

In vitro differentiation of cord blood unrestricted somatic stem cells expressing dopamine-associated genes into neuron-like cells

Mohammad Fallahi-Sichani ^{a,d}, Masoud Soleimani ^{b,d,*}, Seyed Mahmoud Arab Najafi ^c,
Jafar Kiani ^{b,d}, Ehsan Arefian ^{b,d}, Amir Atashi ^{b,d}

^a Department of Biotechnology, University College of Science, University of Tehran, Tehran 14155-6455, Iran

^b Department of Hematology, Faculty of Medical Sciences, Tarbiat Modares University, Tehran 14115-111, Iran

^c School of Biology, University College of Science, University of Tehran, Tehran 14155-6455, Iran

^d Stem Cell Technology Co. Ltd., Tehran, Iran

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Abstract

An intensive study is underway to evaluate different potential candidates for cell therapy of neurodegenerative disorders such as Parkinson's disease (PD). Availability and lower immunogenicity compared to other sources for stem cell therapy such as bone marrow have made human umbilical cord blood stem cells a considerable source for cell therapy. The present study aimed to investigate differentiation of recently introduced pluripotent cord blood stem cells, known as unrestricted somatic stem cells (USSCs), into cells with neural features in serum-withdrawal medium. Using reverse transcription polymerase chain reaction and immunocytochemistry assays, we have shown the expression of neuron-specific genes following a 2 week treatment of USSCs in serum-withdrawal induction medium. In addition, we have found that USSCs and USSC-derived neuron-like cells express transcripts of genes associated with development and/or survival of dopaminergic mesencephalic neurons including En1, En2, Nurr1, Ptx3, Pax2, Wnt1 and Wnt3a. The expression of dopamine-associated genes suggests that these cells may be potential candidates to be used for cell therapy of PD.

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1. Introduction

Parkinson's disease (PD), a progressive neurological disorder of the central nervous system is caused by the degeneration of dopaminergic (DA) neurons in the substantia nigra (SN) and ventral tegmental area (VTA) of the midbrain (Hornykiewicz, 1966; Kish et al., 1988). Although a range of pharmacological options and specific surgeries are currently used to reverse the symptoms of PD (Cotzias et al., 1967; Obeso et al., 2000; Olanow et al., 2000; Pollak et al., 1997), they provide only limited relief and an efficient therapeutic agent to prevent or cure PD is highly desirable.

In recent years, novel therapeutic strategies including stem cell therapy have been targeted for the treatment of central nervous system disorders (Björklund and Lindvall, 2000). Stem cells isolated from different resources such as pluripotent embryonic stem cells (Ben-Hur et al., 2004; Björklund et al., 2002; Kim et al., 2002), neural stem cells (Martino and Pluchino, 2006; Sanchez-Pernaute et al., 2001; Studer et al., 1998), adult bone marrow stem cells (Hellmann et al., 2006; Mezey et al., 2003) and umbilical cord blood stem cells (Sanberg et al., 2005; Buzanska et al., 2002; Sun et al., 2005) have been shown to generate differentiated neuronal and glial cells both *in vitro* and *in vivo*, which can be used for replacement therapy in various neurodegenerative diseases such as PD. However, each stem cell type has its own limitations, which emphasizes the importance of exploring and evaluating different sources of stem cells to identify potential candidates for cell therapy.

* Corresponding author. Tel.: +98 21 88011001x4508; fax: +98 21 88013030.

E-mail address: soleim_m@modares.ac.ir (M. Soleimani).

Umbilical cord blood cells are easily available and less immunogenic than other sources for stem cell therapy such as bone marrow (Sanberg et al., 2005), and they could potentially serve as a routine starting material for isolation and expansion of cells for allogenic as well as autologous transplantations (Buzanska et al., 2002). Human unrestricted somatic stem cells (USSCs) are self-renewing pluripotent cells, recently isolated from human cord blood (CB) (Jager et al., 2003). USSCs have been shown to be CD45 negative, produce functionally significant amounts of hematopoiesis-supporting cytokines (Kögler et al., 2005), and express HLA class II in smaller amounts than other progenitor cells (Kögler et al., 2004). Under lineage-specific stimulation, USSCs have been shown to differentiate into osteo-, chondro- and adipoblasts as well as muscle, hematopoietic and neural cells *in vitro* (Jager et al., 2003, 2004, 2006; Kögler et al., 2004; Wild et al., 2004). *In vivo* differentiation of these pluripotent cells into bone, cartilage, heart and liver has been also investigated (Kögler et al., 2004; Kim et al., 2005). We selected USSCs for our study.

The development of dopaminergic mesencephalic neurons has been shown to require the participation of different genes and factors that are expressed in different stages of dopaminergic neuron development. Expression of some transcription factors, including engrailed-1 (En1), Nurr1 and Ptx3 has been shown to be necessary for the survival and/or differentiation of DA neurons and may regulate the expression of other genes needed to produce dopamine (Alberi et al., 2004; Jankovic et al., 2005; Martinat et al., 2006; Nunes et al., 2003; Saucedo-Cardenas et al., 1998; Simon et al., 2001). In this study, we investigated the expression of genes associated with DA progenitor/precursor cells and developing DA neurons in USSCs. Moreover, we have suggested a multistep protocol for *in vitro* differentiation of USSCs into neuron-like cells in 2 weeks.

2. Materials and methods

2.1. USSC isolation and culture

Collection, isolation and propagation of human USSCs were performed as described by Kögler et al. (2004). In brief, CB was collected from the umbilical cord vein with informed consent of the mother and the mononuclear cell fraction was separated by density centrifugation over a Ficoll-Hypaque gradient (Pharmacia-Amersham; d 1.077 g/ml). In this work, USSCs were successfully generated from 4 out of 11 cord blood samples and studied. Growth and expansion of the cells were performed in low glucose DMEM (Gibco) supplemented with 30% FBS (Gibco). USSCs were cultured in a humidified atmosphere of 95% air with 5% CO₂ at 37 °C and extensively propagated. When cells reached 80% confluency, they were detached with 0.25% Trypsin-EDTA (Gibco) and replated.

2.2. Neural differentiation

Human USSCs were induced to differentiate into neuron-like cells in serum-withdrawal medium in 2 weeks using a multistep protocol. USSCs seeded at a density of 3000–4000 cells/cm², were treated with the first step medium containing DMEM/F12 (Gibco) supplemented with 1% insulin/transferrin/selenium (ITS) supplement (Gibco), 2% B27 supplement (Gibco), 10 ng/ml basic fibroblast growth factor (bFGF; Peprotech) and 5 μM retinoic acid (RA; Sigma). After 1 week, the medium was changed to one containing ITS

and B27 supplements with 1 mM dibutyryl cyclic AMP (dbcAMP; Sigma) and 100 μM ascorbic acid (AA; Sigma) and cells were treated for 4 days. The third step treatment of the cells was performed in medium containing ITS and B27 with 10 μM forskolin (Sigma), 0.1 mM isobutylmethylxanthine (IBMX; Sigma) and 100 μM AA for 3 days. All culture media were changed every 2–3 days.

2.3. Immunocytochemistry analysis

Cells were fixed with 4% paraformaldehyde, permeabilized with Triton X-100 (0.3%) and processed for immunocytochemistry using primary antibodies to βTubulin III 1:50 (mouse monoclonal; Chemicon), and NFM 1:500 (mouse monoclonal; Sigma). For fluorescence, fluorescein isothiocyanate (FITC)-conjugated (Sigma) anti-mouse secondary antibody 1:500 was applied.

2.4. Total RNA isolation and RT-PCR analysis

For RT-PCR analysis, total cellular RNA was extracted using TRI-reagent (Sigma). Synthesis of cDNA was carried out with M-MuLV reverse transcriptase (RT) and random hexamer as primer, according to the manufacturer's instructions (Fermentas). PCR amplification was performed using a standard procedure with Taq DNA Polymerase (Fermentas) with denaturation at 94 °C for 15 s, annealing at 55 °C or 60 °C for 30 s according to the primers, and extension at 72 °C for 45 s. The number of cycles varied between 30 and 40, depending on the abundance of particular mRNA. The primers and product lengths are listed in Table 1.

Table 1
Primers used for reverse transcriptase polymerase chain reaction

Gene	Primer sequences (sense, top; antisense, bottom)	Size (bp)
Beta 2microglobulin	TCTGGGTTTCATCCATCC TACCTGTGGAGCAACCTG	432
Nestin	GCCCTGACCACTCCAGTTTA GGAGTCCCTGGATTTCCTCC	199
NSE	AAGGACAAATACGGCAAGGA TGGACCAGGCAGCCCAATC	327
MAP2	CCATTTGCAACAGGAAGACAC CAGCTCAAATGCTTTGCAACTAT	428
βTubulinIII	ATGTACGAAGACGACGAGGAG GTATCCCCGAAAATATAAACACA	310
EN1	CTGGGTGTACTGCACACGTTAT TACTCGTCTCGTCTTTGTCTCT	356
EN2	GTGGGTCTACTGTACGCGCT CCTACTCGCTGTCCGACTTG	358
Nurr1	GCACTTCGGCAGAGTTGAATGA GGTGGCTGTGTTGCTGTAGTT	491
Ptx3	TGGGAGTCTGCCTGTTGCAG CAGCGAACCCTCTCTGGG	213
Pax2	ATGTTTCGCTGGGAGATTTCG GCAAGTGCTTCCGCAAACCTG	361
Wnt1	TAGCCTCCTCCACGACCTG CAGCCTCGGTTGACGATCTTG	239
Wnt3a	AAGCAGGCTCTGGGCAGCTA GACGGTGGTGCAGTTCCA	234
Wnt5a	ATCCTGACCACTGGAAGCCCTGT GGCTCATGGCGTTCCACCAC	358
AADC	CTCGGACCAAAGTGATCCAT GTCTCTCTCCAGGGCTTCTCT	212
TH	GTCCCCTGGTTCCCAAGAAAAGT TCCAGCTGGGGATATGTCTTC	331

3. Results

In this study, USSCs were successfully generated from 4 out of 11 cord blood samples. After 1 week to 1 month, USSC colonies were detected, which grew into monolayers within 2–3 weeks. After colonies were obtained, cells could be expanded up to 10^{15} cells in 20 passages.

USSC cultures were checked for their mesenchymal multipotency before starting neural studies. The cells were treated with appropriate osteo-, chondro- and adipo-inductive media (Kögler et al., 2004), and their differentiation was confirmed via appropriate staining (data not shown).

Using RT-PCR, we have defined the expression of several mesencephalic genes associated with development and/or survival of DA progenitor cells in undifferentiated USSCs. RT-PCR product without reverse transcriptase was used as negative control. The PCR data indicate that undifferentiated USSCs express transcripts for several key genes associated with dopaminergic neuron development including En1, En2, Nurr1, Ptx3, Pax2, Wnt1 and Wnt3a but not TH and AADC, the more specific markers of dopaminergic neurons (Fig. 1).

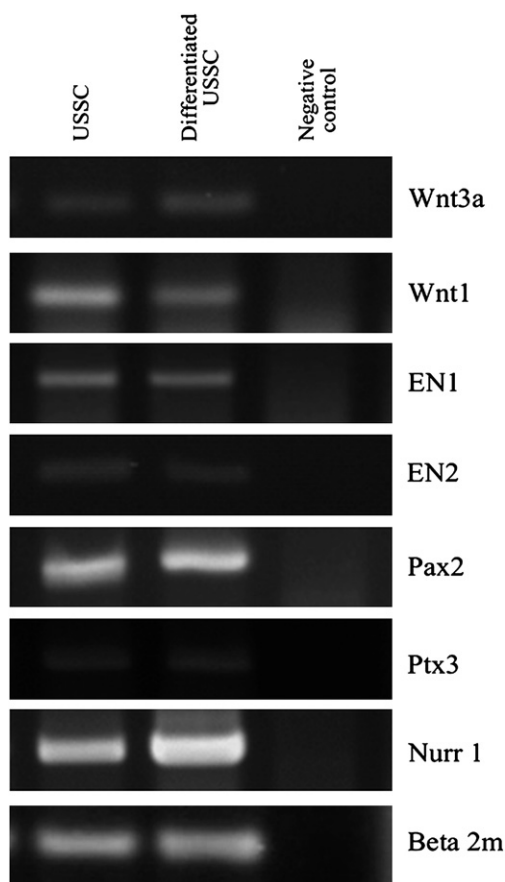


Fig. 1. Reverse transcription polymerase chain reaction analysis of genes associated with dopamine neurons. For negative control, reverse transcriptase was absent during cDNA synthesis. USSCs express transcripts of several key genes in development of DA progenitor cells including En1, En2, Nurr1, Ptx3, Pax2, Wnt1 and Wnt3a. These genes are also expressed in differentiated USSCs.

To further investigate the potential of USSCs for cell therapy of neurological disorders, the cells were induced to differentiate into neuron-like cells *in vitro*. Using the suggested multistep induction method in serum-withdrawal medium, neurogenic potential of human cord blood-derived USSCs was tested. After 2 weeks of differentiation, 61% of cultured cells were morphologically differentiated into neural cells with extended cellular processes and branches.

Using RT-PCR analysis, we have defined the expression of mRNA transcripts of the neural progenitor marker, nestin and several neuron-specific genes, including neuron-specific enolase (NSE), microtubule associated protein 2 (MAP2) and β tubulin III in USSC-derived neuron-like cells differentiated by the suggested method (Fig. 2). In addition to expression of these neuron-specific markers, USSC-derived neuron-like cells express primarily expressed dopamine-associated genes (Fig. 1).

Neuron-specific immunostaining also confirmed neural differentiation of USSCs. Immunofluorescence assays on undifferentiated cells stained negative for MAP2 and NFM, while differentiated cells stained positive for these neuron-specific proteins (Fig. 3). Our observations indicate that the suggested method supports neural differentiation of USSCs within 2 weeks.

4. Discussion

In vitro cultured umbilical cord blood mononuclear cells devoid of hematopoietic stem cells have been reported to be affected by pericellular micro-environments (adherence to plastic dishes) and differentiate spontaneously into neural-like phenotypes (Habich et al., 2006). This high potential of neural differentiation along with using efficient differentiating stimuli may make these cells promising candidates for experimental

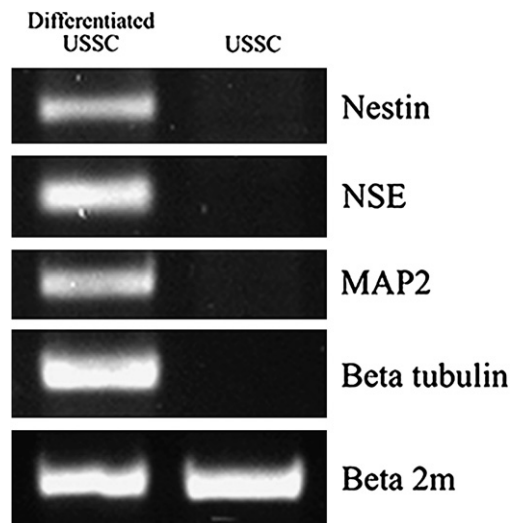


Fig. 2. Reverse transcription polymerase chain reaction analysis of neuron specific markers (NSE, MAP2, β TubulinIII) and a neural progenitor marker (Nestin). USSCs treated by multistep neural induction protocol for 2 weeks express neuron-specific markers.

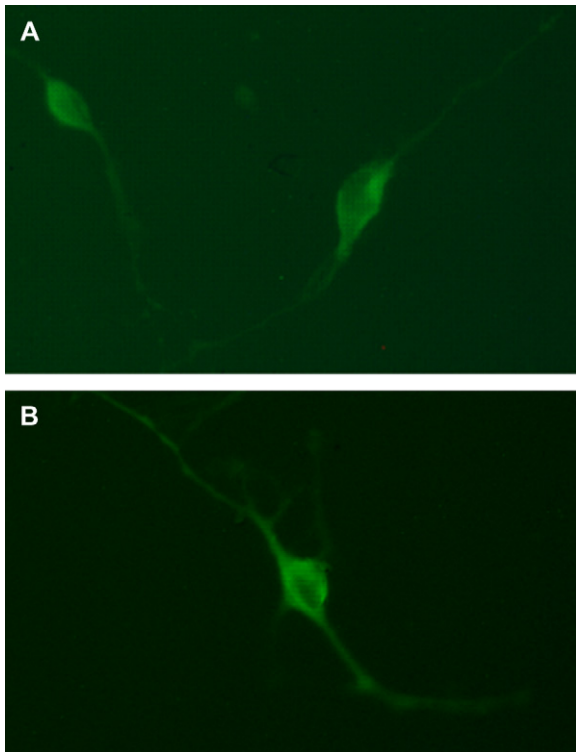


Fig. 3. *In vitro* differentiation of USSCs into neuron-like cells. Differentiated cells showed positive immunoreactivity for the neuron-specific markers NFM (A) and β tubulin III (B).

treatment of neural injuries. Characteristic advantages over other mesenchymal stem cell types such as bone marrow-derived cells, including higher proliferative capacity, availability, lower immunogenicity and high potentiality for differentiation into neural-like phenotypes, suggest that cord blood unrestricted somatic stem cells may be an alternative to other progenitor cells for clinical applications. Therefore, studying different aspects of their potential will be helpful for their future application.

USSCs have been shown to differentiate into neural cells in the presence of chemically defined induction medium containing fetal calf serum (Kögler et al., 2004). The present study examined neural differentiation of USSCs in serum-withdrawal neural induction medium. Thin cellular processes with branches were developed following treatment of USSCs in neurogenic culture medium over 2 weeks, which was further confirmed by immunocytochemistry and RT-PCR assays. Undifferentiated cells did not express neuron-specific markers and did not stain positive for neuron-specific proteins by immunostaining analysis but expressed transcripts for several genes associated with dopaminergic neuron development including *En1*, *En2*, *Nurr1*, *Ptx3*, *Pax2*, *Wnt1* and *Wnt3a*. Differentiated cells express neuron-specific markers as well as primarily expressed dopamine-associated genes. Neither undifferentiated USSCs nor USSC-derived neuron-like cells express specific markers of dopaminergic neurons such as *TH* or *AADC*. Table 2 displays the complete list of genes examined by RT-PCR.

The information available from studies focused on the genesis of mesencephalic DA neurons indicates the participation

Table 2
List of all transcripts examined

Gene	Uninduced USSCs	Neural induced USSCs
<i>Nestin</i>	–	+
<i>NSE</i>	–	+
<i>MAP2</i>	–	+
β -tubulin	–	+
<i>EN1</i>	+	+
<i>EN2</i>	+	+
<i>Nurr1</i>	+	+
<i>Ptx3</i>	+	+
<i>Pax2</i>	+	+
<i>Wnt1</i>	+	+
<i>Wnt3a</i>	+	+
<i>Wnt5a</i>	–	–
<i>AADC</i>	–	–
<i>TH</i>	–	–

of several genes and transcription factors within this complex process during different stages of DA neuron development. Although none of the expressed factors are uniquely expressed in midbrain DA neurons, each of the components is necessary for the maturation and survival of the midbrain DA neurons which indicates the importance of their expression in stem cells which are to be used for PD cell therapy.

The expression of *Nurr1* and some other dopamine-associated genes in undifferentiated stem cells from other resources such as adult rat bone marrow stromal cells and cell lines derived from human amniotic fluid cells was recently reported (Kramer et al., 2006; McLaughlin et al., 2006). *Nurr1* is a nuclear receptor essential for the development of DA neurons. Numerous studies have documented that *Nurr1* is necessary for both the survival and differentiation of the mesencephalic dopaminergic precursor neurons (Chu et al., 2006; Saucedo-Cardenas et al., 1998). In addition, it has been shown that *Nurr1* can regulate the expression of several other genes that are important in the synthesis and storage of dopamine (Jankovic et al., 2005). Recently, it has been reported that *Nurr1* expression is reduced over the course of normal aging, which is one of the significant events in the loss of the dopaminergic neuronal phenotype (Chu et al., 2002, 2006). Therefore, the expression of *Nurr1* in USSCs and USSC-derived neurons may be significant for the future study of USSCs as potential candidates for cell therapy of DA deficiency.

Ptx3 is a transcription factor that is also expressed in mesencephalic dopaminergic neurons. Recently, it has been suggested that *Nurr1* and *Ptx3* synergistically promote terminal maturation to the midbrain neuron phenotype in murine and human ES cell cultures (Martinat et al., 2006). The simultaneous expression of *Nurr1* and *Ptx3* in USSCs may be helpful in maturation of these cells to the midbrain DA neuron phenotype. *Engrailed* homeodomain transcription factors, *EN1* and *EN2*, have been shown to have significant roles in controlling the developmental fate of midbrain dopaminergic neurons and the expression of the genes genetically linked to PD (Simon et al., 2001), which shows the importance of their expression in USSCs more. On the other hand, *Wnts* are essential regulators of proliferation and differentiation of ventral midbrain DA

precursor neurons (Castelo-Branco et al., 2003). Expression of Wnt1 and Wnt3a in USSCs is another advantage of USSC as a candidate for neurological cell therapy.

In conclusion, the expression of important genes associated with dopaminergic precursor cells in unrestricted somatic stem cells isolated from human cord blood, together with their potential for differentiation into neuron-like cells, suggest that USSCs and neuron-like cells derived from these novel stem cells may be important candidates to be used for cell therapy of neurological diseases such as Parkinson's disease. Further investigation is underway to examine long-term cultures and their application to replace degenerated neurons of Parkinson's disease.

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