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A PROTOCOL FOR THE DIFFERENTIATION OF HUMAN EMBRYONIC STEM CELLS INTO DOPAMINERGIC NEURONS USING ONLY CHEMICALLY DEFINED HUMAN ADDITIVES: STUDIES IN VITRO AND IN VIVO

Lorraine Iacovitti*, **Angela E. Donaldson**, **Cheryl E. Marshall**, **Sokreine Suon**, and **Ming Yang**
Farber Institute for Neurosciences, Department of Neurology, Thomas Jefferson University Medical College, 900 Walnut Street, Philadelphia, PA. 19107

Abstract

Our ability to use human embryonic stem (hES) cells in cell replacement therapy for Parkinson's disease depends on the discovery of ways to simply and reliably differentiate a dopaminergic (DA) phenotype in these cells. Although several protocols exist for the differentiation of DA traits in hES, they involve the prolonged use of complex media with undefined components, cell conditioned media and/or co-culture with various cells, usually of animal origin. In this study, several well-characterized (H9, BG01) and several new uncharacterized (HUES7, HUES8) hES cell lines were studied for their capacity to differentiate into DA neurons in culture using a novel rapid protocol which uses only chemically-defined human-derived media additives and substrata. Within 3 weeks, cells from all 4 cell lines progressed from the undifferentiated state to β-tubulin III positive cells expressing DA markers in vitro. Moreover, transplantation of these cells into the striata of 6-hydroxydopamine-treated rats at the neuronal progenitor stage resulted in the appearance of differentiated DA traits in vivo 2–3 weeks later.

Keywords

human; embryonic stem cells; dopamine; differentiation; tissue culture; transplantation

Introduction

Because of their self-renewal capacity and pluripotentiality, human embryonic stem (hES) cells are thought to hold enormous promise as potential replacement tissue in neurodegenerative diseases such as Parkinson's (PD). Fulfilling that promise, however, requires the identification of a reliable source of hES cells and a workable strategy for differentiating cells into dopaminergic (DA) neurons capable of surviving and functioning after transplantation into the brain. Currently, there are a number of established hES lines available from NIH-approved sources (Wicell, Bresagen) and a number of new lines provided by the Howard Hughes Institute of Harvard University. All of these cell lines were originally propagated on mouse feeder layers,

*Correspondence to: Lorraine Iacovitti, Ph.D., Associate Director, Farber Institute for Neurosciences, Thomas Jefferson University Medical College, Suite 462-Jefferson Hospital for Neuroscience, 900 Walnut Street, Philadelphia, PA. 19107, Phone: 215-955-8118, Fax: 215-955-2993, e-mail; lorraine.iacovitti@jefferson.edu

although several have recently been transferred to feeder free support systems (1,3,7,9,12, 21). While the differentiation of DA traits in some hES lines has been described previously (4,5,16,17,18,26), with the exception of one study (19) these protocols involve the prolonged use of complex media containing serum or other undefined reagents and/or cell conditioned media (CM) or co-culture with these cells (ie. PA6 mouse stromal cells) (4,5,10,13,16,17,18, 22,26). Since animal cells contain immunogenic antigens that can be incorporated into hES cells (14), they ultimately can cause immune rejection after their transplantation into the brain. Even in cases where animal sera, CM and cells have been eliminated from the hES differentiation protocol (9,10,13,23), media additives (B27, Matrigel, etc.) have been used which contain undefined, often proprietary, components as well as hormones and growth products of animal origin.

Another potentially complicating feature of most current DA differentiation protocols is the prolonged time course required for cells to adequately develop expression of DA traits in culture. During this period, whether cells are attached to a substrate or differentiated in suspension culture, they can elicit an extensive highly branched network of processes that make harvest untenable without irreparable mechanical damage to cells. Ultimately, few, if any, of these cells survive transplantation into the brain (19,24).

In this study, we compared hES cells from different sources for their capacity to differentiate into DA neurons in culture using a 3-week, four-stage protocol of our own devising which employs serum free media with chemically-defined human-derived media additives and substrates. In addition, we harvested cells at early stages in the protocol with minimal cellular damage and assessed their ability to continue the DA differentiation process in vivo following engraftment.

Propagation of ES lines

A timeline and flow chart of the protocol used here for the propagation and differentiation of hES cells is shown in Fig. 1. H9 cells (passage 22/23 – 46 XX karyotype) were purchased from Wicell (University of Wisconsin, Madison, WI) and maintained according to the supplier's instructions. Briefly, cells were grown on a monolayer of primary mouse fibroblasts (MEFs) (Specialty Media) in DMEM/F12 media (Gibco #11330) supplemented with 1% Non-Essential Amino Acids (NEAA) (Gibco #11140), 1mM l-glutamine (Gibco #25030), 0.1mM 2-mercaptoethanol, and 4ng/ml bFGF (BD Biosciences #356060) and 20% Knockout Serum Replacer™ (KOSR; Gibco #10828). KOSR contains bovine serum albumin and porcine transferrin; neither we nor Gibco (personal communication) have succeeded in keeping cells alive after replacing these reagents with the human-derived substances. Continual propagation of cells required colonies to be manually lifted, dissected into multiple small pieces and transferred onto fresh MEF feeders once per week. BG01 cells (passage 24 – 46 XX karyotype) were purchased from Bresagen (Athens, GA) and essentially were carried as above in DMEM/F12, supplemented with 20% KOSR, 2mM l-glutamine, 0.1mM 2-mercaptoethanol, 1% NEAA, 50U/ml penicillin, 50 ug/ml streptomycin, and 4ng/ml bFGF. For H9 and BG01 lines, there was a very low success rate (3 of 12 attempts) establishing undifferentiated colonies from thawed cell aliquots. HUES7 (passage 11 – 46 XY karyotype) and HUES8 (passage 21 – 46 XY karyotype) cells were provided through the generosity of Dr. Douglas Melton (Howard Hughes Institute, Harvard University, Boston, MA). Undifferentiated cells were readily established from thawed aliquots and were maintained on a layer of MEFs in Knockout DMEM™ (Gibco #10829) supplemented with 10% KOSR, 10% human Plasmanate (Bayer #NDC0026-0613-20), 50U/ml Penicillin, 50ug/ml Streptomycin, 1mM Glutamax™ (Gibco #35050), 55uM 2-mercaptoethanol, 12ng/ml human LIF (Chemicon #LIF1010), and 10ng/ml bFGF (Becton Dickinson #356060). Colonies were passaged once per week using 0.05% trypsin, EDTA in HBSS-CMF (Gibco #25300) at room temperature until the MEF layer

retracted and edges of colonies began to pull up under the microscope (about 1 min). As expected, RT-PCR analysis of HUES8 (Fig. 2A; PCR primers and methods as described in legend) and H9 (data not shown) cells revealed the presence in Stage I undifferentiated cells of the pluripotent cell markers Oct-3/4 and Sox-2, as well as fucosyltransferase 4 (FUT4) which catalyzes synthesis of the sialyl Lewis X determinant (SSEA) during early human embryogenesis (6,15).

Directed Differentiation—In order to initiate the differentiation process in all cell lines, colonies were manually lifted with a pulled Pasteur pipette or after gentle dissociation with collagenase (1mg/ml in DMEM/F12) and grown in suspension on uncoated plastic dishes in the cell line-specific media described above where they formed embryoid bodies (EBs). Media was changed on day 2 of this 3-day stage. Although all lines formed EBs in stage II, H9 cells did so more readily than BG01 cells or HUES cells which grew in flattened monolayers. By the end of Stage II, RT-PCR analysis revealed a decline in stem cell markers (Fig. 2A) as well as markers of all three germ layers (Fig. 2B) and the first appearance of the neural progenitor marker nestin (Fig. 2A).

EBs were then seeded onto collagen-coated tissue culture flasks (100ng/cm^2 human Collagen IV, BD Biosciences #354245) in defined media (DM). DM consisted of DMEM/F12 (Gibco #11320-033), 100U/ml penicillin, 100ug/ml streptomycin, 1.5mM L-glutamine, 6mg/ml glucose and 1% ITS Premix (BD Biosciences #354350), containing human recombinant insulin, human transferrin, and selenous acid, all at 5g/ml. While published protocols have utilized gelatin-coated dishes at this stage, we found that relatively few (5%) EBs actually attached to the dish, thus limiting the number neural progenitor rosettes at later times. If instead we substituted type IV human collagen, we could greatly increase this number such that 95% of EBs were adherent. To induce formation of neural progenitor rosettes (1,4,9) in stage III, we further supplemented DM with 5ug/ml human plasma fibronectin (Sigma #F2006) and 200ng/ml recombinant human noggin (RDI/Fitzgerald Industries #RDI1210). With developmental progression in stage III, endodermal (α-fetoprotein [αFP], HNF3-α) mesodermal (GATA-2, Mix-1) and epidermal (keratin-5) markers disappeared by RT-PCR (Fig. 2B) while expression of the neural marker β-tub III was first detected (Fig. 2A) although at levels too low to be detected immunocytochemically (data not shown).

During stage IV, rosettes were expanded in DM supplemented with 20ng/ml recombinant human bFGF (BD Biosciences), fed fresh media every 2–3 days and passaged every 7–10 days. Larger rosettes were fragmented using a P100 pipette. Although no enzymes were used in passaging during this stage, after one week, rosettes were gently lifted with dispase (0.5mg/ml in DMEM/F12 basal media) and seeded onto uncoated tissue culture dishes. At Stage IV, stem cell markers seen in stage I (Fig. 3A inset) were no longer evident either by RT-PCR (Fig. 2) or after immunocytochemical staining for SSEA-4 or Oct-4 (Fig. 3A,B) (for methods see Fig. 3 legend). Instead, many cells expressed the neural progenitor marker nestin (Fig. 3A–C) and the proliferation antigen ki67 (Fig. 3C), indicating that they were still capable of cell division. In addition, cells at this stage also first began to express markers of a DA phenotype. Thus, the DA–related transcription factors ptx-3 and Nurr-1 were seen in many cells which co-labeled for a human nuclear antigen (Fig. 2, 3D–F). More mature markers of a DA phenotype, such as the DA biosynthetic enzymes, tyrosine hydroxylase (TH) and aromatic L-amino acid decarboxylase (AADC) (data not shown) the midbrain-associated G-protein-regulated inwardly rectifying potassium channel (GIRK2) (Fig. 3F), however, were not spontaneously expressed at this stage although the dopamine transporter (DAT) could be detected by RT-PCR (Fig. 2). At this point, stage IV cells could be carried for 2–3 weeks as suspended spheres before further differentiation in culture or transplantation into the brain.

Additional DA differentiation

Further differentiation of DA traits in Stage V was achieved by incubating cells seeded onto poly-ornithine coated slides for an additional 1–7 days in DM supplemented with 1mM dibutyryl cAMP (dbcAMP) as described in earlier studies (20,23,24). Following this treatment, no cells expressed stem cell markers like Oct-4 (Fig. 2; Fig. 4A). Although some hES cells remained in the progenitor nestin-positive stage (Fig. 4A, B), there was no evidence of cell division in this population as there had been at Stage IV; all Stage V cells were ki67 negative (Fig. 4B). In fact, for all four hES lines examined, the majority of hES cells were β-tubIII$^+$ neuronal cells (Fig. 4E–H), many of which robustly expressed the DA transcription factor Nurr1 (Fig. 2; Fig. 4C) and the DA biosynthetic enzyme TH (Fig. 2; Fig. 4D–H). However, as shown in a low power view (Fig. 4D; 4X), the percentage of TH$^+$ cells varied from one colony to another and one plating to another. Some colonies were comprised nearly entirely of TH$^+$ neurons (Fig. 4E) while others were devoid of TH$^+$ neurons. Often TH and other DA markers appeared in cell clusters within colonies. It is unclear whether this was due to clonal expansion of a single DA-specified progenitor cell giving rise to a cluster of DA neurons or because of local differentiation factors signaling nearby cells. In order to assess the efficiency of DA differentiation across the four cell lines, all colonies were counted in all 20X microscopic fields on the dish and the number which contained TH$^+$ β-tubIII$^+$ neurons were expressed as a percentage of the total number of colonies on the dish. We found that all four lines produced a comparable degree of TH differentiation: H9:60% + 9; HUES7:78% + 12; HUES8:81% + 14; and BG01:56% + 17 (N = 4–7 platings; not significantly different by ANOVA; Statview software). Importantly, TH expression was maintained in cells 5 days after removal of dbcAMP from the media, suggesting that cells had permanently adopted a DA phenotype. In addition, many other known DA-related markers, including Ptx-3, Lmx-1b, AADC, GIRK2 and DAT (Fig. 2) were also expressed in the differentiated cells. When stage V media was supplemented with other growth substances of importance in DA development (FGF1, FGF2, GDNF), no further increase in the number of DA cells over dbcAMP-induced levels was observed (data not shown).

Transplantation of hES cells into the 6-OHDA rat striatum

Although many hES expressed traits of a terminally differentiated DA phenotype by stage V in culture, these cells were in fact so mature that they could not be successfully harvested from the culture dish and survive transplantation into the brain. Consequently, fewer that 2% of Stage V TH$^+$ cells survived a second re-plating in culture (6422 + 63 TH$^+$ cells before re-plating versus 32 + 6 TH$^+$ cells after re-plating; N = 3). Therefore, to learn whether the DA differentiation process could be initiated in culture with the current protocol but continued in situ in the brain, hES cells from H9 (N=3), HUES7 (N=2) and HUES8 (N=5) lines were harvested at the neural progenitor stage (Stage IV) for transplant. Approximately 2–5 × 10^5 cells were stereotaxically implanted into the striata rats with unilateral 6-OHDA lesions as described previously (24,25). Upon sacrifice 2–3 weeks later, robust cell survival was seen in 7 of the 10 engrafted animals (no viable hES cells were found in one H9 and two HUES8 grafts). In all viable cases, HNA$^+$ hES-derived nestin$^+$ progenitor cells were present in abundance in the host rat brain regardless of the cell line (Fig. 5A inset). A number of these progenitor cells continued to divide as indicated by the appearance of the nuclear proliferation antigen Ki67 (Fig. 5A) but did not revert to expressing earlier stem cell markers, such as Oct-4 or SSEA-4 (data not shown). When the grafts were stained for cell-type specific markers of differentiation, many engrafted HNA$^+$ cells expressed markers of differentiated neurons like β-tubIII (Fig. 5B) and MAP2 (data not shown). Importantly, no HNA$^+$ cells co-stained for the glial marker glial fibrillary acidic protein (GFAP); instead all GFAP$^+$ cells were of host origin (Fig. 5C). Remarkably, many of these neurons continued the DA differentiation process begun in vitro, expressing the DA biosynthetic enzymes TH (Fig. 5D, E) and to a lesser extent, AADC (Fig. 5F) but not other neurotransmitter markers (5-HT, ChAT, DBH; N=3) (data not shown).

Discussion

The present study establishes that it is indeed possible to differentiate a significant number of DA neurons from several different hES cell lines, including the previously unstudied HUES7and 8 lines, both in vitro and in vivo, using a protocol of defined human-derived reagents in a simple serum-free media. According to this procedure, hES cells will differentiate into β-tubIII^{+} neurons, many of which express DA traits such as TH, AADC, Ptx-3, Lmx-1b, Nurr-1 and DAT after only three weeks in culture with several chemically-defined media additives and substrates, all of human derivation. Alternatively, cells harvested from culture at earlier stages in the protocol can continue the DA differentiation process in vivo, expressing differentiated DA traits within several weeks of transplantation into the brain.

This protocol differs from previously published procedures in a number of important ways. In earlier studies, DA differentiation of hES cells required incubation in culture with other cell types (PA6 mouse stromal line; human HepG2 liver line) or in cell-CM, usually containing fetal calf serum and/or other undefined components (4,5,16,17,18,22,26). In several recent studies, efforts have been made to move to a serum-free defined protocol for neuronal (9,10, 13) or DA differentiation (19,22) of hES cells. With only one exception (19), however, media was supplemented with serum substitutes, such as B27, and often cells were grown on Matrigel, both of which contain undefined proprietary components and growth substances of animal origin. Only in the case where hES cells were grown as spheres in suspension was a simple unsupplemented serum-free media sufficient to support the differentiation of DA neurons (19), possibly due to the local production of necessary factors in the spheres. However, DA neurons differentiated in this manner did not survive in adherent culture and only rarely survived engraftment in vivo (19). Importantly, neural progenitor cells, also present in these spheres, did not go on to develop into DA neurons after transplantation. The current protocol likewise employs a relatively simple serum-free media but maintains cells for the most part on adherent substrates. Unlike hES suspension cultures, these attached cells did not develop beyond the neural progenitor stage to produce DA neurons until further differentiation with dbcAMP in culture or transplantation in vivo where some cells carried on the DA differentiation process. Interestingly, mouse ES-derived progenitors sharing similar DA potentiality in the graft have also recently been reported (8,11). Whether these cells represent those which express DA fate determinant genes (2) and are normally committed to a DA fate in the ventral midbrain remains to be determined. Regardless, the findings of this study, unlike those of previous hES studies, establish that a simple media containing several defined human additives can foster the DA developmental potential of hES-derived progenitor cells. Studies to determine the long-term survival and function of these transplanted hES-derived progenitors cells are still ongoing.

It will be important to develop feeder-free support systems not only for the propagation but also the initial establishment of undifferentiated ES cells so that exposure of human cells to mouse antigens can be entirely eliminated. Although a number of feeder-free systems have been described (1,3,7,9,10,12,13,21), all hES lines to date have been established and repeatedly passaged on mouse feeders prior to transfer to a feeder free-system for cell propagation. Thus far, the feeder free protocols which we have tried (3,21), have not been compatible with the DA differentiation of hES cell lines developed in this protocol. Finally, it is essential that the bovine serum albumin and porcine transferrin found in KOSR basal media be replaced with human products that can support hES development; currently our efforts (unpublished findings) and those of Gibco (personal communication) in this regard have not been successful. Advances such as these however are critical for the translation of these technologies from the bench to the clinical treatment of patients with diseases such as Parkinson's.

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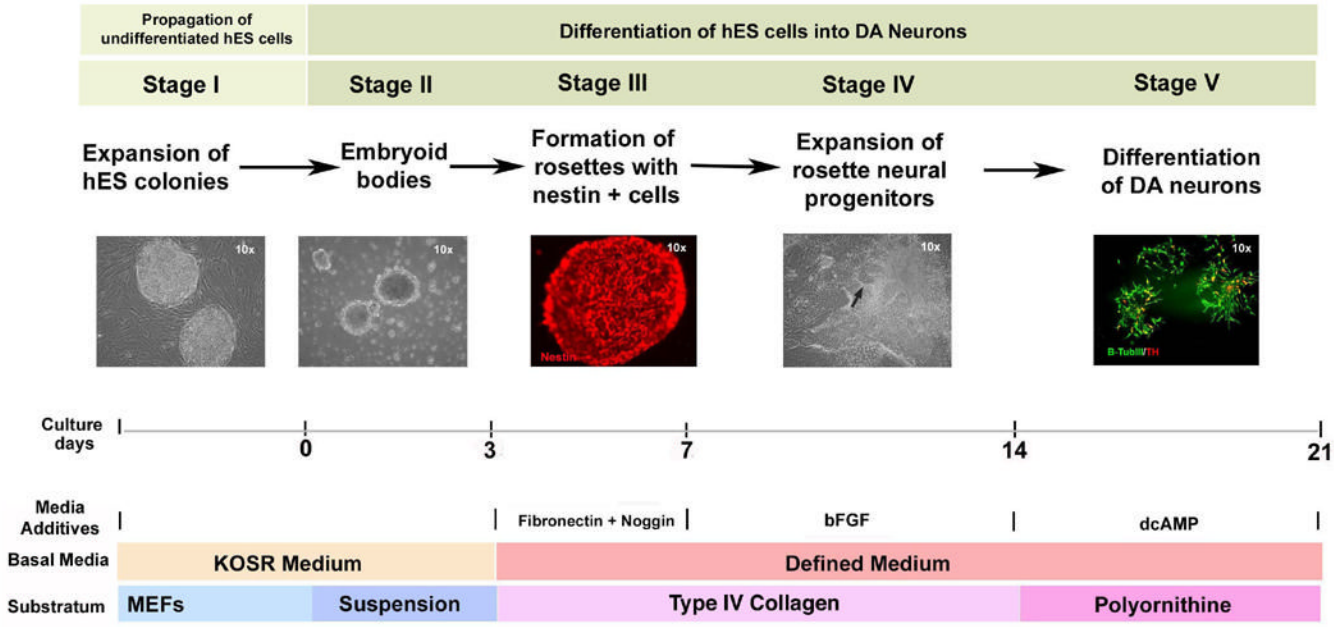

Figure 1.

Depiction of the various stages and treatments used to differentiate hES cells into DA neurons in culture. In brief, H9, BG01, HUES7 and HUES8 cells lines were propagated as colonies on MEFs (see Stage I). H9 and Bresagen colonies were dissected and manually transferred into new dishes while HUES7 and 8 cells were lifted using a mild trypsin dissociation. Colonies were harvested in Stage II and grown in suspension for 3 days in KOSR medium to generate EBs. To promote the formation of nestin^{+} neural progenitor rosettes in Stage III, cells were plated onto type IV human collagen coated dishes on DM containing fibronectin and noggin. Four days later, the DM is replaced with media containing bFGF to promote the expansion of neural progenitor rosettes. One week later, cells were either harvested for transplant or plated onto polyornithine-coated tissue culture dishes and treated with 1mM dbcAMP for 1 week. The vast majority of cells treated in this fashion differentiated into β-tubIII^{+}of hES, many of which expressed the DA biosynthetic enzyme TH.

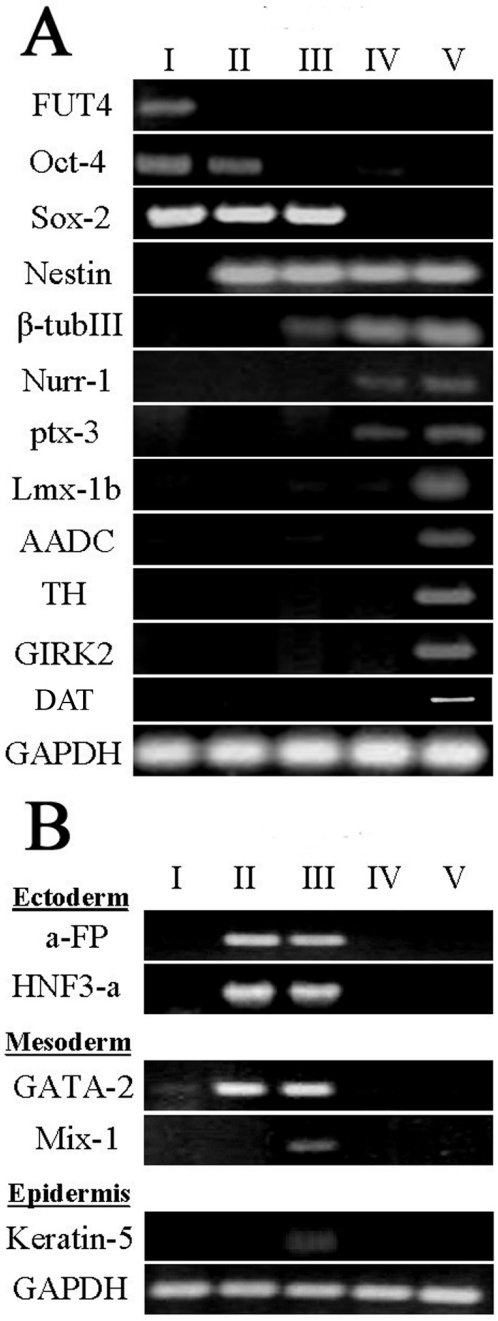

Figure 2.

RT-PCR analysis of various stages in hES differentiation. Stem cell, neuroectodermal, and DA-specific markers (A) as well as markers other germ layers (B) were examined. The HUES8 line (which is also representative of the H9 line results) is shown here. RT-PCR was carried out following the instructions provided in the Cells-to-cDNA™ II kit (Ambion) for reverse transcriptase (RT)-PCR (Promega). Briefly, total RNA was extracted from 10^6 cells of H9 and HUES8 cell lines at all 5 stages and incubated with 100 l of lysis buffer at 75°C for 10 min. DNAse was added into each sample at a final concentration of 0.06 U/l to digest genomic DNA at 37°C for 60 min. The RT reaction was prepared by initially incubating 5 l of cell lysate, 4 l of dNTP mixture, 2 l of Oligo(dT) and 5 l of nuclease-free water. This mixture was incubated

at 70°C for 3 min. At the end of the incubation, the following reagents were added to the mixture: 2 l of 10X RT buffer, 1 l of M-MLV RT and 1 l of RNAse inhibitor. At this point RT was conducted at 42°C for 1 h. cDNA template (1 L) was used in a 50 L reaction volume with the DNA polymerase from AccessQuick RT-PCR System (Promega). The number of cycles varied from 25 to 40 cycles depending on the particular RNA abundance. The cycling parameters were: 94°C, 1 min; 55°C or 60°C, 1 min; 72°C, 1 min. The PCR cycle was preceded by an initial denaturation of 5 min at 94°C and followed by a final extension of 10 min at 72°C. Primers are as follows: GAPDH (5′- TGACATCAAGAAGGTGGTGAAGC-3′, 5′-CCCTGTTGCTGTAGCCGTATTC-3′); FUT4: (5′-GTGGTGAGACTGAATTCAGC-3′, 5′-CTTGGCACCGTCCTTCTCTG-3′) and Girk2 (5′-GGAACTGGAGATTGTGGTCAT-3′, 5′-CATCACCATTCCTCTCTGTCA-3′). The primers were as described previously for AADC, Nurr-1, Ptx-3, Lmx-1b, TH (1); Oct-3/4, Sox2, Nestin, beta;-tub III (13); Gad67 (14); αFP, HNF3-agr;, GATA-2, Mix-1, keratin-5 (10) and DAT (26).

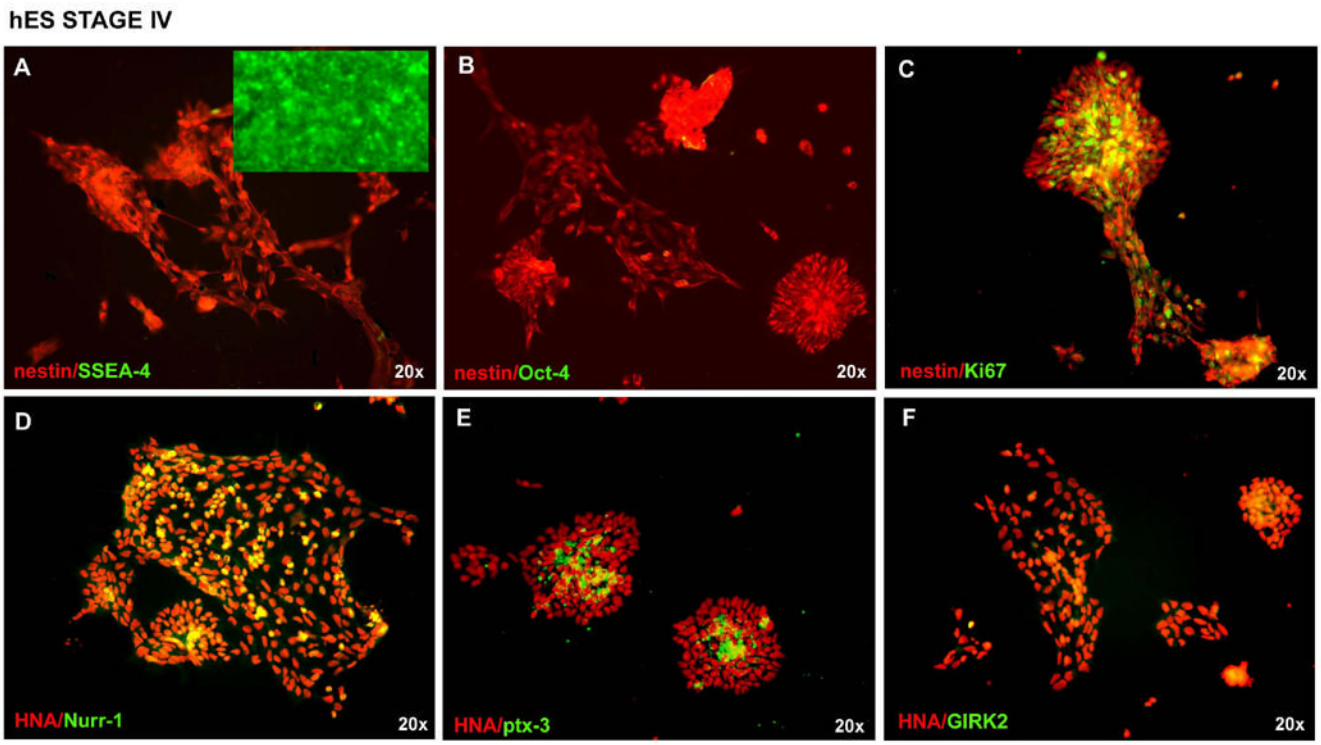

Figure 3.

Immunocytochemical localization of phenotypic markers in stage IV differentiated HUES7 hES cells. Cells were co-stained for nestin and SSEA-4 (A); Oct-4 (B); ki67 (C); HNA and Nurr-1 (D); HNA and ptx-3 (E) and HNA and Girk2 (F) as described below. Inset in A is positive staining control for SSEA4 in undifferentiated stage I cells in culture. The first antibody in each pair was developed with rhodamine (red) and the second with FITC (green) labeled secondary antibodies; overlapping staining in the same cells appears yellow. Cultures were rinsed twice and fixed with 4% paraformaldehyde and stained with rabbit polyclonal antibodies from Chemicon to nestin (1:100); stage specific embryonic antigen 4 (SSEA-4; 1:25); ki67 (1:50), human nuclear antigen (HNA; 1:50), Ptx-3 (1:100), from Alomone Labs, GIRK2 (1:50), and mouse monoclonal antibodies to Oct-4 (1:25); from Santa Cruz Biotechnology, rabbit antibodies to a peptide mapping at the N-terminus of Nurr-1 (1:200); from Sigma, rabbit antibodies to β-tubIII (1:200). All secondary antibodies were purchased from Jackson Immunoresearch: donkey anti-rabbit rhodamine (1:100), donkey anti-mouse FITC (1:100), donkey anti-rabbit FITC (1:100), donkey anti-mouse rhodamine (1:100), donkey anti-goat FITC (1:100).

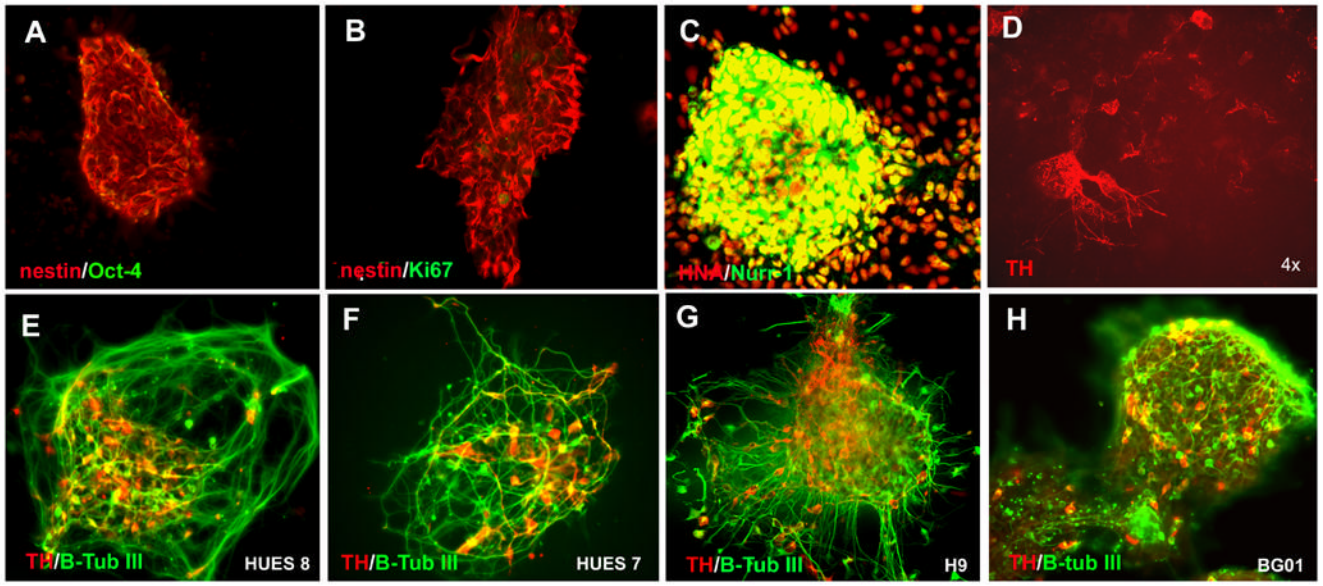

Figure 4.

Immunocytochemical localization of phenotypic markers in stage V DA-differentiated HUES7 hES cells (A-C, F), HUES8 (D, E), H9 (G) and BG01 (H). Cells were single (D) or double labeled for nestin and Oct-4 (A); nestin and ki67 (B); HNA and Nurr-1 (C); β-tubIII and TH (E-H). The first antibody in each pair was developed with rhodamine (red) or FITC (green), and the second with the converse labeled secondary antibodies; overlapping staining in the same cells appears yellow/orange. Methods for staining were as described in legend 3 with the addition of rabbit antibodies to TH from Pel-Freez, (1:100).

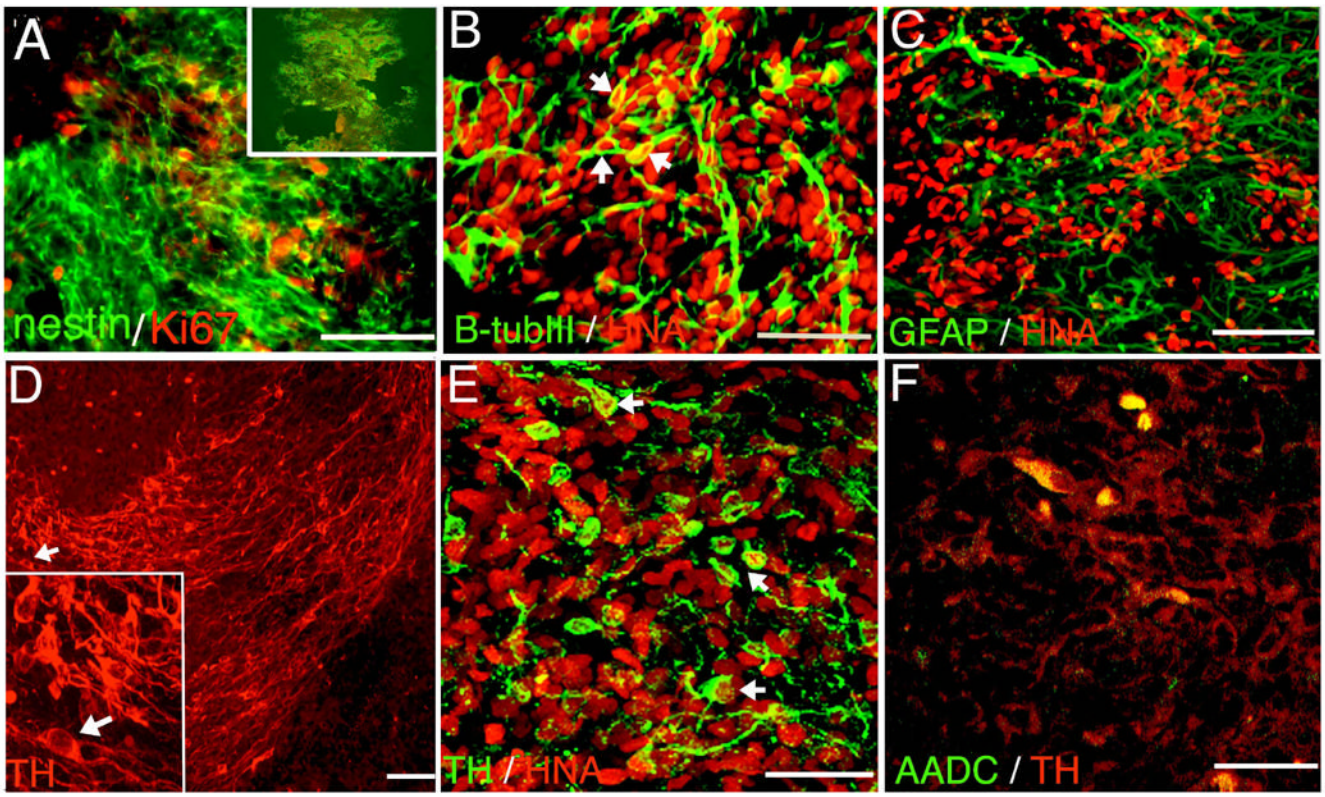

Figure 5.

Immunocytochemical localization of phenotypic markers in stage IV H9 hES cells 2-3 weeks after transplantation into the striata of 6-OHDA treated rats. Cells were single or double labeled for: Ki67 and nestin (A; bar = 150 M); HNA and nestin (A inset); β-tubIII and HNA (B; bar = 100 M); GFAP and HNA (C; bar = 150 M); TH (D; bar = 200 M) TH and HNA (E bar = 100 M) and AADC and TH (F bar = 50 M). For double-labeled cells, the first antibody in each pair was developed with rhodamine (red) and the second with FITC (green) labeled secondary antibodies; overlapping staining in the same cells appears yellow. For immunocytochemical staining in transplantation experiments, rats were perfused with 500ml of cold (4°C) periodate-lysine-paraformaldehyde (4%). Brains sections were cut at 30m on a freezing microtome and processed for immunocytochemistry as described previously (23,24,25). Antibodies were used as above except for the following differences in dilution (β-tubIII; 1:200), HNA (1:40); ki67 (1:25); nestin (1:250); and rabbit polyclonal antibodies to serotonin (5-HT) from Chemicon (1:3000); to choline acetyl transferase (ChAT) from Sigma (1:6000) and mouse monoclonal antibodies to dopamine-β -hydroxylase (DBH) from Chemicon (1:50). Sections were additionally stained for GFAP from Chemicon (1:200) and AADC from Protos Biotech (1:50). Brain sections were analyzed along the length of the graft using a Nikon-Scanalytics Image System or a Zeiss LSM510 Confocal Image System.