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Dopaminergic differentiation of human mesenchymal stem cells—Utilization of bioassay for tyrosine hydroxylase expression

Inna Kan [a,*], Tali Ben-Zur [a], Yael Barhum [a], Yossef S. Levy [a], Alex Burstein [a], Tirza Charlow [a], Shlomo Bulvik [b], Eldad Melamed [a], Daniel Offen [a,*]

[a] *Laboratory of Neurosciences, Felsenstein Medical Research Center, Rabin Medical Center, Beilinson Campus, Tel Aviv University, Petah Tiqwa 49100, Israel*
[b] *Laniado Hospital, Kiryat Sanz, Netanya 42150, Israel*

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Abstract

Parkinson's disease (PD) is a neurodegenerative disorder, caused by a selective loss of dopaminergic neurons in the substantia nigra. In PD, the best therapeutic modalities cannot halt the degeneration. The selective hallmark pathology and the lack of effective treatment make PD an appropriate candidate for cell replacement therapy. Adult autologous bone-marrow-derived mesenchymal stem cells (MSCs) have been investigated as candidates for cell replacement strategies. Several laboratories, including ours, have induced MSCs into neuron-like cells demonstrating a variety of neuronal markers including dopaminergic characteristics, such as the expression of tyrosine hydroxylase (TH). This project aimed to induce MSCs into mature dopamine secreting cells and to generate a bioassay to evaluate the induction. For that purpose, we created a reporter vector containing a promoter of TH, the rate-limiting enzyme in the dopamine synthesis and red fluorescent protein DsRed2. Transfection of human neuroblastoma, dopamine synthesizing, SH-SY5Y cells confirmed the reliability of the constructed reporter plasmid. Following dopaminergic differentiation of the transfected human MSCs cells, TH expressing cells were identified and quantified using flow cytometry. Further study revealed that not only did the differentiated cells activate TH promoter but they also expressed TH protein and secreted dopamine. The reported results indicate that MSCs may be primed in vitro towards a dopaminergic fate offering the promise of innovative therapy for currently incurable human disorders, including PD.

Keywords: Mesenchymal stem cells; Dopaminergic differentiation; Tyrosine hydroxylase; L-Dopa; Dopamine

Parkinson's disease (PD) is a neurodegenerative disorder, attributed to the degeneration of dopaminergic neurons in the substantia nigra. Although several treatments have been shown to modify the course of the disease, none successfully halted the degeneration [1]. Owing to these factors, cell replacement therapy to replace the degenerated dopaminergic cells may serve as a valuable alternative to achieve significant clinical improvement.

Adult autologous bone-marrow-derived mesenchymal stem cells (MSCs) have been proposed as candidates for cell replacement therapy of neurodegenerative diseases. These cells are able to differentiate beyond tissues of mesodermal origin into neuron-like cells, demonstrating a variety of neuronal markers and transcription factors [2,3,5,7,12,14,15,18,19]. To our knowledge, there has been only one published study in which non-transformed human MSCs were induced in vitro into mature neuron-like cells showing dopamine synthesis [5]. In the above-mentioned study, dopamine production required supplementation of tetrahydrobiopterin (BH4), a cofactor for dopamine synthesis, potentially limiting the clinical relevance of the differentiated cells. Therefore, the major limiting factor of MSC-derived replacement therapy of PD is the difficulty in obtaining sufficient functional dopamine secreting cells.

This project aimed to induce MSCs into mature dopamine secreting cells and to generate a bioassay to evaluate the induction. For this purpose, we successfully generated a vector of red fluorescent protein (RFP) under the promoter of human tyrosine hydroxylase (TH), the rate-limiting enzyme in the dopamine synthesis. Following the induction of dopaminergic differentiation the activation of TH promoter was evaluated and quantified

* Corresponding authors. Tel.: +972 3 9376130; fax: +972 3 9211478.
E-mail addresses: innakan@post.tau.ac.il (I. Kan), doffen@post.tau.ac.il (D. Offen).

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utilizing the generated assay. In addition, we addressed the expression of TH protein and the secretion of dopamine in the differentiated cells.

TH vector was generated from the promotorless plasmid pDsRed2-1 (Clontech, Palo Alto, CA) and pHGTLI-190 plasmid (kindly given by Karen L. O'Malley, School of Medicine, Washington University). The plasmids were cloned, extracted and digested with *Hin*dIII restriction enzyme (MBI Fermentas, Hanover, MD). Human TH promoter 5′-flanking region of about 2420 bp, obtained by *Hin*dIII digestion of the PHGTLI-190, was cloned directly into multiple cloning site of pDsRed2-1 vector, digested by *Hin*dIII, upstream of the promotorless reporter gene encoding a variant of RFP-DsRed2 to generate TH190. TH190 was transformed into competent bacteria and grown on agar plates with 50 μl/ml Kanamicin antibiotics. Restriction analysis and PCR reaction using 22 bases sense primer 5′ tgg gcc ata gag ggg ctg agt g 3′, homological to TH promoter sequence, and 22 bases antisense primer 5′ gtc ccc tcc atg cgc acc ttg a 3′, homological to pDsRed2-1 sequence, followed by sequencing verified the incorporation and the correct orientation of the insert.

Human neuroblastoma cells, SH-SY5Y (ATCC, Manassas, VA), were maintained in Dulbecco's modified Eagle's medium (DMEM; Biological Industries, Bet-Haemek, Israel) supplemented with 100 μg/ml streptomycin, 100 U/ml penicillin, and 12.5 U/ml nystatin (SPN; Biological Industries), 10% fetal calf serum (FCS; Biological Industries) and 2 mM L-glutamine (Biological Industries). TH190 and pDsRed2-1 (control) plasmids were transfected into SH-SY5Y cells with LipofectAMINE Plus reagent (Invitrogen, Carlsbad, CA). The expression of DsRed2 protein was observed by Olympus IX70-S8F2 microscope and pictures were taken by Olympus digital camera (DP50) with ViewfinderLite™ software (Olympus). Picture analysis was carried out using StudioLite™ software (Olympus).

The study was approved by the Helsinki ethical committee of the Israeli Ministry of Health and Tel-Aviv University, and individual informed consent was obtained from donors. MSCs were isolated as previously described [2] and cultured in growth medium. Growth medium consisted of DMEM supplemented with 15% FCS, 2 mM L-glutamine and SPN. The cell cultures were maintained at 37 °C in a humidified 5% CO_2 and 20% O_2 incubator. When cells reached 70–90% confluency, cultures were harvested with trypsin–EDTA solution (0.25% trypsin and EDTA 1:2000 in Puck's saline) (Biological Industries). MSCs were transfected with TH190 and pDsRed2-1 (control) plasmids with LipofectAMINE 2000 reagent (Invitrogen). Following transfection, cells were induced into dopaminergic neurons and the expression of DsRed2 was evaluated by fluorescent microscope and fluorescence-activated cell sorting (FACS) analysis. For the quantification of cells activating TH promoter, MSCs were transfected with TH190 with LipofectAMINE 2000 reagent followed by G418 sulfate (Calbiochem, La Jolla, CA) antibiotics selection and dopaminergic differentiation. DsRed2 expression was evaluated by fluorescent microscope and quantified by FACS analysis.

Dopaminergic differentiation was induced by a previously described protocol [2]. Briefly, MSCs were cultured for at least 14 days, as described above. Growth medium was replaced with differentiation medium I consisting of DMEM supplemented with 10% FCS, 2 mM glutamine, SPN, 10 ng/ml basic fibroblast growth factor (bFGF; R&D Systems, Minneapolis, MN), 10 ng/ml epidermal growth factor (EGF; R&D systems) and N2 supplement (5 μg/ml insulin, 20 nM progesterone, 100 μM putrescine, 30 nM selenium, 100 μg/ml transferrin). Forty-eight hours later, the medium was replaced with differentiation medium II containing DMEM supplemented with SPN, 2 mM L-glutamine, N2 supplement, 200 μM butylated hydroxyanisole (BHA; Sigma, St. Louis, MO), 1 mM dibutyryl cyclic AMP (dbcAMP; Sigma), 3-isobutyl-1-methyl-xanthine (IBMX; Sigma) and 1 μM all-trans-retinoic acid (RA; Sigma) for 48 h. For high-performance liquid chromatography (HPLC) analysis, following 48 h incubation in differentiation medium I, cells were exposed to differentiation medium II supplemented with 10 ng/ml human glial cell line-derived neurotrophic factor (GDNF; R&D systems) for 96 h.

For FACS analysis, the cells were harvested by incubation in trypsin/EDTA solution resuspended in 0.5 ml PBS and studied by a FACSCaliburTM flow cytometer utilizing CELLQuestTM version 3.0 software (Becton Dickinson, San Jose, CA). Specific staining was measured from the cross-point of control curve and treatment curve.

Protein extraction and Western blotting were prepared as described previously [7]. In short, 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 30 min incubation on ice, the mixture was centrifuged and supernatants were collected. Protein content was determined by BCA protein assay kit (Pierce, Rockford, IL). Twenty-five micrograms of protein from each sample was subjected to SDS-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) followed by goat anti-mouse horseradish peroxidase conjugated antibody (1:10,000) (Jackson, West Grove, PA) and rabbit anti-emerin (1:5000) (Santa Cruz, Delaware Avenue, CA), followed by goat anti-rabbit horseradish peroxidase conjugated secondary antibody (1:10,000) (Sigma). Proteins of interest were detected using the enhanced Super Signal® chemiluminescent detection kit (Pierce).

Reverse-phase HPLC coupled with an electrochemical detector (ECD) was used to measure dopamine levels. Briefly, the conditioned mediums of MSCs (growth medium) and differentiated neuron-like cells (differentiation medium) were harvested and cells were incubated in KCL depolarization buffer containing 56 mM KCL in Hank's balanced salt solution (HBSS; Biological Industries) for 30 min. Following the collection all the samples were stabilized with 0.1 M perchloric acid/metabisulfite (2 mg/ml) (Sigma), and extracted by aluminum adsorption (Bioanalytical Systems, West Lafayette, IN). An aliquot of filtrate was applied to HPLC/ECD (Bioanalytical Systems) equipped with a catecholamine C18 column (125 mm × 4.6 mm)

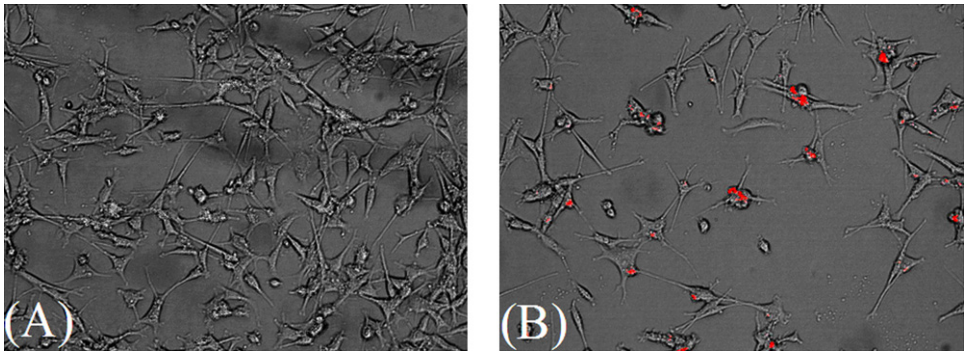

Fig. 1. Transfection of human neuroblastoma cells (SH-SY5Y). SH-SY5Y cells were transfected with promotorless pDsRed2-1 plasmid (negative control) (A) and with TH190 plasmid (B). Fluorescent microscope detected the appearance of DsRed2 color, expressed under the control of TH promoter.

(Hichrom, Berkshire, UK) and with the electrode potential set at +0.65 V versus 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. Flow rate was 1.2 ml/min. L-3,4-Dihydroxyphenylalanine (L-dopa) and dopamine were identified by retention time and were validated by co-elution with cathecolamine standards under varying buffer conditions and detector settings.

In order to verify the reporter activity of the constructed TH reporter vector—TH190, TH190 and pDsRed2-1 plasmid (negative control) were transfected into human dopaminergic neuroblastoma cells—SH-SY5Y. Two days following the transfection the expression of the RFP was identified only in the TH190 transfected cells (Fig. 1).

Following the transfection of SH-SY5Y cells, we transfected MSCs with TH190 or pDsRed2-1 plasmid (negative control) in order to provide evidence that the RFP expression was not the outcome of the transfection process itself. The transfected cells underwent induced dopaminergic differentiation. FACS analysis revealed an increase in the amount of RFP expressing cells within the differentiated cells transfected with TH190 versus cells transfected with pDsRed2-1 (Fig. 2).

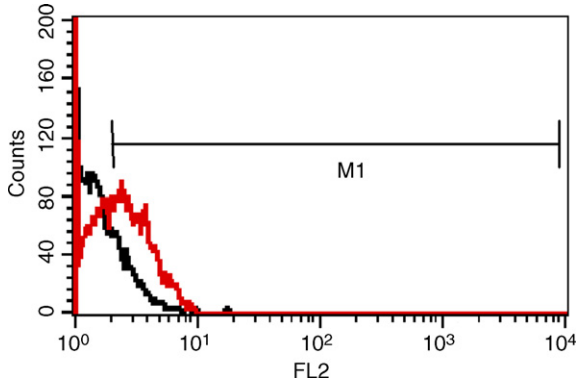

Fig. 2. Detection of TH promoter activation in dopaminergic neuron-like cells. MSCs were transfected with TH190 plasmid or pDsRed2-1 plasmid (control) and induced to differentiate into dopaminergic neurons. Flow cytometry determined the expression of DsRed2 in cells transfected with TH190 (red line) or pDsRed-1 (black line). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

To quantify the elevation in the amount of RFP expressing cells following induced dopaminergic differentiation of MSCs we achieved stable transfection of MSCs with TH190 vector. A part of the stably transfected cells underwent dopaminergic induction. Fluorescent microscope revealed a small number of MSCs expressing RFP before differentiation (Fig. 3A). Following differentiation, we observed a distinct increase in the amount of the RFP expressing cells (Fig. 3B). FACS analysis indicated more specific data: induction of dopaminergic differentiation resulted in an increase of 31% in the amount of RFP expressing cells (Fig. 3C). A repetition of the experiment yielded an increase of 38% in the amount of RFP expressing cells (data not shown).

Western blot analysis of TH protein expression normalized to the nuclear membrane protein, emerin, showed that MSCs expressed basal levels of TH and raised the TH expression levels following dopaminergic induction (Fig. 4A). The experiment was repeated more than three times. HPLC analysis of L-dopa secretion showed that prior to induction, MSCs secreted 14.4 pg/ml per 2×10^5 cells before KCL depolarization and 31.0 pg/ml per 2×10^5 cells after incubation with 56 mM KCL. Following induction, dopaminergic neuron-like cells secreted 152.2 pg/ml per 2×10^5 cells of L-dopa before KCL induced depolarization and 33.6 pg/ml per 2×10^5 cells after depolarization (Fig. 4B). Dopamine was not detectable in the MSCs medium with or without KCL depolarization. Following dopaminergic induction, only the cells incubated with 56 mM KCL, secreted dopamine (123.8 pg/ml per 2×10^5 cells) (Fig. 4C). Additional experiments with MSCs obtained from several human donors confirmed the secretion of dopamine following GDNF enriched dopaminergic induction and KCL depolarization (data not shown).

In the present study, we generated a reporter plasmid that expressed RFP following the activation of the TH promoter. By using the latter assay we revealed that the dopaminergic induction of MSCs resulted in 31% elevation in the number of cells activating the TH promoter. Further analysis pointed out that undifferentiated MSCs express TH protein and produce relatively small amounts of dopamine precursor—L-dopa. Following the in vitro induced dopaminergic differentiation, the cells elevated the expression levels of TH protein, raised the amounts of produced L-dopa and secreted dopamine.

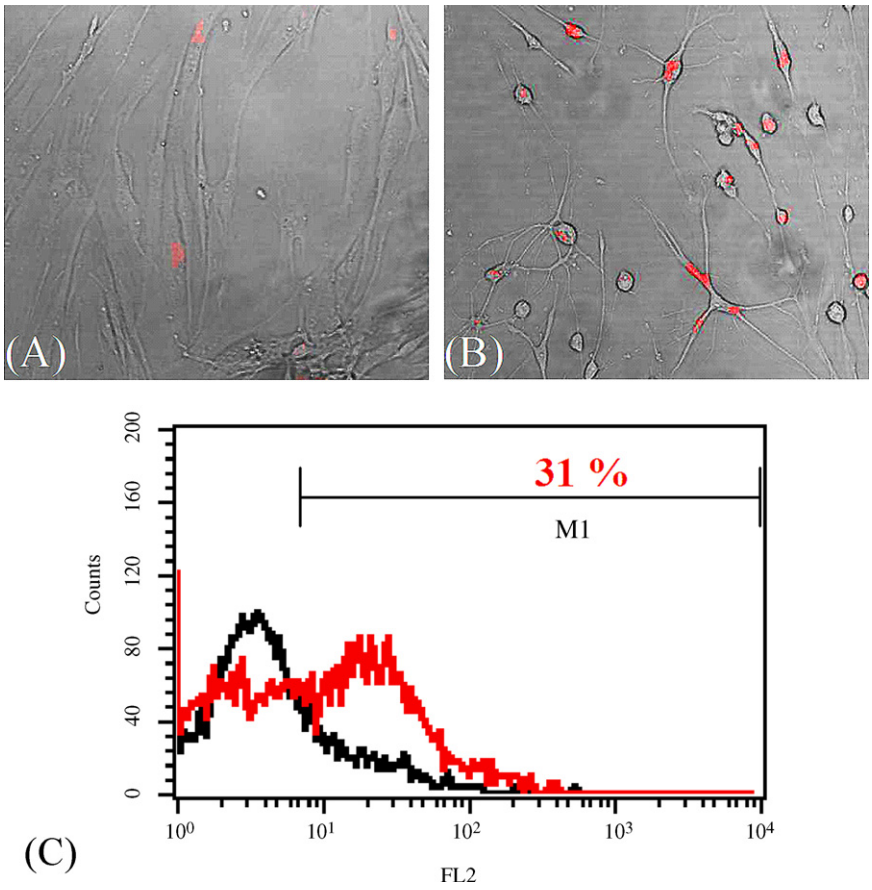

Fig. 3. Quantification of MSCs activating TH promoter following dopaminergic induction. MSCs were stably transfected with TH190 vector and partially induced to differentiate into dopaminergic neurons. DsRed2 expression was revealed by fluorescent microscope in MSCs (A) and in differentiated dopaminergic neuron-like cells (B). Flow cytometry detected the DsRed2 expressing cells (C). Quantification was made from the cross-point of the MSCs graph (black line) with the differentiated cells graph (red line).

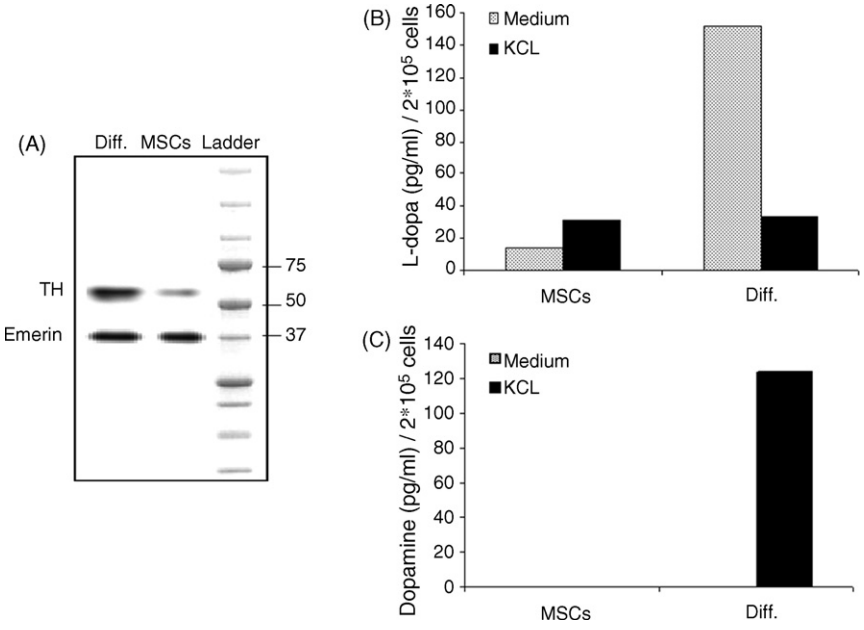

Fig. 4. Functional attributes of MSCs following dopaminergic induction. (A) Western blot analysis of TH and emerin expressions in MSCs and in differentiated dopaminergic neuron-like cells (Diff.). HPLC analysis of L-dopa (B) and dopamine (C) secreted by MSCs and differentiated cells to the conditioned medium (growth medium/differentiation medium II) and to the KCL HBSS depolarization buffer.

TH is the initial and rate-limiting enzyme in the catecholamine synthesis pathway, and considered the 'principal regulator' of dopamine biosynthesis in the central nervous system. TH catalyzes the conversion of tyrosine to the L form of dopa. L-Dopa is converted to dopamine by amino acid decarboxylase (AADC). Nagatsu et al. [9] showed that post-mitotic differentiation of dopamine progenitors is marked by the upregulation of mature dopaminergic markers, such as TH. The study of Sawamoto et al. [13] demonstrated TH expression in the developing dopaminergic neurons of the ventral mesencephalon in transgenic mice expressing green fluorescent protein (GFP) under the control of the rat TH gene promoter. Isolation and transplantation of the labeled cells into the rat model of PD had beneficial effects on clinical outcomes. Several studies of dopaminergic induction reported TH expression in MSCs after induced differentiation [2,3,5,18]. In view of the fact that many studies exploit TH expression for the evaluation of dopaminergic differentiation, there is a rising need to label, quantify and finally sort the TH positive cells. In this study, we generated a reporter assay of red fluorescent protein under human TH gene promoter. By using this newly generated system we discovered that a small amount of undifferentiated MSCs activates the TH promoter. These findings correlate with the reported results of MSCs expressing neuronal markers [2,16,18]. Following the induced differentiation, we detected an elevation of 31% in the number of cells that activated the TH promoter. Western blot analysis supported our findings showing that not only does the dopaminergic differentiation of MSCs elevate the number of cells that activate the TH promoter but it also elevates the production of TH protein. Since the expression of TH is an essential but not a sufficient requirement of dopaminergic differentiation, we explored the functional properties of the dopaminergic cells, mainly the production and the release of dopamine and its precursor L-dopa. Our study reveals, for the first time to our knowledge, that undifferentiated MSCs express TH and produce and secrete L-dopa, supporting previously proposed theory of neural predisposition of MSCs [2]. The levels of the secreted L-dopa did not change significantly before and after KCL induced depolarization. In addition, MSCs did not secrete any recognizable amounts of dopamine. Following the induction of dopaminergic differentiation, elevated levels of L-dopa were detected in the conditioned medium. However, further KCL induced depolarization, reverted the levels of the secreted L-dopa to baseline (i.e. pre-differentiation levels). We hypothesize that since the produced L-dopa was released through a different mechanism than depolarization-dependent mechanism used by vesicle-bound neurotransmitters, the incubation time of 30 min was the cause for the low levels of L-dopa recorded in the depolarization buffer of the differentiated cells. Because L-dopa is an amino acid, the most probable form of L-dopa release is by the use of constitutive amino acid transport mechanisms. The secretion of dopamine was recorded only after KCL induced depolarization of the differentiated cells, stressing the terminal and functional induction of the MSCs. However, when the induction medium did not include GDNF, no dopamine secretion was recorded (data not shown). GDNF is one of the most potent trophic factors for nigral DA neurons [8], playing a role in development and survival [4,6,8]. It is not surprising, therefore, that GDNF has a major contribution in the dopaminergic induction of MSCs into mature dopamine secreting cells.

To conclude, the TH190 reporter vector provides a basic evaluation tool that may further be employed in order to investigate the mechanisms underlying MSC differentiation into dopamine secreting neurons and to optimize the induction procedure. In addition, our study implies that MSCs may be employed for autologous therapy of PD, avoiding the risks of obtaining neural stem cells from the brain, the ethical concerns and the issue of immunological rejection associated with the use of fetal tissues [10,11,17]. Therefore, clinical application of MSC-derived dopaminergic neurons offers numerous advantages and exhibits a broad array of possible applications.

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