Published in final edited form as:

Stem Cells. 2012 September ; 30(9): 1852–1862. doi:10.1002/stem.1162.

Increasing Doublecortin Expression Promotes Migration of Human Embryonic Stem Cell-Derived Neurons

Radmila Filipovic, Saranya Santhosh Kumar, Chris Fiondella, and Joseph Loturco

Department of Physiology and Neurobiology, University of Connecticut, Storrs, Connecticut, USA

Abstract

Human embryonic stem cell-derived neuronal progenitors (hNPs) provide a potential source for cellular replacement following neurodegenerative diseases. One of the greatest challenges for future neuron replacement therapies will be to control extensive cell proliferation and stimulate cell migration of transplanted cells. The doublecortin (DCX) gene encodes the protein DCX, a microtubule-associated protein essential for the migration of neurons in the human brain. In this study, we tested whether increasing the expression of DCX in hNPs would favorably alter their proliferation and migration. Migration and proliferation of hNPs was compared between hNPs expressing a bicistronic DCX/IRES-GFP transgene and those expressing a green fluorescent protein (GFP) transgene introduced by *piggyBac*-mediated transposition. The DCX-transfected hNPs showed a significant decrease in their proliferation and migrated significantly further on two different substrates, Matrigel and brain slices. Additionally, a dense network of nestin-positive (+) and vimentin+ fibers were found to extend from neurospheres transplanted onto brain slices, and this fiber growth was increased from neurospheres containing DCX-transfected hNPs. In summary, our results show that increased DCX expression inhibits proliferation and promotes migration of hNPs.

Keywords

Human embryonic stem cells; Neuronal progenitor; Proliferation; Migration; Radial glia

Introduction

Several studies have demonstrated successful generation of cerebral cortical neurons from human embryonic stem cells (hESC) [1–3] giving hope that hESC therapies may eventually be developed to treat central nervous system disorders that involve the loss of neurons. Transplantation studies have demonstrated successful integration of hESC-derived neuronal progenitor cells (hNPs) into the striatum [4], hippocampus [5], and cerebral cortex [6, 4, 7]. While appropriate dendritic and axonal connectivity of transplanted neural progenitors has been reported for transplanted mouse ES-derived neurons into rat cortex ([8], to date, neuronal progenitor cells derived from human ESCs have shown more limited potential to migrate into parenchyma of cerebral cortex [6, 4, 7] suggesting that methods to enhance

© AlphaMed Press

Correspondence: Radmila Filipovic, Ph.D., Department of Physiology and Neurobiology, University of Connecticut, Storrs 06268, Connecticut, USA. Telephone: 1-860-48703283; Fax: 1-860-486-3033; radmila.filipovic@uconn.edu.

Disclosure of Potential Conflicts of Interests

The authors indicate no potential conflict of interest.

Author contributions: J.L.: conception and design, data analysis and interpretation, manuscript writing, financial support, and final approval of manuscript.; R.F.: conception and design, collection and/or assembly of data, data analysis and interpretation, financial support, manuscript writing, and final approval of manuscript; S.S.K.: collection and assembly of data; C.F.: plasmid construction.

migration may be required for migration of hESC-derived neurons into mature brain tissue. Doublecortin (DCX) is a microtubule-associated protein [9, 10] that is expressed in the leading processes of migrating neurons [11]. DCX is essential for normal neuronal migration in humans. It was first identified as a gene critical for neuronal migration through the discovery that mutations in DCX cause severe neuronal migration disorders in humans [9, 10]. In adult brain, DCX is expressed in two neurogenic niches, the subventricular zone and hippocampus, and throughout the rostral migratory stream (RMS) a pathway of continued neuronal migration in the mature brain [12, 13]. Dcx is required for the migration of neurospheres that give rise to cells in the RMS, and mouse knockouts of Dcx show alterations in the morphology of migrating neurons [10, 14]. Similarly interneurons of the cerebral cortex require Dcx function for neuronal migration [11].

DCX has more recently been implicated in cell proliferation. Mutation of Dcx in mice causes defects in spindle positioning and decreases proliferation of neuronal progenitor cells in developing mouse neocortex [15]. Similarly, two reports have shown that ectopic expression of DCX inhibits proliferation and self-renewal of glioma cells [16, 17]. This raises the possibility that in addition to enhancing migration, induced DCX expression may also decrease proliferation or neural progenitors.

In this study, we hypothesized that ectopic expression of DCX in neural progenitors derived from hESCs would promote migration and decrease proliferation. We used a binary *piggyBac* transposon system to nonvirally introduce a DCX/ green fluorescent protein (GFP) or GFP transgenes into H9 derived human neural progenitors. Our results show that induced DCX expression has effects on differentiation of human neural progenitors. DCX decreases proliferation of hNPs, facilitates their migration in cell culture and across neural tissue, and noncell autonomously stimulates the invasion of radial glia scaffolds into neural tissue. These changes in hNP behavior are consistent with previously known roles for DCX and suggest that increasing DCX expression may be one possible way to limit proliferation and enhance migration of human neural progenitors.

Materials and Methods

hESC Propagation and Derivation of hNP

H9 (WiCell Research Institute, Wisconsin, <http://www.wicell.org>) and CT-2 hESC lines (Stem Cell Core, University of Connecticut Health Center) were received from the University of Connecticut Health Center Stem Cell Core (Farmington, CT) at passages 38–46 (H9) and passages 35–37 (CT-2). Cells were maintained according to modified protocols (Fig. 1A, [1, 2]). Karyotype analysis of G-banded metaphase chromosomes was performed regularly at Chromosome Services in Stem Cell Core at University of Connecticut and showed normal XX karyotype. hESC colonies (Fig. 1B) were grown on feeder layers of γ -irradiated mouse embryonic fibroblasts in hESC media containing Dulbecco's modified Eagle's medium (DMEM)/F12 (Cat. No. 11330), 20% knockout serum replacer (KSR) (Cat. No. 10828-028), 1% minimum essential medium (MEM) nonessential amino acids (Cat. No. 11140), 0.5% L-Glutamine (Cat. No. 25030), 6.5 μ M beta mercaptoethanol (β -ME) (Cat. No. 21985), fibroblast growth factor (FGF-2) (4 ng/ml; Cat. No. 13256) (all from Invitrogen, San Diego, CA, <http://www.invitrogen.com>). γ -Irradiated mouse embryonic fibroblasts were prepared from E15 CD-1 mice (Charles River, Willmington, MA), according to published protocols [18]. Animal protocol was approved by IACUC at University of Connecticut. Alternately, we used commercially available γ -irradiated mouse embryonic fibroblasts (GlobalStem Inc., Rockville, MD, Cat. No. GSC-6001G, <http://www.globalstem.com/>). Embryoid bodies (EBs, Fig. 1C) were isolated either by mechanical isolation or using enzyme collagenase IV (1 mg/ml; Invitrogen, Cat. No. 17104) and cultured in low-adhesion plates in cell suspension. EBs were kept in hESC medium for 4

days in the presence of FGF-2 (10 ng/ml; Invitrogen) followed by incubation for 3 days in neural induction medium (NIM), containing DMEM/F12 supplemented with MEM nonessential amino acids (1:100; Invitrogen), heparin (2.5 mg/ml; Sigma, St. Louis, MO; Cat. No. H3393, <http://www.sigmaaldrich.com/>), FGF-2 (10 ng/ml; Invitrogen), N2 (1:100; Invitrogen; Cat. No. 17502-048), and B27 (1:100; Invitrogen; Cat. No. 17504-044) and penicillin/streptomycin (1:100, Invitrogen; Cat. No. 15140). After NIM treatment, cell aggregates were allowed to attach to laminin-coated coverslips until they formed flattened neural rosettes (Fig. 1D, 1E). After 15 days in vitro (div), rosettes were manually dissected under dissecting microscope, from the plate using a 28G 1/2 U-100 insulin syringe needle (BD Biosciences, Franklin Lakes, NJ, <http://www.bdbiosciences.com>; Cat. No. 329461). The core of the rosette structure, which typically consisted of human neuronal progenitor cells, was removed from surrounding flat cells and collected. Human neuronal progenitor cells (hNPs) were dissociated using accutase (Sigma; Cat. No. A 6964) and either transfected for the experiments or plated for immunostaining (Fig. 1F, 1G). For differentiation, hNPs were switched to neuronal differentiation medium (NDM) containing DMEM/F12 (Invitrogen) supplemented with MEM nonessential amino acids (1:100; Invitrogen), N2 (1:100; Invitrogen), B27 (1:100; Invitrogen), brain derived growth factor (BDNF, 10 ng/ml; Peprotech, Rocky Hill, NJ, <http://www.peprotech.com>; Cat. No. 450-02), glial cell-derived neurotrophic factor (GDNF, 10 ng/ml; Peprotech; Cat. No. 450-10), ascorbic acid (20 μ M; Sigma; Cat. No. A4403), cyclic adenosine monophosphate (1 μ M; Sigma; Cat. No. A9501), and laminin (20 μ M; Invitrogen; Cat. No. 23017). Medium was changed every other day.

Nucleofection of hNP with *piggyBac* Plamid Transposon System

For stable transgenesis of hNPs, we used a binary *piggyBac* transposon system, which consists of two plasmids: the helper plasmid, providing the enzyme *piggyBac* transposase, which catalyzes genomic transposition, and a donor plasmid, which has the recognition sequence and terminal repeats (TRs) flanking a transgene for genomic insertion [19]. In this system, genes that are flanked by two TRs in the donor plasmids are inserted into the genome. Both the 3'- and 5'-*piggyBac* TRs (3'-TRs and 5'-TRs) were amplified from *piggyBac* transposable element pZGs [20]. In order to make pPBCAG-GFP, 3'-TRs was cloned into pCAG-GFP [21] using Sall and SpeI sites, and 5'-TRs was cloned into the same vector using PstI and HindIII sites. Helper plasmid pCAG-PBase was constructed by replacing eGFP with PBase sequence [20] in pCAG-GFP using EcoRI and NotI. Similarly, pPBCAG-DCX-IRES-GFP was made by inserting plasmid sequence DCX-IRES-GFP into XbaI and Bgl II sites of plasmid pPBCAG-GFP, from which GFP was removed. Sequence DCX-IRES-GFP was obtained by cutting from the plasmid pCAG-DCX-IRES-GFP made in our laboratory, pCAG-DCX-IRES-GFP, which was made by polymerase chain reaction cloning of DCX into pCAGIG [22]. Transgenic hNPs were made by transfecting hNPs after dissecting out a core of neurorosettes (Fig. 1D). The cores of the neurorosettes were dissociated with accutase (Sigma), and single cell suspensions (1.5×10^6 cells per nucleofection) were nucleofected using Amaxa II nucleofector (Program setting A-023), using Amaxa Human stem cell Nucleofector kit (Cat. No. VPH-5022, Lonza, Cologne AG, Germany, <http://www.lonza.com/>). Cells were transfected with pPBCAG-GFP or pPBCAG-DCX-IRES-GFP, along with pCAG-PBase (10 μ g of each plasmid). Cells were first plated as adherent cells for 2 days and after that mechanically detached and grown as neurospheres for 5 days in NIM in the presence of FGF-2 (10 ng/ml; Invitrogen), Rho-associated kinase (ROCK) inhibitor (10 μ M; EMD Chemicals, Billerica, MA, <http://www.emdmillipore.com/chemicals>; Cat No. 68800), before they were used for migration and EdU incorporation assays.

EdU Incorporation Assay

To assess the percentage of cells within neurospheres in S-phase of mitotic cycle, EdU (10 μ M, Invitrogen) was added during last 12 hours of culture. hNPs were then fixed at 12, 24, and 48 hours with 4% paraformaldehyde, and EdU incorporation was developed using Invitrogen Click-iT EdU Fluor 647 imaging kit (Invitrogen, Cat. No. C 10340), according to manufacturer's instructions.

Neurosphere Formation

hNPs transfected with either pPBCAG-GFP or pPB-DCX-IRES-GFP, along with pCAG-PBase, were detached from poly-D-lysine/ laminin surface by treatment with accutase (Sigma), and neurospheres were formed in 12-well low-adhesion plates (Thermo Fisher Scientific, Rochester, NY, <http://www.fishersci.com/>). The number and sizes of neurospheres was determined using inverted microscope from five fields of view of three repeats of experiments ($n = 3$).

Migration Assays

After 7 days of transfection, neurospheres were selected under a dissecting microscope, and similar sized neurospheres ($\sim 200 \mu$ m in diameter) were plated onto chamber four-well chamber slides (Thermo Fisher Scientific), coated with growth factor-reduced Matrigel (Cat. No. 35231, BD Pharmigen, <http://www.bdbiosciences.com/>). Cultured neurospheres were allowed to attach and processed for image analysis at various times after plating. For the slice overlay assays, coronal brain slices (300 μ m thick) from P7 Wistar rats (Charles River, Willmington, MA) forebrain tissue were cut on a Vibratome (Leica VT 1200S), and cultured in NIM medium, on inserts with 30 μ m pores (Cat. No. PICM 03050, Millipore, Billerica, MA, <http://www.millipore.com>) using the interface method [23, 24]. Slices were allowed to recover in the incubator for 3 days before neurospheres were overlaid. Three to four neurospheres were placed in a small volume (10 μ l) under the dissecting microscope below cortex of each hemisphere of the brain slices and cultured in NIM (without FGF-2). After 1–5 days, tissue slices were fixed in 4% paraformaldehyde for 2–3 hours and processed for immunofluorescence.

Immunofluorescence and Microscopy

The neurospheres plated on Matrigel (BD Pharmigen), or overlaid onto slices, were fixed using 4% paraformaldehyde, rinsed with PBS, and incubated with blocking solution containing 0.2% Triton X-100 and 10% normal goat or donkey serum for 1 hour before overnight incubation with following primary antibodies: mouse anti-Nestin human specific (Cat. No. MAB 5326), rabbit anti-Sox2 (Cat. No. AB 5603), rabbit anti-Otx2 (Cat. No. AB 9566), mouse anti-human nuclear antigen (Cat. No. MAB 1281; all from Millipore), rabbit anti-Pax6 (Santa Cruz, Santa Cruz, CA; Cat No. sc-11357, <http://www.scbt.com/>), goat anti-DCX (Santa Cruz, Cat No. sc-8066), mouse anti- β III tubulin (Sigma, Cat No. T8578), mouse anti-vimentin (Sigma, Cat. No. V6630), rabbit anti-GFP (Invitrogen; Cat. No. A11122), mouse anti-glial fibrillary acidic protein (GFAP, sc-52333, Santa Cruz), and followed by appropriate fluorescent-conjugated secondary antibodies (goat anti-mouse or anti-rabbit IgG 488, 568, or 637, donkey anti-goat IgG 588 [all from Invitrogen]). Nuclei were stained with DAPI (Hoechst 33342 stain, 2 μ g/ml; Sigma). For better visualization of cells in brain slices, whole mounts were dehydrated in serial dilutions of ethanol (30%, 50%, 70%, 100%, 15 minutes in each dilution) and cleared in clearing solution (one part of benzyl alcohol and two parts of benzyl benzoate, Cat. No. 108006, B 6630, Sigma), according to published protocol [25]. Tissue was mounted with Prolong-Gold anti-fade reagent (Cat. No. P36930, Invitrogen) and coverslipped prior to imaging.

Image Acquisition and Analysis

Images were acquired using either Leica TCS SP2 confocal laser scanning microscope or Zeiss Axio imager M2 microscope equipped with Apotome. Image stacks were acquired sequentially and maximum projection was performed using Leica Confocal software. An average distance of hNPs from the center of neurosphere, placed either on slice or Matrigel surface was quantified in images taken using Zeiss Axio imager M2 microscope equipped with Apotome, under $\times 10$ magnification and analyzed with Image J 1.45 software (NIH). The distance of migrating cells was analyzed from predetermined center of neurosphere, in four repeats ($n = 4$) of experimental groups (GFP-, DCX-transgenic, or untransfected neurospheres). Within each neurosphere, 50–60 cells from five different fields of view (FOVs) were evaluated.

Statistics

Statistical comparisons were made by using Student's t test for unpaired data. For experiments with more than two groups, ANOVA was performed with Tukey's post hoc. Results are expressed as the mean $\pm$ SEM for n given samples.

Results

Generation of Dorsal Forebrain hNPs

Our goal in this study was to examine whether migration and/ or proliferation of forebrain hNPs could be altered by transgenic expression of human DCX. We derived neuronal progenitor cells from the H9 cell line (passages 38–46), using a protocol previously described to generate mostly forebrain glutamatergic neurons [1, 2]. An outline of the steps and timing for this protocol is shown in Figure 1A. From colonies (Fig. 1B), EBs (Fig. 1C) and neurospheres (Fig. 1D, 1E) were made, as described in Materials and Methods section. Previous studies have shown that in the absence of sonic hedgehog (a ventralizing factor) and retinoic acid (a caudalization factor), hESCs are directed toward dorsal forebrain neuron fates in vitro [2]. To confirm dorsal forebrain fates of hNPs generated in our culture conditions, we performed immunofluorescence analysis by staining rosettes with antibodies against neural stem cell-associated markers, nestin and Sox2, and antibodies against forebrain neuronal transcription factors, Otx2 and Pax6. After 4 days of attachment, at 11 div cells within the rosette core were nestin+/Sox2+, while cells in the periphery of rosettes expressed only nestin (Fig. 1E). Quantification of cells revealed that $65.5\% \pm 8.5\%$ of nestin+ cells in the core were also positive for early neuronal progenitor marker, Sox2. To further investigate identity of cells within rosette core, cells were dissociated and plated on coverslips. Quantification showed that $75.5\% \pm 8.5\%$ of nestin+ cells were positive for the forebrain specific marker, transcription factor, Otx2 at 15 div ($n = 3$) (Fig. 1F). After an additional week in NDM, in presence of growth factors, BDNF and GDNF, $75.5\% \pm 9.4\%$ of cells in culture expressed the dorsal forebrain transcription factor Pax6 (Fig. 1G, $n = 3$). Based on this marker profile, we can conclude that hNPs generated in our culture conditions have identities primarily of dorsal forebrain neuronal progenitors.

DCX Transgenesis Decreases hNP Proliferation

Results from studies using Dcx knockout mice and brain tumor cells indicate that DCX plays a role in cell proliferation [15–17]. Thus, we examined whether increasing DCX expression would alter proliferation of dorsal forebrain hNPs in vitro. In order to produce hNPs which express more DCX, cells from the neurosphere stage were dissociated and transfected with a binary *piggyBac* transposon system, a viral-free method for stable transgene integration [19]. *PiggyBac* transposon system was reported as an excellent vehicle to deliver genetic elements into mammalian cells, due to extremely efficient vector–

chromosome transposition [26]. This transposon system has been used in studies to generate induced pluripotent stem cells [27–29], in gene therapy models [30], in neuronal development studies in *Drosophila* [31], chicken [32], and in rat [33]. Importantly, recently *piggybac* system was used for transgene expression in H9 hESC, and the stability of the *piggyBac*-mediated transgene expression was subsequently evaluated by differentiating transfected clones toward neural lineage [34]. We chose to transfect hNPs rather than pluripotent hESCs to avoid any potential earlier effects of DCX on hESC differentiation or proliferation. Cell suspensions were transfected by nucleofection with pPBCAG-GFP or pPBCAG-DCX-IRES-GFP (donor plasmids), along with pCAG-PBase (transposase expression plasmid, helper plasmid) (Fig. 2A). Because the transposase expression plasmid is an episomal plasmid it is lost with cell divisions leaving the integrated transgene from the donor plasmid in the genome of the previously transfected cells. The transfection efficiency of hNPs using nucleofection method in this study was $56.5\% \pm 7.5\%$ (Fig. 2B), which allowed us to assess both transgenic and nontransgenic cells in the same experiments. As shown in Figure 2, the cells containing pPBCAG-DCX-IRES-GFP expressed both DCX, nestin, and GFP (Fig. 2D), whereas the pPBCAG-GFP-transfected hNPs expressed GFP and nestin, but not DCX (Fig. 2C). After 2 days of transfection cells were detached from poly-D-lysine/laminin-coated coverslips and neurospheres formed in suspension in low-adhesion plates (Fig. 2E). The expression of GFP in neurospheres could be observed even 30 days after transfection with either pPBCAG-GFP or pPBCAG-DCX-IRES-GFP along with pCAG-PBase (data not shown), suggesting the stability of *piggyBac* transgenesis in hNP. To determine the effect of increased DCX expression on proliferation of hNPs, we tested whether EdU incorporation, growth rates, and sizes of transfected neurospheres are altered by DCX. For the EdU assay, neurospheres ($n = 4$), generated from transfected hNPs (Fig. 2E) were plated onto Matrigel, and $10 \mu\text{M}$ EdU was added for the last 12 hours in vitro. Cells were fixed at 12, 24, and 48 hours after plating onto Matrigel. The percentage of EdU+ cells of total GFP+ cells was determined. We observed a significant decrease in the percentage of EdU+ hNPs transfected with pPBCAG-DCX-IRES-GFP (Fig. 2G, 2H), relative to hNPs transfected with pPBCAG-GFP (Fig. 2F, 2H). The percentage of GFP+ cells that were EdU+ was significantly decreased in the pPBCAG-DCX-IRES-GFP-transfected cells for all times tested (12 hours, $37.8\% \pm 5.59\%$ pPBCAG-DCX-IRES-GFP vs. $74.06\% \pm 4.03\%$ pPBCAG-GFP, ***, $p < .005$; 24 hours, $22.6\% \pm 4.27\%$ pPBCAG-DCX-IRES-GFP vs. $60.16\% \pm 6.3\%$ pPBCAG-GFP, ***, $p < .005$; 48 hours, $15.5\% \pm 2.12\%$ pPBCAG-DCX-IRES-GFP vs. $46.65\% \pm 5.6\%$ pPBCAG-GFP, ***, $p < .005$). Interestingly, although only small percentage of DCX transgenic cells fixed at 48 hours after plating on to Matrigel incorporated EdU ($15.5 \pm 2.12\%$), nearly all remained positive for nestin, suggesting that these DCX-transfected hNPs retained some features of neural progenitors.

To further assess the effect of DCX transgenesis on cellular proliferation, we measured the growth rates and sizes of floating neurosphere cultures prepared from transfected hNPs ($n = 3$). The number of primary neurospheres forming after 3, 5, and 7 div was determined. In comparison to pPBCAG-GFP-transfected hNPs (Fig. 3A), the number and size of neurospheres formed by pPBCAG-DCX-IRES-GFP-transfected hNPs (Fig. 3B) was significantly decreased (Fig. 3C). These data are consistent with the decrease in EdU incorporation and together show that DCX induces a decrease in cell proliferation of hNPs.

Increased DCX Expression Promotes hNP Migration out of Neurospheres

To assess the effect of DCX on migration of hNPs, we performed two separate assays: migration across a Matrigel substrate and migration in brain slice cultures. Similar sized hNPs prepared from H9 (Fig. 4A–4D), and CT-2 (Fig. 4E–4G) hESC (diameter approximately $200 \mu\text{m}$, $n = 4$) were plated and the distance from the center, to the edge of

the neurosphere, was measured at the start of each experiment 1, 12, 24, and 48 hours later. Within each neurosphere, 50–60 cells from five different FOVs were evaluated for their migration away from the neurosphere. By 1 hour after plating cells had not yet migrated away from either pPBCAG-GFP (Fig. 4A) or pPBCAG-DCX-IRES-GFP neurospheres (Fig. 4B). In contrast, 12 hours after plating GFP⁺ cells migrated out of neurospheres and onto the Matrigel in both transfection conditions. In GFP transgenic neurospheres, without a DCX transgene, GFP⁺/DCX[−] cells (box 1) migrated for a shorter distances than GFP⁺/DCX⁺ cells (box 2) (Fig. 4D). In the DCX transgenic neurospheres, we observed a significantly larger number of GFP expressing cells migrating out onto the Matrigel (Fig. 4B), and the cells that migrated onto the Matrigel traveled farther than cells from the GFP-transfected neurospheres (Fig. 4C, 4D). The average distance of cells from the center of neurosphere 12 hours after plating was $288.8 \pm 16.6 \mu\text{m}$ for DCX-transfected hNPs and $125.2 \pm 11.5 \mu\text{m}$ for GFP-transfected hNPs (***, $p < 0.005$). DCX⁺/GFP⁺ cells from the GFP-transfected neurospheres migrated significantly shorter distances ($188.2 \pm 20.45 \mu\text{m}$) than the GFP⁺/DCX⁺ cells from hNPs transfected with the DCX expressing transgene ($288.8 \pm 16.6 \mu\text{m}$) (Fig. 4D).

To test whether the same effect of DCX on migration of hNPs could be replicated using another source of hESC, we repeated the same experiment using CT-2 hESC ($n = 2$; Fig. 4E–4G). Importantly, although hNPs migrated at smaller distances 12 hours after plating in both GFP- and DCX-transfected hNPs, as compared with H9-derived hNPs (Fig. 4D), increasing expression of DCX in CT-2-derived hNPs promoted their further migration out of neurospheres (Fig. 4G), showing that the effect we observed is not cell line-specific. Together, these results indicate that endogenous DCX expression is correlated with enhanced migration out of neurospheres, and that increased expression of DCX further increases migration.

Dcx Expression Enhances Migration of hNPs Across Neocortical Brain Slices

To test for migration on neocortical tissue, we used an organotypic interface slice culture method to produce a tissue substrate [23, 24]. The organotypic brain slice culture substrate contains the cell–cell and cell–extracellular matrix interactions that transplanted cells may encounter within tissue. Similar to the Matrigel assay described above, neurospheres of similar sizes ($200 \mu\text{m}$ in diameter, $n = 5$) were selected and placed (in volume of 10–15 μl) just below the neuronal layers of neocortex, proximal to the region near the ventricular surface. Unlike the Matrigel assay where cells migrated significantly within 12 hours out of the neurospheres, in brain slices cells remained largely confined to the neurospheres in both experimental conditions even after 24 hours (Fig. 5B). This indicates that compared to Matrigel, neural tissue is a less permissive substrate than is Matrigel. After 48 hours on brain slices, hNPs migrated away from neurospheres onto slices. Cells from the DCX-transfected neurospheres migrated significantly further than did cells from GFP-transfected neurospheres [Fig. 5C; $130.2 \pm 15.1 \mu\text{m}$ in nontransfected cells, which are also nestin⁺ (Fig. 6C, 6F), $28.4 \pm 13.5 \mu\text{m}$ in GFP-transfected and $180.9 \pm 19.5 \mu\text{m}$ in DCX-transfected cells]. After 5 div the difference in migration between GFP- and DCX-transfected neurospheres was more pronounced. hNPs from neurospheres transfected with DCX migrated $360.97 \pm 36.2 \mu\text{m}$, while those from GFP-transfected neurospheres migrated $188.45 \pm 20 \mu\text{m}$, and similarly, cells from untransfected neurospheres migrated $200.5 \pm 16.6 \mu\text{m}$ (***, $p < .005$). These results show that increased DCX expression significantly increases the migration of hNPs across a neocortical tissue substrate.

Increased Dcx Expression Enhances Formation of a Radial Glial Fiber Network

During development, neocortical progenitors in vivo migrate along cellular scaffolds that are formed by radial glia [35]. The brain slice cultures used here do not contain host tissue radial

glia because slices were prepared from P7 rat forebrain tissue after radial glia had transformed into astrocytes and astrocyte progenitors. Therefore, we hypothesized that the migration through brain slices may be associated with the growth of radial glial fibers originating from the human neurospheres. To detect radial glial fibers of human origin, we used antibodies that specifically recognize human nestin protein and antibodies against vimentin [36, 37] and GFAP [38] that label radial glial fibers in humans. In neurospheres transfected with pPBCAG-GFP or pPB-DCX-IRES-GFP, nestin⁺ cells with short fibers were observed as soon as 12 hours after overlay onto brain slices. Similar length of nestin fibers was observed in both pPBCAG-GFP- and pPB-DCX-IRES-GFP-transfected neurospheres, within the first days after overlay (Fig. 6A, 6D). The same nestin fibers expressed radial glial markers vimentin and GFAP (data not shown). After 5 days of plating neurospheres onto brain slice cultures, nestin⁺ fibers were qualitatively different and were significantly longer in the DCX transgenic condition after 5 days (Fig. 6B, 6E, 6G). In both experimental conditions (after GFP or DCX transfection of neurospheres), GFP⁺ cells were associated along nestin⁺ fibers. In pPBCAG-GFP-transfected neurospheres one to two nestin fibers were typically arranged close to each other but did not show fasciculation (Fig. 6C), whereas in pPBCAG-DCX-IRES-GFP-transfected neurospheres several nestin fibers formed thick fiber bundles (Fig. 6F). In GFP transgenic hNP neurospheres, the length of nestin fibers was typically longer than maximal distance of GFP⁺ neuroblasts (Fig. 6C), while in the DCX transgenic hNP neurospheres GFP⁺ cells were always found at the end of nestin⁺ and vimentin⁺ fibers growing into the host tissue (Fig. 6F). The difference in the length of the nestin fibers invading neocortical tissue between the different transfection conditions emerged from 1 to 5 days after plating. The average length of nestin⁺ fibers emerging from DCX-transfected neurospheres was similar to nontransfected or GFP-transfected neurospheres 24 hours ($120.4 \pm 14.5 \mu\text{m}$ vs. $109.66 \pm 17.07 \mu\text{m}$ vs. $117 \pm 15.58 \mu\text{m}$), and 48 hours ($165.6 \pm 17.5 \mu\text{m}$ vs. $156.66 \pm 17.5 \mu\text{m}$ vs. $188.33 \pm 21.36 \mu\text{m}$) after neurospheres were overlaid onto brain slices (Fig. 6G). However, by 5 days after overlay, nestin⁺ fibers extended from untransfected neurospheres $230.7 \pm 25.5 \mu\text{m}$, from GFP-transfected neurospheres $220.83 \pm 27.64 \mu\text{m}$, and $371.66 \pm 33.1 \mu\text{m}$ from neurospheres transfected with DCX ($p < 0.05$, Fig. 6G). These data show that increased migration into the tissue induced by DCX expression is accompanied by an invasive growth of a cellular scaffold known to be permissive to neuronal migration.

Discussion

hESC-derived neuronal progenitors (hNPs) provide a potential source for cellular replacement following neurodegenerative diseases. One of the greatest challenges for future neuron replacement therapies will be to define conditions which will enable better migration of neuronal progenitors into host brain. Our study demonstrates that increasing DCX expression in hNPs by *piggyBac*-mediated transgenesis alters the developmental trajectory of hNPs, supportive to their migration into host brain tissue. Specifically, increased expression of DCX in hNPs: (a) decreased their proliferation, (b) increased cell migration on Matrigel and across the brain tissue, and (c) promoted the generation and extension of radial glial scaffolds from hNPs.

DCX Expression Decreases the Proliferation of hNPs

Previously, Dcx was reported to be expressed mostly in migrating postmitotic neurons [39, 10], but its role in neurogenesis and proliferation of neuronal progenitor cells was not described until recently. However, recent data suggest that Dcx may play an important role in maintaining a correct balance between neurogenic and stem cell-like mitotic divisions, and that loss of Dcx contributes to depletion of progenitor pool during cortical development [15]. A role of DCX in proliferation and terminal differentiation of brain tumor cells was

also reported [16, 17, 40]. Ectopic expression of DCX in tumor cells inhibited their self-renewal and induced terminal differentiation by interaction with neurabin II [17]. In accordance with these data, our results show that when DCX expression was increased in hNPs, there is a significant decrease in their proliferation, as shown by numbers and sizes of neurospheres and EdU incorporation.

Promoted Migration of hNPs After DCX Expression

There are few studies which demonstrate genetic manipulations that enhance migration of hNPs. Increased expression of glycoprotein M 6A, important for filopodium formation in neuronal cells, had enhanced migration of hESC-derived neurons [41]. Similarly, inhibiting the expression of microRNA-9 (microRNA-9), a negative regulator of migration and proliferation in hNPs, suppressed proliferation and promoted migration of hNPs in a model of stroke [42]. In a nongenetic manipulation, recent data showed that migration of hNPs can be guided by electric fields in vitro [43]. The degree to which these different manipulations share pathways is not immediately apparent; however, future studies that combine these and perhaps other approaches may result in even greater migration through neural tissue. Our study shows that ectopic expression of DCX gene in hNPs significantly improves their motility and is in congruence with previous results that reported that *Dcx* plays an important role in migration of neuronal progenitors in rodents [14, 44, 45]. Furthermore, our data demonstrate that in hNPs, a stable integration of DCX gene by *piggyBac* transposition improves their initial migration into organotypic forebrain cultures. After 5 days of overlay, cells migrated significantly further in DCX- than in GFP-transfected hNPs. Recent studies have shown that in *Dcx* knockout cells a cortical migration is multidirectional with frequent changes of direction in the cortex and branching [15]. Future studies should address the effect of DCX expression on branching and directionality of hNPs. Knockout and RNAi studies in rodents suggest both cell-autonomous and noncell autonomous mechanisms of *Dcx* on neuronal migration [14, 44, 45]. Noncell autonomous effects could be due to the cell interactions between radially migrating cells in the neocortex [44, 46]. Results in our study also show a noncell autonomous action of increased DCX expression in hNP on increased organization of radial glial fibers.

Creating Scaffolds for Migration of hNPs into the Host Cortex

Biodegradable scaffolds have been used in several studies where neurons were transplanted in models of brain trauma. For example, it was shown recently that a Matrigel scaffold promotes survival of transplanted neuronal progenitor cells [47], and that three-dimensional scaffolds of hyaluronic acid, native components of extracellular matrix, could be used to mimic native neuronal tissue and promote survival of transplanted cells [48]. The first indication that transplanted cells are integrated into tissue is their ability to migrate within host tissue. During development, neocortical progenitors in vivo typically migrate along cellular scaffolds that are formed by radial glia [35]. Recently, it was shown that hESC-derived neurons radially migrated when overlaid onto neocortex in embryonic rat brain slice cultures [49]. In contrast to embryonic brain in which radial glial is present in cortex, the brain slice culture substrate used in this study do not contain host radial glia, because slices were prepared from P7 rat forebrain tissue after radial glia had transformed into astrocytes [50, 51]. As radial glia are absent from adult cortex, a self-organizing structure containing both migrating neurons and radial glia may be necessary for full integration and migration into parenchyma of mature cortex. Recent studies which illustrate that maintaining the integrity of the radial glial scaffolds is crucial for neuronal migration [52] are in congruence with this study. Our finding that hNPs are able to form radial glial scaffolds in the host brain could be important for future design of transplantation experiments. Furthermore, results presented here show that DCX promoted formation of dense network radial glia which may enable migration of hNPs into host brain tissue. Namely, nestin+ and vimentin+ fibers, of

human origin, and not transgenic for DCX, were promoted to fasciculate and invade neocortical tissue when growing from neurospheres containing hNPs with increased DCX expression. We hypothesize that enhanced radial glial fiber fasciculation and growth may promote neuronal migration, and in turn, DCX⁺ migrating neurons may promote additional radial glial cell growth. A reciprocal interaction between migrating neurons and radial glial scaffolds has been shown previously for both migrating cerebellar cells [53] and for migrating neocortical neurons [54]. Importantly, our results indicate that given the right conditions, neurospheres are capable of organizing a similar radial glia-neuronal cellular niche that is permissive to enhanced migration into neocortex. Interestingly, DCX effect on bundling of radial fibers was observed only in slice culture overlay assay, and not when hNPs were plated on Matrigel surface, indicating complex interactions with host tissue which need to be explored in the future.

Conclusion

Together, our findings demonstrate that induced DCX expression in hNPs decreased proliferation of hNPs and facilitated their migration on Matrigel substrates and on cortical rat slice cultures. Furthermore, DCX expression induced, in a noncell autonomous manner, an organized network of cell processes which may serve as a cellular scaffold for neuronal migration.

Acknowledgments

This work was supported by grants from Connecticut Stem Cell Initiative to Dr. Joseph LoTurco and to Dr. Radmila Filipovic. Authors thank Fuyi Chen for a help in cloning, and Dr. Anne Booker and Matthew Girgenti for critical reading of the manuscript.

References

1. Zhang SC, Wernig M, Duncan ID, et al. In vitro differentiation of transplantable neural precursors from human embryonic stem cells. *Nat Biotechnol.* 2001; 19:1129–1133. [PubMed: 11731781]
2. Li XJ, Zhang X, Johnson MA, et al. Coordination of sonic hedgehog and Wnt signaling determines ventral and dorsal telencephalic neuron types from human embryonic stem cells. *Development.* 2009; 136:4055–4063. [PubMed: 19906872]
3. Eiraku M, Watanabe K, Matsuo-Takasaki M, et al. Self-organized formation of polarized cortical tissues from ESCs and its active manipulation by extrinsic signals. *Cell Stem cell.* 2008; 3:519–532. [PubMed: 18983967]
4. Tabar V, Panagiotakos G, Greenberg ED, et al. Migration and differentiation of neural precursors derived from human embryonic stem cells in the rat brain. *Nat Biotechnol.* 2005; 23:601–606. [PubMed: 15852001]
5. Rota Nodari L, Ferrari D, Giani F, et al. Long-term survival of human neural stem cells in the ischemic rat brain upon transient immunosuppression. *PLoS One.* 2010; 5:e14035. [PubMed: 21124963]
6. Wernig M, Benninger F, Schmandt T, et al. Functional integration of embryonic stem cell-derived neurons in vivo. *J Neurosci: Off J Soc Neurosci.* 2004; 24:5258–5268.
7. Steinbeck JA, Koch P, Derouiche A, et al. Human embryonic stem cell-derived neurons establish region-specific, long-range projections in the adult brain. *Cell Mol Life Sci.* 2012; 69:461–470. [PubMed: 21779868]
8. Ideguchi M, Palmer TD, Recht LD, et al. Murine embryonic stem cell-derived pyramidal neurons integrate into the cerebral cortex and appropriately project axons to subcortical targets. *J Neurosci: Off J Soc Neurosci.* 2010; 30:894–904.
9. Francis F, Koulakoff A, Boucher D, et al. Doublecortin is a developmentally regulated, microtubule-associated protein expressed in migrating and differentiating neurons. *Neuron.* 1999; 23:247–256. [PubMed: 10399932]

10. Gleeson JG, Lin PT, Flanagan LA, et al. Doublecortin is a microtubule-associated protein and is expressed widely by migrating neurons. *Neuron*. 1999; 23:257–271. [PubMed: 10399933]
11. Friocourt G, Liu JS, Antypa M, et al. Both doublecortin and double-cortin-like kinase play a role in cortical interneuron migration. *J Neurosci: Off J Soc Neurosci*. 2007; 27:3875–3883.
12. Lois C, Alvarez-Buylla A. Long-distance neuronal migration in the adult mammalian brain. *Science*. 1994; 264:1145–1148. [PubMed: 8178174]
13. Luskin MB. Restricted proliferation and migration of postnatally generated neurons derived from the forebrain subventricular zone. *Neuron*. 1993; 11:173–189. [PubMed: 8338665]
14. Ocbina PJ, Dizon ML, Shin L, et al. Doublecortin is necessary for the migration of adult subventricular zone cells from neurospheres. *Mol Cell Neurosci*. 2006; 33:126–135. [PubMed: 16931042]
15. Pramparo T, Youn YH, Yingling J, et al. Novel embryonic neuronal migration and proliferation defects in Dcx mutant mice are exacerbated by Lis1 reduction. *J Neurosci: Off J Soc Neurosci*. 2010; 30:3002–3012.
16. Santra M, Zheng X, Roberts C, et al. Single doublecortin gene therapy significantly reduces glioma tumor volume. *J Neurosci Res*. 2010; 88:304–314. [PubMed: 19681167]
17. Santra M, Santra S, Buller B, et al. Effect of doublecortin on self-renewal and differentiation in brain tumor stem cells. *Cancer Sci*. 2011; 102:1350–1357. [PubMed: 21477071]
18. Amit M, Carpenter MK, Inokuma MS, et al. Clonally derived human embryonic stem cell lines maintain pluripotency and proliferative potential for prolonged periods of culture. *Dev Biol*. 2000; 227:271–278. [PubMed: 11071754]
19. Fischer SE, Wienholds E, Plasterk RH. Regulated transposition of a fish transposon in the mouse germ line. *Proc Natl Acad Sci USA*. 2001; 98:6759–6764. [PubMed: 11381141]
20. Wu A, Oh S, Ericson K, et al. Transposon-based interferon gamma gene transfer overcomes limitations of episomal plasmid for immunogene therapy of glioblastoma. *Cancer Gene Therapy*. 2007; 14:550–560. [PubMed: 17415381]
21. Matsuda T, Cepko CL. Electroporation and RNA interference in the rodent retina in vivo and in vitro. *Proc Natl Acad Sci USA*. 2004; 101:16–22. [PubMed: 14603031]
22. Matsuda T, Cepko CL. Controlled expression of transgenes introduced by in vivo electroporation. *Proc Natl Acad Sci USA*. 2007; 104:1027–1032. [PubMed: 17209010]
23. Stoppini L, Buchs PA, Muller D. A simple method for organotypic cultures of nervous tissue. *J Neurosci Methods*. 1991; 37:173–182. [PubMed: 1715499]
24. Filipovic R, Zecevic N. The effect of CXCL1 on human fetal oligodendrocyte progenitor cells. *Glia*. 2008; 56:1–15. [PubMed: 17910053]
25. Dodt HU, Leischner U, Schierloh A, et al. Ultramicroscopy: Three-dimensional visualization of neuronal networks in the whole mouse brain. *Nat Methods*. 2007; 4:331–336. [PubMed: 17384643]
26. Wang W, Lin CY, Lu D, et al. Chromosomal transposition of Piggy-Bac in mouse embryonic stem cells. *Proc Natl Acad Sci USA*. 2008; 105:9290–9295. [PubMed: 18579772]
27. Lacoste A, Berenshteyn F, Brivanlou AH. An efficient and reversible transposable system for gene delivery and lineage-specific differentiation in human embryonic stem cells. *Cell Stem cell*. 2009; 5:332–342. [PubMed: 19733544]
28. Woltjen K, Michael IP, Mohseni P, et al. piggyBac transposition reprograms fibroblasts to induced pluripotent stem cells. *Nature*. 2009; 458:766–770. [PubMed: 19252478]
29. Yusa K, Rad R, Takeda J, et al. Generation of transgene-free induced pluripotent mouse stem cells by the piggyBac transposon. *Nat Methods*. 2009; 6:363–369. [PubMed: 19337237]
30. Wilson MH, Coates CJ, George AL Jr. PiggyBac transposon-mediated gene transfer in human cells. *Mol Ther: J Am Soc Gene Ther*. 2007; 15:139–145.
31. Schuldiner O, Berdnik D, Levy JM, et al. piggyBac-based mosaic screen identifies a postmitotic function for cohesin in regulating developmental axon pruning. *Dev Cell*. 2008; 14:227–238. [PubMed: 18267091]
32. Lu Y, Lin C, Wang X. PiggyBac transgenic strategies in the developing chicken spinal cord. *Nucleic Acids Res*. 2009; 37:e141. [PubMed: 19755504]

33. Chen F, Loturco J. A method for stable transgenesis of radial glia lineage in rat neocortex by piggyBac mediated transposition. *J Neurosci Methods*. 2012; 207:172–180. [PubMed: 22521325]
34. Chen YT, Furushima K, Hou PS, et al. PiggyBac transposon-mediated, reversible gene transfer in human embryonic stem cells. *Stem Cells Dev*. 2010; 19:763–771. [PubMed: 19740021]
35. Levitt P, Rakic P. Immunoperoxidase localization of glial fibrillary acidic protein in radial glial cells and astrocytes of the developing rhesus monkey brain. *J Comp Neurol*. 1980; 193:815–840. [PubMed: 7002963]
36. Pixley SK, de Vellis J. Transition between immature radial glia and mature astrocytes studied with a monoclonal antibody to vimentin. *Brain Res*. 1984; 317:201–209. [PubMed: 6383523]
37. Hartfuss E, Galli R, Heins N, et al. Characterization of CNS precursor subtypes and radial glia. *Dev Biol*. 2001; 229:15–30. [PubMed: 11133151]
38. Zecevic N. Specific characteristic of radial glia in the human fetal tel-encephalon. *Glia*. 2004; 48:27–35. [PubMed: 15326612]
39. des Portes V, Pinard JM, Billuart P, et al. A novel CNS gene required for neuronal migration and involved in X-linked subcortical laminar heterotopia and lissencephaly syndrome. *Cell*. 1998; 92:51–61. [PubMed: 9489699]
40. Santra M, Santra S, Roberts C, et al. Doublecortin induces mitotic microtubule catastrophe and inhibits glioma cell invasion. *J Neurochem*. 2009; 108:231–245. [PubMed: 19094064]
41. Michibata H, Okuno T, Konishi N, et al. Human GPM6A is associated with differentiation and neuronal migration of neurons derived from human embryonic stem cells. *Stem Cells Dev*. 2009; 18:629–639. [PubMed: 19298174]
42. Delaloy C, Liu L, Lee JA, et al. MicroRNA-9 coordinates proliferation and migration of human embryonic stem cell-derived neural progenitors. *Cell Stem cell*. 2010; 6:323–335. [PubMed: 20362537]
43. Feng JF, Liu J, Zhang XZ, et al. Guided migration of neural stem cells derived from human embryonic stem cells by an electric field. *Stem Cells*. 2012; 30:349–355. [PubMed: 22076946]
44. Bai J, Ramos RL, Ackman JB, et al. RNAi reveals doublecortin is required for radial migration in rat neocortex. *Nat Neurosci*. 2003; 6:1277–1283. [PubMed: 14625554]
45. Kerjan G, Gleeson JG. Genetic mechanisms underlying abnormal neuronal migration in classical lissencephaly. *Trends Genet*. 2007; 23:623–630. [PubMed: 17997185]
46. Ramos RL, Bai J, LoTurco JJ. Heterotopia formation in rat but not mouse neocortex after RNA interference knockdown of DCX. *Cereb Cortex*. 2006; 16:1323–1331. [PubMed: 16292002]
47. Jin K, Mao X, Xie L, et al. Transplantation of human neural precursor cells in Matrigel scaffolding improves outcome from focal cerebral ischemia after delayed postischemic treatment in rats. *J Cereb Blood Flow Metab: Off J Int Soc Cereb Blood Flow Metab*. 2010; 30:534–544.
48. Suri S, Han LH, Zhang W, et al. Solid freeform fabrication of designer scaffolds of hyaluronic acid for nerve tissue engineering. *Biomed Microdevices*. 2011; 13:983–993. [PubMed: 21773726]
49. Shi Y, Kirwan P, Smith J, et al. Human cerebral cortex development from pluripotent stem cells to functional excitatory synapses. *Nat Neurosci*. 2012; 15:477–486. [PubMed: 22306606]
50. Cameron RS, Rakic P. Glial cell lineage in the cerebral cortex: a review and synthesis. *Glia*. 1991; 4:124–137. [PubMed: 1827774]
51. Tramontin AD, Garcia-Verdugo JM, Lim DA, et al. Postnatal development of radial glia and the ventricular zone (VZ): A continuum of the neural stem cell compartment. *Cereb Cortex*. 2003; 13:580–587. [PubMed: 12764031]
52. Manent JB, Beguin S, Ganay T, et al. Cell-autonomous and cell-to-cell signalling events in normal and altered neuronal migration. *Eur J Neurosci*. 2011; 34:1595–1608. [PubMed: 22103417]
53. Edmondson JC, Hatten ME. Glial-guided granule neuron migration in vitro: a high-resolution time-lapse video microscopic study. *J Neurosci: Off J Soc Neurosci*. 1987; 7:1928–1934.
54. Anton ES, Cameron RS, Rakic P. Role of neuroglial junctional domain proteins in the maintenance and termination of neuronal migration across the embryonic cerebral wall. *J Neurosci: Off J Soc Neurosci*. 1996; 16:2283–2293.

Figure 1.

Generation of human forebrain neurons from hESC. **(A)**: Timeline and media used for differentiation of hESC to human neuroprogenitors. **(B–G)**: Stages of differentiation of hESC at given time points. **(B)**: Phase contrast image of H9 hESC grown on irradiated mouse fibroblasts. **(C)**: Phase contrast image of EBs made from hESC colonies. **(D)**: When grown in NIM and attached to laminin/poly-D lysine-coated plates, EBs flatten and form neurorosettes. A distinct core of neurorosette is selected for further enrichment of central nervous system neuronal progenitors (encircled). **(E)**: Neurorosettes stained with nestin (in red) and Sox2 (in green) antibodies. **(F)**: Nestin⁺ neuronal progenitors (in green) express forebrain marker, Otx2 (in red). **(G)**: When cells are differentiated in NDM, a majority of cells express neuronal markers β III tubulin (in green) and Pax6 (in red). Scale bars = 100 μ m. Abbreviations: DAPI, 4',6-diamidino-2 phnylindole; EB, embryoid body; hESC, human embryonic stem cell; NDM, neuronal differentiation medium; NIM, neuronal induction medium;

Figure 2.

Decreased proliferation is observed after DCX transfection in human neuroprogenitor (hNP). **(A)**: Plasmids used in experiments. hNPs are transfected either with pPBCAG-DCX-IRES-GFP or pPBCAG-GFP, along with pCAG-PBase. **(B)**: Phase contrast (left) and fluorescence image (right) of hNPs transfected with pPBCAG-GFP. **(C)**: After 2 days of transfection, all hNPs transfected with GFP (left, in green, arrows) express nestin (right, in blue, arrows), while cells are not DCX positive (in red). **(D)**: hNPs transfected with pPBCAG-DCX-IRES-GFP are colabeled with GFP (left, in green, arrows) and nestin antibodies (right, in blue, arrows). Upregulation of DCX expression is noted in transfected cells (in red, right, arrows). **(E)**: Phase contrast (left) and fluorescence image (right) of neurospheres 2 days after pPBCAG-GFP transfection. **(F)**: After 12 hours of EdU pulse, majority of nestin/GFP+ cells within GFP transgenic neurospheres incorporate EdU (in blue). **(G)**: After 12 hours of EdU pulse, majority of migrating cells within DCX-transfected neurospheres does not incorporate EdU. **(H)**: Graph shows quantification of EdU+/GFP+ cells of total GFP+ cells. 10 μ M EdU was applied for last 12 hours of time points indicated at y-axis. Significantly smaller percentage of cells within DCX transgenic neurospheres proliferate, as compared to GFP transgenic neurospheres at all time points. Asterisks indicate statistical significance with respect to the value of control cells. ***, $p < .005$; $n = 4$. Scale bars = 100 μ m. Abbreviations: DCX, Doublecortin; GFP, green fluorescent protein; EdU, 5-ethynyl-2'-deoxyuridine; IRES, internal ribosome entry site; TRs, terminal repeats.

Figure 3.

The effect of DCX expression on neurospheres formation. Bright-field (left) and fluorescence images (right) of pPBCAG-GFP-(A) or pPBCAG-DCX-IRES-GFP (B)-transfected neurospheres 5 days after transfection. (C): Graph above represents quantification of the number of neurospheres in both conditions 3, 5, and 7 days after transfection. Human neuroprogenitor (hNP) transfected with pPBCAG-DCX-IRES-GFP form significantly fewer neurospheres than hNP transfected with pPBCAG-GFP. Similarly, the average size of neurospheres (graph below) is smaller after pPBCAG-DCX-IRES-GFP transfection at 3, 5, 7 days after transfection. $n = 3$, Asterisk indicates statistical significance with respect to the value of control cells. ***, $p < .005$. Scale bars = 100 μm . Abbreviations: DCX, Doublecortin; GFP, green fluorescent protein.

Figure 4.

Migration of human neuroprogenitor (hNP) from neurospheres on Matrigel surface. **(A–D):** H9 hNP. **(E–G):** CT-2 hNP. **(A)** Neurospheres are of similar size in both conditions (pPBCAG-GFP-PB, **A**, **E**, left) and pPBCAG-DCX-IRES-GFP (**B**, **F**, left) when plated at Matrigel at the beginning of experiment. After 12 hours while many hNPs transfected with pPBCAG-GFP-PB are still found within the core of neurospheres (**A**, **E**, right), neurospheres transfected with pPBCAG-DCX-IRES-GFP cells migrated further away from the center of neurospheres (**B**, **F**, right). **(C):** Graph shows an average distance of GFP+ hNPs in neurospheres transfected with either pPBCAG-DCX-IRES-GFP or pCAG-GFP-PB. **(D):** An average distance of hNPs 12 hours after plating on Matrigel is shown in graph. Distance of both DCX+/GFP– and GFP+ cells after pPBCAG-GFP is significantly smaller when compared to distance of GFP+ cells after pPBCAG-DCX-IRES-GFP transfection. In each experiment ($n = 4$), a migration is assessed in three neurospheres. Asterisk indicates statistical significance with respect to the value of control cells. ***, $p < .005$. **(G):** An average distance of CT-2 derived hNPs was determined at 12 hours after plating neurospheres on Matrigel. In each experiment ($n = 2$), a migration was assessed in three neurospheres. Scale bars = 150 μm . Abbreviations: DAPI, 4',6-diamidino-2-phenylindole; DCX, Doublecortin; GFP, green fluorescent protein; IRES, internal ribosome entry site.

Figure 5.

Migration of human neuroprogenitor (hNP) in organotypic slice cultures of rat brain. **(A):** Schematic diagram of experiment. Neurospheres are transfected either with pPBCAG-GFP or pPBCAG-DCX-IRES-GFP and placed under dissecting microscope below cortex in P7 rat brain slices. Orientation of images is indicated on the top. **(B):** Images of the left show that 24 hours after slice overlay, migration of hNPs is similar between pPBCAG-GFP and pPBCAG-DCX-IRES-GFP conditions. However, 5 days after slice overlay (right), many cells are positioned at long distances away from center of neurosphere. **(C):** Quantification of migration 24 hours, 48 hours, and 5 days after neurosphere overlay. Asterisk indicates statistical significance with respect to the value of control cells. $n = 4$, ***, $p < 0.005$. Scale bars = 500 μm . Abbreviations: Cx, cortex; DCX, Doublecortin; GFP, green fluorescent protein; lv, lateral ventricle; WM, white matter.

Figure 6.

Human embryonic stem cell-derived neuronal progenitor migrate along nestin/vimentin+ fibers. (A–C): Neurospheres transfected with pPBCAG-GFP stained with GFP (in green) and nestin antibodies (in red). (A) Neurospheres transfected with pPBCAG-GFP 12 hours after overlay. (B) 5 days after overlay, long nestin fibers extend from neurospheres. (C): GFP+ human neuroprogenitors (hNPs) are found along nestin fibers, which are extending further. (D–F): Neurospheres transfected with pPBCAG-DCX-IRES-GFP stained with GFP (in green) and nestin antibodies (in red). (D) 12 hours after overlay. (E, F) After 5 days of overlay, hNPs transfected with pPBCAG-DCX-IRES-GFP are always found at the end of nestin fibers. (G) Graph shows average length of nestin fibers 12 hours, 24 hours, and 5 days after overlay in untransfected, pPBCAG-GFP, or pPBCAG-DCX-IRES-GFP-transfected neurospheres. Scale bars = 100 μm. Abbreviations: GFP, green fluorescent protein; DCX, Double-cortin; IRES, internal ribosome entry site.