

Short communication

**THE DIFFERENTIATION OF HUMAN PLACENTA-DERIVED  
 MESENCHYMAL STEM CELLS INTO DOPAMINERGIC CELLS *in vitro***

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**Abstract:** Mesenchymal stem cells (MSCs) constitute an interesting cellular source to promote brain regeneration after Parkinson's disease. MSCs have significant advantages over other stem cell types, and greater potential for immediate clinical application. The aim of this study was to investigate whether MSCs from the human placenta could be induced to differentiate into dopaminergic cells. MSCs from the human placenta were isolated by digestion and density gradient fractionation, and their cell surface glycoproteins were analyzed by flow cytometry. These MSCs were cultured under conditions promoting differentiation into adipocytes and osteoblasts. Using a cocktail that includes basic fibroblast growth factor (bFGF), all trans retinoic acid (RA), ascorbic acid (AA) and 3-isobutyl-1-methylxanthine (IBMX), the MSCs were induced *in vitro* to become dopamine (DA) neurons. Then, the expression of the mRNA for the Nestin and tyrosine hydroxylase (TH) genes was assayed via RT-PCR. The expression of the Nestin, dopamine transporter (DAT), neuronal nuclear protein (NeuN) and TH proteins was determined via immunofluorescence. The synthesized and secreted DA was determined via ELISA. We found that MSCs from the human placenta exhibited a fibroblastoid morphology. Flow cytometric analyses showed that the MSCs were positive for CD44 and CD29, and negative for CD34, CD45, CD106 and HLA-DR. Moreover, they could be induced into adipocytes and osteocytes. When the MSCs were induced with bFGF, RA,

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Abbreviations used: AA – ascorbic acid; bFGF – basic fibroblast growth factor; DA – dopamine; DAT – dopamine transporter; DMEM/F12 – Dulbecco's modified Eagle's medium/F12; ESCs – embryonic stem cells; FBS – fetal bovine serum; IBMX – 3-isobutyl-1-methylxanthine; MSCs – mesenchymal stem cells; NeuN – neuronal nuclear protein; PBS – phosphate-buffered saline; RA – all trans retinoic acid; TH – tyrosine hydroxylase

AA and IBMX, they showed a change in morphology to that of neuronal-like cells. The induced cells expressed Nestin and TH mRNA, and the Nestin, DAT, NeuN and TH proteins, and synthesized and secreted DA. Our results suggest that MSCs from the human placenta have the ability to differentiate into dopaminergic cells.

**Key words:** Mesenchymal stem cell, Human, Placenta, Differentiation, Dopamine

## INTRODUCTION

Dopaminergic (DA) cell replacement therapy is a potentially successful therapy for patients with Parkinsonism. A few reports have been published on the derivation of DA neurons from mesenchymal stem cells (MSCs) and embryonic stem cells (ESCs) [1-4]. The disadvantages of ESCs are that they can form teratomas, and that the use of fetal or embryonic SCs poses major ethical concerns. The characteristics and potential of MSCs are such that using them might get around these issues and open new lines of application [1]. It was reported that MSCs could differentiate into various lineages of mesoderm cells, such as osteoblasts, adipocytes, islet-like cells and nerves [5-8] *in vitro*. MSCs from the human placenta are similar to those derived from adult bone marrow [9]. It is not known whether MSCs from the human placenta could differentiate into dopaminergic cells *in vitro*. The human placenta is a complex tissue consisting of vessels, mesenchyma and trophocytes derived from the extraembryonic mesoderm. In this project, by means of *in vitro* manipulation with basic fibroblast growth factor (bFGF), all trans retinoic acid (RA), ascorbic acid (AA) and 3-isobutyl-1-methylxanthine (IBMX), we induced MSCs from the human placenta to transform into dopamine (DA) neuronal cells. We showed that the induced MSCs not only express DA neuron-specific markers but also secrete DA.

## MATERIALS AND METHODS

### **The isolation and culture of mesenchymal stem cells from human placentae**

The use of human placentae was approved by the first affiliated hospital of Jinan University. Placentae were obtained by vaginal delivery or caesarean section from women with uncomplicated full-term pregnancies. The placentae were dissected carefully, and the harvested pieces of tissue were washed several times in phosphate-buffered saline (PBS), and then mechanically minced and enzymatically digested with 0.1% collagenase II (Gibco-Invitrogen) for approximately 30 min at 37°C. The digested tissue was filtered twice through a 100- $\mu$ m nylon membrane to eliminate undigested fragments. A cell suspension was collected by density gradient fractionation at 1000 r/min for 5 min. The mononuclear cells were suspended in 5 ml Dulbecco's modified Eagle's medium/F12 (DMEM/F12; Gibco-Invitrogen) supplemented with 10% fetal

bovine serum (FBS; PAA Laboratories, Austria), cultured at 37°C in 5% CO<sub>2</sub> in fully humidified air. 50% of the medium was replaced with fresh medium when the adherent cells reached 80% confluence.

#### **Analysis of the MSC surface glycoproteins by flow cytometry**

Cultured cells were harvested after three cell passages. The cells were incubated with the appropriate fluorochrome-conjugated antibodies for the various cell surface glycoproteins: CD44, CD29, CD34, CD45, CD106 (BD Biosciences, San Diego) and HLA-DR (Immunotech, Marseille, France). These are commonly used for the positive and negative detection of MSCs. The cells were incubated with the antibody for 30 min on ice, and immediately analyzed by flow cytometry (FACScan, BD Biosciences).

#### **Adipogenic and osteogenic differentiation of MSCs**

The cultured MSCs were assessed after the third passage. Adipogenic differentiation was assessed using DMEM/F12 supplemented with 100 ml/l FBS, 1 µmol/l dexamethasone and 5 U/ml insulin (Sigma, USA). Cells with adipogenic differentiation were identified by the cellular accumulation of neutral lipid vacuoles, which stained with Oil Red O (Sigma, USA). Osteogenic differentiation was assessed by incubating the cells using DMEM/F12 with 10% FBS, 0.1 µmol/l dexamethasone, 10 mmol/l β-glycerophosphate and 50 µmol/l ascorbate acid (Sigma, USA). Osteoblasts were identified by immunocytochemical staining with alkaline phosphatase.

#### **The induction of dopaminergic cell differentiation**

When the cultured MSCs grew at 70% confluence, the cells were induced with a cocktail of 10 ng/ml bFGF, 5 µM RA, 100 mM AA (Sigma, USA), and 0.1 mM IBMX (R&D Systems, Minneapolis, MN). The medium was not replaced during the induction period. As a control, MSCs were continuously cultured in DMEM/F12 supplemented with 10% FBS without the induction cocktail.

#### **Immunofluorescence staining**

The induced and uninduced MSCs were collected, washed in PBS, fixed with 4% paraformaldehyde (pH 7.4) for 10 min, and rinsed again with PBS. They were then permeabilized with 0.1% Tween-20 in PBS for 5 min and 3% bovine serum albumin (BSA, Sigma) for 30 min. The cells were incubated overnight at 4°C with the primary mouse anti-human antibody. The dilutions were as follows: Nestin, neuronal nuclear protein (NeuN), TH, 1/300; DAT 1:500 (Santa Cruz Biotechnology). The cells were washed twice in PBS and incubated for 1.5 h with the secondary fluorescence-conjugated goat anti-mouse antibody (DAKO, Denmark, Cy3, FITC, both dilutions at 1:1,000). The DNA-specific fluorescent dye Hoechst33258 (Sigma) was used to stain the cell nuclei. The fluorescence was visualized using microscopy (Leica, Germany). The cells were photographed with a Nikon camera (magnification, ×200).

### Reverse transcription-polymerase chain reaction

RNA was extracted from the cultured cells using a Trizol extraction Kit (Invitrogen). cDNA synthesis was performed using AMV reverse transcriptase (Takara, Japan) following the manufacturer's instructions. The primer pairs specific for Nestin were: forward primer: 5'-GCCCTGACCACTCCAGTTTA-3' and reverse primer: 5'-GGAGTCCTGGATTCCTTCC-3', and for TH were: forward primer: 5'-GTCCCCTGGTTCCCAAGAAAAGT-3' and reverse primer: 5'-TCCAGCTGGGGGATATTGTCTTC-3'. Polymerase chain reaction (PCR) amplification was done with the cycling parameters: 95°C for 30 s, 60°C for 30 s, and 72°C for 60 s for 40 cycles. The PCR products were separated by electrophoresis on a 1% agarose gel containing ethidium bromide.

### The dopamine enzyme-linked immunosorbent assay

The DA levels were quantitated using an enzyme-linked immunosorbent assay (ELISA) kit (IBL, Germany) according to the manufacturer's instructions. After the MSC differentiation, the extracted whole cell protein and collected culture supernatant were used for DA quantitation through ELISA.

### Statistical analysis

The data of quantitation was expressed as mean  $\pm$  SD. The results were analyzed by one-way ANOVA.  $P < 0.05$  was considered statistically significant.

## RESULTS

### The expression of MSC marks

Immunophenotype analysis revealed MSCs from the human placenta to be positive for CD44 and CD29, but negative for CD34, CD45, CD106 and HLA-DR (Tab. 1).

Tab. 1. The expressed markers of MSCs from the human placenta, as assessed by flow cytometric analysis (% mean  $\pm$  SD).

Marker	CD44	CD29	CD34	CD45	CD106	HLA-DR
Positive rate (%)	99.83 $\pm$ 2.26	99.76 $\pm$ 3.03	4.82 $\pm$ 1.63	0.90 $\pm$ 2.31	2.34 $\pm$ 2.19	1.02 $\pm$ 1.52

### The induction of differentiation into osteocytes and adipocytes

To verify that MSCs from the human placenta are multipotent, we analyzed their ability to differentiate into osteocytes and adipocytes. After a 28-day induction, it was shown that the MSCs differentiated into osteocytes (detectable increase in alkaline phosphatase levels) and adipocytes (detectable by Oil Red O staining) (Fig. 1).

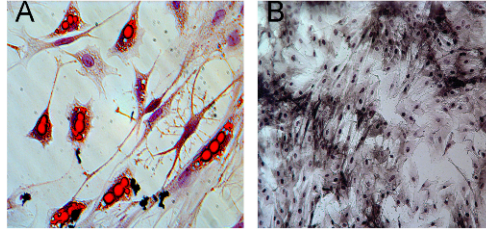


Fig. 1. Images of cell multilineage differentiation. A – Adipogenic differentiation of the MSCs by Oil Red O staining. B – Osteogenic differentiation of MSCs by alkaline phosphatase (AP) staining.

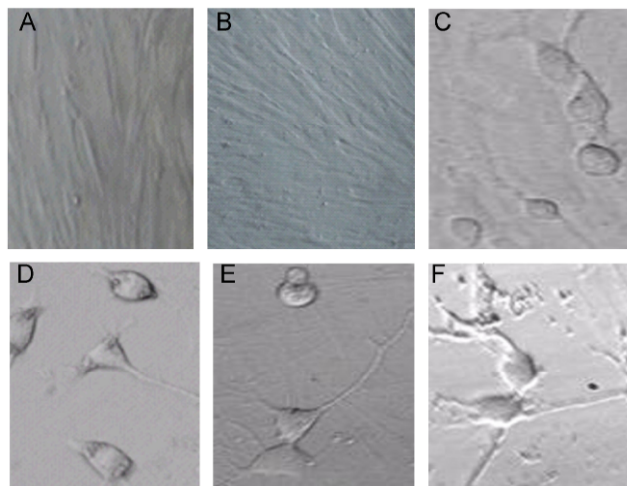


Fig. 2. The morphology of MSCs in culture. A – MSCs were isolated from the human placenta and cultured for 12 days. B – MSCs after three cell passages. C-F – MSCs induced as described in the main body of the text for 5 (C), 10 (D), 15 (E) and 20 days (F). Magnification, A, B  $\times 100$ , C-F  $\times 200$ .

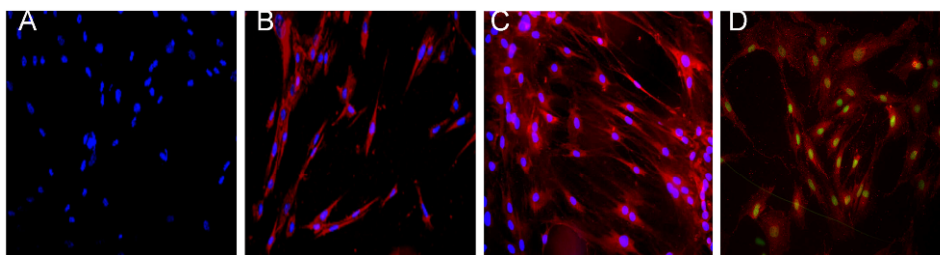


Fig. 3. Representative images of the immunofluorescence staining of MSCs that had been induced to dopaminergic differentiation for 0, 5, 7 and 10 days. A – MSCs stained on day 0 for TH displaying only the nuclear label Hoechst33258 (blue). B – The expression of Nestin (Cy3, red) on day 5. C – The expression of TH (Cy3, red) on day 7. D – A merged image (from three staining images) of MSCs that had been induced for 10 days and then stained for dopamine transporter (DAT) in the cytoplasm (Cy3, red), NeuN in the nucleus (FITC, green) and Hoechst33258 for nucleus (blue).

### Morphological characterization of the MSCs differentiation into DA cells

Uninduced MSCs are very flat, symmetrical, and spindle-shaped (Fig. 2A, B). A cocktail that included bFGF, RA, AA, IBMX was used to induce differentiation in the MSCs. After 5 days, they only showed a slight change in morphology (Fig. 2C). The round cells became gradually larger by day 10 (Fig. 2D). On day 15 (Fig. 2E), a characteristic neuronal morphology with contracted cytoplasm and a condensed nucleus was observed. Cytoplasmic connections between cells were observed after 20 days (Fig. 2F).

### Immunofluorescence staining

The levels of Nestin, DAT, NeuN and TH protein were measured after the differentiation via immunofluorescence staining. In undifferentiated MSCs, only the nucleus showed a blue color, which was the Hoechst33258 fluorescent signal (Fig. 3A). There was no fluorescent signal in the cytoplasm. Differentiated MSCs were positive for Nestin on day 5 (Fig. 3B), positive for TH on day 7 (Fig. 3C) and positive for DAT and NeuN on day 10 (Fig. 3D).

### The differentiated MSCs express DA-specific genes

To determine whether the induction protocol could induce MSCs to differentiate into neuronal cells with DA characteristics, we tested for the expression of the mRNA of genes known to be associated with DA neuron differentiation. DA neurons are classically identified by their expression of TH mRNA (TH is the rate-limiting enzyme in the biosynthesis of DA). As shown in Fig. 4, after 7 days of induction, the MSCs expressed TH and Nestin mRNA. Uninduced MSCs did not express TH mRNA, and only expressed low levels of Nestin on day 7.

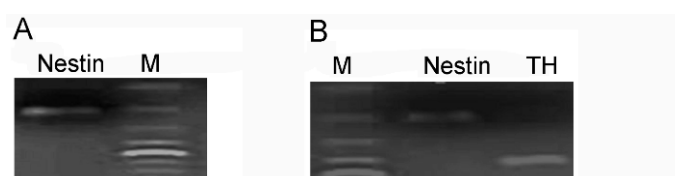


Fig. 4. The expression of dopamine-specific genes as assessed by RT-PCR. The expected sizes of the products of the Nestin and TH mRNA are respectively 199 bp and 331 bp. M is a 100-bp standard DNA ladder. The results are from a representative experiment. A – Nestin, weakly expressed before induction. B – Nestin and TH as expressed after induction.

### Differentiated MSCs secrete DA

To determine whether the induced MSCs synthesized and released DA, we measured the levels of DA. An ELISA assay of dopamine secretion showed that prior to induction and on day 1, dopamine was not detectable in the MSC medium. Fig. 5 shows that MSCs induced for 5, 10, 15 and 20 days continuously released DA into the extracellular medium. This level is significantly enhanced ( $P < 0.05$ ) compared with that for uninduced MSCs. The synthesized intracellular levels of DA were:  $12.86 \pm 2.25$  (day 5),  $18.15 \pm 3.87$  (day 10),

$25.29 \pm 4.29$  (day 15) and  $19.53 \pm 3.58$  ng/ml (day 20), which was a significant increase ( $P < 0.05$ ), compared with the untreated MSCs. The elongation of the induction process led to the decrease in DA level. This suggests that the differentiated MSCs synthesized and released DA continuously.

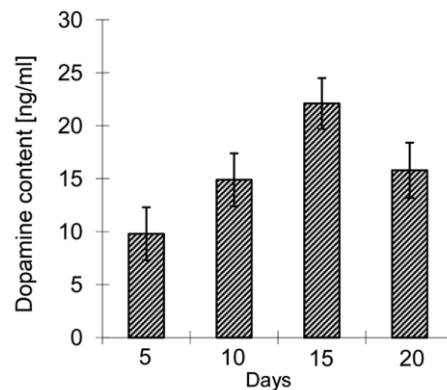


Fig. 5. The levels of dopamine secreted by the induced MSCs on days 5, 10, 15, and 20. The error bars show the standard deviation. The results are the average of three separate experiments

## DISCUSSION

Our results indicated that human placental MSCs are similar to those present in adult human bone marrow. Their morphology and immunophenotype are similar to those of adult bone marrow MSCs. Like bone marrow-derived MSCs, placental MSCs also express a large number of adhesion molecules, including CD29 and CD44, while being negative for CD106 and HLA-DR. That the isolated cells were indeed MSCs was verified by inducing them to differentiate into adipocytes and osteoblasts. Our results indicated that the isolated cells had the characteristics of MSCs.

Our results show that MSCs from the human placenta could differentiate into dopaminergic cells when exposed to an induction cocktail containing bFGF, RA, AA and IBMX. We also provide novel evidence that DA cells are generated by the *in vitro* induction of MSCs from the human placenta. Previous studies reported on the transdifferentiation of human bone marrow MSCs to DA neuronal cells [1, 10]. We used induction cocktails containing bFGF, RA, AA and IBMX to induce MSCs to TH-expressing cells. Prior studies done by Trzaska *et al.* [1] showed that human bone marrow MSCs gave a 67% yield of cells with DA markers after sequential induction with SHH, FGF8, and bFGF. The induced MSCs also synthesize and secrete DA in a dlarization-independent manner. Fu *et al.* [10] showed that MSCs were derived from the umbilical cord. However, they did not report on DA secretion.

In our study, during the 15-day differentiation, bFGF, RA, AA and IBMX induced MSCs to differentiate into cells with the morphological and functional characteristics of dopaminergic cells. The differentiated dopaminergic cells could produce and secrete dopamine. From the 5<sup>th</sup> day, the level of dopamine increased significantly compared with that from the controls, indicating that the differentiated MSCs were secreting dopamine. On the 15<sup>th</sup> day, the concentration of dopamine was highest, suggesting that MSCs had differentiated into mature dopaminergic cells. In our study, we found that Nestin protein could be detected on the 5<sup>th</sup> day after differentiation, TH protein could be detected on the 7<sup>th</sup> day after differentiation, and DAT and NeuN protein could be detected on the 10<sup>th</sup> day after differentiation. We also observed the expression of Nestin and TH mRNA after differentiation. The data suggests that human placental MSCs can be induced to differentiate into dopaminergic cells.

Retinoic acid regulates neuronal differentiation in the developing nervous system, in embryonic stem cells, and in adult neural progenitors [11-13]. Recent reports indicate that bFGF has significant effects on neurogenesis [14-15]. The induction factor IBMX is a PKC activator that increases ascorbic acid, which is directly linked to dopaminergic differentiation [16].

In conclusion, MSCs from the human placenta can be isolated, expanded, and differentiated into dopaminergic cells. Our study implies that MSCs from the human placenta might be useful cells for the generation of MSC-derived dopaminergic neurons. Further studies are necessary to test the functional properties of DA-secreting cells from induced human placental MSCs in animal models of Parkinson's disease.

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