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Original Research Report

Induction of Human Mesenchymal Stem Cells into Dopamine-Producing Cells with Different Differentiation Protocols

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ABSTRACT

Several reports have shown that human mesenchymal stem cells (MSCs) are capable of differentiating outside the mesenchymal lineage. We sought to induce MSCs to differentiate into dopamine-producing cells for potential use in autologous transplantation in patients with Parkinson's disease (PD). Following cell culture with various combinations of differentiation agents under serum-free defined conditions, different levels of up-regulation were observed in the protein expression of tyrosine hydroxylase, the rate-limiting enzyme in dopamine synthesis. Further analysis of selected differentiation protocols revealed that the induced cells displayed a neuron-like morphology and expressed markers suggesting neuronal differentiation. In addition, there was an increase in Nurr 1, the dopaminergic transcription factor gene, concomitant with a decrease in γ-aminobutyric acid (GABA)ergic marker expression, suggesting a specific dopaminergic direction. Moreover, the induced cells secreted dopamine in response to depolarization. These results demonstrate the great therapeutic potential of human MSCs in PD.

INTRODUCTION

PARKINSON'S DISEASE (PD) is a neurodegenerative disorder affecting more than 1.5% of the elderly population [1]. It is characterized pathophysiologically by severe degeneration of the dopaminergic neurons in the midbrain, leading to impaired motor function. The specificity of the damaged cells makes PD an attractive candidate for cell transplantation therapy [2]. This concept was supported by pioneer works involving the successful engraftment of fetal tissues in patients with PD [3,4]. However, although the embryonic stem (ES) cell field has made impressive progress in establishing methods to generate dopaminergic neurons in vitro and has even had limited success in animal models [5,6], teratoma formation remains a great concern [7]. Moreover, the use of fetal or ES cells, although ideal, not only requires allograft transplantation with its inherent risk of immune rejection [8], it also poses major ethical concerns.

Bone marrow mesenchymal stem cells (MSCs) are known for their ability to adhere to cell culture plastic surfaces and to expand and proliferate extensively [9]. Under specific conditions, MSCs can differentiate into various mesenchymal phenotypes, such as bone, cartilage, and

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fat [10,11]. Several studies have shown that both human and rodent MSCs are also capable of differentiating into neuron-like cells in vitro [12,13]. Previously, we found that even in the absence of induction medium, MSCs express a basal level of neural genes and proteins, some of which are associated with dopaminergic phenotypes [14].

Developmental neurobiology and ES cell research on the extrinsic signals involved in dopaminergic cell differentiation and survival have suggested the importance of a wide range of factors, such as retinoic acid (RA), estrogen, and a spectrum of cytokines and neurotrophic factors [15–17]. Most reports implied that the elevation in intracellular cAMP levels plays a crucial role in the differentiation process [18]. However, the compatible dopaminergic differentiation of bone marrow-derived cells requires that they cross from a mesodermal to a neuroectodermal lineage. The possibility of such transdifferentiation is still being debated [19,20].

In the present study, the primary objective was to develop a controlled system that efficiently yields dopamine-producing MSCs under defined conditions. Specifically, we examined the effect of multiple combinations of induction agents on the protein expression level of tyrosine hydroxylase (TH), the rate-limiting enzyme in dopamine synthesis. The protocols that induced the most robust TH expression were then further analyzed and were found to drive the cells toward a neuronal phenotype. Two different combinations yielded dopamine-secreting cells, which may, in the future, serve as a compatible source for cell therapy in patients with PD.

MATERIALS AND METHODS

MSC isolation, culture, and characterization

Fresh bone marrow aspirates harvested from the iliac crests of adult donors (age 19–76) were diluted with Hanks balanced salt solution (HBSS; Biological Industries). Mononuclear cells were isolated by centrifugation in Unisep-Maxi tubes (Novamed, Jerusalem, Israel) by density gradient. The cells were then plated in Dulbecco's modified Eagle medium (DMEM) supplemented with 15% fetal bovine serum (FBS), 2 mM glutamine, 100 μg/ml streptomycin, 100 U/ml penicillin, and 12.5 U/ml nystatin (Biological Industries, Beit HaEmek, Israel) in polystyrene plastic 75-cm^2 tissue culture flasks. After 24 h, nonadherent cells were removed. The medium was changed every 3–4 days. Adherent cells were cultured to 70–90% confluence and reseeded at a density of 5,000–10,000 cells/cm^2. The cells were maintained at 37°C in a humidified 5% CO_2 incubator. The MSCs were characterized for their cell-surface phenotype and their mesenchymal differentiation capacity, as previously described [14].

Induction of differentiation

The differentiation protocol was composed of two steps. In step 1, cells were transferred to serum-free medium (DMEM) supplemented with 2 mM glutamine, 100 μg/ml streptomycin, 100 U/ml penicillin, 12.5 U/ml nystatin, N2 supplement (Invitrogen, New Haven, CT), and 20 ng/ml fibroblast growth factor-2 (FGF-2) and epidermal growth factor (EGF) (both from R&D Systems, Minneapolis, MN) for 48–72 h. In step 2, the medium was changed to basic induction medium containing Neurobasal and B27 (both from Invitrogen), in addition to 1 mM dibutyryl cyclic AMP (db-cAMP), 3-isobutyl-1-methylxanthine (IBMX), and 200 μM ascorbic acid (all from Sigma, St Louis, MO). The following factors were added to the basic induction medium in different combinations: brain-derived neurotrophic factor (BDNF, 50 ng/ml), glial-derived neurotrophic factor (GDNF, 10 ng/ml), neurturin (100 ng/ml), neurotrophin 3 (NT3, 50 ng/ml), and fibroblast growth factor-8 (FGF-8, 100 ng/ml) (all from Cytolab, Rehovot, Israel); transforming growth factor-β 3 (TGF-β3, 2 ng/ml), sonic hedgehog (Shh. 500 ng/ml) (both from R&D), estrogen (0.1 μM, Sigma), and all-*trans*-retinoic acid (RA, 0.1 μM; Sigma).

Western blot

Total protein was extracted by suspending the harvested cells in lysis buffer containing 10 mM Tris base (U.S. Biochemical Corporation, Cleveland, OH), 5 mM EDTA (Merck, Whitehouse Station, NJ), 140 mM sodium chloride (NaCl; BioLab, Jerusalem, Israel), 10 mM sodium fluoride (NaF; Sigma), 0.5% NP-40 (U.S. Biochemical Corporation), and 1 μM phenylmethylsulfonyl fluoride (PMSF; Sigma). Following incubation on ice for 30 min, the mixture was centrifuged and the supernatants were collected. Protein content was determined by the BCA protein assay kit (Pierce, Rockford, IL). Twenty five micrograms of protein from each sample was subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SCD-PAGE) (12.5% acrylamide), followed by electrophoretic transfer to nitrocellulose membrane (Schleicher & Schuell, Dassel, Germany). Membranes were probed by mouse anti-TH (1:10,000; Sigma) and mouse anti β-actin (1:10,000; Chemicon, Billerica, MA) and then by goat anti-mouse HRP-conjugated antibody (1:10,000; Jackson, West Grove, PA) and rabbit anti-emerin (1:5,000; Santa Cruz Biotechnology, Santa Cruz, CA), followed by goat anti-rabbit HRP-conjugated secondary antibody (1:10,000; Sigma). Adequate controls included HeLa cells (ATCC, Manassas, VA) as negative control and neuroblastoma M17 cells (BE(2)-M17, American Type Culture Collection) as positive control. Proteins of interest were detected using the enhanced Super Signal® chemiluminescent detection kit (Pierce).

Immunocytochemistry

Cells were fixed with 4% paraformaldehyde, blocked, and permeabilized in 5% goat serum (Biological Industries), 1% bovine serum albumin (BSA; Sigma), and 0.5% Triton-X in PBS for 1 h at room temperature. Primary antibodies included mouse anti-TH and mouse anti-β3-tubulin (both 1:1,000; Sigma), followed by goat anti-mouse Alexa-488 or Alexa-568 (both 1:1,000; Molecular Probes). DNA-specific fluorescent dye 4′,6-diamidino- 2-phenylindole (DAPI; Sigma) counterstain was used to detect cell nuclei. Cells were photographed

with a fluorescence Olympus IX70-S8F2 microscope with a fluorescent light source (excitation wavelength, 330–385 nm; barrier filter, 420 nm) and a U-MNU filter cube (Olympus, Center Valley, PA).

RNA isolation and cDNA synthesis

Total RNA was isolated by a commercial TriReagent (Sigma). The amount and quality of RNA was determined with the ND-1000 spectrophotometer (NanoDrop, Wilmington, DE). First-strand cDNA synthesis was carried out with Super Script II RNase H-reverse transcriptase (Invitrogen) using a random primer.

Real-time quantitative reverse transcription PCR

Real-time quantitative (Q) PCR of the desired genes was performed in an ABI Prism 7700 sequence detection system (Applied Biosystems, Foster City, CA) using Syber Green PCR Master Mix (Applied Biosystems) and the following primers: glyceraldehyde-3-phosphate dehydrogenase (GAPDH), sense, CGACAGTCAGCCGCATCTT, GAPDH, antisense, CCAATACGACCAAATCCGTTG; Nurr1, sense, GGATGGTCAAAGAAGTGGTTCG, Nurr1, antisense, CCTGTGGGCTCTTCGGTTT; glutamic acid decarboxylase 67 (GAD67), sense, CGAGGACTCTGGACAGTAGAGG, GAD67, antisense, GATCTTGAGCCCCAGTTTTCTG. The GAPDH gene served as an internal control. For each gene, the specificity of the PCR product was assessed by verifying a single peak in the melting curve analysis. The PCR was performed in a total volume of 20 μl containing 1 μl of the previously described cDNA, the 3′ and 5′ primers at a final concentration of 500 nM each, and 10 μl of Syber Green Mix. The amplification protocol was 40 cycles of 95°C for 15 sec followed by 60°C for 1 min each. Quantitative calculations of the gene of interest versus GAPDH were done according to the $\Delta\Delta$CT method, as instructed in the user bulletin of the ABI Prism 7700 sequence detection system (updated 10/2001).

Intracellular fluorescence-activated cell sorter analysis

Cells were harvested from the tissue culture flasks, centrifuged, and resuspended in phosphate-buffered saline (PBS). Intracellular staining was performed with the IntraCyte kit (Orion Biosolutions, Vista, CA), according to the manufacturer's instructions. TH staining was performed with mouse anti-TH antibody (1:1,000; Sigma) followed by donkey anti-mouse phycoerythrin (PE)-conjugated immunoglobulin G (IgG; Jackson Laboratories, Bar Harbor, ME). The results were analyzed with CellQuest software. A PE-conjugated isotype control was included in each experiment. To verify specific detection of TH expression, we employed HeLa cells as a negative control and PC12 (ATCC) cells as a positive control.

High-performance liquid chromotography

Reverse-phase high-performance liquid chromatography (HPLC) coupled with an electrochemical detector (ECD) was used to measure dopamine levels. Briefly, the conditioned medium of the MSCs (growth medium) and the differentiated neuron-like cells (differentiation medium) were harvested, and the cells were incubated in KCl depolarization buffer containing 56 mM KCl in HBSS for 30 min. Following collection, all samples were stabilized with 0.1 M perchloric acid and metabisulfite (2 mg/ml) (Sigma) and extracted by aluminum adsorption (Bioanalytical Systems, West Lafayette, IN). An aliquot of filtrate was applied to the HPLC/ECD (Bioanalytical Systems) equipped with a catecholamine C18 column (125 mm $\times$ 4.6 mm) (Hichrom, Berkshire, UK), with the electrode potential set at +0.65 V relative to the Ag/AgCl reference electrode. The mobile phase consisted of a monochloroacetate buffer (150 mM, pH 3) containing 10% methanol, 30 mg/L sodium 1-octanesulfonate, and 2 mM EDTA. The flow rate was 1.2 ml/min. Dopamine was identified by retention time and validated by co-elution with catecholamine standards under varying buffer conditions and detector settings.

Statistics

Statistical significance was determined by one-way anlaysis of variance (ANOVA) followed by Dunnett's multiple comparison tests.

RESULTS

Phenotypic characterization of human MSCs

The MSCs were characterized according to the previously published position paper by Dominici et al. [21]. They expressed mesenchymal markers such as $CD29^+$, $CD44^+$, and $CD105^+$ and did not express the hematopoietic markers $CD45^-$ and $CD34^-$. Moreover, the cells were sufficiently induced to differentiate into adipocytes and osteoblasts (data not shown).

Various differentiation protocols up-regulate TH expression

Western blot analysis of TH protein expression, normalized to the housekeeping gene actin, revealed that the basic induction medium was associated with a moderate increase in TH protein level; the addition of neurotrophic factors further facilitated this effect. In particular, supplementation of the basic induction medium with Shh, TGF-β3, GDNF, BDNF, and estrogen directly affected the TH protein expression level. However, adding more than one differentiation-inducing factor did not necessarily facilitate TH up-regulation. The results of 12 different differentiation protocols are summarized in Fig. 1A. The supplementation of antioxidants, such as butylated hydroxyanisole (BHA) and *N*-acetylcysteine (NAC), did not facilitate up-regulation, but rather led to higher levels of cell death in the culture (data not shown).

Of the 12 differentiation protocols, we selected the two that yielded the highest TH expression: one contained

A

Differentiation agents	TH/actin ratio
Basic induction medium	+
+*Shh*	++++
+*Shh* + *FGF8*	+++
+*TGFβ3*	+++++
+*GDNF*	++++
+*GDNF* + *TGFβ3*	++++++
+*BDNF*	+++++
+*Neurturin* + *NT3*	++++
+*Neurturin* + *NT3* +*TGFβ3* +*GDNF* + *BDNF*	+++++
+*Neurturin* + *NT3* + *TGFβ3* +*GDNF* + *BDNF* + *Shh* + *FGF8*	++++
+*Estrogen*	++++
+*GDNF* + *TGFβ3* + *BDNF* +*Estrogen*	++++

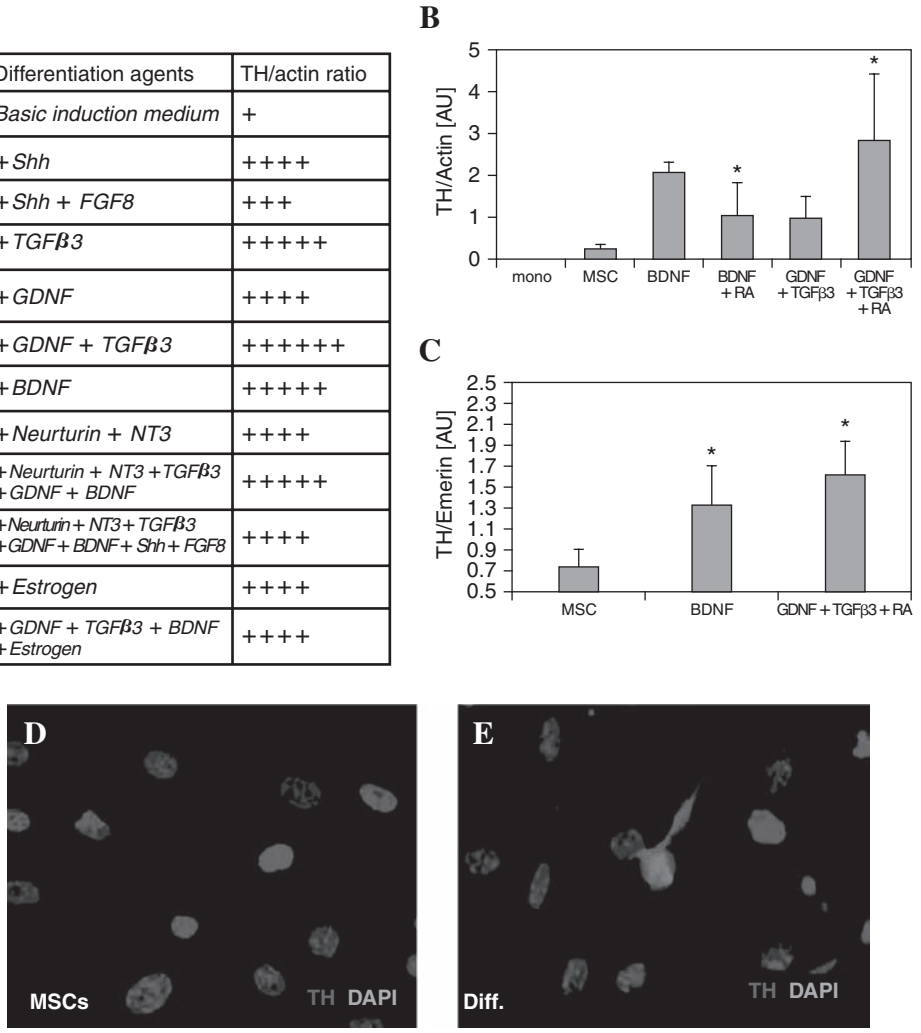

FIG. 1. Various induction protocols induce up-regulation of TH. (**A**) Summary of western blot analyses for TH expression normalized to β-actin following induction protocols based on basic induction medium and various combinations of growth factors, as specified in Materials and Methods. (**B**) Western blot results of TH expression normalized to β-actin following differentiation protocols that included either BDNF or GDNF and TGF-β3, with or without the addition of RA (means $\pm$ SD,* $p < 0.05$). (**C**) Western blot results of TH expression normalized to emerin following differentiation protocols that included either BDNF or GDNF, TGF-β3 and RA (means $\pm$ STD,* $p < 0.05$). (**D–E**) Immunocytochemistry of TH and DAPI nuclear stain in untreated MSCs and in cells differentiated in BDNF containing medium (DIFF).

GDNF, TGF-β3, and RA, and the other, BDNF and RA. To determine the importance of RA, both protocols were carried out with and without its addition. Western blot analysis of TH protein expression normalized to actin revealed that MSCs expressed basal levels of TH and elevated the TH expression levels following four induction protocols. Although all four induction protocols up-regulated TH expression, significant results were obtained with BDNF alone and with the combination of GDNF, TGF-β3, and RA (Fig. 1B). In no experiment did we observe TH expression in our starting bone marrow mononuclear cell sample. Conversely, in most experiments, untreated MSCs expressed basal levels of TH.

Several reports have questioned the ability of β-actin to serve as an internal standard in stem cell differentiation [22]. Therefore, we further verified our results of TH expression normalized to the nuclear envelope protein emerin as a standard (Fig. 1C). We found that at 7 days, both differentiation protocols yielded a significant increase in TH expression compared to untreated MSCs. Elongation of the induction process that included incubation in medium containing Shh and FGF8 for 72 h did not lead to a further increase in TH expression (data not shown). Immunocytochemistry for TH expression confirmed the Western blot analysis (Fig. 1D,E).

Induction results in neuronal phenotype

We next sought to examine whether the induction of TH up-regulation is correlated with neuronal differenti-

ation of the MSCs. Bright-light microscopy revealed that the use of an induction medium containing BDNF was associated with a morphological change in the cells, from the characteristic MSC fibroblast shape to a neuron-like appearance (Fig. 2A,B). Immunocytochemistry revealed that the induced cells expressed higher levels of Tuj1, a neuronal progenitor marker, than the untreated MSCs (Fig. 2C,D). In addition, the induced cells stained positively for the mature neuronal nuclear specific antigen NeuN (Fig. 2E,F). We found no $NeuN^+$ cells following induction with GDNF, TGF-β3, and RA (data not shown).

BDNF-mediated cell differentiation is dopaminergic-specific

The characteristics of the MSCs following incubation with the BDNF-containing differentiation medium were further investigated. Quantitative real-time PCR revealed a significant up-regulation of the expression of Nurr1, a transcription factor involved in dopaminergic neuron differentiation and maintenance [23,24], compared to untreated MSCs (Fig. 3A). Further study indicated down-regulation of GAD67, a GABAergic marker, indicating that the induction was dopaminergic-specific (Fig. 3B).

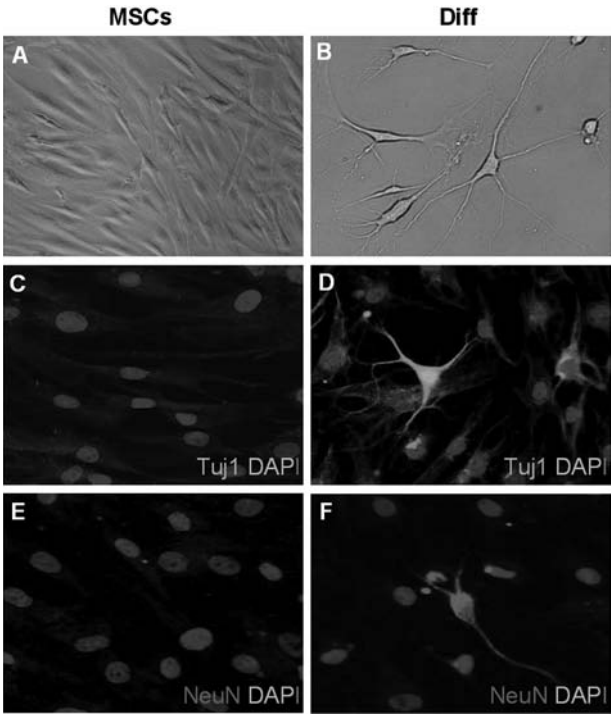

FIG. 2. MSCs express neuronal proteins following BDNF-mediated differentiation. (**A**) Fibroblast-like morphology of MSCs. (**B**) Neuronal morphology of MSCs following differentiation (**C–F**). Immunocytochemistry for neuronal markers Tuj1 and NeuN in differentiated cells and untreated MSCs.

To quantify the yield of the dopamine-directed cells, we performed intracellular FACS analysis of TH expression. More than 30% of the induced cells expressed the TH protein at a detectable level compared to none of the untreated cells (Fig. 3C). Although we found a basal level of TH expression in Western blot analysis, we speculated that the different sensitivities of the methods may have accounted for the different results. HPLC analysis of dopamine secretion showed that prior to induction, dopamine was not detectable in the MSC medium, with or without KCl depolarization. Following induction of differentiation, the cells secreted a mean of 673.69 pg/ml of dopamine per 10^6 cells to the conditioned medium and 1,127.86 pg/ml of dopamine per 10^6 cells to the depolarization buffer (Fig. 3D).

DISCUSSION

The use of differentiated human bone marrow MSCs for the treatment of PD would eliminate the concerns associated with embryonic cells [25]. They do not present an ethical dilemma, their malignant potential after transplantation is relatively low, and they pose no threat of immune rejection. However, fully compatible dopaminergic differentiation of MSCs has yet to be reported. The first studies of the neural differentiation of MSCs were received with great expectations [12,13], but were followed by concerns of some researchers that the plasticity of MSCs is limited and that processes such as cell fusion or sheer stress could have accounted for what was initially interpreted to be neural transdifferentiation [20,26].

Nevertheless, efforts are still being made to generate functional neuronal cells from bone marrow MSCs under defined conditions [27,28]. A few reports described the generation of dopamine-producing cells from MSCs [29–31], but none managed to achieve the fully functioning dopaminergic neuron in terms of electrophysiology and in vivo integration in the brain network. Recently, our laboratory has described the dopaminergic differentiation of MSCs using a defined medium in the absence of neurotrophic factors [32]. However, the induction protocol resulted in significant cell death, and secretion of dopamine was observed only on addition of GDNF to the medium. This work, as well as the report of Tao et al. [33], prompted us to seek a differentiation protocol that would rely more heavily on neurotrophic factors than on chemical agents such as dimethyl sulfoxide (DMSO), β-mercaptoethanol, or BHA, and which would allow cell incubation for a longer time with less cell death to improve future in vivo applications.

The objective of the present study was to develop a serum-free controlled differentiation protocol that would yield dopamine-producing cells from bone marrow-de-

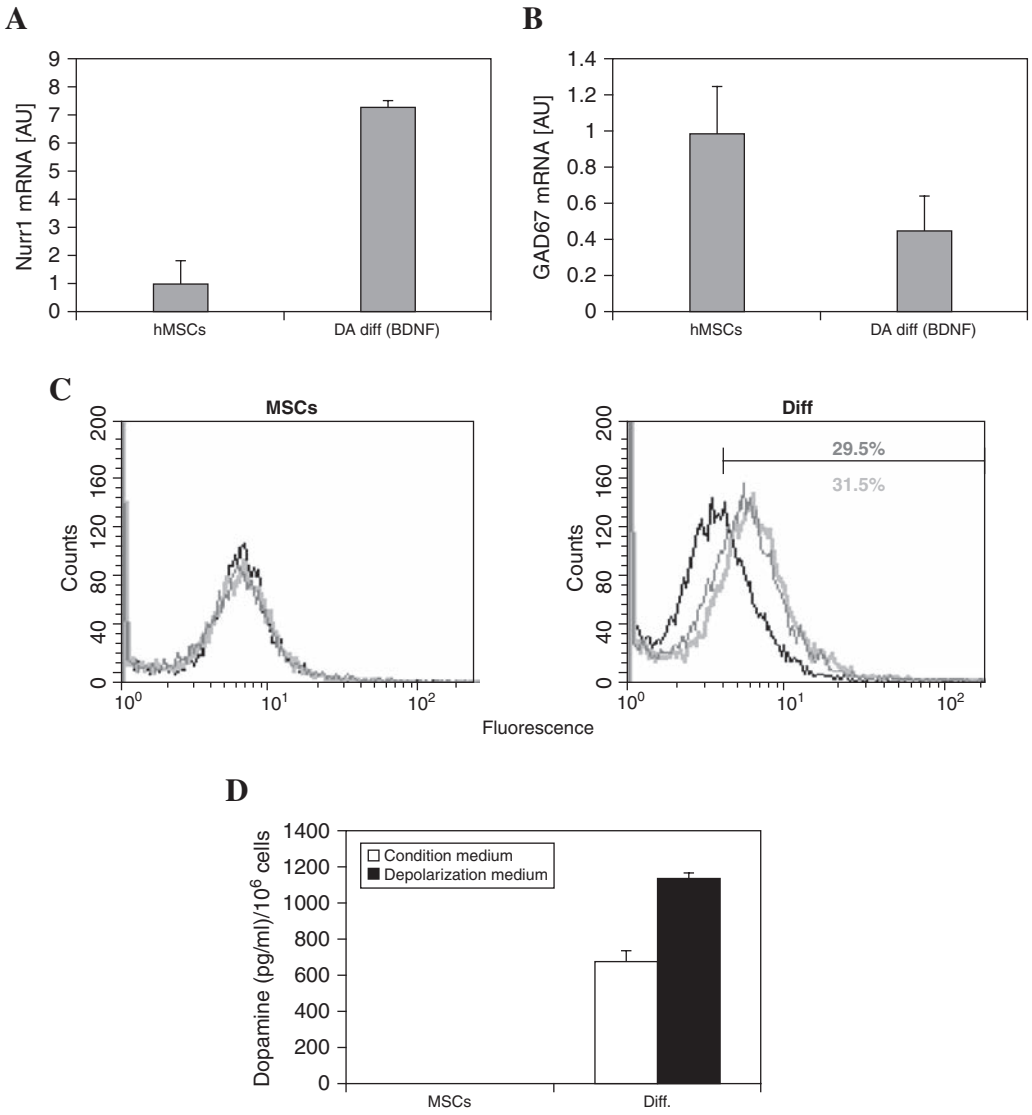

FIG. 3. Dopaminergic characteristics of differentiated MSCs. (**A** and **B**) Real-time PCR analysis of the dopaminergic transcription factor Nurr1 and the GABAergic marker GAD67. (**C**) Intracellular FACS analysis of TH in untreated and differentiated MSCs (black line represents PE-conjugated isotype control; green and pink lines represent anti-TH staining). (**D**) HPLC analysis of dopamine secreted by MSCs and differentiated cells to the conditioned medium (growth medium/differentiation medium) and to the KCL HBSS depolarization buffer (means ± STD).

rived cells. We employed a basic two-step induction procedure previously developed in our laboratory [32]. In the first step, MSCs were transferred to a serum-free medium containing EGF and FGF-2 for 48–72 h. This was followed by the removal of mitogens and incubation in an induction medium containing cAMP and IBMX, which increases intracellular cAMP levels and ascorbic acid, for another 48–72 h. High cAMP levels have been reported to be involved in neuronal differentiation [20], and ascorbic acid is directly linked to dopaminergic differentiation [34]. The induction medium was enriched with several neurotrophic factors, such as, BDNF, GDNF, neurturin, and NT3, all of which have been found to play a role in dopaminergic differentiation [17,35]. We also tested the effect of estrogen and TGF-β3, which are involved in both the differentiation and the survival of dopamine neurons [36]. Other agents included Shh and FGF-8, which are known to be involved in the development of the mesencephalic dopaminergic neurons [37]. Our analysis revealed that all the induction protocols up-regulated the basal level of TH expression. The most significant up-regulation was obtained with BDNF alone or with the combination of GDNF, TGF-β3 and RA.

Further analysis by immunocytochemistry for neuronal markers and by quantitative PCR for neuronal transcripts suggested neuronal induction. The BDNF-mediated pro-

tocol resulted in cells that both expressed dopaminergic markers and secreted dopamine.

These results are in line with previous reports showing the significance of BDNF in the dopaminergic differentiation of MSCs [29,38]. Furthermore, our findings for the combination of GDNF, TGF-β3, and RA, which induced the highest level of TH expression and the secretion of dopamine (data not shown), are in accordance with reports suggesting that these factors act in concert to allow dopaminergic differentiation [39].

We conclude that the use of bone marrow-derived MSCs to generate dopamine secretion in the brain is a realistic goal. Although we still cannot claim to have obtained mature functional dopaminergic neurons from MSCs, the expression of some neuronal markers by the induced cells supports the feasibility of this approach. We assume that in addition to increasing intracellular cAMP levels and inducing the presence of selected neurotrophic factors, more steps need to be taken for functional neuronal transdifferentiation. The addition of polyunsaturated fatty acids [39] is one possibility. Another is genetic manipulation of the MSCs to reprogram their molecular code and direct them to the neuronal lineage via overexpression of an inserted intrinsic signal in the form of a dopaminergic differentiation-associated transcription factor.

In summary, we present, for the first time, a comprehensive comparative analysis of the effect of more than 20 different induction protocols of dopaminergic differentiation on human MSCs. Two different protocols resulted in the generation of dopamine-secreting cells, with more than 30% of the cells expressing significant levels of TH. Future studies are necessary to facilitate dopaminergic differentiation and to test the functional properties of induced MSCs in animal models of PD.

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