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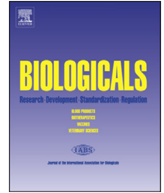

# Differentiation of mesenchymal stem cells -derived trabecular meshwork into dopaminergic neuron-like cells on nanofibrous scaffolds

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Parkinson's disease (PD) is a neurodegenerative disorder of the brain which is produced by the damage to dopaminergic neurons. Stem cell transplantation with a nanofibrous scaffold is one of the encouraging strategies for Parkinson's disease therapy. In this study, human mesenchymal stem cells (MSCs) from eye trabecular meshwork (TM) were differentiated into dopaminergic neurons on nanofibrous scaffold.

After Trabecular meshwork biopsy, MSCs were isolated, cultivated on Poly-L-Lactide Acid (PLLA) nanofibrous scaffold (fabricated by electrospinning methods) and treated with medium containing DMEM supplemented with RA, IBMX and forskolin for 7 days. Scanning electron microscopy imaging, qPCR and immunostaining were used to analyze differentiated TM-MSCs on scaffold and their expression of dopaminergic-specific markers such as TH and Nurr-1.

qPCR analysis revealed the expression of dopaminergic neuron genes such as TH, Nurr-1 on fibrous scaffold as well as TCPS. Immunostaining revealed that the differentiated TM-MSCs on TCPS and Scaffold not only express TH and Nurr-1 genes, but also express TH protein.

In conclusion, the results indicate that TM-MSCs might be a suitable source for cell transplantation therapy. In addition the nanofibrous scaffold reported herein could be used as a potential cell carrier for the central system diseases such as PD.
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## 1. Introduction

Parkinson's disease (PD) is a neurodegenerative disorder of the brain which is produced by damage to dopaminergic neurons and a reduction of dopamine in the substantia nigra and in the striatum, respectively [1–3]. Although many treatment strategies such as administration of drugs, have been designed for this disease, they have shown an imperfect efficacy [4]. In recent years, stem cell therapy has emerged as a promising alternative treatment for Parkinson's disease (PD) [5].

Despite the fact that human embryonic stem cells (ESCs) and neural stem cells (NSCs) are considered well-accepted cell sources with the capacity to differentiate into dopaminergic neurons [6], The clinical use of ESCs and NSCs has presented problems including teratoma formation as well as eliciting immune rejection after transplantation, low efficiency of differentiation and lineage polarization [7]. Based on these facts, additional studies are necessary to identify other stem cell sources with the ability to produce dopaminergic neurons with high efficiency. Mesenchymal stem cells (MSCs) have been shown to be a reliable cell source for PD treatment [8]. MSCs are multipotent stem cells that have been isolated from a wide variety of tissues and can be differentiated into neurons [9]. MSCs are currently in clinical trials for a number of disorders such as multiple sclerosis [10,11] and PD [11].

Human brain microenvironments are highly complex, with three-dimensional (3D) structures involving the organized interaction

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of multiple neural cell types with other cells (microglia and endothelial cells) [12].

One option for improving the physiological relevance of human neuronal culture models is to use these cells in combination with 3D scaffolds. Several studies have indicated that cell transplantation with a biocompatible polymer scaffold increases survival [13] and differentiation [14] of the cells. Scaffolds are the key constituents of tissue engineering for stem cells delivery. To date, several studies have been conducted on the effects of 3D electrospun scaffolds of a variety of biomaterials to support neural differentiation of stem cells [15].

While 3D scaffolds have been used to engineer neurocyte cell-type specific constructs, there have been few reports on the successful application of a single 3D scaffold to support cell differentiation into specific types of neurocyte cells [16].

In the present study, Trabecular meshwork Mesenchymal stem cells (TM-MSCs) were differentiated into dopaminergic-like neuron on a nanofibrous scaffold in vitro.

Taken together, a combination of TM-MSCs (as a new source of cells) and a nanofibrous scaffold could provide a strategy for Parkinson disease therapy.

## 2. Materials and methods

### 2.1. Isolation and differentiation into mesenchymal lineage cells

This research was performed in accordance with the Helsinki Declaration and with approval of the ethics board of the Zanjan University of medical Sciences. Eye globes were obtained from the Central Eye Bank of Iran. TM-MSCs were isolated according to a protocol modified by Nadri et al. [17]. TM biopsy was incubated in PBS (GIBCO-BRL, Grand Island, NY) supplemented with 40 mg/mL BSA and 4 mg/mL collagenase for 1 h. The isolated stromal tissue segment was cultured in low glucose DMEM (GIBCO-BRL, Grand Island, NY) supplemented with 20% serum (GIBCO-BRL, Grand Island, NY), 200 ng/mL basic-FGF (Peprotech, Rocky Hill, NJ), and was incubated at 37 °C with 5% CO2 in a humidified chamber. After 2 weeks, the cells were trypsinized (0.25% Trypsin – GIBCO-BRL, Grand Island, NY) and expanded by two passages.

To demonstrate the multipotent nature of the isolated cells, the cells were differentiated down three lineages over a period of 21 days as follows: osteogenic (DMEM including 50 mg/mL ascorbic acid 2-phosphate (Sigma Chemical Co. St Louis, MO), 10 nM dexamethasone (Sigma Chemical Co.), 10 mM b-glycerol phosphate (Sigma Chemical Co.), adipogenic (DMEM supplemented with 50 mg/mL indomethacine (Sigma Chemical Co) and 100 nM dexamethasone (Sigma Chemical Co), and chondrogenic (DMEM supplemented with 10 ng/mL transforming growth factor- β3 (TGF-β3; Sigma Chemical Co), bone morphogenetic protein- 6 (BMP-6), $10^{-7}$ M dexamethasone (Sigma Chemical Co.), 50 mg/mL ascorbate-2-phosphate (Sigma Chemical Co.), and 50 mg/mL insulin-transferrin- selenium (ITS; GIBCO- BRL) medium.

### 2.2. Fabrication of electrospun nanofibrous PLLA scaffolds

Nanofibrous PLLA scaffolds were fabricated using an electrospinning technique [18]. PLLA was dissolved in chloroform (9% w/w) and added to the chloroform/N,N-dimethylformamide (DMF) (Sigma, Steinheim, Germany) solution (10:1). Aligned nanofibrous scaffolds were obtained using high (3000 rpm) speed rotating disk.

### 2.3. In vitro differentiation of TM-MSCs to dopaminergic neuron on PLLA scaffold

For sterilization, the scaffolds were placed in 70 °C alcohol for 20 min and treated under UV irradiation for 10 min. Nanofibrous PLLA scaffolds were placed in 70 $mm^2$ culture dishes and $3 \times 10^5$ of the TM-MSCs were cultivated on them.

For differentiation, the cells were incubated in an induction medium that consisted of DMEM (low glucose), supplemented with 10 μM retinoic acid (RA, sigma), 0.5 mM IBMX and 10 μM Forskolin for 7 days.

Cultivated cells on a tissue culture polystyrene (TCPS) plate were maintained in induction medium and culture medium as a two dimensional culture group (as a control group). At the end of this period, the cells were used for qPCR and Immunofluorescence analysis. The expression levels of the genes and proteins were compared with untreated cells.

### 2.4. Immunofluorescence analysis

The cells were fixed (with 4% paraformaldehyde) and permeabilized with 0.5% Triton X-100 for 10 min. Then the cells were reacted with primary antibodies for TH (SANTA CRUZ BIOTECHNOLOGY,INC) at 4 °C for 24 h, and subsequently reacted with the FITC-conjugated IgG as the secondary antibody (SANTA CRUZ BIOTECHNOLOGY,INC) at room temperature for 1 h. In addition, the cells were incubated with diaminobenzidine (DAB) solution (Sigma Chemical Co.) for 30 s for nuclear staining.

### 2.5. Gene expression

The total RNA was extracted by RNXPLUS (Sinaclon, Iran). Quantification and purity of RNA was determined using a spectrophotometer (nanodrop 2000, Wilmington, USA). cDNA was synthesized using PrimeScript 1st strand cDNA synthesis Kit (Takara. Japan) according to the manufacturer's procedure. Real-time PCR was performed with an Applied BiosystemsTM Real-Time PCR System (Life Technologies Corporation, USA). All genes were subjected to 40 cycles, which consisted of a 15 min for initial denaturation at 95 °C, followed by forty cycles of 15 s at 95 °C, 30s annealing at 57 °C, and a final 30s extension at 72 °C. Related specific primers are presented in Table 1. The rest software from the rotor-gene Q, based on Pfaffl mathematical methods, was used for statistical analysis of real time data and relative gene expression [18].

## 3. Results

Cell culture and In vitro differentiation of trabecular meshwork mesenchymal stem cells (TM-MSCs) into mesenchymal lineages.

After 2 weeks, fibroblast-like cells with spindle-shaped morphology appeared on TCPS dishes. These cells were utilized for future experiments. To confirm their mesenchymal nature, the isolated cells were treated with appropriate osteo-, chondro- and adipoinduction media, and their differentiation was confirmed via appropriate staining including, alizarin red (for osteogenic differentiation), alcian blue (for chondrogenic differentiation) and oil red (for adipogenic differentiation) staining (Fig. 1 A–D).

### 3.1. Dopaminergic differentiation of TM-MSCs

Next, we studied the potential of TM-MSCs to differentiate into dopaminergic neural cells. To elucidate the dopaminergic neural differentiation potential of CJMSCs, these cells were cultured in neuroinductive medium including DMEM supplemented With RA, IBMX and Forskolin. During differentiation more morphological changes in cells were observed (Fig. 2). Following induction, bi and multipolar cells with multiple dendrites and cytoplasmic branches were observed 2, 5 and 7 days after induction in TM-MSCs (see Fig. 2). The SEM results indicated that the TM-MSCs were well

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