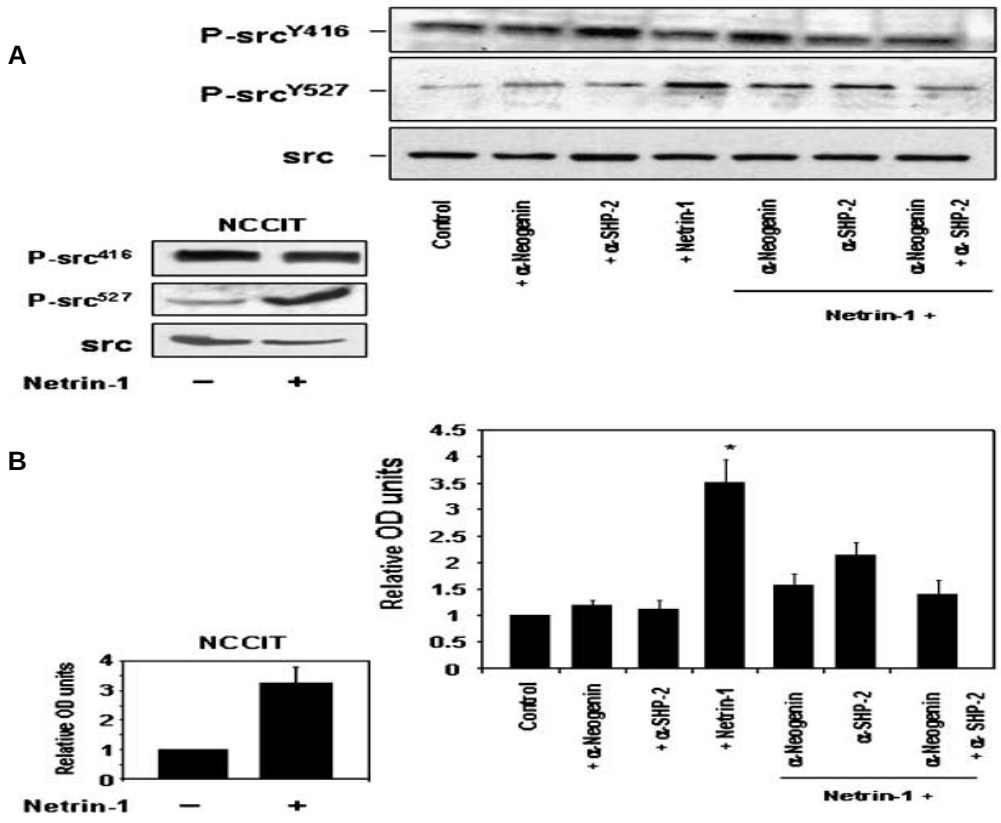

Fig 14 (A) Western blot analysis shows reduced expression of active c-src(Y416) with concomitant increase in the expression of the inactive c-src(Y527) in Netrin-1–treated NTERA/2 and NCCIT (inset) EC cells. (B densitometric analysis of the results in A shows a significant increase (*, $P < 0.05$) in 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.

We found that this Netrin-1 induced effect on c-src was significantly reduced when NTERA/2 cells were pretreated with anti-Neogenin blocking antibody (1μg/ml) or with the SHP-2 inhibitor (50μM), alone or in combination (Fig 12 A-B). 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 (Fig 15A) or repress the expression of Nanog, Oct4 and CR-1 (Fig 15B).

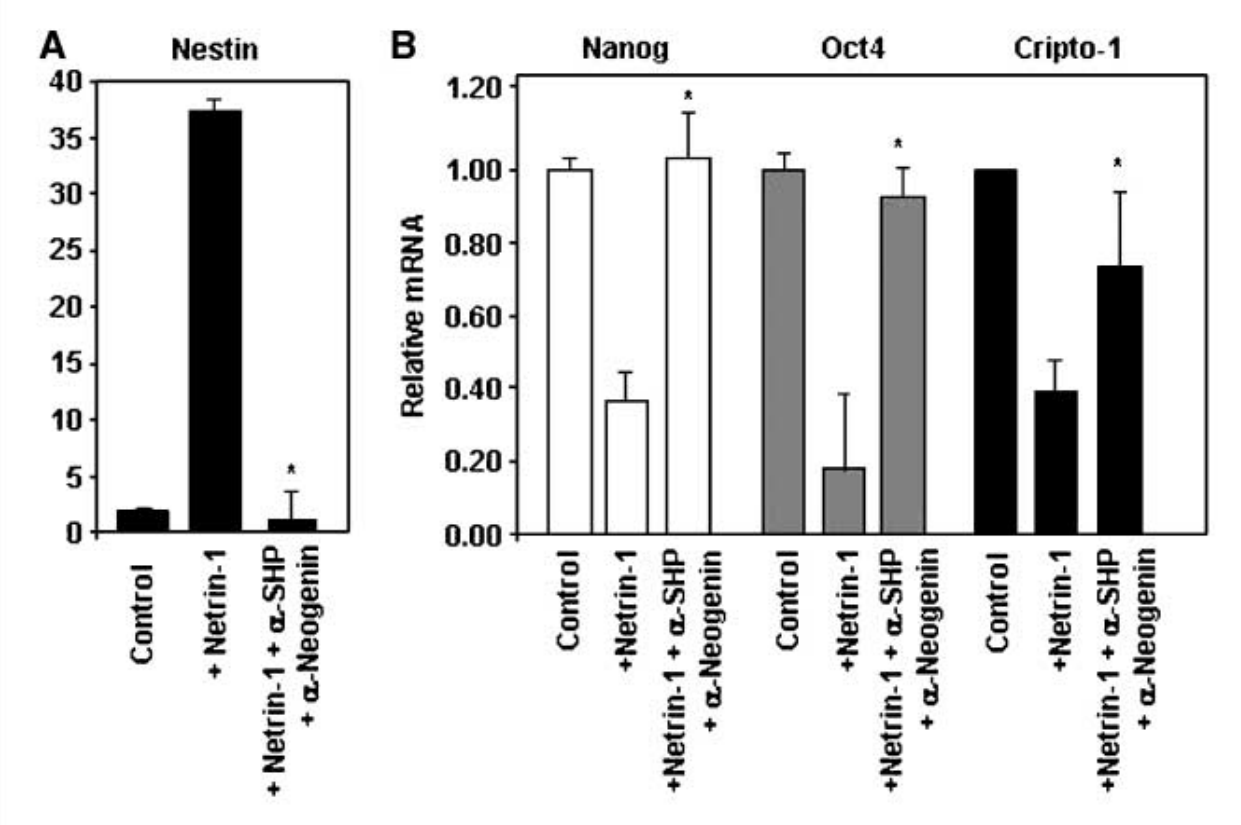

Fig 15 Antagonizing the Netrin-1 effect in NTERA/2 EC cells by treating the cells with a specific anti-Neogenin functional blocking antibody (1 Ag/mL) and with a synthetic SHP-2 inhibitor (50 Amol/L) not only significantly inhibited Netrin-1–dependent induction of

Nestin (A) but also significantly inhibited Netrin-1–dependent reduction of Nanog, Oct4, and Cripto-1 (B). *, $P < 0.05$.

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 which could result in the modification of the levels of active c-src.

DISCUSSION

Extracellular environmental factors play important roles during the regulation of cell growth and differentiation (97). They can significantly. influence cell fate by activating specific signaling pathways and can affect the activity of different transcription factors and therefore gene expression. Netrin-1, a neuronal guidance protein, was among several genes that were identified in ES cells using a modified gene-trap approach which analyzed the in vivo function of these genes encoding secreted and membrane proteins and considered critical for normal or abnormal in vivo development (98). Netrin-1 was found to promote adhesion between cap cells and body epithelial cells, which is necessary for proper development of the mouse mammary TEB (90) and to affect branching and development of extraneuronal systems like lung and vasculature (83,99). Moreover, Netrin-1 was identified as a potential regulator of Cripto-1 function both in vitro and in vivo (12). Significant expression of Cripto-1 mRNA and or protein has been demonstrated to occur with high frequency in different types of human malignancies, as compared to lower levels of Cripto-1 expression observed in corresponding normal tissues (9, 100). Interestingly, Cripto-1 expression is also increased in premalignant

lesions, such as colon adenomas and intestinal metaplasia of the gastric mucosa and has been detected in normal colon mucosa specimens from individuals with increased familial risk for developing colon carcinomas (101-103). Cripto-1 expression, therefore, gradually increases during the multistage process that evolves from normal mucosa to premalignant lesions and carcinoma. Furthermore, repression of Cripto-1 is associated with differentiation of EC cells toward a neuroectodermal lineage(7). Together with Nanog and Oct4, Cripto-1 has been suggested to play a role in self-renewal and maintenance of pluripotency (8). It has been shown to be a direct target gene in ES cells for Nanog and Oct4 suggesting that many of the functions of Nanog and/or Oct4 may be mediated in part through expression of Cripto-1 (54).

Our data demonstrate for the first time that Netrin-1 can inhibit migration and induce differentiation of human EC cells by influencing signaling pathways in which the genes, above mentioned, are involved.

Human EC cells can assume morphological and biochemical features common to early neuronal precursor cells when treated with exogenous soluble Netrin-1. We show that Netrin-1 can activate SHP-2 resulting in

increased levels of inactive c-src. In fact, previous work has shown that Netrin-1 was capable of regulating c-src-dependent signaling in Cripto-1 expressing mammary epithelial cells (13). Furthermore, activation of SHP-2 is associated with differentiation of ES cells (104) and activity of src family tyrosine kinases, such as c-src, Fyn and c-Yes have been previously shown to play a role during the maintenance of pluripotency (105). Our data suggest that c-src is most likely the more essential tyrosine kinase involved in the Netrin-1 induced effects observed in the human EC cells that were utilized in our study. The members of the src family of tyrosine kinases which might have been important in mediating Netrin-1 signaling is Fyn (96). However, Fyn or activated P-Fyn was not detected in either control or Netrin-1 treated NTERA/2 or NCCIT EC cells. Furthermore, active FAK, which is a downstream mediator of Netrin-1 induced Fyn activity (106), could not be detected in Netrin-1 treated EC cells. Netrin-1 treated NTERA/2 and NCCIT EC cells also showed reduced levels of Nanog, Oct4 and Cripto-1. 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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