Endometrial stem cell transplantation restores dopamine production in a Parkinson’s disease model

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Abstract

Parkinson’s disease (PD) is a neurodegenerative disorder caused by the loss of dopaminergic neurons. Adult human endometrial derived stem cells (HEDSC), a readily obtainable type of mesenchymal stem-like cell, were used to generate dopaminergic cells and for transplantation. Cells expressing CD90, platelet derived growth factor (PDGF)-Rβ and CD146 but not CD45 or CD31 were differentiated in vitro into dopaminergic neurons that exhibited axon projections, pyramidal cell bodies and dendritic projections that recapitulate synapse formation; these cells also expressed the neural marker nestin and tyrosine hydroxylase, the rate-limiting enzyme in dopamine synthesis. Whole cell patch clamp recording identified G-protein coupled inwardly rectifying potassium current 2 channels characteristic of central neurons. A 1-methyl 4-phenyl 1,2,3,6-tetrahydro pyridine induced animal model of PD was used to demonstrate the ability of labelled HEDSC to engraft, migrate to the site of lesion, differentiate in vivo and significantly increase striatal dopamine and dopamine metabolite concentrations. HEDSC are a highly inducible source of allogenic stem cells that rescue dopamine concentrations in an immunocompetent PD mouse model.

Keywords: stem cells • adult stem cells • MSC • endometrium • Parkinson’s disease • transplantation

Introduction

Stem cell research has vastly expanded in recent years, with the promise of revolutionizing medical therapy. An active target of this research has been Parkinson’s disease (PD), a chronic, progressive, degenerative disease of the central nervous system that debilitates both motor function and speech due to the insufficient production of dopamine by pigmented cells in the substantia nigra. 1-methyl 4-phenyl 1,2,3,6-tetrahydro pyridine (MPTP) is a selective neurotoxin of dopaminergic cells that induces PD in both animals and human beings.

Initial excitement about the therapeutic potential of embryonic stem cells for PD was damped by ethical concerns and technical difficulties, including tumour formation. Similarly, the initial enthusiasm for foetal tissue transplantation for PD was tempered in double-blind clinical trials with poor long-term results [1, 2]. The role of mesenchymal stem cells (MSC) in neuro-transplantation has shown recent promise due to the ability of this subtype of stem cells to migrate to sites of damaged neural tissue following both intravenous and intracranial transplantation of bone marrow derived MSC [3–5] or amnionic fluid derived stem cells [6] in preclinical studies. MSC have the additional advantage of being readily manipulated for use as delivery vehicles for gene therapy in PD models [7, 8].

Adult human endometrial derived stem-like cells (HEDSC) are a type of MSC that have only recently been characterized [9–16]. The endometrium displays tenacious regeneration ability due to the demands of menstruation and pregnancy, which make this tissue a promising source of dynamic stem cells suitable for use in regenerative medicine therapies. In vitro transdifferentiation of HEDSC into cartilage, bone, fat and muscle has recently been demonstrated [10, 17, 18]; however, neither transdifferentiation in vivo, transplantation, nor differentiation into a neurogenic cell type has previously been demonstrated. Here we demonstrate the
ability of HEDSC to differentiate into dopamine-producing neurons. We also demonstrate their ability to be used for transplantation, where HEDSC engraft, migrate to the site of lesion, and are spontaneously differentiated in vivo. Furthermore, we show a therapeutic benefit, where transplantation rescues dopamine concentrations in an immunocompetent PD mouse model. To our knowledge this study is the first to demonstrate dopamine improvement using MSC in the absence of gene therapy, which could indicate a particular penchant for HEDSC use in PD transplantation.

**Results**

**Flow cytometry**

Flow cytometry was performed to characterize the HEDSC used in this study. After two passages in culture, HEDSC displayed the following surface markers: CD31^+ 1.4%, αSMA^+ 5.5%, CD90^+ 99.6%, CD45^+ 0.3%, which is consistent with a non-haematogenously derived endometrial stromal cell. HEDSC were strongly positive for both PDGF-Rβ^+ 99.7% and CD146^+ 99.7%, which have been shown to isolate HEDSC from fresh endometrial samples.

**Neurogenic in vitro differentiation of HEDSC**

*In vitro* transdifferentiated HEDSC exhibited neurogenic morphology including long axon projections, pyramidal cell bodies and dendritic projections that appear to recapitulate synapse formation in culture (n = 3) (Fig. 1). Neurogenic cell identity was demonstrated using immunostaining. Cytoplasmic expression of the neural stem cell marker nestin was observed in *in vitro* differentiated cells using a human nestin antibody. Dense staining in the soma and axon hillock region is evidence in some neurons, which appear to overlay the nucleus in other cells. In addition, almost all HEDSC that remained adherent after neurogenic differentiation in *in vitro* expressed the rate-limiting enzyme involved in dopamine production tyrosine hydroxylase (TH). The presence of TH production suggests a functional phenotype, specifically dopamine synthesis. Control cells not differentiated with neurogenic media failed to demonstrate any of these indicators of neuronal identity (Fig. 1).

**Electrophysiological properties of in vitro differentiated cells**

In addition to morphological and immunostaining characteristics, *in vitro* differentiated cells expressed electrophysiological properties of neurons. A whole cell patch clamp recording method was used to measure the current characteristics of individual cells to look for evidence of barium sensitive potassium channels, which are characteristic of central neurons, including dopaminergic cells. The experiments were performed on 10 separate experiments derived from samples differentiated from three separate patients. In the differentiated cells, a series of voltage steps from −60 mV to −120 mV induces inward currents, which were dramatically decreased in the presence of barium (200 μM), a non-specific blocker of the inwardly rectifying potassium current (Kir). The Kir current, resembling the G-protein coupled inwardly rectifying potassium current (GIRK), was only present in differentiated cells, therefore no Ba^2+-sensitive inward currents were present in undifferentiated cells (Fig. 2).

**Transplantation of HEDSC in Parkinson’s disease mouse model**

HEDSC were successfully transplanted into both immunodeficient and immunocompetent MPTP lesioned mice, where engraftment was demonstrated up to 5 weeks following transplantation using multiple techniques. First, human genomic DNA was detected within transplanted mouse brains using PCR (Fig. 3A). Next, engrafted cells were visualized within the mouse brain using four different techniques. A human mitochondrial antibody, which does not cross react with the mouse antigen, was used to detect human cells in mice brains. Human cells were found around the transplantation site in the striatum; however, they were also found to have migrated to the substantia nigra (Fig. 3B). In contrast, when transplantations were performed with differentiated HEDSC, localization to the substantia nigra was not observed.

The transplanted human cells were shown to exhibit neural stem cell markers by staining with the human nestin antibody (Fig. 3B). Mice that were transplanted with HEDSC were also found to express human TH by RT-PCR, whereas sham transplanted animals did not (data not shown). As cells were observed to successfully engraft in both immunodeficient as well as immunocompetent mice, wild-type mice were used for subsequent experiments.

Engraftment and migration was confirmed using two different types of fluorescently labelled HEDSC for transplantation: PKH26 was used for surface labelling and whereas green fluorescent protein (GFP) transfection was used for cytoplasmic labelling. First, PKH26 red labelled HEDSC were identified at the site of transplantation in the striatum; however, they also demonstrated the ability to migrate to the site of lesion, localizing in the substantia nigra. Furthermore, these migrating cells were able to differentiate from an endometrial phenotype into a neurogenic phenotype *in vivo* (Fig. 3B). GFP transfected HEDSC were also able to be visualized within the mouse brains, but this method was limited by the low transfection efficiency of approximately 10% of HEDSC in culture prior to use for transplantation.

Intracranial transplantation with HEDSC resulted in a significant improvement of striatal dopamine (DA) and dihydroxyphenylacetic acid (DOPAC) concentrations in this MPTP mouse model of PD as measured by high-performance liquid chromatography (HPLC). Mean DA concentrations (ng/ml) were significantly higher in MPTP lesioned mice after HEDSC transplant (n = 8, 113.1 ± 5.5 S.E.M.) compared to MPTP lesioned mice treated with sham phosphate buffered saline (PBS) transplant (n = 14, 78.6 ± 7.0 S.E.M.).
Fig. 1  In vitro neurogenic differentiation of HEDSC. HEDSC cultured in control media demonstrate typical stromal cell morphology (A), whereas cells cultured in neurogenic media demonstrated both pyramidal and dendritic cell morphology as is pictured using light microscopy (B, C). Differentiated cells visualized using: differential interference contrast (D), IF for neural stem cell marker nestin expression (E), and a merge of both (F). Differentiated cell cultures, also express TH (H), DAPI nuclei staining (G), and merge of both (I).
whereas mean concentrations of unlesioned mice were 134.6 ± 3.2 (n = 5), P < 0.0001. Mean DOPAC concentrations (ng/ml) were also significantly higher in HEDSC transplanted (n = 8, 5.5 ± 0.3 S.E.M.) versus sham mice (n = 14, 4.0 ± 0.4 S.E.M.), whereas unlesioned mice exhibited baseline concentrations of 5.3 ± 0.2 S.E.M. (n = 5), P = 0.008 (Fig. 4).

Discussion

Here we demonstrate the ability of HEDSC to differentiate into dopamine-producing neurons, where in vitro cultures demonstrate characteristic neuron morphology, express markers of neural cell phenotype and enzymatic function, and display electrophysiological properties specific to dopamine-producing neurons. Furthermore, we demonstrate the ability of HEDSC to be used for transplantation for the first time, even in immunocompetent animals. This was shown by detecting human DNA in mouse brains after HEDSC transplantation, visualizing human HEDSC in mouse brains using antibodies specific to human cells, identifying HEDSC labelled with red fluorescent dye and identifying GFP fluorescing human HEDSC. These cells survive in the location they are transplanted, but also spontaneously migrate to areas of damage and spontaneously differentiate in vivo. HEDSC exert a therapeutic benefit by rescuing dopamine concentrations in this PD animal model.

HEDSC represent an important source of stem cells that can be obtained from a routine office procedure. Further, they can be used as an autologous or allogenic stem cell source, thereby obviating concerns regarding rejection in human beings. The lack of rejection of human stem cells in this murine model could be due to several possibilities. First it is possible this is a result of immune-privilege provided by the blood–brain barrier. Lack of rejection after neuro-transplantation has been demonstrated in several studies [3, 4, 19–27]. However, evidence suggests that MSC have immuno-privileged properties themselves compared to other types of stem cells [26, 28–30], lending additional support to the potential of stem cells from foetal cells is well established in the setting of pregnancy [31, 32] and following blood transfusions. In addition, MSC can home to and engraft mouse bone marrow [33]. It is also possibly that MSC, or HEDSC in particular, display a particular penchant for neural regeneration such as PD treatment due to an underlying disposition of these cells towards neural cells [34]. An additional advantage of MSC is
their low potential for teratoma formation. It is also possible that PD disease is particularly amenable to exogenous stem cells transplantation due to the lack of gliosis, which impedes MSC influx in pathological disease pathologies like stroke.

To date, stem cells derived from the endometrium that demonstrate the ability to transdifferentiate have been isolated in two ways: (1) by performing flow cytometry to select for cells that are both PDGF-Rα<sup>+</sup> and CD146<sup>+</sup> [9, 18] and (2) by passaging routine stromal cell endometrial cultures [10, 11, 17]. It is interesting that merely passaging cells in a routine manner selects cells that are strongly positive for the same markers shown to prospectively isolate HEDSC, and serves to mutually validate both reports. However, the differences in cells collected in these two manners remains to be more fully characterized.

As our understanding of stem cell biology grows, we are re-evaluating their role in tissue repair. It now appears that stem
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or progenitor cells are present in most tissues [35]. An expanding 
body of evidence suggests that stem cells play a role in processes that were previously unrecognized; for instance, it was once thought that neurons in the brain did not undergo regeneration after completing development, when in fact neurons are involved in adult tissue remodelling [36]. It is possible that stem cells may actually be involved in a dynamic state of disease and repair relevant to many chronic disease processes. In fact, it is possible that chronic disease may actually be an indicator of the lifetime burden of regeneration and stem cell deple-
tion. This theory can be illustrated by the cases of early onset PD in high regenerative burden states such as boxing, post-concus-
sion syndrome or drug abuse.

Stem cells are defined by the ability to clonally proliferate and self-replicate, but do exhibit a terminal, albeit extremely long pro-
liferation capacity in vivo. Variations in observed cell culture may reflect a similar naturally occurring variation in vivo. The life time burden of regeneration may help to explain the high levels of vari-
ation observed in culture between patients; e.g. tissue donors with low burdens produce cell cultures with robust replication and dif-
ferentiation potential, while cultures derived from donors with high burdens of regeneration produce cultures with decreased activity. This could also help explain the remarkable plasticity of newborn brains, but relatively limited neural plasticity in adults. Perhaps endogenous MSC do not treat diseases like PD well in vivo because the PD phenotype occurs after a long burden of auto-

transplantation with endogenous MSC, which then are depleted. The therapeutic potential of MSC, in particular HEDSC, will likely vary depending on the lifetime regeneration burden of the donor source. Based on the therapeutic potential we demonstrate here, HEDSC may become an important source of allogenic stem cells to be used for regenerative medicine.

Materials and methods

Sample collection

Human endometrial tissue was collected by curettage from nine reproduc-
tive aged women undergoing surgery for benign gynaecological conditions. Standard endometrial stromal cell cultures were generated in a routine fashion, which produced an unfractionated stromal cell population. Briefly, endometrial tissue was minced and then digested in Hank's balanced salt solution (HBSS) (Gibco, Invitrogen, Carlsbad, CA, USA) containing 4-(2-hydroxyethyl)-1-piperazineneethanesulfonic acid (HEPES) (25 mM), collagenase B (1 mg/ml, Roche Diagnostics, Indianapolis, IN, USA) and DNase I (0.1 mg/ml, Sigma-Aldrich, St. Louis, MO, USA) for 30–45 min. at 37°C with agitation. Resultant dispersed cell solutions were then passed through a 70 μM sieve (BD Biosciences, Beford, MA, USA) to remove glandular epithelial components. Filtered cell solutions were then centrifuged, supernatant decanted and resuspended in Dulbecco’s modified Eagle’s medium: Ham’s F12 (DMEM, Gibco, Invitrogen, Carlsbad, CA, USA) containing 1% antibiotics-antimycotics (ABAM, Gibco, Invitrogen) and 10% foetal bovine serum (FBS, Gibco, Invitrogen). Resuspended cells were then plated in plastic flasks, maintained at 37°C in a humidified chamber (5% CO2). Thereafter, cells were passaged using standard trypsinization methods.

Flow cytometry

Human endometrial derived stromal cultures were characterized using flow cytometry after passage two. Cells were trypsinized and washed with staining buffer, which comprised PBS with 5% FBS and 0.05% sodium azide. Cells were passed through a 70 μM sieve to minimize cell clumping for analysis, centrifuged and supernatant decanted. Cell pellets were incubated on ice with the following antibodies for 1 hr: CD90 directly conjugated with antigen-presenting cell (APC; BD Pharmingen, San Jose, CA, USA), CD146 directly conjugated with phycocyanin (PE) (BD Pharmingen), CD45 directly conjugated with fluorescein isothiocyanate (FITC) (BD Pharmingen), CD31 directly conjugated with FITC (BD Pharmingen) and platelet derived growth factor receptor β directly conjugated with APC (PDGF-Rβ, R&D Systems,
Minneapolis, MN, USA) for 1 hr. For intracellular staining, cells were treated using BD Cytofix/Cytoperm Fixation/Permeabilization Kit before incubation with α smooth muscle actin antibody directly conjugated with Cy3 (α-SMA, Sigma, St. Louis, MO) for 1 hr. Cells were then washed and resuspended with sorting buffer, which comprised PBS with 0.1% bovine serum albumen. Directly conjugated isotype controls were used set electronic gates to <3% positive cells. Cells were then analysed on the BD FACSVantage SE Cell Sorter using FACSDiVa (BD Biosciences, San Jose, CA, USA).

**Neurogenic in vitro differentiation**

After the second passage, cells were treated with a two-step dopaminergic differentiation protocol adapted from Blondheim et al. [34]. HEDSC were first treated with differentiation medium I for 24–48 hrs, which consisted of DMEM with 10% FBS, 1% ABAM, 2 mM L-Glutamine (Invitrogen), recombinant human fibroblast growth factor (rhFGF) (10 ng/ml, rhFGF, R&D Systems), recombinant human epidermal growth factor (rhEGF) (10 ng/ml, rhEGF, R&D Systems) and N2 supplement-B (StemCell Technologies, Vancouver, CA, USA). Cells were then changed to differentiation medium II for up to 96 hrs, which consisted of DMEM with 2 mM L-glutamine, 1% ABAM, N2 supplement-B, butylated hydroxyanisole (200 μM, Sigma), dibutyryl cyclic AMP (1 mM, Sigma), 3-isobutyl-1-methyl-xanthine (10 ng/ml, rhFGF, R&D Systems) and all-trans-retinoic acid (1 μM, Sigma). Undifferentiated endometrial stromal cell culture in DMEM w/10% FBS were grown until limited by confluence, then harvested or fixed and used as controls.

**In vitro immunostaining**

For in vitro immunofluorescent experiments, cells were cultured in chamber slides and then fixed with methanol. To examine for evidence of nestin production (a neural stem cell marker), cells from in vitro experiments were washed with PBS, permeabilized in PBS containing 0.2% Triton X-100 (PBS-TX) for 10 min., blocked with 10% normal goat serum in PBS. The following day cells were incubated with FITC-labelled anti-rabbit IgG prepared in goats (Vector Laboratories, Burlingame, CA, USA) for 1 hr. Slides were mounted with VECTASHIELD Mounting Medium with 4’,6-diamidino-2-phenylindole (DAPI) (Vector Laboratories).

To examine for evidence of TH production (the rate-limiting enzyme in dopamine synthesis), fixed in vitro cell cultures were washed with PBS and blocked using donkey antimouse serum for 1 hr. Cells were then stained using a mouse monoclonal TH antibody (DiaSorin, Stillwater, MN, USA) diluted at 1:5000 in PBS-TX overnight. After a series of washes, sections were incubated with the fluorescent secondary antibody donkey antimouse IgG 488 Alexafluor (1:200; Molecular Probes, Carlsbad, CA, USA) for 1 hr to visualize TH-immunoreactive cells. Slides were mounted with VECTASHIELD Mounting Medium with DAPI.

**In vitro electrophysiology**

Whole cell patch clamp recording was then performed on in vitro differentiated and undifferentiated endometrial cells from three different patients to examine for evidence of GIRK2 channels, which are characteristic of central neurons including dopaminergic cells. Briefly, cells were maintained in a recording chamber with artificial cerebrospinal fluid (bubbled with 5% CO2 and 95% O2) containing (in mM): NaCl 124, KCl 3, CaCl2 2, MgCl2 2, NaH2PO4 1.23, NaHCO3 26, glucose 10, pH 7.4 with NaOH. Whole-cell voltage clamp (at –60 mV) was performed to observe inwardly rectifier potassium currents with a Multiclamp 700A amplifier (Axon Instruments; Molecular Devices Corporation, Sunnyvale, CA, USA). The patch pipettes with a tip resistance of 4–6 MΩ were made of borosilicate glass (World Precision Instruments, Sarasota, FL, USA) with a Sutter pipette puller (P-97) and filled with pipette solution containing (mM): K-glucolate 135, MgCl2 2, HEPES 10, ethylene glycol tetraacetic acid (EGTA) 1.1, Mg-ATP 2, Na2-phosphocreatine 10, and Na2-GTP 0.3, pH 7.3 with KOH. After a giga-Ω (GΩ) seal and whole-cell access were achieved, the series resistance (between 20 and 40 MΩ) was partially compensated by the amplifier. A series of voltage steps from −60 mV to −120 mV was applied to recorded cells under voltage clamp in the presence of high concentration of K+ (60 mM) to monitor inwardly rectifier potassium current. The existence of inwardly rectifier potassium currents was verified by applying Ba2+ (200 μM) containing bath solution to the recorded neurons. Both input resistance and series resistance were monitored throughout the experiments. Only those recordings with stable series resistance and input resistance were accepted. All data were sampled at 3–10 kHz and filtered at 1–3 kHz with an Apple Macintosh computer using Axograph 4.9 (Axon Instruments). Electrophysiological data were analysed with Axograph 4.9 and plotted with Igor Pro software (WaveMetrics, Lake Oswego, OR, USA).

**HEDSC fluorescent labelling**

Fluorescent labelling of HEDSC was performed in two ways. Trypsinized cells after passage two were labelled with PKH26 (Sigma) according to the manufacturer’s instructions prior to use for transplantation. Alternately, cells were transfected with a GFP plasmid using Lipofectamine for 24 hrs. Cell media was then changed and cultures continued for an additional 48 hrs prior to trypsinization and transplantation. Cell labelling with PKH26 and GFP transfection was confirmed by visualization immediately prior to transplantation.

**HEDSC transplantation into mice**

For HEDSC transplantations, an established PD mouse model was generated by injecting 8-week-old male mice with 30 mg/kg intraperitoneal MPTP on two consecutive days in both immunocompetent (C57-Black 6) and immunodeficient (non-obese diabetic severe combined immunodeficiency knockout on a Black 6 background) mice. All mice were maintained under standard laboratory conditions with water and food available ad libitum; lights were maintained on a 12 hr light/dark cycle. Transplantations were performed with undifferentiated as well as differentiated HEDSCs. Five days after MPTP treatment, anesthetized mice underwent transplantation using a stereotaxic frame. Using an aseptic technique, a burr hole (0.5 mm) was made on both sides of the skull. Each mouse received a total of four injections: 2 mm lateral to Bregma at both 0.5 mm rostral and caudal of Bregma. Mice were transplanted with either 103 undifferentiated HEDSC in PBS, 105 differentiated HEDSC in PBS, or PBS control over 5 min. at a depth of 4, 3.5 and 3 mm. The needle remained in the striatum for an additional 5 min. interval before slowly being retracted to avoid HEDSC reflux.

Mice were killed 5 weeks later and one striatum was used for DNA analysis and the other striatum was used to measure dopamine concentrations.
Detection of human DNA in mouse brains

DNA was harvested using the QIAamp DNA MiniKit (Qiagen, Valencia, CA, USA). Human DNA was amplified using the genomic primers: forward 5’-GCTTGACACGAGGTGAG-3’ and reverse 5’-TCCTGAAAGCTGAGGSAAG-3’ at 65°C annealing temperature using high-fidelity Taq polymerase (Invitrogen). Human genomic DNA was used as a positive control. To detect expression of human TH in mice that received transplantation, mRNA was extracted from mouse brains using the RNAeasy kit (Qiagen). cDNA was generated using iScript cDNA synthesis kit (Bio-Rad Laboratories, Inc., Hercules, CA, USA). Primers used for TH were specific for human and for mouse (NIH U54 HD052668) and were post-fixed overnight in fixative without glutaraldehyde. Serial 50 μm coronal sections were cut through mouse brains using a vibratome and were collected in microtitre plates.

To detect all human cells present in the mouse brains, a generic antibody against human cells that does not cross react with mouse tissue was used: human 60 kD mitochondrial antibody (Millipore, Bedford, MA, USA). Brain sections were then counterstained with the fluorescent secondary antibody donkey antimouse IgG 488 Alexafluore (1:200; Molecular Probes) and were collected in microtitre plates.

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