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Induced *in vitro* differentiation of neural-like cells from human exfoliated deciduous teeth-derived stem cells

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ABSTRACT Stem cells from human exfoliated deciduous teeth (SHED) are highly proliferative, clonogenic and multipotent stem cells with a neural crest cell origin. Additionally, they can be collected with minimal invasiveness in comparison with other sources of mesenchymal stem cells (MSCs). Therefore, SHED could be a desirable option for potential therapeutic applications. In this study, SHEDs were established from enzyme-disaggregated deciduous dental pulp obtained from 6 to 9 year-old children. The cells had typical fibroblastoid morphology and expressed antigens characteristic of MSCs, STRO1, CD146, CD45, CD90, CD106 and CD166, but not the hematopoietic and endothelial markers, CD34 and CD31, as assessed by FACS analysis. Differentiation assessment revealed a strong osteogenic and adipogenic potential of SHEDs. In order to further evaluate the *in vitro* differentiation potential of SHED into neural cells, a simple short time growth factor-mediated induction was used. Immunofluorescence staining and flow cytometric analysis revealed that SHED rapidly expressed nestin and β-III tubulin, and later expressed intermediate neural markers. In addition, the intensity and percentages of nestin and β-III tubulin and mature neural markers (PSA-NCAM, NeuN, Tau, TH, or GFAP) increased significantly following treatment. Moreover, RT-PCR and Western blot analyses showed that the neural markers were strongly up-regulated after induction. In conclusion, these results provide evidence that SHED can differentiate into neural cells by the expression of a comprehensive set of genes and proteins that define neural-like cells *in vitro*. SHED cells might be considered as new candidates for the autologous transplantation of a wide variety of neurological diseases and neurotraumatic injuries.

KEY WORDS: *dental pulp stem cell, Stem cells from Human Exfoliated Deciduous Teeth (SHED)*

Introduction

Since the adult mammalian central nervous system (CNS) has a limited ability of regeneration after injury or disease, it has become an important target of regenerative medicine. Different strategies, including implementation of neurotrophic factors, gene therapy and cell therapy have been suggested to overcome this limited regeneration (Lindvall and Kokaia, 2010; Manfredsson *et al*., 2009). The growth of undifferentiated stem cells *in vitro* and inducing them toward the desired differentiated neural cells with subsequent cell transplantation is one important approach for cell

Abbreviations used in this paper: MSC, mesenchymal stem cell; SHED, Stem cells from Human Exfoliated Deciduous Teeth.

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therapy. In this regard, autologous stem cells collected from dental pulp of extracted wisdom teeth or from pulpectomy of teeth left *in situ* are a chance that should be improved in clinical trial. Up to now different stem/progenitor cells derived from dental tissue including dental pulp stem cells (DPSCs), stem cells from exfoliated deciduous teeth (SHED), periodontal ligament stem cells (PDLSCs), stem cells from apical papilla (SCAP), and dental follicle progenitor cells (DFPCs) (Huang *et al.*, 2009). SHED cells are a population of highly proliferative multipotent cells capable of differentiating into functional osteoblasts, odontoblasts, endothelial cells, adipocytes, and neural cells *in vitro* and *in vivo* (Chadipiralla *et al.*, 2010; Laino *et al.*, 2006; Miura *et al.*, 2003; Sakai *et al.,* 2010). Obtaining SHED is simple and convenient, with little or no trauma. Every child loses primary teeth which creates the perfect opportunity to recover and store this convenient source of stem cells; should they be provided to treat future injuries or ailments. This presents a far better alternative to simply discarding the teeth or storing them as mementos from the past. Therefore, there are many interests in banking person-specific tooth-derived stem cells (Arora *et al.*, 2009; Perry *et al.*, 2008). The proliferation rate of SHED is significantly higher than that of DPSCs and BM-MSCs (Nakamura *et al.*, 2009; Takeda *et al.*, 2008). It is interesting to note that DPSC isolation is possible for at least five days after tooth extraction, which implies that immediate processing after extraction may not be necessary for successful DPSC banking. Further, the recovery of viable DPSCs after cryopreserving intact teeth suggests that minimal processing may be needed for the banking of samples that have no immediate plans for expansion and use (Perry *et al.*, 2008). Even after cryopreservation these cells were capable to differentiate into neural, osteocytes/odontocytes, adipocytes, myocytes, and chondrocytes (Zhang *et al.*, 2006) under related condition. The transplantation of these cells improved critical-size calvarial or large cranial defects in animal models (de Mendonca Costa *et al.*, 2008; Seo *et al.*, 2008). Additionally, the implantation of human SHED cells to golden retriever muscular dystrophy dogs showed no signs of immune rejection and presented significant engraftment and better clinical conditions (Kerkis *et al.*, 2008). SHED transplantation is also capable of effectively reversing SLE-associated disorders in mice by increasing the ratio of regulatory T cells (Yamaza *et al.,* 2010). It is supposed that these cells have immunosuppressive activity (Pierdomenico *et al.*, 2005) which is so important in the field of regenerative medicine (Yalvac *et al.*, 2009).

Therefore, based on the potentials of SHED and considering their neural crest origin, this study was undertaken to propose a simple method for SHED isolation and their differentiation into neural-like cells, as characterized by the use of a variety of experimental approaches for future transplantation in neurodegenerative diseases or neurotraumatic injuries.

Results

Characteristics of isolated and in vitro expanded SHED

After single step enzymatic treatment, viable cells with elongated shapes were detected one day after the onset of culture. These cells formed colonies upon further culture (Figs. 1A and 1B). Cells from a single colony were isolated and subcultured mechanically (first passage). Subcultured cells gradually became flattened and acquired a fibroblast-like morphology (Fig. 1C) and in this study the presented results are from one single colony. Moreover, the cultured SHEDs expressed stromal marker ALP (Fig. 1D). The ex vivo-expanded SHEDs expressed the cell surface molecules STRO-1 and CD146 (MUC18), two early MSC

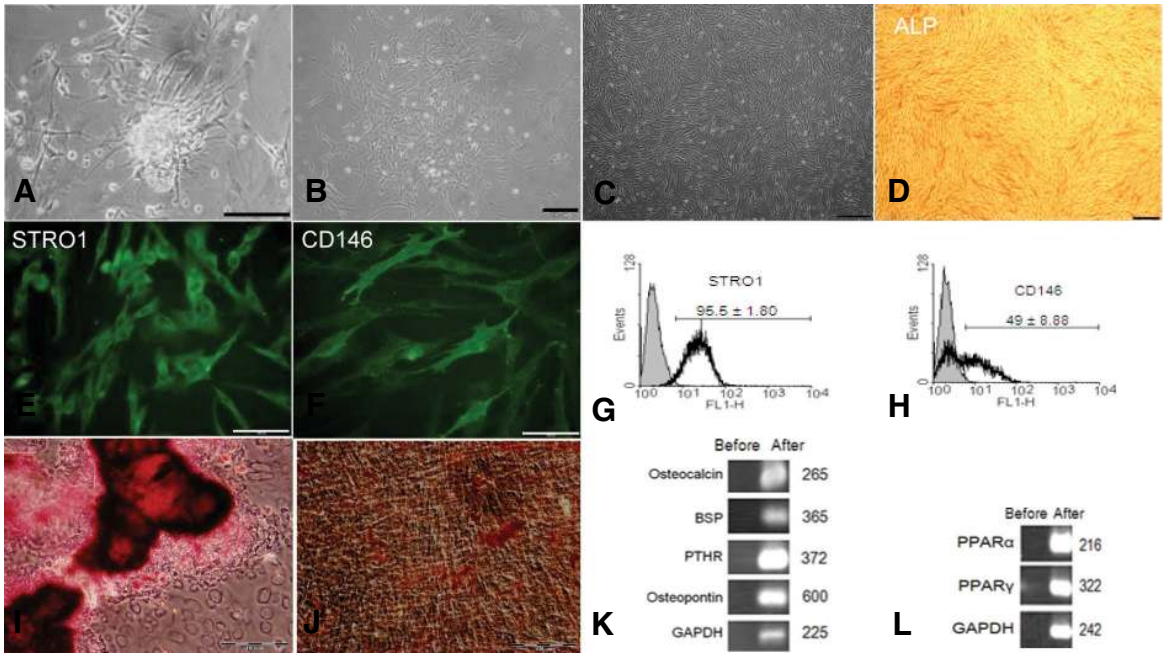

Fig. 1. Characteristics of isolated and *in vitro* expanded SHED. *Deciduous dental pulp, 24 hours after culture* **(A)**, *colony derived from SHED at the first passage* **(B)**, *subconfluent culture* **(C)**, *SHED cells stained for alkaline phosphatase (ALK,* **D**), *mesenchymal markers STRO-1* **(E)** *and CD146* **(F)** *and their corresponding flow cytometries* **(G,H)**, *Oil red staining of adipogenic induced SHED* **(I)**, *Alizarin red staining of osteogenic induced SHED* **(J)**, *RT-PCR analysis of SHED before and after induction for adipogenesis* **(K)**, *and osteogenesis* **(L)**.

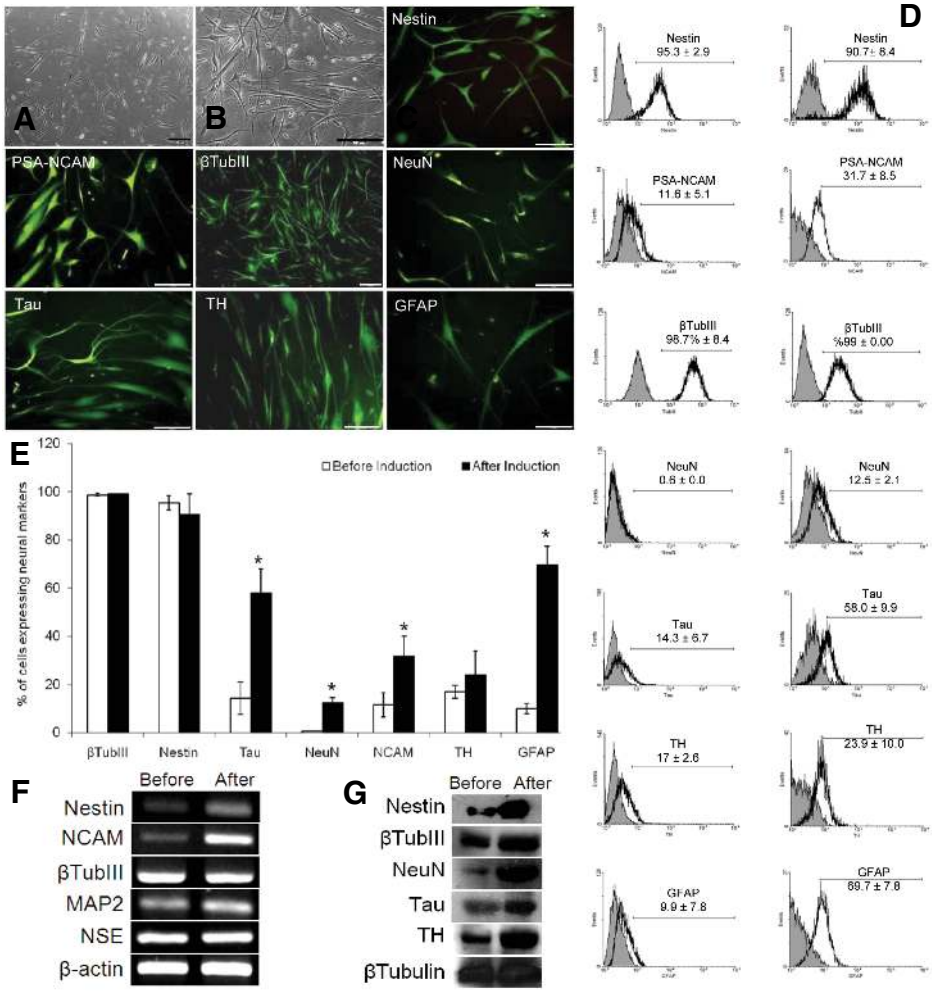

Fig. 2. Characteristics of differentiated cells. *Phase contrast of SHED cells five* **(A)** *and ten* **(B)** *days after neural induction. Immunofluorescence staining for neural markers after ten days of neural induction* **(C)**. *Flow cytometry histograms of neural markers assessed before and after ten days of differentiation* **(D)**. *The results are summarized in Fig. E as mean ± SD. Asterisks show significant difference (at least p<0.05). RT-PCR* **(F)** *and Western blot* **(G)** *analyses of neural markers before and ten days after neural induction.*

markers previously found to be present in bone marrow MSCs and DPSCs (Figs. 1E and 1F). Flow cytometric analysis revealed that 96.5±1.8% and 49.0±8.9% of the *in vitro* expanded SHEDs expressed STRO-1 and CD146, respectively (Figs.1G and 1H). SHEDs positively expressed certain important MSC markers, including CD106, CD166 and CD90; while they showed a negative expression for CD31, an endothelial marker, in addition to CD34 and CD45, two hematopoietic markers (Supplementary Fig. 1).

In order to evaluate the potential of SHED cells to differentiate into mineralized cells, established secondary SHED cultures were supplemented with L-ascorbate-2-phosphate, dexamethasone, and inorganic phosphate. Alizarin red-positive nodules formed in the SHED cultures after ten days of induction (Fig. 1I), indicating calcium accumulation *in vitro*.

Accordingly, RT-PCR analysis revealed that various bone markers, osteocalcin, bone sialoprotein (BSP), parathyroid hormone receptor (PTHR), and osteopontin were expressed under the induction (Fig. 1K). Moreover, the cells which contained oil red positive small lipid droplets appeared three weeks after induction by dexamethasone and indomethacin (Fig. 1J). RT-PCR analysis of the induced cells showed that the transcripts of adipogenic markers, peroxisome proliferator-activated receptor-α (PPARα) and PPARγ were expressed (Fig. 1L). These data indicated that SHEDs possessed the ability to differentiate into bone and adipose cells *in vitro*.

Neural differentiation

We used a growth factor-mediated induction for SHED at passages 8, 14, and 18. During the induction procedure, the cytoplasm in the flat cells initially retracted towards the nucleus and formed a contracted multipolar cell body which left membranous processes much like peripheral extensions (Fig. 2A). Over the subsequent days, the cell bodies became increasingly spherical, retractile, and exhibited a typical neuronal perikaryal appearance. Gradually, SHED cell-derived neuron-like cells displayed distinct neuronal morphologies that ranged from simple bipolar to large, extensively branched multipolar cells which made connections via their processes (Fig. 2B) and saved their morphology up to day 30. In order to verify that the observed morphological changes were not just an incipient *in vitro* neurogenesis which resulted from serum starvation; neural induction medium at day ten post-induction was replaced with mesenchymal medium (DMEM+10% FCS). In this condition, the majority of cells maintained their neural-like morphology following medium exchange.

The protein expression patterns of early (nestin or PSA-NCAM), intermediate (β-III tubulin), and late (NeuN, Tau, TH, or GFAP) neural-associated markers expressed by SHEDs cultured in the SHED and neural inductive media were shown by immunofluorescence (Fig. 2C) and flow cytometry analyses (Figs. 2D and 2E). The data revealed that SHEDs were strongly positive for nestin and βTubIII before and after induction (90.7±8.4% vs. 95.3± 2.9% and 99.0±0.0% vs. 98.7±8.4%, respectively, Figs. 2D and 2F). Of interest, these markers were highly expressed both before and after induction. The shift in the histogram of flow cytometry analysis suggested a higher intensity for both neural markers (Figs. 2D and 2E). Moreover, a progressive enhancement in the PSA-NCAM (31.7±8.5% vs. 11.6±5.1%, p<0.05), NeuN (12.5±2.1% vs. 0.6±0.1%, p<0.002), tau (58.0±9.9% vs. 14.3±6.7%, p<0.01), TH (23.9±10.0% vs. 17±2.6%, p<0.3), or GFAP (69.7±7.8% vs. 9.9± 7.8%, p<0.0001) (Figs. 2D and 2E).

Supportive RT-PCR analysis demonstrated that the SHEDs expressed nestin, PSA-NCAM, β-III tubulin, MAP2, and neuron-

specific enolase (NSE) neural markers at the mRNA level before and after culture in neural inductive media (Fig. 2F). Furthermore, western blot analysis revealed that when SHEDs were cultured in neural inductive media, the protein level of the neural markers, nestin, β-III tubulin, NeuN, tau, and TH appeared to be upregulated (Fig. 2G). Collectively, these results suggested that in response to the neural inductive stimuli, SHEDs had acquired a phenotype resembling neural-like cells.

Discussion

In this study we have investigated the neural potential of stem cells from SHED. These cells are highly proliferative MSCs, located in dental pulp and potentially derived from neural crest cells, may be an optimal source of postnatal stem cells for neural differentiation and therefore transplantation (Chai *et al.*, 2000). Our isolated cells were colony forming and could go through more than 25 passages. We showed that SHED did not express CD34 and CD45, hematopoietic stem cell markers, and CD31, an endothelial marker, yet they expressed some important CD markers that included CD146, CD106, CD166, and CD90 (which belong to the collection of MSC CD markers) (d'Aquino *et al.*, 2007). Additionally, in our experiment a high percentage of SHED cells expressed STRO-1, a stromal cell marker, at higher levels than previously reported by others (Miura *et al.*, 2003) and its expression was maintained with further cell passages. These discrepancies may be attributed to cell origin, isolation procedure, passaging time, or cell-culture techniques. For example, Nagatomo *et al.,* reported 63% STRO expression in cells derived from human periodontal ligament (Nagatomo *et al.*, 2006). These cells presented their ability to differentiate into osteocyte-like and adipocyte-like cells after treatment with osteogenic and adipogenic induction medium as confirmed by alizarin red, oil red-O staining and RT-PCR.

Various groups have studied the potential of dental pulp cells (both adult and deciduous) to differentiate into neural, osteocytes/odontocytes, adipocytes, myocytes, and chondrocytes under related condition (de Mendonca Costa *et al.*, 2008; Seo *et al.*, 2008; Stevens *et al.,* 2008; Zhang *et al.*, 2008). However, these researchers have mainly focused on adult rather than deciduous DPSCs or SHED. To evaluate the neuroplasticity of SHEDs, bFGF, a neural inducer was added to the SHED monolayer culture on poly-L-lysine coated dishes for five days in the absence of serum. The cells gradually lost their mesenchymal appearance and obtained a more neural appearance including neurite-like outgrowth. Further addition of SHH/FGF8 resulted in neural appearing cells with elongated and elaborated axon or dendrite-like structures. It is notable that that this neural induction procedure has not been used for the induction of such cells or SHEDs. Considering previous reports, the neural structure obtained in this procedure was more elaborate in terms of morphology and the numbers of neural extensions or axons observed (Miura *et al.*, 2003; Morsczeck *et al.*, 2009). Additionally, the duration of neural induction was relatively short. Furthermore, the morphology of differentiated cells did not initially change to mesenchymal cells by the replacement of differentiation medium with mesenchymal medium (data not shown) which may further confirm the neural identity of these cells, as previously stated (Bertani *et al.*, 2005).

Further analysis of these cells by immunostaining, flow cytometry, western blot and RT-PCR analyses, revealed that neural cell markers increased upon neural induction of these cells. The differentiation of human DPSCs that have been exposed to the appropriate environmental cues into functionally active neurons has been previously reported (Arthur *et al.*, 2008). Unlike the previous study (Miura *et al.*, 2003), this study also presented quantitative data to support the neural properties of the induced SHED cells. The high expression of Nestin and β-Tub III in SHED cells before induction was in concordance with previous reports (Arthur *et al.*, 2008) which may be related to the neural crest-cell origin of the dental pulp (Bronner-Fraser, 1994; Chai *et al.*, 2000). The expression of nestin is also reported in other tissues such as the pancreas, testes, and bone marrow (Delacour *et al.*, 2004; Yang *et al.*, 2008), which are believed to contain multi-lineage progenitor cells with early embryonic origin (Wiese *et al.*, 2004). However, flow cytometric analysis revealed a shift in their expression, which suggested a prominent intensity for two markers post-neural induction and western blot analysis showed an increase in their expression after induction.

bFGF, SHH, and FGF8 which were used in this study have been shown to have fundamental roles in neurogenesis. Indeed, bFGF is known as a regulator of proliferation and differentiation (Dono *et al.*, 1998) while SHH is secreted by notochord during development. SHH initially induces a general ventral cell fate and is required for the induction of floor plate and motor neuron differentiation (Ericson *et al.*, 1996). FGF8, a member of the fibroblast growth factor family, is responsible for dorsalization of the anterior neural tube (Garel *et al.*, 2003). The protocol of neural induction has already been used for dopaminergic differentiation (Kim *et al.*, 2002). Therefore evaluation of TH expression, as an enzyme that only expresses at catecholamine-containing neurons, has revealed a 14% positivity of these cells for TH which increased upon neural induction to 25%. This observation was further confirmed at the mRNA and protein levels and was in accordance with previous studies that showed DPSC when exposed to neurotrophic factors supported dopaminergic neurons *in vitro* (Nosrat *et al.*, 2004). More recently, it was demonstrated that after incubation with a cocktail of cytokines including Shh, FGF8, glial cell line-derived neurotrophic factor, and forskolin, SHED cells differentiated into a cell population that contained specific dopaminergic neurons. Moreover, transplantation of growth factor-treated SHED cells into the striatum of parkinsonian rats partially improved the apomorphine-evoked rotation of behavioral disorders. (Wang *et al.,* 2010).

It was demonstrated that gangliosides may play a role in the neural differentiation process of DPSCs when cultured in neurogenic medium (Ryu *et al.*, 2009). Interestingly, a large number of regulatory genes in these differentiation interact or crosstalk via Notch, Wnt, transforming growth factor beta (TGF-beta)/bone morphogenic protein (BMP), and cadherin signaling pathways to play a crucial role in their determination (He *et al.*, 2009; Liu *et al.*, 2009). Moreover, it was reported that implantation of DPSCs induce endogenous axon guidance and induced neuroplasticity within a receptive host nervous system (Arthur *et al.*, 2009).

In conclusion, we have demonstrated that stem cells derived from deciduous DPSCs are a type of MSCs that express a variety of early and intermediate neural markers *in vitro*. They gain neural-like morphology and significantly over-express most neural markers in an appropriate microenvironment. This neurogenic potential of SHED cells, besides their accessibility, makes them a valuable resource in clinical applications.

Materials and Methods

Isolation of dental pulp MSCs

In this study, ten normal human exfoliated deciduous incisors were collected from six to nine year-old children under approved guidelines set by Royan Institute and the Isfahan University of Medical Sciences, Faculty of Dentistry. The pulps were gently separated from the crown and digested in a solution of 4 mg/ml collagenase type I (Invitrogen, 17100-017) containing 5% fetal calf serum (FCS, Invitrogen, 16141-079) for one hour at 37°C. The single cells were initially cultivated in α-MEM medium (Sigma, M 0644) supplemented with 10% FCS in a 24-well plate (TPP, 92424). After one day, non-adherent cells were removed by washing with PBS (with Ca^{+2} and Mg^{+2}, Invitrogen, 14287).

Osteogenic and adipogenic differentiation

Osteogenic and adipogenic differentiation of DPSCs were induced as previously described (Eslaminejad *et al.*, 2006). To achieve osteogenic differentiation, cells were seeded at a density of $3◊10^3$ cells/cm^2 and then cultured for ten days in the presence of 10 nM dexamethasone, 50 μg/ml ascorbic acid 2-phosphate and 10 mM β-glycerophosphate. At the end of the cultivation period, the cells were fixed with 4% paraformaldehyde for 10 minutes and stained with alizarin red for 15 minutes at room temperature. This procedure allowed examination of the mineralized bone matrix.

For adipogenic differentiation, cells were grown until they reached confluence and were then induced by three cycles of induction/maintenance with 100 nM dexamethasone and 50 μg/ml indomethacin. Adipogenesis was assayed by staining of intracellular lipid droplets with oil red O (Sigma) as previously described (Eslaminejad *et al.*, 2006).

Alkaline phosphatase

The SHED from each line, having been plated at 100 cell/cm^2 in a 6-well plate, were incubated in DMEM (Invitrogen, 12800-116) supplemented with 15% ES-FCS for seven days until confluence was achieved. The cultures were then kept in an incubator at 37°C and 5% CO_2 for 21 days with media changes three times per week. Alkaline phosphatase (ALP) staining was performed based on the manufacturer's recommendations (Sigma, 85L3).

Neural induction

Induction of neural differentiation was initiated by plating the cells on poly-L-lysine (Sigma, P4707)-coated glass cover slips at a concentration of 5000 cells/cm^2 in neurobasal medium containing 1% ITS (Invitrogen, 41400), and cytokines including 100 ng/ml basic fibroblast growth factor (bFGF, Sigma, F0291) for five days followed by 100 ng/ml bFGF, 10 ng/ml FGF8 (Sigma, F6926), and 100 ng/ml sonic hedgehog (SHH, Sigma, S0191) for an additional five days. This protocol is a modified protocol that has been previously reported for neural differentiation of multipotent adult progenitor cells (MAPCs) (Jiang *et al.*, 2003). During the first five days of induction, media were half refreshed and bFGF was added with the full concentration. Following media exchange on day five, the culture was continued without any media renewal.

Flow cytometry analysis

The passage-4 cells from each line were harvested and the expression of cell surface markers which included: STRO1, a stromal marker and CD146, a perivascular marker, CD45, CD90, CD106 and CD166 (markers of MSCs), CD34, a hematopoietic stem cell marker, and CD31, an endothelial marker were evaluated by flow cytometry. SHEDs before and after induction were washed twice in PBS (free Ca^{+2} and Mg^{+2}) and fixed in 4% paraformaldehyde. For permeabilization 0.2% Triton X-100 was used. Non-specific antibody binding was blocked with the combination of 10% goat serum in primary antibodies. The primary antibodies were against the following: negative control (MCA928PE Serotec, 1:50), CD31 (MCA1738PE, Serotec, 1:50), CD90 (MCA90A647, Serotec, 1:50), CD45, (R7087, Dako, 1:100), CD34-FITC (F7166, Dako, 1:200), CD106 (MCA1557PE, Serotec, 1:50), CD166-FITC (MCA1926F, Serotec, 1:500), CD146 (MAB16985, Chemicon, 1:500), STRO-1 (MAB4315, Chemicon, 1:500), β- Tubulin III (βTubIII, ab7751, Abcam, 1:200), nestin (MAB5326, Chemicon, 1:200), tau (T5530, Sigma, 1:200), neuronal nuclei (NeuN, MAB377, Chemicon, 1:200), polysialic acid-neural cell adhesion molecule (PSA-NCAM, AB5032, Chemicon, 1:200), tyrosine hydroxylase (TH, T1299, Sigma, 1:300), glial fibrillary acidic protein (GFAP, MAB3402, Millipore, 1:200) and IgG negative isotype control (CBL600, Chemicon, 1:200). The primary antibodies were incubated for 2 hours at room temperature. Binding of primary antibodies was revealed with specific secondary antisera fluorescein isothiocyanate (FITC)-conjugated antimouse IgG (AP124F, Chemicon, 1:50) for 1 hour at room temperature. Flow cytometric analysis was performed with a BD-FACS calibur flow cytometer (Becton Dickinson). The experiments were triplicated and acquired data were analyzed by using the WinMDI (2.9) software.

Immunofluorescence staining

Immunofluorescence staining was performed in cells grown on glass coverslips in relevant conditions and fixed with 4% paraformaldehyde. The following antibodies against βTubIII, nestin, tau, NeuN, PSA-NCAM, TH, and GFAP were used for 2 hours at room temperature. Cells were washed with PBS and incubated for 1 hour, at 37°C with the FITC-conjugated antimouse IgG. The cells were rinsed three times with PBS and analyzed using fluorescence microscopy (Nikon, Olympus, BX51, Tokyo, Japan).

Relative reverse transcription–polymerase chain reaction (RT-PCR) analysis

Total RNA was collected from the cells induced to differentiate into neural, osteoblast and adipocyte lineages as detailed above, using the

TABLE 1

PRIMES AND THE REACTION CONDITIONS OF RT-PCR

Name	Sense Primer (5'-3')	Antisense Primer (5'-3')	Accession No.	Prod. Length (bp)	Ann. T (°C)
Osteopontin	GCTCATTGCTCTCATCATTGGC	GGC TAA ACCCTGACCCATCTC	NM_000582.2	600	57
Osteocalcin	GACCATCTTTCTGCTCACTCTG	GTGATACCATAGATGCGTTTGTAG	NM_007541.2	275	65
PTHR*	GACAAGCTGCTCAAGGAAGTTCTG	GGAATATCCCACGGTGTAGATCATG	NM_001083935.1	372	68C
Sialoprotein	GGCAGTAGTGACCATCCGAAGAA	GGTACTGGTGCCGTTTATGCCTTG	NM_007541.2	359	68C
PPAR-α	TGCTATCATTTGCTGTGGAG	ACTCCGTCT TCT TGATGAT	NM_001003093.1	216	68C
PPAR-γ	CTAAAGAGCCTGCGAAAG	TGTCTGTCTCCGTCTTCTTG	NM_138711.3	330	61C
NCAM	TGGCAGGAGATGCCAAAGAT	CTCGGCCTTTGTGTTTCCAG	NM_000615	466	66
βTub III	AGATGTACGAAGACGACGAGGAG	GTATCCCCGAAAATATAAACACAAA	NM_006086	313	60
Nestin	CTCTGACCT GTCAGAAGAAT	CCCACTTTCTTCCTCATCTG'	NM_006617	172	62
MAP2	CTGGGTCTACTGCCATCACTC	CCCCTTTAGGCTGGTATTTGA	NM_002374.3	282	66
NSE	CTTGGAGCTGGTGAAGGAAG	TTTTGGGTTGGTCACTGTCA	NM_001975	310	66
GAPDH	CAGGTGGTCTCCTCTGACTTCAAC	AGGGTCTCTCTCTTCCTCTTG	NM_002046.3	225	68C
β-actin	CGTGACATTAAGGAGAAGCTGTGC	CTCAGGAGGAGCAATGATCTTGAT	NM_01101.3	374	65

RNeasy Mini Kit (Qiagen, Spain). Prior to RT, RNA samples were digested with DNase I (Fermentas; EN0521) to remove contaminating genomic DNA. Standard RT was performed using 2 μg total RNA, oligo (dT) and the RevertAid™ Minus First Strand cDNA Synthesis Kit (Fermentas; K1622) according to the manufacturer's instructions. The cDNA samples were subjected to PCR amplification using primers (Table 1). The PCR products were analyzed by gel electrophoresis on a 1.7% agarose gel and stained with ethidium bromide (0.5 μg/ml) and visualized and photographed on a UV transilluminator (SynGene, UK).

Western blotting

Western blot analysis was carried out as previously described (Henkel and Bieger, 1994; Nasrabadi *et al*., 2009). Briefly, cell pellets were treated with 10% (w/v) trichloroacetic acid in acetone with 0.07% (w/v) dithiothreitol (DTT) at -20 ˚C for 1 hour. The suspension was centrifuged for 15 minutes at 16000 g. The pellets were washed with ice-cold acetone, incubated at -20˚C for 30 minutes and centrifuged again at 4˚C at 12000 g for 15 minutes. The pellets were lyophilized. The sample powder was then solubilized in lysis buffer [9.5 M urea, 2% (w/v) CHAPS, 0.8% (w/v) pharmalyte pH 3-10, 1% (w/v) DTT] and protein concentration was assessed by the Bradford assay (Bio-Rad) using BSA as the standard. The prepared samples were electrophoresed under reducing conditions on 10% SDS-PAGE gels (Bio-Rad, Munich, Germany) and then transferred to PVDF membranes (Bio-Rad, Munich, Germany). Page ruler prestained protein ladder (SM 0671; Fermentas, Germany) was applied for the evaluation of molecular weights. Membranes were blocked overnight in 10% skim milk (Merck, Germany) in PBS and incubated with primary different antibodies including βTubIII (1:200), nestin (1:200),TH (1:200), NeuN (1:200), tau (1:200) and β-Tubulin (T5293, Sigma-Aldrich, 1:200, housekeeping gene or internal control), for 90 minutes. The blots were washed once for 15 minutes and then 3 times, each time for 5 minutes using tris buffer that contained 0.05% Tween 20 (Sigma, USA). For detection of primary antibodies blots were incubated with the appropriate horseradish peroxidase (HRP, Dako P0447) conjugated secondary antibodies for 1 hour. The washing procedure was repeated and bound secondary antibodies were detected using ECL advanced western blotting detection (GE Healthcare) and visualized by x-ray films (Agfa, Mortsel, Belgium).

Statistical analysis

The experiments were repeated at least three times and the values expressed as mean ± SD. One-way ANOVA followed by the Tukey post hoc test multiple group comparison was used to analyze group differences of the data collected from flow cytometric analysis. The difference between groups was then considered to be statistically reliable if a value of $p< 0.05$ was obtained.

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