Induction of dopaminergic neuronal-like cells from CD44+ human amniotic fluids that are ameliorative to behavioral recovery in a Parkinson's disease rat model

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Abstract. Parkinson's disease (PD) is a common age-associated neurodegenerative disorder. To date, stem cell transplantation therapy has been developed to replace lost or damaged neural cells in PD patients, in whom dopaminergic neuron cells are lost. Here, we show that CD44+ human amniotic fluid cells (HuAFCs) can be induced to become functional dopaminergic neuronal-like cells *in vitro*. Furthermore, when CD44+ or CD44- HuAFCs were transplanted into 6-hydroxydopamine (6-OHDA)-treated PD rats, the results indicated that CD44+ HuAFCs expressed multiple dopaminergic neuron cell markers and were ameliorative to behavioral recovery in PD rats after induction both *in vitro* and *in vivo*. CD44- HuAFCs did not fully differentiate into dopaminergic neuronal-like cells. When compared with CD44- HuAFCs, CD44+ HuAFCs showed increased activity in regeneration of dopaminergic neuron cell-like cells, increased migration distances, and improvement of animal behavior in the PD rat model. Therefore, CD44+ HuAFCs could be a source of dopaminergic neuronal-like cells with a potential use in cell-replacement therapy for PD.

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Abbreviations: 6-OHDA, 6-hydroxydopamine; AKP, alkaline phosphatase; bFGF, basic fibroblast growth factor; BMP-4, bone morphogenetic protein 4; DAPI, 4',6-diamidino-2-phenylindole; DAT, dopamine transporter; EGF, epidermal growth factor; ESCs, embryonic stem cells; FCM, flow cytometry; GFP, green fluorescent protein; HuAFCs, human amniotic fluid cells; ICH, immunohistochemistry; IF, immunofluorescence; iPS, induced pluripotent stem cells; MFB, medial forebrain bundle; NSCs, neural stem cells; NSE, neuron-specific endolase; PD, Parkinson's disease; qRT-PCR, quantitative real-time polymerase chain reaction; TGF-α, transforming growth factor α; TGF-β, transforming growth factor β; TH, tyrosine hydroxylase; VTA, ventral tegmental area

Key words: cells transplantation, dopaminergic neurons, human amniotic fluid cells, Parkinson's disease, rat model

Introduction

Parkinson's disease (PD) is a major neurodegenerative disease that affects almost 2% of the population above the age of 65. Midbrain dopamine neurons in the substantia nigra project into the dorsolateral striatum, caudate and putamen forming the nigrostriatal pathway and release dopamine, an important neurotransmitter that controls body movement (1,2). Currently, available therapies for the treatment of PD address symptoms but are not cures. However, at present, cell-replacement therapies may provide the most promising curative treatment for PD (3). If cells can be placed in the brain to produce suitable, controlled levels of dopamine release in the striatum, then there is hope of attenuating the disease process and restoring motor function in patients. From a scientific perspective, successful cell-replacement therapy for PD has to meet at least the following goals (4): i) long-term survival in host tissue; ii) differentiation into mature dopaminergic neurons that exhibit the right molecular and morphological characteristics of mature dopaminergic neurons; iii) release of dopamine in a regulated manner; iv) long-term efficacy in reversing the behavioral deficits in PD patients; and v) minimum risk for tumor development and minimum side effects such as dyskinesias. To date, there are two approaches for cell-based therapy in PD. One is transplantation of exogenous cells into the diseased brain to replace lost cells or support the remaining cells. The other strategy is to enhance the proliferation, differentiation, migration of endogenous stem or progenitor cells, such as neural stem cells (NSCs), embryonic stem cells (ESCs), and induced pluripotent stem (iPS) cells (1-8). Previous studies have indicated that not only NSCs but also ESCs and iPS cells could be induced to differentiate into dopaminergic neurons

and provide behavioral improvement in 6-hydroxydopamine (6-OHDA)-legioned rat models (1-8). These results suggest that these stem cells have potential for cell therapy for neurodegenerative diseases. For many years, scientists have been looking for mechanisms that regulate the differentiation of stem cells, the answer is likely to be complex and involve a variety of contributing factors (7). Despite these stem cells having been studied intensively in the last few decades, not much is known about how their self-renewal properties are maintained or how cell fate is determined (1,9). In addition, some stem cells, such as the ESCs, have not been used clinically due to ethical limitations. In fact, some studies have reported that iPS cells seeded into the mouse brain could result in highly malignant teratocarcinomas that do not migrate away from the transplantation site (4,10-12). Collectively these factors underscore the importance of finding safe and effective ways for stem cells to be used as potential therapies.

Human amniotic fluid cells (HuAFCs) are currently used for routine prenatal genetic diagnosis of a wide range of fetal abnormalities (13-17). However, amniotic fluid contains multiple cell types derived from the developing fetus. These cells can give rise to various cell types, including adipose, muscle, bone and neuronal cells (18-21). The $CD44^+$ subpopulation can be sorted directly from human amniotic fluid, and can be cultured for *ex vivo* expansion (22-25). Previous studies have demonstrated the ability of these cells to differentiate into ectodermal, endodermal, mesodermal, hepatic and cardiac muscle cells (18-21). Therefore, the $CD44^+$ human amniotic fluid subpopulation can differentiate into all three germ layers.

Our previous studies have indicated that HuAFCs express many growth factors, including epidermal growth factor (EGF), basic fibroblast growth factor (bFGF), transforming growth factor α (TGF-α), transforming growth factor β (TGF-β) and bone morphogenetic protein 4 (BMP-4), as well as the stem cell markers Nanog, Oct-4 and Nestin. More importantly, these cells lack major histocompatibility complex class II antigens and only express low levels of major histocompatibility complex class I antigens. In addition, these cells are more easily accessible than other adult stem cells, making them a potential autologous donor source for stem cell therapy. In this study, therefore, we set out to determine which subpopulation of HuAFCs can be induced to differentiate into dopaminergic neuron cell-like cells and whether the $CD44^+$ subpopulation can influence the speed and effectiveness of dopaminergic neuron cell induction.

Materials and methods

Isolation and in vitro expansion of CD44 phenotype cells by a magnetic activated cell sorting system. Human amniotic fluid was obtained by ultrasound-guided amniocentesis performed on pregnant women for routine prenatal diagnosis purposes at gestational ages ranging from 18-22 weeks. All of the human samples were obtained after approval from the Ethical Review Board of the Shanghai First Maternity and Infant Hospital (Shanghai, China) and after obtaining written informed consent from the subjects (25). $CD44^+$ subpopulation cells were isolated from human amniotic fluid using 4 μl of the primary monoclonal antibodies (rabbit anti-rat $CD44^-$ PE, eBioscience) stored at 4˚C in PBS for 30 min in a volume of 1 ml as previously described (26). After reaction, the cells were washed twice in PBS, and were put the secondary monoclonal antibodies were added (goat anti-mouse or goat anti-rabbit coupled to magnetic microbeads, Miltenyi Biotec, Auburn, CA), incubated at 10˚C in PBS for 15 min and then washed twice in PBS. Single cells were plated at 1000 cells/ml in α-MEM (HyClone), supplemented with 10 ng/ml bFGF, 10 ng/ml EGF (all from Sigma-Aldrich, St. Louis MO), 10% fetal bovine serum and 2 mM L-glutamine (all from HyClone). All $CD44^+$ cells were cultured in a humidified incubator, at 37˚C with 5% CO_2, until 80% confluent. In this experiment, all cells had been cultured on the same conditions until passage 4 before performing subsequent experiments.

Induction and expansion of the CD44 phenotype HuAFCs and differentiation into dopaminergic neuron cell-like cells. To induce differentiation into dopaminergic neuron cells, expanded CD44 phenotype HuAFCs ($1x10^6$) were seeded in a 10-cm dish and cultured in serum-free DMEM:F12 (1:1) containing 20 ng/ml EGF and 1 ng/ml bFGF supplement (all from Gibco), 1% B27 and N_2 supplement (Sigma-Aldrich), and 1.0 μmol/l all-trans retinoic acid (Sigma-Aldrich) for 10 days. The medium was replaced twice a day for 10 days. All cells were cultured in a humidified incubator, at 37˚C with 5% CO_2, until 80% confluent according to the manufacturer's instructions (2,7,8).

Alkaline phosphatase (AKP) staining. The AKP activity of HuAFCs was determined using the AKP substrate kit (Sigma-Aldrich) according to the manufacturer's instructions (27).

Immunofluorescence staining analysis and relative protein expression. The cultured cells were washed 3 times with PBS and fixed with 4% paraformaldehyde (Sigma-Aldrich) for 30 min. After blocking, the cells were incubated first with rabbit anti-rat neuron-specific endolase (NSE) polyclonal antibody (1:200; Santa Cruz Biotechnology, Inc., Santa Cruz, USA) and rabbit anti-rat tyrosine hydroxylase (TH) polyclonal antibody (1:200; Santa Cruz Biotechnology, Inc.) overnight at 4˚C, and then with Cy3-conjugated goat anti-rabbit IgG antibody (1:200; Abcam, Cambridge, UK) and 5 mg/ml 4',6-diamidino-2-phenylindole (DAPI) (Sigma-Aldrich) at room temperature for 30 min. Subsequently, the cells were thoroughly washed with TBST and viewed through a fluorescence microscope (DMI3000; Leica, Allendale, NJ, USA).

Flow cytometric analysis of CD44 expression. HuAFCs (after and before sorting) were washed 3 times with FBS and fixed with 4% paraformaldehyde (Sigma-Aldrich) for 30 min. After blocking, the cells were incubated first with rabbit anti-rat $CD44^-$ PE at 4˚C for 30 min, which was hidden from light. Then the cells were thoroughly washed with TBST 3 times. While, these stained cells were analyzed using the FACS analyzer (FCM-500, Beckman Coulter), a total of 10,000 events were acquired for analysis using the CellQuest software.

Quantitative real-time PCR (qRT-PCR) analysis of the expression dopaminergic neuron cell like cell markers. Total-RNA from each cells was isolated using TRIzol reagent (Invitrogen) according to the manufacturer's protocol. The RNA samples were treated with DNase I (Sigma-Aldrich), quantified, and

reverse-transcribed into cDNA using the ReverTra Ace-α First Strand cDNA Synthesis kit (Toyobo). qRT-PCR was conducted using a RealPlex4 real-time PCR detection system from Eppendorf Co., Ltd. (Germany), with SYBR-Green real-time PCR Master mix (Toyobo) used as the detection dye. qRT-PCR amplification was performed over 40 cycles with denaturation at 95˚C for 15 sec and annealing at 58˚C for 45 sec. Target cDNA was quantified using the relative quantification method. A comparative threshold cycle (Ct) was used to determine gene expression relative to a control (calibrator) and steady-state mRNA levels are reported as an n-fold difference relative to the calibrator. For each sample, the marker genes Ct values were normalized using the formula $\Delta Ct = Ct_{markers}-Ct_{18S\ rRNA}$. To determine the relative expression levels, the following formula was used $\Delta\Delta Ct = \Delta Ct_{cells\text{-}induced}-\Delta Ct_{cells\text{-}not\ induced}$. The values used to plot the relative expression of marker genes were calculated using the expression $2^{-\Delta\Delta Ct}$. The mRNA levels were calibrated based on levels of 18S rRNA. The cDNA of each stem cell marker was amplified using primers as previously described (2,7,8).

Western blot analysis. After induction for 10 days, total protein extracts of each group (transfected siRNA-PDX-1 or siRNA-MOCK) and non-induced cells (as blank control) were resolved by 12% SDS-PAGE and transferred on PVDF (Millipore) membranes. After blocking, the PVDF membranes were washed 4 times for 15 min with TBST at room temperature and incubated with primary antibody (rabbit anti-rat NSE polyclonal antibody (1:500); rabbit anti-rat TH polyclonal antibody (1:500) and rabbit anti-rat dopamine transporter (DAT) polyclonal antibody (1:500) (all from Santa Cruz Biotechnology). Following extensive washing, membranes were incubated with secondary peroxidase-linked goat anti-rabbit IgG (1:1000, Santa Cruz Biotechnology) for 1 h. After washing 4 times for 15 min with TBST at room temperature once more, the immunoreactivity was visualized by enhanced chemiluminescence (ECL kit, Pierce Biotechnology).

Rat model of Parkinson's disease (PD). Adult female Sprague-Dawley rats (n=18), weighing 250-300 g, were obtained from the Shanghia Tongji University. Experiments were conducted with Institutional Animal Care and Use Committee approval in accordance with institutional guidelines. All rats were maintained for 15 days, 3 to 4 per cage, in a temperature-controlled colony room under standard light-dark cycle with free access to food and water. The study protocol was according the manuscript (2,7,8). The animals were divided into three groups: a vehicle group (6 animals) grafted with PBS, a negative control groups (6 animals) grafted with $CD44^-$ HuAFCs, and an experimental group (6 animals) grafted with $CD44^+$ HuAFCs. All animals received unilateral stereotaxic injections of 6-hydroxydopamine (6-OHDA) (Sigma-Aldrich) into the right medial forebrain bundle (MFB) (AP, 4.4 mm; L, 1.2 mm; DV, 7.8 mm) and ventral tegmental area (VTA) (AP, 4.8 mm; L, 1.0 mm; DV, 7.8 mm) in order to make lesions. The anesthetized animals were injected unilaterally in the intrastriatal region with 6-OHDA using a 10 μl Hamilton microsyringe. Upon completion of the injection, the needle was left in place for an additional 5 min and then withdrawn at a rate of 1 mm/min. The rotation behavior of all the rats in this PD model was examined and the animal was selected for experiments if the testing result showed a rate of over 6 turns/min in response to 0.5 mg/kg of apomorphine (Sigma-Aldrich) after 4 weeks.

Cell transplantation. Four weeks after surgery, each experimental model animal received an injection of 5 μl of HuAFCs ($1x10^5$ cell spheres/μl), which harbored a green fluorescent protein (GFP) by lentivirus, or PBS into the right MFB and VTA, using a 10 μl Hamilton microsyringe. A 5-min waiting period allowed the cells to settle before the needle being removed.

Immunohistochemistry analysis. Three rats from each group were sacrificed and examined by immunohistochemistry (ICH). First, brains were fixed by perfusion with fixative (4% paraformaldehyde) for 2 days. Afterward, 30 μm frozen sections (near the injection tract) were cut with a cryostat at -20˚C, and sections were processed for ICH. The slides were incubated for 48 h at 4˚C with a primary antibody specific for rabbit anti-rat TH polyclonal antibody (1:100; Santa Cruz Biotechnology), followed by reaction with biotin-conjugated anti-rabbit immunoglobulin G (Vector Laboratories). Subsequently, sections were washed in PBS and incubated with ABC. The preparations were stained using a Vectastain ABC kit (Vector Laboratories) then developed with DAB (2,28). Immunohistochemical studies were repeated at least three times.

Behavioral assay. This procedure was performed as previously described (2,7,8). The rotational behavior of the animals in both groups was examined 2, 4, 6 and 8 weeks after transplantation. In this test, the animals were allowed to habituate for 5 min in a cylindrical container of a 33 cm diameter and a 35 cm height. After the injection of 0.5 mg/kg (i.p.) apomorphine hydrochloride (Sigma-Aldrich), full contralateral rotations were counted for 30 min in a quiet isolated room.

Statistical analysis. Each experiment was performed as least three times and data are shown as the mean ± SE. The differences were evaluated using Student's t-tests. The probability of <0.05 was considered to be statistically significant.

Results

Isolation, enrichment and pluripotency of $CD44^+$ HuAFCs. Previous studies have suggested that the $CD44^-$ expressing subpopulation is relatively small in HuAFCs. Therefore, we used a magnetic activated cell sorting system to isolate and enrich this specific subpopulation. After isolation, the cells were detected by flow cytometry (FCM), and the $CD44^+$ cells were found to represent 78.37±2.28% of HuAFCs after sorting, compared with 22.14±2.68% of HuAFCs prior to sorting (Fig. 1). These results indicated that $CD44^+$ cells could be successfully enriched from human amniotic fluids using magnetic activated cell sorting.

To compare the pluripotency of $CD44^+$ HuAFCs to the full HuAFC population, we performed an AKP staining assay and found that the number of AKP-positive cells increased in the $CD44^+$ group when compared with the total HuAFC population (Fig. 1). This finding suggests that the enriched $CD44^+$ population is more pluripotent than the HuAFCs population as a whole.

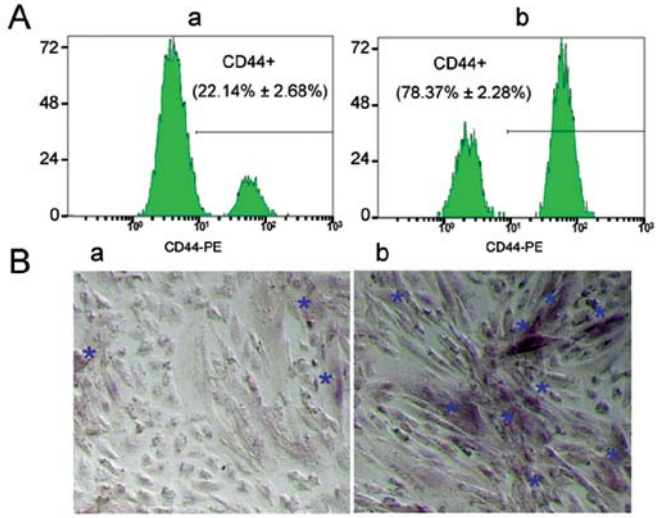

Figure 1. Isolation, enrichment and pluripotency assay of CD44+ human amniotic fluid cells (HuAFCs) from full amniotic fluids. (A) Flow cytometry (FCM) results indicated that CD44+ cells represented 78.37±2.28% of HuAFCs after sorting (b), compared with 22.14±2.68% of unsorted HuAFCs (a); $P<0.05$, n=3. (B) Alkaline phosphatase (AKP) staining assay of sorted and unsorted HuAFCs. (a) A small number of AKP-positive HuAFCs was present in the unsorted group, (b) but many AKP-positive cells were found in the sorted group. *AKP-positive cells. Original magnification, x100.

CD44+ HuAFCs express dopaminergic neuron markers after induction. In the first step, CD44+ and CD44- HuAFCs were divided into two groups. At 10 days after induction, some neuron cell-like cells could be found in the CD44+ HuAFCs group. However, few cells of this type could be found in the CD44- HuAFCs group. To determine if these cells were dopaminergic neuronal-like cells, cell markers were assayed by immunofluorescence (IF) staining, qRT-PCR and Western blotting. IF staining was performed 10 days after induction to compare the expression levels of NSE and TH in CD44+ and CD44- HuAFCs. Expression levels of both proteins were increased in CD44+ HuAFCs compared with CD44- HuAFCs (Fig. 2). These results suggest that CD44+ HuAFCs have great pluripotential activity, and are easier to differentiate into dopaminergic neuronal like cells by induction *in vitro* than are CD44- HuAFCs. qRT-PCR was used to compare the expression levels of several important dopaminergic neurons cell factors in each group. We found that when the levels of DAT, TH and NSE expression were measured, all were expressed at significantly higher levels in the CD44+ HuAFCs group than in the CD44- HuAFCs group (Table I, Fig. 3). Relative mRNA expression is shown after normalization to 18S rRNA, which served as an internal control. In the CD44+ HuAFCs group, Western blotting revealed that levels of DAT, TH and NSE proteins were 1.205±0.075, 1.015±0.065 and 0.900±0.01 compared with control β-actin expression levels, respectively. These values were significantly higher than levels for the CD44- HuAFCs group (0.325±0.045, 0.235±0.043 and 0.13±0.03 of β-actin levels, respectively). These data indicate that DAT, TH and NSE are abundant and highly expressed in ordinary CD44+ HuAFCs during the induction of dopaminergic neuron cells differentiation (Fig. 3).

Survival, migration and differentiation of transplanted CD44+ HuAFCs in the PD rat model brain. The brain injection tracts were examined (Fig. 4) to confirm the presence of transplanted

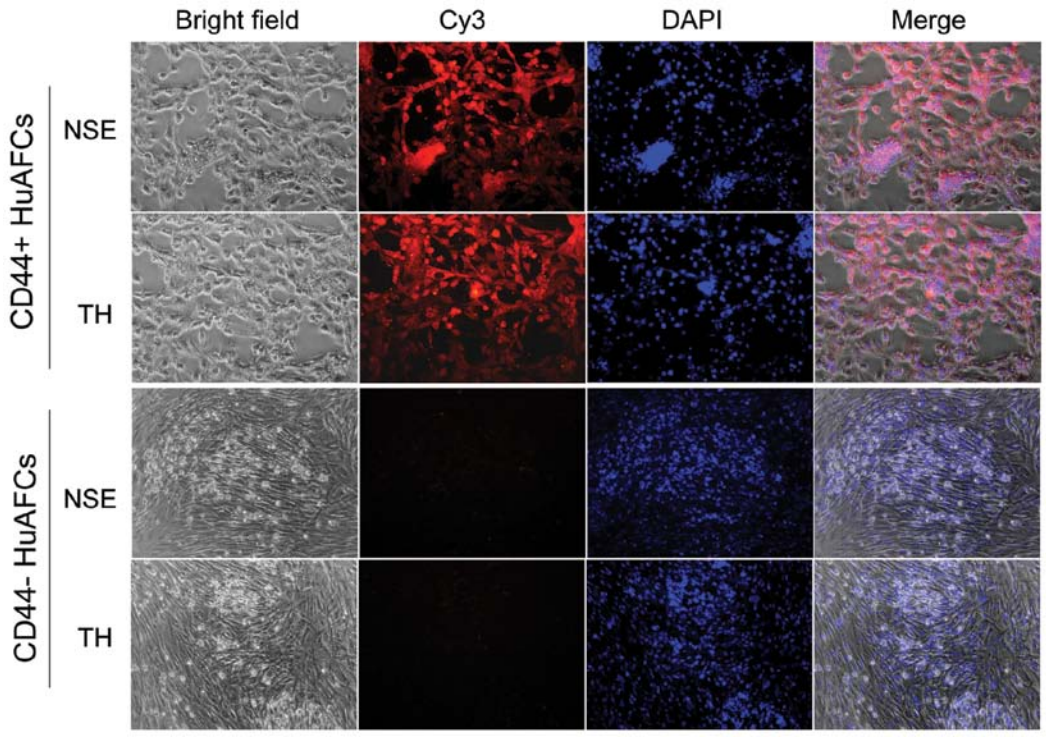

Figure 2. Tyrosine hydroxylase (TH) and neuron-specific endolase (NSE) expression in dopaminergic neuronal-like cells in CD44+ or CD44- human amniotic fluid cells (HuAFCs). In CD44- HuAFCs, neither NSE nor TH was expressed as assayed by immunofluorescence (Cy3, red). However, after induction both NSE and TH expressed in CD44+ HuAFCs. The intensity of TH and NSE staining was higher in the CD44+ HuAFCs compared with the CD44- HuAFCs. 4',6-diamidino-2-phenylindole (DAPI, blue) was used to stain cellular DNA. Original magnification, x100.

Table I. Expression levels of dopaminergic neuron cell markers examined by qRT-PCR (n=3).

	Not induced groups		Induced groups	
Markers	$CD44^-$ HuAFCs	$CD44^+$ HuAFCs	$CD44^-$ HuAFCs	$CD44^+$ HuAFCs
DAT	1.04±0.01	1.11±0.00	1.03±0.07	9.02±0.33
TH	1.00±0.02	1.04±0.01	1.04±0.01	11.82±0.53
NSE	1.01±0.02	1.06±0.03	1.05±0.01	14.49±0.62
18s rRNA	1.00±0.03	1.01±0.01	0.99±0.01	1.02±0.02

DAT, dopamine transporter; TH, tyrosine hydroxylase; NSE, neuron-specific endolase.

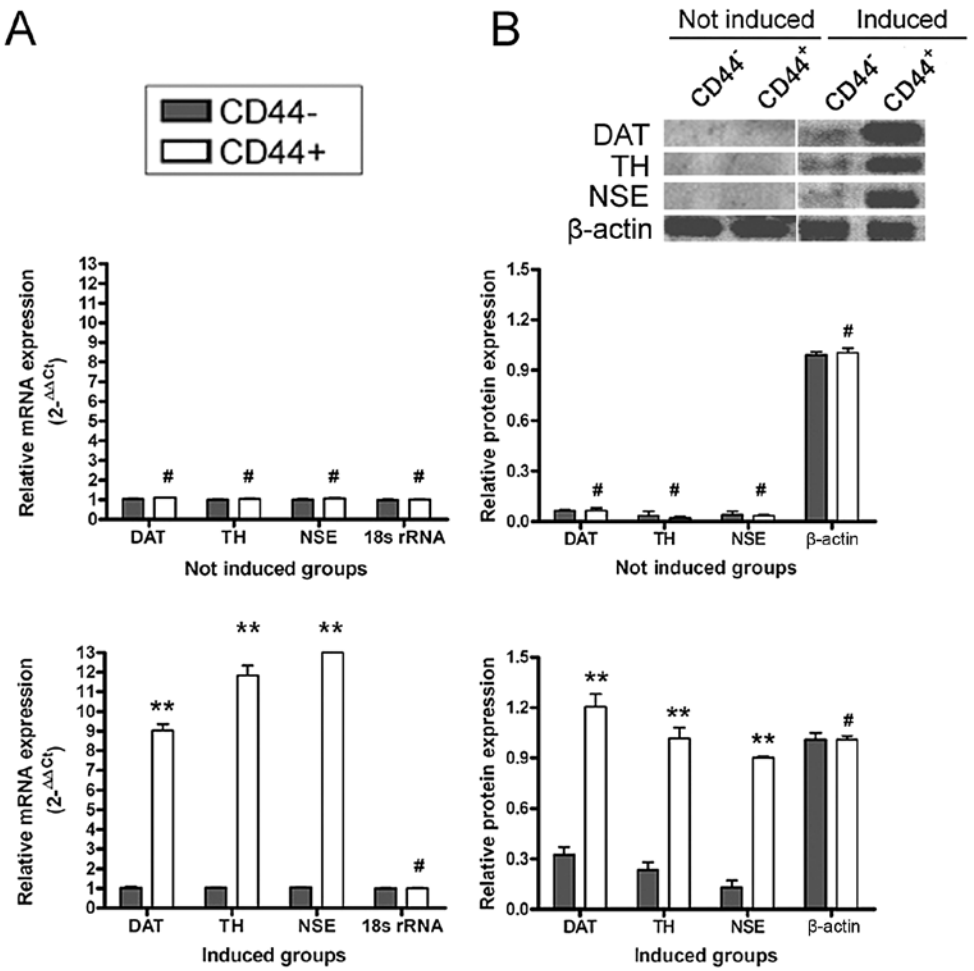

Figure 3. Analysis of dopaminergic neuron marker expression by quantitative real-time polymerase chain reaction (qRT-PCR) and Western blotting assay. (A) The results of the qRT-PCR experiment indicated there was little to no expression of mRNAs of dopaminergic neuron markers [dopamine transporter (DAT), tyrosine hydroxylase (TH) and neuron-specific endolase (NSE)] in $CD44^-$ human amniotic fluid cells (HuAFCs) during induction. However, levels of dopaminergic neuron markers were measured, and all were found to be significantly higher in $CD44^+$ HuAFCs than in $CD44^-$ HuAFCs. Relative mRNA expression is shown after normalization to 18S rRNA, which served as an internal control. (B) The protein expression of DAT, NSE and TH was tested by Western blotting between $CD44^-$ HuAFCs group and $CD44^+$ HuAFCs group. In $CD44^+$ HuAFCs group, levels of DAT, TH and NSE were 1.205±0.075, 1.015±0.065 and 0.900±0.01 of β-actin levels, respectively. These values were significantly higher than DAT, TH and NSE in the $CD44^-$ HuAFCs group (0.325±0.045, 0.235±0.043 and 0.13±0.03 of β-actin control levels, respectively). $^{**}P<0.01$; $^{\#}P>0.05$.

HuAFCs. GFP-expressing cells were transplanted and were observed for 3 consecutive weeks. As anticipated, we found that GFP-positive $CD44^+$ HuAFCs could be found along the injection tract in the PD rat model brain (Fig. 4), demonstrating that the transplanted $CD44^+$ HuAFCs can survive within the PD rat brain for at least 3 weeks *in vivo*, and morphological changes can be seen after that. However, GFP-positive $CD44^-$ HuAFCs could not be found along the injection tract in the PD rat brain. This finding demonstrated that the grafted $CD44^+$ HuAFCs survived. For cell migration, we found that $CD44^+$

Table II. The average rotation numbers (rotations/30 min, n=5).

Time (weeks)	CD44+ HuAFCs group	CD44- HuAFCs group	Vehicle group
0	245±10	240±15	250±14
2	165±13	225±13	235±18
4	150±13	205±12	220±10
6	155±12	195±13	205±16
8	130±10	180±8	185±14

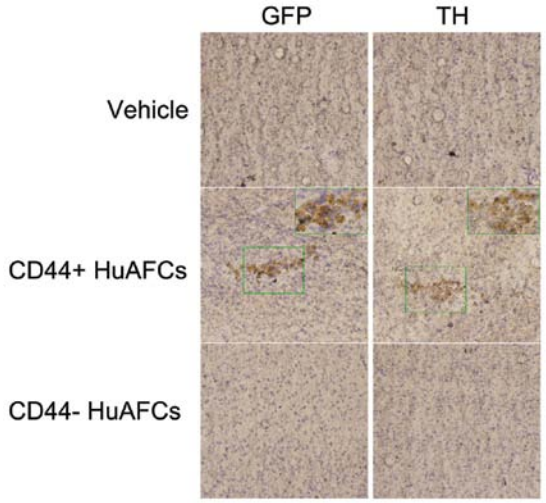

Figure 4. ICH analysis of survival and differentiation of transplantation of CD44+ or CD44- human amniotic fluid cells (HuAFCs) *in vivo*. The results of an ICH assay on tyrosine hydroxylase (TH) and green fluorescent protein (GFP) expression demonstrated that the TH and GFP were highly expressed in the CD44+ HuAFCs transplanted in the Parkinson's disease (PD) rat model brain, and they could survive within the PD rat brain for at least 3 weeks *in vivo*. However, CD44- HuAFCs could not be found along the injection tract in the PD rat brain. This assay indicated that grafted CD44+ HuAFCs could be observed to survive and differentiate into dopaminergic neurons at the ventral tegmental area (VTA) and in the medial forebrain bundle (MFB) regions of injured rats. Original magnification, x100.

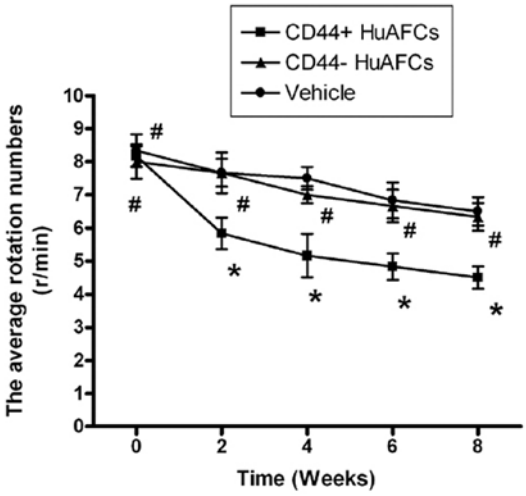

Figure 5. Behavior analysis of a Parkinson's disease (PD) rat model by apomorphine-induced rotations after transplantation of CD44+ or CD44- human amniotic fluid cells (HuAFCs). Apomorphine-induced rotations decreased significantly in CD44+ HuAFCs transplantation group compared with the vehicle group (P<0.05, n=5). However, there was no significant difference in the behavior of vehicle group compared with CD44- HuAFCs transplantation group (P>0.05, n=5). This result suggests that CD44+ HuAFCs can aid recovery.

HuAFCs migrated from the graft for 3 consecutive weeks after transplantation (Fig. 4), and that GFP-transduced CD44+ HuAFCs showed that the migrated cells were present in the PD rat brain for 3 consecutive weeks. When the insets are enlarged the neuronal-like cells can be detected 3 weeks later.

We found some rat-specific TH antibody-labeled CD44+ HuAFCs in the ischemic area (Fig. 4) and in the vicinity of the infarct area; there were no positive cells in CD44- HuAFCs-treated PD rats or in PBS-treated PD rats. These results suggest that CD44+ HuAFCs migrate in the PD rat brain. To determine the pattern of differentiation of transplanted HuAFCs in the PD rat brain, we immunostained the PD rat brain tissue with rat-specific anti-TH antibodies. We found that TH-positive cells were found along the injection tracts (Fig. 4) at 3 weeks after transplantation in CD44+ HuAFCs-treated PD rat; there were no positive cells in CD44- HuAFCs-treated PD rats or PBS-treated PD rats. Thus, transplanted CD44+ HuAFCs express the dopaminergic neuron marker of TH, and may retain the potential to differentiate into dopaminergic neurons. However, CD44- HuAFCs could not be found.

CD44+ HuAFCs stimulate behavioral and functional recovery of the PD rat model. To assess the functional effects of the CD44+ or CD44- HuAFCs transplantation in PD model rats, the rotational behavior of the rats was examined for over 30 min following i.p. injection of apomorphine (Table II, Fig. 5). Apomorphine-induced rotations decreased significantly in the CD44+ HuAFCs transplantation group compared with the vehicle group (P<0.05, n=5). Furthermore, in the CD44+ HuAFCs transplantation group, the rotational behavior was varied significantly between the different weeks (P<0.05, n=5). However, there was no significant difference in the behavior of the vehicle group compared with the CD44- HuAFCs transplantation groups (P>0.05, n=5). These results suggest that treatment with CD44+ HuAFCs is a more effective approach towards recovery and that CD44+ HuAFCs is favorable for behaviorial and functional recovery of the PD rat model.

Discussion

PD is a widespread neurodegenerative disorder, affecting 1% of those over the age of 60 years (3,6). To date, no curative therapies have been developed that prevent disease onset or protect the remaining neurons, therefore drug or surgical therapies to

reduce and alleviate symptoms remain the treatment of choice. At present, laboratory-based cell-replacement therapies may provide the most promising curative treatment for PD. Out of all the cell types, the question still to be answered is which stem cells have the greatest potential for dopamine neuron replacement in PD. At present it seems that the ESCs, iPS and NSCs show the greatest efficiency in generating functional midbrain dopaminergic neurons (1,3-5). However, the safety aspects must also be considered. It is not ethically acceptable to inject cells to humans when they may retain the potential to divide *in vivo*; therefore, there is a need to generate a cell source that is free from proliferating cells (3).

Our previous studies have indicated that HuAFCs express many growth factors, as well as the stem cell markers Nanog, Oct-4 and Nestin. These cells only express low levels of major histocompatibility complex class I antigens and are more easily accessible than other adult stem cells, making them a potential autologous donor source for stem cell therapy. Therefore, we set out to determine which subpopulation of HuAFCs differentiate into dopaminergic neuronal-like cells, and whether the $CD44^+$ subpopulation could influence the speed and effectiveness of dopaminergic neuron cell induction.

First, we isolated and enriched the $CD44^+$ subpopulation of HuAFCs from human amniotic fluids and determined they were pluripotent, in agreement with previous reports (22). Induced differentiation activated a number of genes related to dopaminergic neuron cell development and function in HuAFCs, indicating that these cells have the capacity to differentiate as dopaminergic neuron cells with neuron factor-sensing ability. To prove that HuAFCs can be induced to differentiate into the dopaminergic neuron cell-like cells, and that the $CD44^+$ subpopulation has an important role in this process, dopaminergic neuron cell markers were detected by Western blotting, qRT-PCR and IF. We found that dopaminergic neuron cell markers were more highly expressed in the $CD44^+$ HuAFCs-transfected group than in the $CD44^+$ HuAFCs-transfected group, indicating that the CD44 factor-positive subpopulation is indeed important in this process. Furthermore, the $CD44^+$ or $CD44^-$ HuAFCs were transplanted into 6-OHDA-treated PD rats. The results of this approach indicated that $CD44^+$ HuAFCs can differentiate into dopaminergic neurons in the VTA and MFB, and migrate around the lesion site. In addition, these cells were conducive to behavior recovery of PD rats *in vivo*. However, $CD44^-$ HuAFCs did not fully differentiate into dopaminergic neuronal-like cells. These observations implicate that tissue reconstruction produced by the transplanted $CD44^+$ HuAFCs is needed before there is functional recovery. Such a tissue repair mechanism is probably required for stable recovery. Potential problems with this $CD44^+$ HuAFCs therapy include developing techniques designed to promote better graft survival.

In conclusion, we have provided an insight into how HuAFCs and PD rat brain tissue interact with each other to promote the recovery in the PD rat model. Analysis of the transplanted cells and the host tissue indicated that $CD44^+$ HuAFCs play a critical role for cell survival, migration and differentiation into dopaminergic neuron cells. Behavioral tests further showed that $CD44^+$ HuAFCs can significantly improve injury repair. Based on this data, we conclude that $CD44^+$ HuAFCs can be induced to differentiate into cells with the function and characteristics of dopaminergic neuron cells and they could be used as a neuron-replacement population. Combined cell therapy and pharmacological treatment at the cellular and organismal levels may provide an in-depth understanding of theoretical mechanisms and clinical applications in PD recovery.

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