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Trans-Differentiation of Rat Mesenchymal Stem Cells into Dopaminergic Neurons for Cell Transplantation

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Abstract

Objective: Transplantation of human embryonic dopaminergic progenitors within the striata of PD patients has provided encouraging results, but ethical concerns and tissue availability limit this approach. The use of mesenchymal stem cells (MSCs) provides a readily available source of cells, as they are derived from adult tissue. This *in vitro* study explored the use of MSCs as a cell source for DA neuronal induction utilizing a single adenovirus.

Methods: Our lab developed a novel adenovirus expressing multiple viral 2A genes allowing for the polycistronic expression of multiple genes (*Ascl1, Lmx1a, and Nurr1*) for transcription factors that are involved in DA neuron differentiation and used the gene for green fluorescent protein (*gfp*) to track transfection. MSCs were cultured with the adenovirus, monitored morphological changes as well as expression of *gfp* as evidenced by fluorescence microscopy. The presence of the viral DNA within the transfected cells was confirmed with PCR, Immunocytochemistry and RT-PCR.

Results: MSCs cultured with the adenovirus, resulted in morphological changes as well as expression of gfp as evidenced by fluorescence microscopy. The presence of the viral DNA within the transfected cells was confirmed with PCR. Immunocytochemistry and RT-PCR analyses revealed that, cells expressing gfp have nuclear co-labeling of translated transcription factors LMX1a, and NURR1, as well as an up-regulation of these genes, along with an up-regulation of downstream gene targets, such as tyrosine hydroxylase (TH), and the dopamine transporter (DAT).

Keywords: Mesenchymal stem cells; Stem cell therapy; Mesenchymal stem cell differentiation; Dopaminergic neuron differentiation; Parkinson's disease treatments; Parkinson's disease; Cell transplantation

Introduction

Parkinson's disease (PD) is the second most common neurodegenerative disease, with approximately 1% of the population of 65 years and older being afflicted [1]. Primary symptoms, which often appear around 60 years of age, are caused by the death of dopaminergic (DA) neurons within the nigrostriatal pathway [2]. Currently, there is no cure for PD and treatments are only palliative in nature. To date, the most popular treatment is the use of the chemical, levodopamine (L-dopa), which is a precursor to dopamine, and is administered to PD patients to alleviate symptoms. However, the effectiveness of L-dopa diminishes over years of therapy [3]. Therefore, more effective strategies to ameliorate the loss DA are needed.

One potential new strategy for treating PD is cell replacement therapy [4]. The first clinical trials for cell replacement therapy utilized ventral mesencephalon tissue from aborted human embryos and proved to reduce some PD symptoms in open-label trials with about 4 to 8 patients in each trial. Along with reduction in motor deficits, effective duration of L-dopa treatment and a decrease in the reliance on the drug was reported [5-8]. However, double-blind clinical trials in the United States [9,10], did not reveal significant decreases in symptoms between control and treatment groups, and some patients actually developed dyskinesia related to the transplants. Olanow and colleagues [10] reported that 56.5% of the patients developed these dyskinesias at 6-12 months after transplantation. The cause of dyskinesia, which became known as graft-induced dyskinesia (GID), has been attributed to the lack of specificity in the types of cells transplanted and their improper integration into the host tissue [11].

An alternative to the use of embryonic cells, the use of adult stem cells has been proposed. To this end, mesenchymal stem cells (MSCs), which have been shown to Trans-differentiate into DA neurons, provide a readily accessible source of adult stem cells. Further, MSCs that have been semi-differentiated into dopaminergic neuronal-like progenitors have been proposed as a potentially effective means of augmenting cellular therapy for PD [12,13].

Human MSCs have been shown to differentiate into DA neurons, cultured with recombinant protein morphogens, sonic hedgehog (SHH), fibroblast growth factor 8 (FGF-8), basic fibroblast growth factor (BFGF), and brain derived neuron factor (BDNF) [12]. Additionally, these cells exhibit robust action potentials that are phenotypically similar to true DA neurons [12]. Furthermore, Barzilay and colleagues [13] reported a significant increase in tyrosine hydroxylase expression when the transcription factor (TF) Lmx1a was transduced into the MSCs prior to their differentiation, compared to cells that were not

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transduced [13]. These cells also displayed significant increases in other DA TFs. Therefore, transduction methods utilizing TF genes and morphogens, such as SHH, can be utilized to transdifferentiate MSCs.

This *in vitro* study explored the use of MSCs as a cell source induction of DA neurons. To this end, our lab developed a novel method of utilizing an adenovirus for the polycistronic expression of multiple TF genes with the viral gene 2A placed between each TF gene (Ascl1, Lmx1a, and Nurr1) plus the gene for green fluorescent protein (gfp) to track transduction. These TF genes are involved in DA neuron differentiation, and have been shown to directly transdifferentiate fibroblasts into DA-like neurons14. An adenovirus serotype 5 was chosen over other viruses, due to the fact that, unlike lentiviruses and retroviruses, the adenovirus does not integrate with the genome of transduced cells. Furthermore, by utilizing a single adenoviral vector, this method allows for a homogenous transduction of the TF genes, and decreases variability within other protocols that utilize morphogens in addition to viral vectors.

Methods

Gene cloning

RNA for Complementary DNA (cDNA) synthesis was isolated from a brain hemisphere of a 1 day old rat pup. RNA was isolated and purified by using the RNeasy kit (Qiagen©). cDNA was synthesized from the RNA utilizing cDNA synthetase (Qiagen©). The amplified genes (Table 1, for primer sequences) were then purified with a PCR product purification kit (Qiagen©), single gene products and the pGEM T-easy vector were ligated with T4 ligase (NEB), and JM109 competent cells were transformed with the ligated plasmid. Ampicillin-resistant colonies were purified using a miniprep plasmid purification kit (Qiagen©). The pGEM T-easy vector plasmids containing each TF, gfp, and 2A genes for viral construction were verified by PCR, restriction endonuclease digestion, and DNA sequencing.

Adenovirus production

For virus production, the HEK 293 cell line was cultured until 90% confluent across the bottom of a 75 cm^2 culture flask in growth media (Gibco® DMEM, with 20% fetal bovine serum and 1% penicillin and streptomycin). Once 90% confluence had been obtained, 1.0 × 106 cells were replated in a 60 mm^2 culture dish. The following day, the media was removed from the dish and replaced in growth media, without serum, and containing the transfection reagent, extreme gene HP (Promega), and the viral vector and viral backbone (Cell Biolabs). At 7 to 10 days, the cells were removed from the flask and the viral product was purified, than expanded.

MSC transfection

The MSCs were extracted from the rat bone marrow of the femur and tibia, following the method described by Rossignol and colleagues [14,15]. Following aspiration, the bone marrow was suspended in 10 mL of DMEM, 15% fetal bovine serum (DMEM, Invitrogen; FBS, Invitrogen), and 1% penicillin/streptomycin. The MSCs were selected by plastic adherence. Following further verification by flow cytometry, the MSCs were cultured for 72 hours in neuronal induction medium, which consisted of DMEM/F12 Ham with 2% fetal bovine serum, 1% B27 serum and the adenovirus expressing the TFs. After 72 hours, the medium was changed. The transfection efficiency was measured by the expression of gfp. Cells were counted with a hemocytometer prior to transfection, and following transfection the cells were removed from the flask and recounted with a hemocytometer and a flow cytometer.

Flow cytometry

Cells were counted and evenly distributed into a 96 well plate. The cells were then washed twice with PBS, containing 1% bovine serum albumin (Invitrogen), and then centrifuged at 2,500 rpm at 4°C for one minute. Following centrifugation, the supernatant was removed. After the two PBS washes, the cells were resuspended in the primary antibody (Table 2, for list of primary antibodies) and incubated overnight at 4°C. The cells were then centrifuged at 2,500 rpm at 4°C for one minute, and the supernatant was removed. Following removal of the supernatant,

Gene	Primer sequence	5' restriction site	3' restriction site
Ascl1	F 5'-ATCGAT CTCGAGAGTGAGAGCTCTGGCAAGATGG-3' R 5'-ACTAGTTGGGCCAGGATTCTCCTCGACGTCACCGCATGTTAGCAGA CTTCCTCTGCCCTCTCCACTGCCGAACCAGTTGTTGGTAAAGTCCAGC-3'	ClaI and XhoI	SpeI
Lmx1a	F 5'-ACTAGTATGTTGGACGGCCTGAAGATGG-3' R 5'-AAGCTTAGAGGTGAAATAGGAATTCTGCATGG-3'	SpeI	HindIII
Nurr1	F 5'-ATCGATATGCCTTGTGTTCAGGCGCAG-3' R 5'-AAGCTTGAAAGGTAAGGTGTCCAGGAAAAG-3'	ClaI	HindIII
2A	F 5'-AAGCTTGGCAGTGGAGAGGGCAGA-3' R 5'-CTCGAGTGGGCCAGGATTCTCCTC-3'	HindIII	XhoI
gfp	F 5'-CTCGAGATGGTGAGCGTGAGCAAGGGCGAGGAG-3' R 5'-GCGGCCGCTTACTTGTACAGCTCGTCCATG-3'	XhoI	NotI

Table 1: Primer sequences for cloning.

Primary Anti-bodies	Isotype	Target	Concentration	dilution	company
Mouse Anti-CD29	IgG	CD29	1 mg/mL	1:500	Abcam
Mouse Anti-CD44	IgG	CD44	1 mg/mL	1:500	Abcam
Mouse Anti-CD90	IgG	CD90	1 mg/mL	1:500	Abcam
Mouse Anti-SSEA4	IgG	SSEA4	1 mg/mL	1:500	Abcam
Mouse Anti-MHCII	IgG	MHCII	1 mg/mL	1:500	Abcam
Mouse Anti-CD105	IgG	CD105	1 mg/mL	1:500	Abcam
Rat Anti-SCA1-FITC	IgG	SCA1	1 mg/mL	1:500	Abcam
Rabbit Anti-CD45	IgG	CD45	1 mg/mL	1:500	Abcam
Rabbit Anti-MHC I	IgG	MHC I	1 mg/mL	1:500	Abcam

Table 2: Primary anti-bodies utilized to verify MSCs.

the cells were then incubated in the secondary antibody for one hour (Table 3, for list of secondary antibodies). The cells were then washed three times, centrifuged after each wash, and placed in .1 M PBS with 4% paraformaldehyde. After 10 minutes, cells were washed once, resuspended in 300 µL of PBS, and were stored at 4°C for flow cytometry analysis. Flow cytometry was conducted utilizing a BD LSR II flow cytometer (BD Biosciences) with forward scatter (FSC) and side scatter (SSC) emissions calibrated using BD calibration beads. The BD FACSdiva software was utilized to manage the flow cytometry system and data collection. Data was presented in a scatter plot as percentage of cells per wavelength emission of secondary antibody fluorophore.

Immunocytochemistry

Transduced cells were grown on coverslips, fixed with 4% paraformaldehyde in 0.1M PBS, followed by three washes in 0.1 M PBS. The cells were then incubated in normal goat serum (Jackson ImunnoResearch Laboratories) to block non-specific antibody binding. Following incubation in the blocking serum, the cells were incubated with primary antibody (Table 4, for list of primary antibodies) overnight or 48 hours (for the TFs) at 4°C. Following primary antibody incubation, the cells were washed in 0.1 M PBS, three times, and then incubated in the secondary antibody (Goat anti-Rb Alexa 594) for one hour at room temperature. The cells were then washed, three times in 0.1 M PBS and mounted with Fluoromount™ aqueous mounting media (Sigma-Aldrich).

Western blot

Cells were removed from media and homogenized within RIPA buffer. After extraction, protein samples were measured with BCA analysis and diluted to equal concentrations. The samples were placed in loading and were denatured by boiling at 100°C for five minutes. Protein was separated with a SDS-PAGE gel and transferred to a nitrocellulose membrane (Invitrogen). Following transfer the membrane was incubated in 5% non-fat milk overnight at 4˚C. The next day the membrane was incubated in the primary antibodies for the transduced TFs (ASCL1, LMX1a, and NURR1,) for 1 hour at room temperature. The membrane was washed in PBS three times, followed by incubation in the secondary antibody (Goat anti-Rb HRP conjugated) for 30 minutes. The secondary antibody was detected using PierceTM enhanced chemiluminescence solution (Thermofisher Scientific, St. Louis, MO), and developed with X-ray film.

RT-PCR

All of the culture media was removed from the culture dish and approximately 500,000 cells were subsequently washed three times in 10 mL 0.1 M phosphate buffered saline (PBS). Following washing, TRIzol® (Invitrogen) reagent was added to the flask and the cells were incubated for five minutes to lyse the cells. RNA from the lysate was purified according to the protocol provided by the manufacture (Invitrogen). Complimentary DNA (cDNA) was then transcribed from the RNA utilizing the Quantitech Reverse Transcriptase kit (Qiagen) following the protocol provided by the manufacturer. The cDNA was subsequently utilized in a RT- PCR reaction to assess the relative mRNA expression levels of certain genes. The RT-PCR reactions were performed in replicates of three and repeated three times. RT-PCR reactions consisted of 1 µL of cDNA, 1 µL of the 20 µM reverse and forward primers (Table 5 for sequences of primers), and 23 µL RT-PCR SYBR green master mixes. The thermal cycler was set with a 10 minute long incubation at 95°C to activate the polymerase enzyme for the first step. The second step consisted of a 15 second incubation period at 95°C, followed by 1 minute incubation at 60°C and this process was repeated for a total of 40 cycles. Results were analyzed utilizing the $2^{-\Delta\Delta CT}$ method. The Student's t-test was used to compare the mean mRNA expression of each gene within the transfected and control MSCs at individual time points (3 and 5 days).

Secondary Anti-bodies	Target	Concentration	Dilution	Company
Anti-mouse-alexa 488	Mouse IgG	2 mg/mL	1:500	Life Technologies
Anti-rabbit-alexa 488	Rabbit IgG	2 mg/mL	1:500	Life Technologies

Table 3: Secondary anti-bodies utilized to label primary anti-bodies.

Primary Anti-bodies	Isotype	Target	Concentration	dilution	company
Rabbit Anti-LMX1a	IgG	TH	1 mg/mL	1:500	Abcam
Rabbit Anti-NURR1	IgG	CD44	1 mg/mL	1:500	Abcam
Rabbit Anti-TH	IgG	CD90	1 mg/mL	1:500	Abcam
Rabbit Anti-DAT	IgG	SSEA4	1 mg/mL	1:500	Abcam
Rabbit Anti-GIRK2	IgG	MHCII	1 mg/mL	1:500	Abcam
Mouse Anti-ASCL1	IgG	CD105	1 mg/mL	1:500	Abcam

Table 4: Primary anti-bodies utilized to verify DA-like Cells.

Gene	Primer sequence
Ascl1	F 5'-CCTACGACCCCCTTAGTCCA-3' R 5'-TGCCATCCTGCTTCCAAAGT-3'
Lmx1a	F 5'-TCTCTGCACAGCCCACATAG-3' R 5'-TCCATCTACCATGCCTCTCC-3'
Nurr1	F 5'-GGTTCATGTCTCCCTTCTGTAG-3' R 5'-GAGCCAAAATGCCCTTTCAC-3'
FoxA2	F 5'-GTGGGTAGCCAGAAAAAGGC-3' R 5'-CAGCATACTTTAACTCGCTGGC-3'
Pitx3	F 5'-CATGGAGTTTGGGCTGCTTG-3' R 5'-CCTTCTCCGAGTCACTGTGC-3'
Msx1	F 5'-TGCTAAGGCCAAGAGACTGC-3' R 5'-CAAGAGGAAAGGAGAGGCCG-3'
TH	F 5'-TGTGTCCGAGAGCTTCAATG-3' R 5'-GGGCTGTCCAGTACGTCAAT-3'
Dat	F 5'-GCTCCAGGAAGGGTAACTCC-3' R 5'-GCTCCAGGAAGGGTAACTCC-3'

Table 5: RT-Primer sequences.

HPLC

To quantify DA levels in reprogrammed cells, cell pellets were homogenized in 100 µl 0.1 N HClO4 and analyzed by using HPLC with electrochemical detection (ESA). To measure dopamine concentrations in the supernatants, cells were exposed to DMEM media for 30 min followed by DMEM media with 50 mM KCl for 30 min, then 0.9 ml of supernatants were collected with the addition of 0.1 ml of 1 N HClO4, and analyzed by HPLC. Dopamine was separated on a reverse-phase column (Capcell PAK, 3 µm, 50 × 1 mm) with a mobile phase consisting of 150 mM phosphate buffer, 4.76 mM citric acid, 3mM SDS, 0.05 mM EDTA, 10% methanol, 15% acetonitrile (pH 6.0) at a flow rate of 200 µL min. Dopamine was detected by a Decade II electrochemical detector

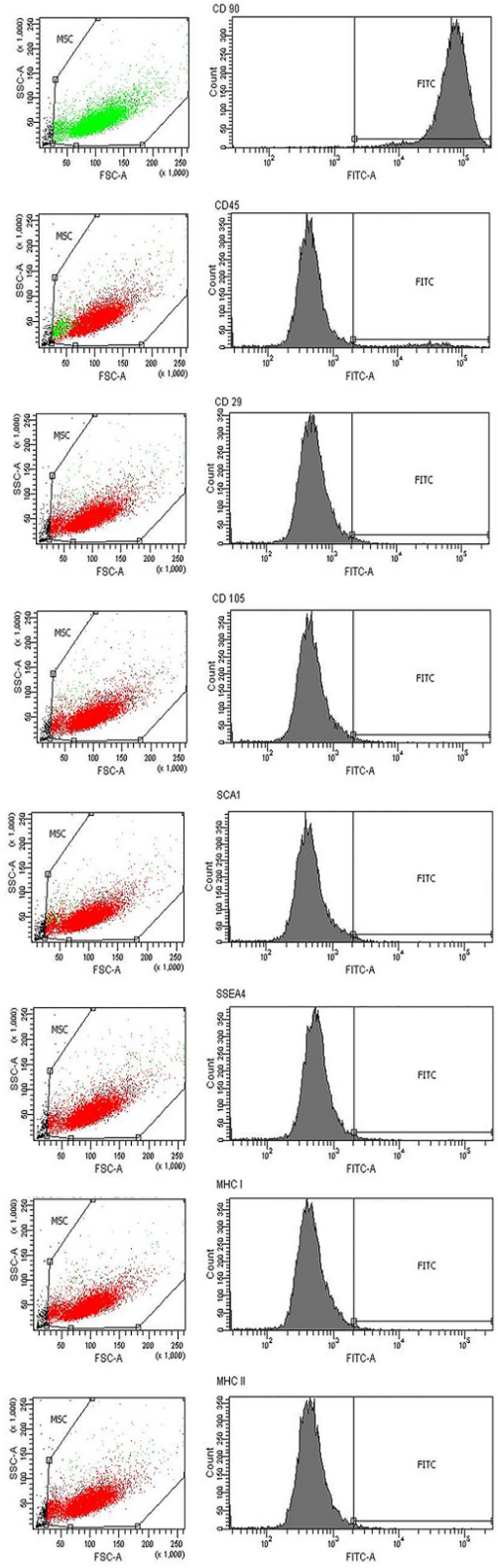

Figure 1: MSC Cell Scatter Plot Graph. Graphs represent the MSC cell population based on non-MSC markers, and MSC marker CD90. Cells were only positive for CD90.

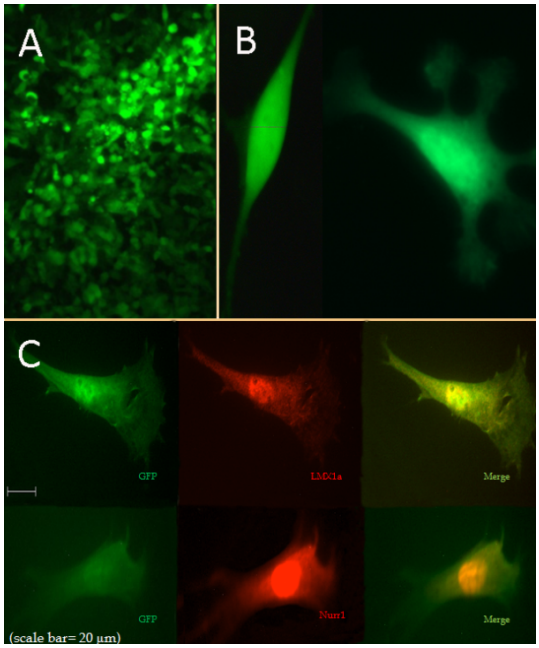

Figure 2: A. plaque formation seen after 10 days post-transfection indicating viral production B. Cellular Morphology; A representation of the typical morphological change seen in transduced MSCs at days three and five respectively. C. LMX1a and Nurr1 Fluorescent Images: these images illustrate the presence of LMX1a and NURR1 within the nucleus of transfected MSCs. Scale bar=20 µL.

equipped with a electrochemical flow cell and a 0.7-mm-diameter glassy carbon electrode (ESA). The volume of the injection was 20 µL. Results were compared to known DA standards.

Results

Flow cytometry was performed on the MSC population to verify stem cell lineage through the use of immunocytochemistry labeling of extracellular non-MSC cell and MSC markers. The MSCs were negative for extracellular, non-stem cell markers while 99.7% of the MSCs were positive for the extracellular stem cell marker CD90 (Figure 1).

Following the cloning process for the genes 2A, Ascl1, gfp, Lmx1a, and Nurr1, production of each plasmid was confirmed with PCR and restriction endonuclease digestion. All of the plasmids were positive for the genes cloned, which were verified by the expected product size. Following confirmation with PCR amplification and restriction digest, plasmids were sequenced from the 5' to the 3' end using the T7 primer and sequenced from the 3' to 5' end using the SP6 primer. The construct plasmids were also confirmed and verified in the same manner. Upon completion of viral production, each of the cloned genes, Ascl1, gfp, Lmx1a, and Nurr1 were verified to be present in the viral genome by PCR.

Viral production was confirmed by the formation of a plaque in a culture of HEK 293 cells (Figure 2A) Following transduction of the MSCs, the viral titer 3.195 ×109 3.195 × 109 infectious units/mL was determined to be the most efficient based upon the level of gfp expression, with a transduction efficiency of 90.35%, verified by two methods (utilizing a hemocytometer and flow cytometer). The transduced cells displayed a change in morphology in which the cells became elongated after 3 days post transfection (Figure 2B). Immunocytochemistry results demonstrated nuclear localization of the

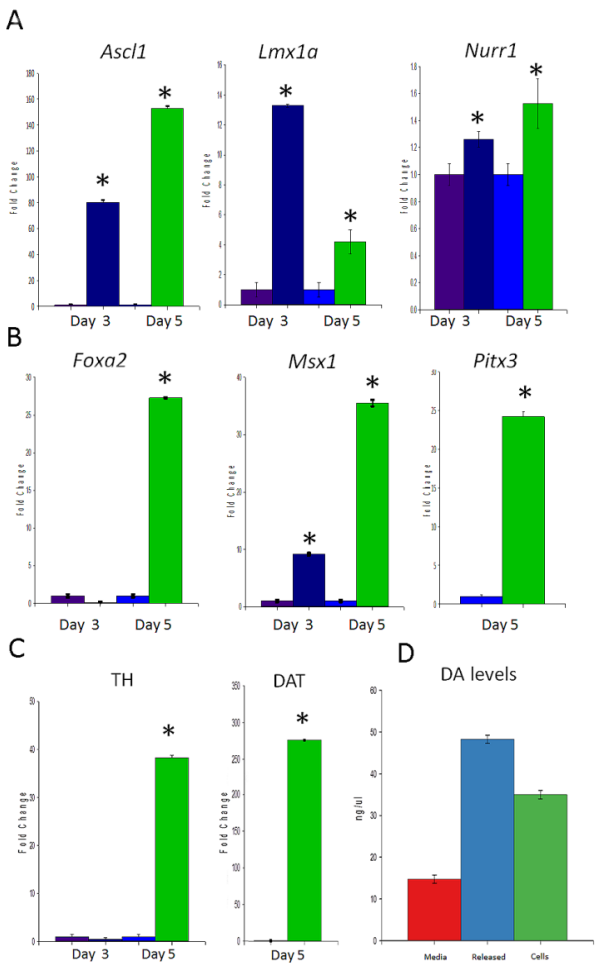

Figure 3: A-C. RT-PCR Results; graphs demonstrating the fold changes in mRNA levels of genes in transfected cells compared to time matched control MSCs ($p<0.05$). D. DA content was measured within the media prior to KCl generated release, DA was measured after release, and the total amount of DA was measured within the cells.

transcription factors NURR1, and LMX1a at 7 days post-transfection (Figure 2C). These the results suggest that the observed morphological changes could be taking place via activated transcription factors. Additionally Western blot analysis at day three demonstrated that the protein expression was observed for all transduced genes (*Ascl1, Lmx1a, Nurr1, gfp*; data not shown).

Utilizing RT-PCR, cells were monitored for gene up-regulation and down-regulation at post-transfection days 3 and 5, and analyzed with the $2^{-\Delta\Delta CT}$ method. Mean fold changes were compared to un-transfected MSCs with Student's t-test. The three transduced TFs (*Ascl1, Lmx1a, and Nurr1*) within the adenovirus were found to be significantly upregulated at both time points, as expected. Ascl1 day 3 ($t(1)=46.797$, $p<0.01$), day 5 ($t(1)=11558.667$, $p<0.0001$); Lmx1a day 3 ($t(1)=13.342$, $p<0.05$), day 5 ($t(1)=351.005$, $p<0.002$); Nurr1 day 3 ($t(1)=173.125$, $p<0.004$), day 5 ($t(1)=555.063$, $p<0.001$) (Figure 3A). The mRNA of the direct and indirect gene targets of these TFs were found to be upregulated as well, suggesting active transcription. These TFs included Msx1, FoxA2, and Pitx. Msx1 day 3 ($t(1)=347.616$, $p<0.002$), day 5 ($t(1)=963.530$, $p<0.001$); FoxA2 day 5 ($t(1)=801$, $p<0.001$); Pitx3 day 5 ($t(1)=73.86$, $p<0.0001$) (Figure 3B). Furthermore mRNA levels for TH and the DAT were found to be upregulated, TH days 5 ($t(1)=1884.872$, $p<0.0001$), DAT day 5 ($t(1)=770.231$, $p<0.001$) (Figure 3C).

Following these results, the induced MSCs into DA like-cells were characterized for DA production utilizing HPLC. The cells were found to spontaneously release DA, release dopamine upon stimulation with high (50 mM) K+ stimulation, and contain DA after release (Figure 3D).

Discussion

The successful cloning of each TF (Ascl1, Lmx1a, and Nurr1), in addition to the other genes included in the virus (2A, gfp), was confirmed by PCR, restriction digest, and nucleotide sequencing. Important concerns that arise with polycistronic expression of numerous genes are: (1) whether the genes will be expressed as a single protein or separate proteins; and (2) if and how protein folding will be affected, based on the function of the 2A peptide. The way in which the 2A peptide cleaves is upon translation of codon, which is located towards the 3' end of the mRNA, which causes translation to stop and start. This action leaves 20 amino acids on the 3' end of the translated gene upstream from the 2A sequence and 2 amino acids on the downstream-translated gene [16-20]. The functionality of the self-cleaving peptide 2A is evident in this work, based upon a few factors. First, GFP was expressed in the transduced MSCs. The expression of GFP would not be observed if the 2A peptide had not cleaved, or had the addition of amino acids by the 2A peptide caused the GFP protein to misfold. It can be argued that the innocuous functionality of the other 2A peptides included in the virus can be indirectly demonstrated by the RT-PCR results that were observed: Lmx1a is significantly up regulated at days 3 and 5, along with the TF, Msx1. This demonstrates that LMX1a is functional, since the mRNA levels of the LMX1a target Msx1 are increased [21]. Additionally we observe upregulation of Nurr1 and, two important downstream targets of NURR1 (TH, DAT) were upregulated, corresponding with the trend in Nurr1 up-regulation [22-24]. Furthermore, at day 5 the transfected MSCs expressed the protein DAT. Therefore, based upon protein and mRNA expression, the functionality of the 2A peptides can be directly and indirectly observed, demonstrating the translation of four genes into functional proteins from a single open reading frame, hereby illustrating a novel method of adenoviral mediated transduction.

For this novel method of adenoviral mediated transduction to bring about the trans-differentiation of MSCs in an efficient manner, the MSCs phenotype was partially verified, utilizing flow cytometry prior to examining the transduction efficiency. These two factors are important to discuss together since the transduction of MSCs and partially differentiated MSCs, known as mesenchymal progenitor cells (MPC), differ due the expression of the coxsackievirus and adenovirus receptor (CAR) [25]. CAR, which is the principle route for adenovirus transduction, is expressed at a high level on MSCs while the expression of CAR is expressed at a low level on MPCs [25,26]. Transduction efficiencies of 90.35% were observed in the MSCs within this study, which are not routinely observed in transduction of cell types that express low levels of CAR, such as MPCs. Further this level of transduction efficiency has been observed in human MSCs, which can also be directly impacted by the blockade of CAR [26]. The flow cytometry assay utilized antibodies accepted as a general marker screening panel. Our population sample of MSCs was 99.7% positive for the protein CD90, which is noteworthy, since it demonstrates a

homogeneous population of cells expressing an accepted MSC marker [27]. These factors shown by transduction efficiency and flow cytometry, together, indicate that the cell type that we successfully modified in our study was more likely MSCs rather than the semi-differentiated MPCs.

MSCs have been shown to express TH and NURR1, suggesting that some expression of NURR1, and downstream targets of the NURR1, should be observed without transduction [28,29]. Despite this expression we still would expect to see significant upregulation in these genes evidenced by RT-PCR. As previously mentioned, Nurr1 was indeed significantly upregulated in differentiated cells compared to undifferentiated control MSCs. We also observed NURR1 labeling with Western blot and within the nucleus of transduced cells that co-expressed GFP. Based on the results of NURR1 expression, we would expect to see up-regulation of the down-stream targets of the TF, such as TH. TH is a target of the NURR1 TF within the rat species rattus norvegicus, and functions by binding to promoter region of the TH gene and eliciting transcription [22,23]. We also observed a significant fold increase (upregulation) in TH, along with up-regulation of additional TFs that target TH indirectly and directly (such as FoxA2, and Pitx3) [30-32], which is indicative of active NURR1, and possible DA neuron-like cellular phenotype. Similar results were also observed by Tondreau and colleagues, upon neuronal induction [28]. Therefore taken together the RT-PCR results and ICC immunocytochemistry results thus far indicate a potential DA neuron-like cellular phenotype.

The argument that the cells in this study are terminally differentiating toward a DA neuron-like cellular phenotype is further supported by the mRNA expression of FoxA2 and Pitx3, which were significantly up-regulated at day 5, compared to controls. FOXA2 has been found to be necessary for the survival of DA neurons, most likely through transcription activation of Pitx3 and Nurr1 [33,34]. None of the transcription factors that were transduced (Ascl1, Lmx1a, and Nurr1) directly up-regulate FoxA2. However SHH, which up-regulates FoxA2, has been shown to be indirectly upregulated by ASCL1 [35-37]. Further Pitx3 has been found to be up-regulated by GDNF, which in turn up-regulates BDNF [38]. Trzaska and colleagues (2009), as well as Peng and colleagues (2011), have shown that BDNF increases DA neuronal cell survival and in fact helps to finalize neuronal status promoting expected calcium-dependent release from neurons [12,38]. In fact, Trzaska and colleagues have shown that BDNF increases the amplitude of action potentials expressed by DA neurons that were derived from MSCs, along with the more neuronal $Ca2^{+}$-dependent) status of release, illustrating that the expression of NURR1, FOXA2, and PITX3, alone will not bring about mature DA neurons through differentiation or trans-differentiation in the absence of BDNF.

The HPLC analysis of dopamine release indirectly demonstrates the TFs LMX1a, FOXA2, NURR1, and PITX3 are functional TF, since the cells released dopamine and exhibited some residual dopamine within the cells (Figure 3D). Potassium-stimulated release suggests mechanisms are in place that supports action potential-derived exocytosis rather than a simple reversal of transport or leakage, which would not be calcium dependent, which is normally observed in the dorsal striatum [39]. In fact, early experiments by Trzaska and colleagues, before the addition of BDNF in their protocol, yielded cells that lacked sufficient electrical activity and did not yield K+-stimulated release [40]. As was shown in Figure 3C, our cell populations were expressing considerable TH and DAT and their demonstrated sensitivities to K+ stimulation indicate that the mechanisms of action potential generation were in place. The presence of DA prior to stimulation suggests that certain levels of spontaneous oscillations may be occurring among these cells, though the osmotic conditions of our assay may deviate somewhat from standard *in-vivo* conditions. It will be important to explore how these cells responded to the typical context in the dorsal striatum, where glutamatergic and GABA-ergic stimulations are routine. Further we confirmed expression and of the G-protein regulated inward rectifier potassium channel 2 (GIRK2; data not shown) which has been shown to be crucial for A9 DA neuronal oscillatory function [41,42]. Moreover, the expression of GIRK2 is utilized as a marker for this phenotype, with this phenotype shown to provide the greatest amount of integration and survival when transplanted within the striatum [41].

Further, *in vitro* and *in vivo* experimentation with this method of viral induction of DA-like cells need to be undertaken. First, the presence of additional proteins involved in the A9 and A10 DA cell function need to be both shown and excluded, such as calbindin and aldh1a [43]. This distinction is crucial since A10 DA neurons or ventral tegmentum area DA neurons have been shown to not integrate within the striatum as efficiently [43]. The *in vivo* transplantation of these cells is needed to demonstrate cell survivability and integration within the host tissue. Nonetheless, our current *in vitro* findings suggest that the trans-differentiation of MSCs into DA like cells is not only possible, but may provide a new means of facilitating cell replacement therapy.

Conclusion

In conclusion, the results of this study suggest the transfected MSCs have a DA-cell-like phenotype, based upon morphology, mRNA, and protein expression. The cellular morphology of some transduced cells within this study demonstrated a circular shaped body with finger-like projections extending from this body, which is similar to immature DA neurons (Figure 2B). Based upon the RT-PCR results the mRNA expression profile resembled that of a DA like cell, and immunohistochemical verification of protein expression (i.e. TH, DAT) confirms said genes are translated, while DA release suggests that they are functional. Furthermore, this body of work demonstrates that a single adenovirus can bring about the poly-cistronic expression of multiple genes with transfection efficiencies as high as 90%, evidenced by the expression of GFP. This is of particular importance in obtaining a purer population of cells that can be utilized for cellular transplantation in PD.

Our research suggests a strategy that provides the potential of two beneficial attributes. First, our strategy seems to drive greater levels of DA phenotype induction compared with similar cells left to their own devices or vulnerable to only local morphogen cues. Secondly, this strategy could theoretically be applied to mesenchymal populations obtained from the patients themselves, avoiding immune rejection and presumably prolonging their effectiveness. Therefore, if side effects such as GIDs do in fact derive from alternative cell type contributions, our strategy may show a comparative reduction in such expressions. Future studies involving transplanting such cells into the dorsal striatum of animals after dopamine-depleting lesions will allow focused explorations with in-vivo behavioral testing, confirming their viability under both spontaneous and stimulated conditions.

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