Differentiation of Dopaminergic Neurons from Mesenchymal-Like Stem Cells Derived from Human Umbilical Cord Vein

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ABSTRACT : One of the most extensively studied populations of multipotent adult stem cells are mesenchymal stem cells (MSCs). MSCs derived from the human umbilical cord vein (HUC-MSCs) are morphologically and immunophenotypically similar to MSCs isolated from bone marrow. HUC-MSCs are multipotent stem cells, differ from hematopoietic stem cells and can be differentiated into neural cells. Since neural tissue has limited intrinsic capacity of repair after injury, the identification of alternate sources of neural stem cells has broad clinical potential. We isolated mesenchymal-like stem cells from the human umbilical cord vein, and studied transdifferentiation-promoting conditions in neural cells. Dopaminergic neuronal differentiation of HUC-MSCs was also studied. Neural differentiation was induced by adding bFGF, EGF, dimethyl sulfoxide (DMSO) and butylated hydroxyanisole (BHA) in N2 medium and N2 supplement. The immunoreactive cells for β-tubulin III, a neuron-specific marker, GFAP, an astrocyte marker, or Gal-C, an oligodendrocyte marker, were found. HUC-MSCs treated with bFGF, SHH and FGF8 were differentiated into dopaminergic neurons that were immunopositive for tyrosine hydroxylase (TH) antibody. HUC-MSCs treated with DMSO and BHA rapidly showed the morphology of multipolar neurons. Both immunocytochemistry and RT-PCR analysis indicated that the expression of a number of neural markers including NeuroD1, β-tubulin III, GFAP and nestin was markedly elevated during this acute differentiation. While the stem cell markers such as SCF, C-kit, and Stat-3 were not expressed after neural differentiation, we confirmed the differentiation of dopaminergic neurons by TH/β-tubulin III positive cells. In conclusion, HUC-MSCs can be differentiated into dopaminergic neurons and these findings suggest that HUC-MSCs are alternative cell source of therapeutic treatment for neurodegenerative diseases.

Key words : Human umbilical cord vein, Mesenchymal stem cells, Dopaminergic neuron, Neural differentiation.

INTRODUCTION

Adult stem cells are rare cells thought to be present in all tissues and responsible for maintaining the specific tissue. Adult stem cells are tissue-specific, may have less replicative capacity, and until recently, were thought to have restricted developmental fates (Paul et al., 2002). Recent studies indicate that tissue-specific stem cells are capable of differentiating into cells of other tissues. One of the most extensively studied populations of multipotent adult stem cells are mesenchymal stem cells (MSCs).

Recently, there has been increasing interest in the MSCs, because they have plastic properties, allowing themselves to differentiate into chondrocytes, osteoblasts, adipocytes, fibroblasts, marrow stroma, and other tissues of mesen-

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chymal origin (Pittenger et al., 1999). Due to this ability, confirmed by the results of either *in vitro* experiments (Schwartz et al., 2002) or *in vivo* studies (Toma et al., 2002), MSCs appear to be an attractive tool in the context of tissue engineering and cell-based therapy.

Recent evidence has suggested that MSCs are capable of changing their commitment to a neuronal phenotype. Bone marrow stromal cells injected into lateral ventricles of neonatal mice differentiated into astrocytes and neurofilament-expressing cells (Kopen et al., 1999). Woodbury et al. (2000) showed that bone marrow stromal cells could be induced to differentiate into neurons, almost exclusively, by using a modified bFGF method that induces embryonic stem cells to form neural cell types. MSCs may have differentiated into early progenitors of neural cells *in vitro* under conditions that increase the intracellular level of cAMP (Deng et al., 2001) and by coculture with rat fetal mesencephalic or striatal cells (Sanchez-Ramos et al., 2000).

Bone marrow had been the main source of MSCs for both experimental and clinical studies (Deans & Moseley, 2000). However, the number of bone marrow (BM) MSCs significantly decreases with age and BM-MSCs can be obtained only by an invasive procedure. In this connection, most attention should be paid to tissues containing cells with higher proliferative potency, capability of differentiation, and lower risk of viral contamination. Recently investigators isolated a cell population from the endothelium/subendothelium layer of the human umbilical cords. The cells are morphologically and immunophenotypically similar to MSCs isolated from bone marrow and also show the ability of adipogenic, osteogenic and endothelial differentiation (Kestendjieva et al., 2008). On the basis of these findings, we investigated that human umbilical cord-derived mesenchymal stem cells (HUC-MSCs) might also differentiate into neuronal cells.

Dopaminergic neurons of the ventral midbrain play important roles in the regulation of motor performances, behavior, and cognition. Neuronal loss results in the functional alterations of dopaminergic systems and dopamine receptors are linked to disorders, such as Parkinson's disease (Hirsch et al., 1988). An approach for restoration of the damaged dopaminergic system is transplantation of cells that synthesize catecholamines (Lindvall, 1989). Clinical trials of transplantation using human fetal nigral tissues have shown symptomatic relief (Mahowald et al., 1987), however, technical and ethical difficulties in obtaining sufficient and appropriate graft tissues have limited the application of this therapy. Using neuroepithelial explant cultures, Ye et al. (1998) have shown that fibroblast growth factor 8 (FGF8) and signal sonic hedgehog (SHH) can specify a dopaminergic fate from neuroepithelial cells. The same principle seems to apply to *in vitro*-generated neuroepithelial cells. Mouse embryonic stem cells are first differentiated into neuroepithelial cells, expanded in the presence FGF2, and further differentiation into dopaminergic neurons in response to FGF8 and SHH (Lee et al., 2000).

In this paper, we report that umbilical cord-derived MSCs are able to rapidly differentiation into cells that express a number of neuron-, glia- and dopaminergic neuron-pecific markers. Our findings implicate that HUC-MSCs could be a suitable cell source for a therapy of various neurodegenerative disease.

MATERIALS AND METHODS

1. Isolation and Culture of MSCs from Human Umbilical Cord Vein

Isolation of cells from umbilical cords was processed within 24 hours after normal deliveries. All experiments were approved by Institutional Review Boards of Seoul Women's University and Inje University. Umbilical vein was flushed with 10 ㎖ of low-glucose type DMEM (DMEM-LG)(Invitrogen, USA). The vein was filled with 0.5% crude collagenase in phosphate-buffered saline (PBS) and both proximal and distal ends were clamped. After incubation at 37℃ for 15 min, the collagenase solution

was collected and centrifuged for 5 min at 300 × g. Cell pellets were resuspended in DMEM-LG containing 20% fetal bovine serum, 2 mM L-glutamine, 100 U/㎖ penicillin G, 100 ㎍/㎖ streptomycin and 10 ng/㎖ EG). Cells were seeded in 25 ㎠ culture flasks at the concentration of 2×10^4 cells/㎠. Cultures were maintained at 37℃ in a humidified atmosphere containing 5% CO_2. After culture for 3 days nonadherent cells were removed and thereafter the medium was changed every 3 days. Well-developed colonies of fibroblast-like cells, designated as CC, appeared 2 weeks later. When these cells filled up the culture flasks, they were detached from the bottom using Hank's balanced salt solution containing 0.125% trypsin and 1 mM EDTA. After several washing, cells were seeded into 75 ㎠ culture flask at 2×10^4 cells/㎠ for the subculture.

2. Induction of Neural Cells from HUC-MSCs

HUC-MSCs were induced to differentiate into neural stem cells and neuronal cells by the modified method of Woodbury et al. (2000) Cells were cultured in DMEM containing 20% FBS, 0.001% β-mercaptoethanol (Sigma, USA), 1X nonessential amino acid (Invitrogen, USA), and 2 mM glutamine (Invitrogen, USA). After 24 to 72 h, the cells were preinduced by overnight treatment with 20 ng/㎖ epithelial growth factor (EGF, Sigma, USA) and 20 ng/㎖ basic fibroblast growth factor (bFGF, Sigma, USA) in N2 medium (Invitrogen, USA). Neuronal differentiation was induced with 2% DMSO (Sigma, USA) and 200 ㎛ butylated hydroxyanisole (BHA) in N2 medium and 1 × N2 supplement. After 5 h, the medium was modified for long-term induction by adding 25 mM KCl, 2 mM valproic acid (Sigma, USA), and 1 ㎛ hydrocortisone (Sigma, USA). A total of 1×10^4 cells/well were seeded onto a cover slip pre-coated with poly-d-lysine, 15 ㎎/㎖ polyornithine (Sigma, USA) and 1 ㎎/㎖ laminin (Sigma, USA).

3. Induction of Dopaminergic Neurons from HUC-MSCs

HUC-MSCs were induced to differentiate into dopaminergic neurons by modified method of Perrier et al. (2004). Cells were cultured in consisting of DMEM, 20% FBS, 0.001% β-mercaptoethanol, 1X nonessential amino acid, and 2 mM glutamine for dedifferentiation. After 24 to 72 hours, the cells were preinduced by overnight treatment with 20 ng/㎖ EGF and 20 ng/㎖ bFGF in N2 medium and 1X N2 supplement. The dopaminergic neuronal differentiation medium was composed of 100 ng/㎖ FGF8, 200 ng/㎖ SHH, 0.2 mM ascorbic acid (AA, Sigma, USA) and 20 ng/㎖ brain-derived neurotrophic factor (BDNF, Invitrogen, USA) in N2 medium. After 7 to 9 d, cells were again cultured in the absence of SHH and FGF8 but in the presence of 20 ng/㎖ BDNF, 20 ng/㎖ glial cell-line neurotrophic factor (GDNF, Invitrogen, USA), 1 mM dibutyryl cAMP (Sigma, USA) and 0.2 mM AA.

4. Immunocytochemistry

Cells cultivated in a medium for the induction of neuronal differentiation were washed with PBS and then fixed with 4% paraformaldehyde (Sigma, USA) in PBS for 20 min at 4℃. This was followed by washing three times with PBS, cells were permeabilized with 1% Triton X-100 for 20 min. Cover slip was blocked with 10% normal blocking serum (derived from same species as the secondary antibody) in PBS for 1 h, washed with PBS, and incubated with primary antibody against the following antigens for 3 h: neuron-specific β-tubulin III (1:400, Sigma, USA), glial fibrillary acidic protein (GFAP, 1:200, Thermo Scientific, USA), galactocerebroside (Gal-C, 1:200, Chemicon, USA), and Tyrosine hydroxylase (TH, 1:250, Pel-Freeze Biochemicals, USA). The cover slips were then washed three times with PBS and incubated with fluorescein isothiocyanate (FITC)-conjugated secondary antibody (1:128, Sigma, USA) for 30 min. Resulting immunoreactive cells were visualized by Axioskop2+ fluorescence microscopy (Carl Zeiss, Germany).

5. Total RNA Extraction and RT-PCR

Total RNA was extracted from 1×10^6 cells by using Tri-Reagent (Sigma, USA). Briefly, total RNA samples (10 ㎍) were reverse-transcribed using 2.5 U MMLV re-

Table 1. Primers used for the RT-PCR analysis and expected size of PCR products

Gene	Forward (5'-3')	Reverse (3'-5')	Size (bp)
SCF	ttacaagggcagttgaaaat	ttccaactgaatcatcc	263
Stat-3	tctcctacttctgctatctttgag	atgggtctcagagaacacatc	117
Wnt-1	atggggctctgggcgctgttgc	cccactcacgctgtgcaggatc	252
Pax-6	agattcagatgaggctcaaa	aattggttggtagacactgg	313
NeuroD1	agtccgccttacggtaccatg	gacagtcactgtaagcacag	448
Nestin	ccagaaactcaagcaccac	ttttccactccagccatcc	398
GPC4	tgggaaaggcaaaagcag	cagaagacagtgaggaggtag	582
β-tubulin III	cctgacaatttcatctttgg	cagtaggtttcatccgtgtt	344
GFAP	tcatcgctcaggaggtcctt	ctgttgccagagatggaggtt	383
MBP	acacgggcatccttgactccatcgg	tccggaaccaggtggttttcagcg	263
GAPDH	acaactgtatcgtggaa	aaattcgttgtcataccagg	456

verse transcriptase and 0.5 μg oligo (dT) primer in the presence of 1 mM dNTP and 1 U RNasin in a final volume of 50 $\mu\ell$. Each cDNA subpopulation was subjected to PCR amplification using gene-specific upstream and downstream primers. PCR was performed in a 25 $\mu\ell$ reaction mixture containing 25 mM $MgCl_2$, 10X PCR buffer, 2 U Taq polymerase, 10 mM dNTP, 100 pM each gene-specific upstream and downstream primers and nuclease-free water. The PCR products were mixed with 6X loading buffer (0.25% bromophenol blue, 0.25% xylene cyanol and 40% sucrose) and separated on 2% agarose gels. After the gel was stained with ethidium bromide, the DNA bands on the gel were imaged under ultraviolet light.

RESULTS

1. Establishment of Primary Culture

When the cells isolated from human umbilical cord vein were cultured, several different types of cells were initially found after 17 d of culture. However, after 40 d without subculture, only one type of cells were observed, whose morphology was very similar to BM-MSCs that filled up the culture flasks. During successive subculture, these cells maintained their MSC-like morphology until 18th passages (Fig. 1).

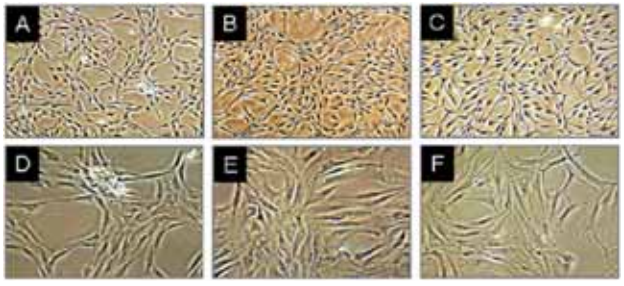

Fig. 1. Morphology of MSC-like cells isolated from human umbilical cord vein. The appearance and growth of MSC-like cells at 2nd (A, D), 7th (B, E), and 13th (C, F) passage are shown. Note a fibroblastic morphology. Magnification: (A-C) ×100, (D-F) ×200.

2. *In Vitro* Neural Differentiation of HUC-MSCs

A number of HUC-MSCs have been shown to differentiate into neurons and glia. To investigate the neurogenic potential of HUC-MSCs, subconfluent cells were incubated for 48 h in a dedifferentiation medium containing β-mercaptoethanol and overnight in a pre-induction medium containing EGF and bFGF, then transferred to induction medium containing BHA and DMSO and maintained up to 4 d. After culture in differentiation medium, the large flat HUC-MSCs were transformed into spindle-shaped cells with long processes (Fig. 2). HUC-MSCs had changed their morphology and adopted neuronal- or glial-like phenotypes, distinguished by highly refractive cell

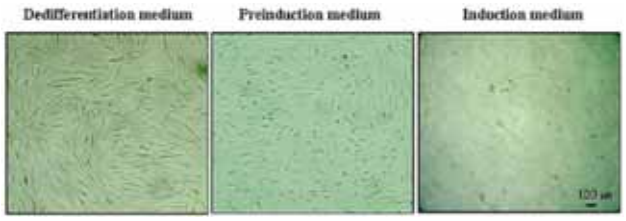

Fig. 2. Changes in cellular morphology after exposure of HUC-MSCs to induction medium. Phase-contrast images show HUC-MSCs before and after incubation with induction medium. Dedifferentiated and preinduced cultures of cells consisted of predominantly spindle-shaped cells and few large and flat cells. After induction, cells exhibited rapidly the multipolar neuron-like morphology. Scale bar = 100 ㎛.

bodies with neurite-like processes terminating in structures resembling growth cones.

3. Immunocytochemistry of Proteins and RT-PCR Analysis of Gene Expression upon Neural Induction

Immunocytochemical staining for neural markers was performed to confirm the cells expressing neural markers among the differentiated HUC-MSCs. According to the neural inducing protocol, cells were fixed and immunostained for specific markers for neural phenotypes. There was no evidence of neural morphology in cells that had not been treated with induction medium, and those cells were not stained for any of the neural antibodies. However, cells cultured with induction medium were stained with antibodies against specific markers for the neuron (β-tubulin III), astrocyte (GFAP) and oligodendrocyte (Gal-C) (Fig. 3). Approximately, 51 ± 7% of the cells treated with the induction medium were positive for β-tubulin III, 17 ± 3% for GFAP and 14 ± 3% for Gal-C (Fig. 3). Markedly elevated expression of neural marker genes during induction was also detected by RT-PCR analysis. The mRNAs for neural markers of β-tubulin III, GFAP and MBP were expressed in neural induced cells. On the other hand, expression of nestin and GPC4, known as markers for neural precursor cells, were detected during treatment with the pre-induction medium but diminished during treatment with the induction medium (Fig. 4).

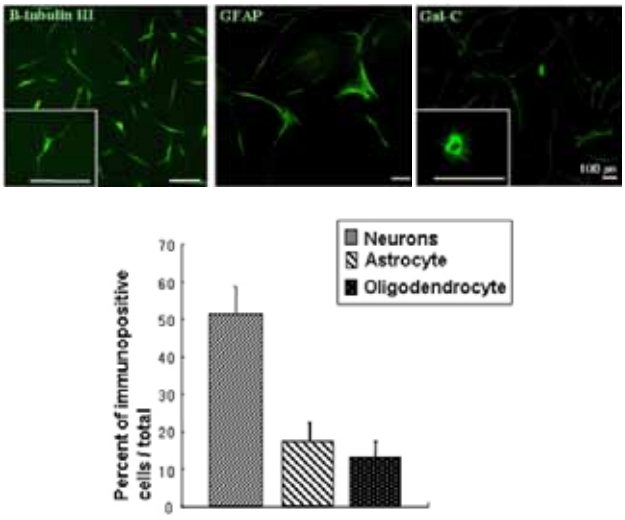

Fig. 3. Cell type-specific immunofluorescence staining of HUC-MSCs cultured in neural induction medium. After incubation for 48 h followed by the treatment with DMSO/BHA, cells were stained for the presence of β-tubulin III, GFAP and Gal-C proteins. β-tubulin III-positive cells displayed neuron-like morphology with long axonal projection and Gal-C-positive cells displayed morphology characteristic of oligodendrocytes, with flat cell body and short or long branched projections. Percentages of neurons, astrocytes and oligodendrocytes were expressed as relative values to the total of cell number per field. Data represent the means ± SEM. Scale bars = 100 ㎛.

4. Dopaminergic Neuronal Differentiation

It was further tested whether TH-positive cells could be generated from HUC-MSCs by treating FGF8 and SHH. Such treatment made HUC-MSCs undergo profound changes in morphology with some cells developing multiple neurites extending from the cell body. The region around a nucleus became narrower and thicker to form a cell-body-like structure while the rest of cytoplasm was elongated to give rise to multiple cellular processes and form complex network (Fig. 5). An immunocytochemistry showed that 38.7 ± 5.7% of cells were TH-immunopositive. The dopaminergic phenotype was confirmed by morphological examination including fluorescence microscopic visualization of TH-positive immunocytochemistry (Fig. 6).

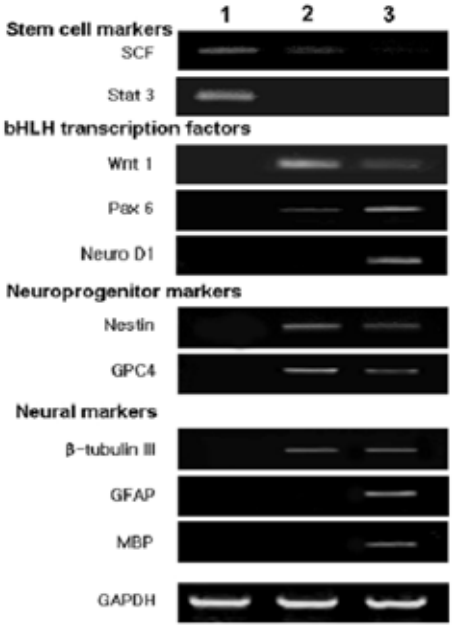

Fig. 4. RT-PCR analysis of cell type-specific genes. Lane 1, dedifferentiated cells; lane 2, preinduced cells; lane 3, cells grown in induction medium. RT-PCR shows a higher expression level of neural markers of β-tubulin III, GFAP and MBP when the cells were treated with both BHA and DMSO compared with undifferentiated cells. Dedifferentiated cells express only stem cell markers of SCF and Stat-3. The expression of bHLH transcription factors related to neurogenesis and neuroprogenitor markers were induced by preinduction medium. GAPDH was used as an internal control.

Fig. 5. Changes in cellular morphology of HUC-MSCs after exposure to induction medium. Phase-contrast microscopic images are showing HUC-MSCs before and after incubation with dopaminergic neuronal induction medium. Dedifferentiated and preinduced cultures of cells consisted predominantly of large and flat cells. Cells cultured in induction medium exhibited the multipolar neuron-like morphology and formed complex networks. Scale bars = 100 ㎛.

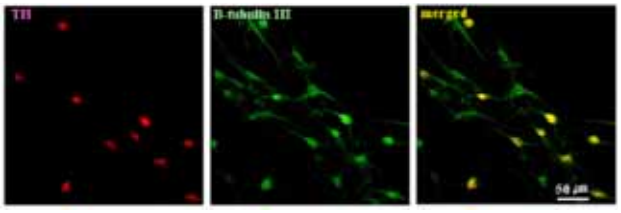

Fig. 6. Dopaminergic neuronal differentiation of HUC-MSCs cultured in induction medium. TH-immunoreactive cells (red) were generated from HUC-MSCs in induction media containing FGF8, SHH, ascorbic acid and BDNF (see materials and methods for details). Beta-tubulin III-immunoreactive cells are shown in green. Double-labeled cells are dopaminergic neurons derived from HUC-MSCs. Scale bars = 50 ㎛.

DISCUSSION

In the present study, mesenchymal like stem cell population was successfully isolated from the subendothelial layers of the human umbilical cord vein. Isolated cells were morphologically similar to bone marrow-derived MSCs. During the successive subcultures, the HUC-MSCs maintained fibroblast-like morphology.

HUC-MSCs underwent changes in morphology and expressed neural-specific proteins when they were cultured with a modified method of Woodbury et al. (2000). Cells with neuronal and glial phenotypes were induced, and many cell type-specific markers for neurons and glial cells were identified in these cells by immunocytochemistry and RT-PCR. Neural induced HUC-MSCs showed positive staining against β-tubulin III, GFAP, and Gal-C antibodies. Therefore, HUC-MSCs maybe easily attainable source of multipotent stem cells that can be cultured *in vitro* and be induced to differentiation to neural cells. This result is consistent with results from bone marrow stromal cells, a source of adult stem cells (Sanchez-Ramos et al., 2000; Woodbury et al., 2000).

Deng et al. (2001) found that about 25% of hMSC differentiated into neural cells by treatment with isobutylmethylxanthine and dibutyryl cyclic AMP. Kim et al.

(2002) observed that approximately 40% of hMSC differentiated into cells expressing neurofilament in the presence of fibroblast growth factor and retinoic acid. In our study, approximately, 51 ± 7% of the differentiated cells were positive for β-tubulin III, 17 ± 3% for GFAP and 14 ± 3% for Gal-C. These observations suggest that the HUC-MSCs are more easily to differentiate into neural cells and thus might be closer to a cell lineage differentiating into nervous and other tissues.

Upon neurogenic stimulations, HUC-MSCs expressed mRNAs of β-tubulin III, GFAP and MBP, suggesting that they could differentiate along a neural fate. The differentiation into mature neural cells was further convinced by a concomitant decrease in expression of nestin and GPC4, because this marker protein was known to be involved in the early but not late stage in neurogenesis. Expression of several transcription factors including Pax6, Wnt1 and NeuroD1 in HUC-MSCs were investigated after neural induction. These bHLH transcription factors are known to regulate neuronal differentiation of embryonic stem cells or neural stem cells and appear in early stages of neural development (Kageyama et al., 1997). Wnt signaling pathways are expressed in developing and mature central nerve system (CNS) and control patterning decisions in embryonic CNS (Patapoutian & Reichardt, 2000). Pax6, a paired box-containing gene family member, is expressed in mitotically active neural stem/progenitor cells in the ventricular zone during early CNS development (Gajovic et al., 1997). Expression of NeuroD1 serves as a particularly useful marker of preneural cells preparing to follow a program of neural differentiation (Lee et al., 1995) and is the first member of the mammalian NeuroD family (D1-D3) to appear during neuronal development (McCormick et al., 1996). In HUC-MSCs, Wnt1 was expressed when they were cultured in preinduction medium but the expression was decreased when they were transferred to the induction medium. Pax6 was weakly expressed during culture in preinduction medium and strongly expressed in induction medium. NeuroD1 was expressed only in induction medium. It seems that bHLH transcription factor genes involved in the genetic program of neuronal differentiation are expressed in HUC-MSCs during culture in neural differentiation medium. Collectively, these data revealed that exposure to specific extrinsic factors could alter the phenotypic and genetic fate of HUC-MSCs towards neural lineages.

An alternative approach that has been targeted using for the repair of the damaged dopaminergic system is application of stem cells (Björklund & Lindvall, 2000). To update, several different stem cells have been investigated for dopaminergic neuronal differentiation, such as embryonic stem cells (Perrier et al., 2004) and MSCs, for treatment of PD disease. Despite the fact the bone marrow is considered to be a well-accepted source of MSCs, the clinical use of MSCs from bone marrow has many problems. This has led many researchers to investigate alternate sources of MSCs for treatment of PD disease. To date, these cells have been differentiated into dopaminergic neurons from Wharton's jelly tissues (Fu et al., 2006).

In this work, we differentiated HUC-MSCs into specific dopaminergic neuron. The development of midbrain dopaminergic neurons requires a complex combination of transcriptional regulators and diffusible signals to control both the acquisition and maintenance of a neurotransmitter-specific phenotype (Maxwell & Li, 2005). The ontogeny of dopaminergic neurons is not clear and remains a focus of intensive research. Studies to date have focused on the genesis of mesencephalic dopaminergic neurons. The generation of ventral midbrain, hindbrain, and spinal cord-type neurons requires the ventralizing SHH in conjunction with factors that define anterior-posterior patterning, such as FGF8, FGF4, and retinoic acid. And several transcription factors, including Lmx1b, engrailed-1, Nurr1, and Ptx3, through independent pathways, are shown to be required for survival and/or maturation of dopaminergic neurons (Smidt et al., 2000). In this study, we differentiated HUC-MSCs into dopaminergic neurons in N2 medium containing FGF8, SHH, GDNF, BDNF and AA in

stage 2. Immunocytochemistry for TH antibody was used for the confirmation of differentiation of HUC-MSCs into dopaminergic neurons. TH protein is the initial and rate-limiting enzyme in the catecholamine synthesis pathway, and is considered the principal regulator of dopamine biosynthesis in the central neuron system. In this work, up to 40% of cells were positive for TH antibody. This showed that HUC-MSCs are differentiated into dopaminergic neurons and are good candidates for neurological cell therapy.

In conclusion, our results show that HUC-MSCs can be easily isolated and expanded in culture and differentiate into neural cells and dopaminergic neurons. HUC-MSCs are more accessible and easier to get in comparison with BM-MSCs, the umbilical cord vessel can serve as a rich source of MSCs which may be used in experimental and clinical demands and these cells may therefore prove to be a new source of cells for cellular therapies. Such studies may have a significant impact on the cell therapy, and potentially help to avoid worrying ethical issues.

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