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Placental mesenchymal stromal cells induced into neurotrophic factor-producing cells protect neuronal cells from hypoxia and oxidative stress

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Abstract

Background aims. Mesenchymal stromal cells (MSC) may be useful in a range of clinical applications. The placenta has been suggested as an abundant, ethically acceptable, less immunogenic and easily accessible source of MSC. The aim of this study was to evaluate the capacity of induced placental MSC to differentiate into neurotrophic factor-producing cells (NTF) and their protective effect on neuronal cells. *Methods.* MSC were isolated from placentas and characterized by fluorescence-activated cell sorting (FACS). The cells underwent an induction protocol to differentiate them into NTF. Analysis of the cellular differentiation was done using polymerase chain reactions (PCR), immunocytochemical staining and enzyme-linked immunosorbent assays (ELISA). Conditioned media from placental MSC (PL-MSC) and differentiated MSC (PL-DIFF) were collected and examined for their ability to protect neural cells. *Results.* The immunocytochemical studies showed that the cells displayed typical MSC membrane markers. The cells differentiated into osteoblasts and adipocytes. PCR and immunohistology staining demonstrated that the induced cells expressed typical astrocytes markers and neurotrophic factors. Vascular endothelial growth factor (VEGF) levels were higher in the conditioned media from PL-DIFF compared with PL-MSC, as indicated by ELISA. Both PL-DIFF and PL-MSC conditioned media markedly protected neural cells from oxidative stress induced by H_2O_2 and 6-hydroxydopamine. PL-DIFF conditioned medium had a superior effect on neuronal cell survival. Anti-VEGF antibodies (Bevacizumab) reduced the protective effect of the conditioned media from differentiated and undifferentiated MSC. *Conclusions.* This study has demonstrated a neuroprotective effect of MSC of placental origin subjected to an induction differentiation protocol. These data offer the prospect of using placenta as a source for stem cell-based therapies.

Key Words: *mesenchymal stromal cells, neuroprotection, placenta*

Introduction

Tissue-specific adult stem cells appear to play an invaluable role in the normal physiology and repair of virtually all tissues (1–5). The presence of neuronal stem cells in the adult human brain was first reported in 1998 (6). Cumulative evidence since then has supported the importance of neurogenesis in the maintenance and, to some extent, replacement of neural cells, especially in pathologic conditions such as stroke (7).

There are two populations of adult stem cells within the bone marrow: the hematopoietic stem cells, and the relatively rare multipotent mesenchymal stromal cells (MSC) that can differentiate into a variety of cell types (1), namely osteoblasts (bone cells), chondrocytes (cartilage cells), and adipocytes (fat cells). Recent *in vitro* studies have shown that MSC can also differentiate into cells outside the mesodermal lineage, into endodermal gut and lung epithelial cells and ectodermal neurons (8).

Multipotent MSC can be isolated from various tissues. The main source for adult MSC currently is bone marrow. In addition to bone marrow, MSC can be isolated from adipose tissue and other adult tissue sources (1). However, the procedures are invasive. Hence researchers are seeking other ethically conducive sources that are easily accessible.

Placental tissue contains abundant maternal and fetal MSC (9). It is readily available and ethically

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acceptable as a source of stem cells because it has no use after birth and is usually discarded. A vast number of cells can be produced from each placenta (10). Furthermore, placental stem cells have the advantages of an almost unlimited self-renewal ability and better differentiation capacity than adult stem cells (10,11). They also have immunosuppressive features that might be useful in treating inflammatory responses (12). However, the issue of immunomodulation by placental MSC is controversial. Some studies have shown immunosuppressive properties of placental MSC under allogeneic settings, while others have not (13). One previous study has shown an immunomodulatory effect of differentiated bone marrow-derived MSC (BM-DIFF) cells, by suppression of myelin oligodendrocyte glycoprotein (MOG)-induced spleen cell proliferation (14). Further studies are needed to explore this.

Previous studies have shown a protective effect of MSC derived from adult bone marrow in animal models of various neurologic diseases, such as Parkinson's disease and multiple sclerosis. Our group found that bone marrow MSC exposed to an induction protocol became astrocyte-like cells that secreted significant levels of neurotrophic growth factors. These induced MSC proved to have a better regenerative potential than untreated parental MSC (15,16,17).

The aim of the present study was to evaluate the capacity of induced placental MSC to differentiate into neurotrophic factor-producing cells and to examine their protective effect on neuronal cells exposed to hypoxic and toxic insults. These findings may have important implications for the treatment of acute neurologic diseases when there is insufficient time to produce autologous stem cells from adult bone marrow.

Methods

Isolation and culture of human placental MSC

The placentas of five healthy women after Caesarean section were collected and processed following acceptance of their written informed consent. The study was approved by the institutional ethics committee.

Several pieces of tissue flanking the umbilical cord were dissected and minced in a Petri dish. The minced tissue was transferred to a 50-mL centrifuge tube containing Hank's balanced salt solution (HBSS; Biological Industries, Beit-Haemek, Israel) with 0.1% bovine serum albumin (BSA; Sigma-Aldrich, St Louis, MO, USA), and digested for 45 min at 37°C with a combination of 0.1% collagenase type IV (Sigma-Aldrich), 200 μg/mL DNAse I (Worthington, Lakewood, NJ, USA) and 0.1% Dispase (Invitrogen, Carlsbad, CA, USA). The tissue was triturated every 10 min using 10-mL plastic serologic pipettes. The slurry was filtered successively through a 100-micron Nytex mesh (BD Falcon, Sparks, MD, USA) placed in a standard laboratory funnel, and the volume was adjusted to 35 mL HBSS (Biological Industries). Thereafter, 15 mL Histopaque (Sigma-Aldrich) were added to the centrifuge tube and layered with the mixture using a 9-inch glass Pasteur pipette. The tube was centrifuged for 20 min at 800 g. The cell layer was recovered and diluted with HBSS (Biological Industries) containing 0.1% BSA (Sigma-Aldrich) to a final volume of 50 mL, and centrifuged at 200 g. The cell pellet was then resuspended twice. The cell suspension was plated on dishes coated with human placental fibronectin (100 ng/mL; Sigma-Aldrich) at a density of 1 million cells/80 cm^2 (i.e. 1×10 cm dish) in Dulbecco's modified Eagle's medium (DMEM; Invitrogen; containing high glucose 4.5 mg/L) with 10% fetal bovine serum (FBS; Biological Industries), 1:100 non-essential amino acids (Biological Industries), 55 μM beta-mercaptoethanol (Sigma-Aldrich), 10 μg/mL ciprofloxacin (Bayer, Leverkusen, Germany) and 10 μg/mL amphotericin B (Invitrogen).

Isolation and proliferation of human MSC

Adult human bone marrow were isolated and characterized as published by Sadan *et al.* (18). Briefly, adult human bone marrow samples were collected from the posterior iliac crest of adult human donors, undergoing bone marrow aspiration, after obtaining informed consent (approved by the Helsinki Committee of the Laniado Medical Center, Netania, Israel). Mononuclear cells were separated by density centrifugation over UNI-SEPMAXI/ UNI-SEP + (polysucrose–sodium metrizoate; NovaMed, Jerusalem, Israel)-containing tubes. Cells were grown in DMEM (Biological Industries) supplemented with 100 μg/mL streptomycin, 100 U/mL penicillin, 12.5 U/mL nystatin (Streptomycin, Penicillin, Nystatin (SPN); Biological Industries), 2 mM L-glutamine (Biological Industries), 2 IU/mL heparin (Trima, Kibbutz Maabarot, Israel), 0.001% 2-mercaptoethanol (Sigma-Aldrich), 1% Minimum Essential Medium (MEM) non-essential amino acid solution (Biological Industries) and 10% platelet lysate. Platelet lysate was processed from frozen–thawed human platelet-rich plasma as described previously (19). Experiments on the cells were performed after 2–7 passages.

Fluorescent-activated cell sorter analysis

Extracellular protein detection was performed with antibodies conjugated with fluorescent isothiocyanote (FITC) or phycoerythrin (PE): anti-CD45

(1:200; eBioscience, San Diego, CA, USA), anti-CD34 (1:10; BD Pharmingen, San Diego, CA, USA), anti-CD90 (1:10; eBioscience), anti-CD105 (1:20; eBioscience), anti-CD29 (1:20; eBioscience) and anti-CD14 (1:10; eBioscience). Control staining was performed by incubation with IgG2b–PE (1:10; eBioscience) or IgG2b–FITC (1:10; eBioscience). After incubation with the antibodies for 45 min at 4°C, the cells were washed in PBS containing 5% FBS (Biological Industries) and 0.1% azide (Sigma-Aldrich). Data were acquired on a FACSCalibur® [Becton Dickinson (BD), New York, NY, USA] and analysis was performed using CellQuest software (BD).

Differentiation protocols to osteoblasts and adipocytes

For induction of MSC differentiation into osteoblasts, the cells were plated on fibronectin (Sigma-Aldrich) (30 000 cells in 24 wells) in differentiation medium containing MEM-alpha with 10% FBS (Biological Industries), 50 µg/mL ascorbic acid, 10^{-7} M dexamethasone and 5 mM β glycerol phosphate (Sigma-Aldrich). The medium was changed every 5 days for 3 weeks. After 3 weeks, the cells were fixed with 4% paraformaldehyde (Sigma-Aldrich) and stained with Alizarine Red (Sigma-Aldrich) 0.5% at pH 4.3.

For induction of MSC differentiation into adipocytes, the cells were plated (30 000 cells in 24 wells) in differentiation medium containing MEM-alpha with 10% FBS (Biological Industries), 50 µM indomethacin and 0.5 mM 1-methyl-3-isobutylxantine (Sigma-Aldrich). The medium was changed every 5 days for 3 weeks. After 3 weeks, the cells were fixed with 4% paraformaldehyde (Sigma-Aldrich) and stained with Oil Red (Sigma-Aldrich) 0.3% in 60% isopropanol and 40% H_2O.

Differentiation of placental MSC into neurotrophic factor-producing cells

Cells at passage 3–10 were used for the differentiation experiments. Growth medium was replaced with pre-differentiation medium consisting of DMEM with 2 mM L-glutamine, 100 µg/mL streptomycin, 100 U/mL penicillin and 12.5 U/mL nystatin (SPN; Biological Industries), supplemented with 20 ng/mL human epidermal growth factor (hEGF; PeproTech Inc., Rocky Hill, NJ, USA), 20 ng/mL human basic fibroblast growth factor (hbFGF; R&D Systems, Minneapolis, MN, USA) and N_2 (Invitrogen). After 72 h, the pre-differentiation medium was replaced with differentiation medium consisting of DMEM containing 2 mM L-glutamine and SPN supplemented with 1 mM dibutyryl cyclic AMP (Sigma-Aldrich), 0.5 mM isobutylmethylxanthine (Sigma-Aldrich), 5 ng/mL human platelet-derived growth factor (PeproTech Inc.) and 50 ng/mL human neuregulin (1-β1 NRG1-β1-GGF-2; R&D Systems).

For neuroprotection assays, the conditioned medium was collected on the sixth day of the induction protocol (PL-DIFF medium). For controls, we collected conditioned serum-free media in which placental MSC were grown for 3 days (PL-MSC medium).

Bone marrow MSC underwent the same induction protocol, and the conditioned medium was collected on the sixth day (BM-DIFF medium). We also collected serum-free conditioned media in which untreated BM MSC were grown for 3 days (BM-MSC medium).

Immunocytochemistry

Cells were fixed with 4% paraformaldehyde (Sigma-Aldrich), then stained with the following primary antibodies: rabbit anti-glial cell-derived neurotrophic factor (GDNF; 1:100; Santa Cruz Biotechnology Inc., Heidelberg, Germany), rabbit anti-brain-derived neurotrophic factor (BDNF; 1:200; Santa Cruz Biotechnology Inc.), mouse anti-glial fibrillary acidic protein (GFAP; 1:500; Santa Cruz Biotechnology Inc.), rabbit anti-vascular endothelial growth factor (VEGF; 1:200; Santa Cruz Biotechnology Inc.) or rabbit anti-insulin-like growth factor (IgF)-1 (1:200; Santa Cruz Biotechnology Inc.). Secondary antibodies were as follows: goat anti-rabbit biotinylated antibody (1:500; Jackson ImmunoResearch Laboratories Inc., West Grove, PA, USA), streptavidine-Alexa-488 antibody (1:200; Molecular Probes, Invitrogen, Carlsbad, CA, USA), goat anti-mouse biotinylated antibody (1:500; Jackson ImmunoResearch Laboratories Inc.) and streptavidin-Alexa-488 (1:200; Molecular Probes, Invitrogen). Nuclear DNA was stained with 4′-6-diamidine-2-phenylindole (DAPI; 1:1000; Sigma-Aldrich). A negative control was performed by using secondary antibody only. Fluorescence mounting medium (DAKO, Glostrup, Denmark) was added, and the slides were visualized with a fluorescence Olympus IX70-S8F2 microscope. Findings were compared between MSC subjected to the differentiation protocol (conditioned media collected on day 6) and untreated MSC grown for 3 days in non-differentiation (serum-free) medium.

RNA isolation and real-time–polymerase chain reaction

Total RNA was extracted from the placental MSC that underwent differentiation and placental MSC without differentiation using TRI reagent (Sigma-Aldrich), according to the manufacturer's instructions. RNA was quantified by spectrophotometer. cDNA production from 0.5-µg RNA samples was

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carried out using 10 U enzyme RT-superscript II (Invitrogen) in a mixture containing 1.3 μM oligo-dT12-18 (Sigma-Aldrich), 1 × buffer supplied by the manufacturer, 10 mM dithiothreitol, 20 μM deoxynucleotide triphosphate and RNase inhibitor (RNAout; Invitrogen). Reverse transcription was performed at 42°C for 2 h. Real-time (RT)–quantitative polymerase chain reaction (PCR) was performed on cDNA using an ABI Prism 7700 sequence detector (Applied Biosystems, Carlsbad, CA, USA) with SYBR green) Invitrogen). The following primers were used: glyceraldehyde-3-phosphate dehydrogenase (GAPDH), 5′-CGACAGTCAGCCGCATCTT-3′ (forward) and 5′-CCAATACGACCAAATCCGTTG-3′ (reverse); BDNF, 5′-AGCTCCGGGTTGGTATACTGG-3′ (forward) and 5′-CCTGGTGGAACTTCTTTGCG-3′ (reverse); GDNF, 5′-TCAAATATGCCAGAGGATTATCCTG-3′ (forward) and 5′-GCCATTTGTTTATCTGGTGACCTT-3′ (reverse); GFAP, 5′-TAGAGGGCGAGGAGAACCG-3′ (forward) and 5′-GTGGCCTTCTGACACAGACTTG-3′ (reverse); VEGF, 5′-TGTGCCCACTGAGGAGTCC-3′ (forward) and 5′-GGTTTGATCCGCATAATC-3′ (reverse); IGF-1, 5′-TCAGCTCGCTCTGTCCGTG-3′ (forward) and 5′-TTGCGTTCTTCAAATGTACTTCCT-3′ (reverse).

Data were calculated with the delta cycle threshold (Ct) ratio. The gene of interest was compared with GAPDH, used as a housekeeping gene. For each gene, the specificity of the PCR product was assessed by verifying a single peak on a melting curve analysis. Findings were compared between MSC grown in differentiation or non-differentiation medium.

Enzyme-linked immunosorbent assay-based measurements of VEGF and GDNF secretion

At the end of the differentiating induction process, human BDNF, GDNF and VEGF concentrations were measured by a sandwich enzyme-linked immunosorbent assay (ELISA; DuoSet; R&D Systems) according to the manufacturer's instructions. The absorbance at 450 and 570 nm was recorded on a microplate reader (Multiscan MS; Labsystems, Barcelona, Spain). The results were calculated for 1 mL medium.

Cell proliferation assay

For the proliferation experiments, motor neuron NSC-34 cells (kindly provided by Dr. N. Cashman of the Montreal Neurological Institute (Montreal, Canada)) were cultured in basal medium consisting of DMEM with 2 mM L-glutamine, SPN (Biological Industries) and 10% FCS. The cells were then exposed to different media: PL-DIFF, PL-MSC, BM-DIFF and BM-MSC. The proliferation rate was

assessed by Alamar Blue and confirmed by 5-bromo-2′-deoxyuridine (BrdU) assay (Millipore Billerica, MA, USA), which quantifies the newly synthesized DNA in the actively proliferating cells. The assay was conducted in quadruplet, according to the manufacturer's protocol, and the results were read at 450/550 wavelength using an ELISA reader (Powerwave X; Biotek Instruments, Winooski, VT, USA).

Cell cultures

For the protection experiments, we used the NSC-34 cell line and the SH-SY5Y neuroblastoma cell line (ATCC, Manassas, VA, USA). Cells were cultured in basal medium consisting of DMEM supplemented with 2 mM L-glutamine, SPN and 10% FCS (Biological Industries). The SH-SY5Y cells were planted in 96-well plates and treated with 10 μM retinoic acid (Sigma-Aldrich) daily for 10 days, to induce neuronal differentiation. NSC-34 and the SH-SY5Y cell lines were exposed to placental MSC conditioned media (the medium collected on day 6 of the induction protocol and medium of MSC that were not exposed to the induction protocol). As control, we used the differentiation medium incubated without any cells at 37°C for 3 days, and serum-free medium (DMEM supplemented with L-glutamine and SPN).

In addition, primary neuronal cultures were prepared from newborn mice (24–48 h) as reported previously by Brewer & Torricelli (20). Cells were cultured on poly D-lysine (Sigma-Aldrich)-coated plates in growth medium (Neurobasal A) containing 2% B27 (Invitrogen), 0.5 mM glutamine SPN and 1% bovine serum (Biological Industries).

One hour after incubation with the different media, cells were exposed to hydrogen peroxide (0–50 μM; Sigma-Aldrich) or 6-hydroxidopamine (6-OHDA; 0–25 μM; Sigma-Aldrich) for 24 h, or incubated at 1% oxygen for 48 h (hypoxia). Metabolic activity was analyzed by adding 10% Alamar Blue solution (Invitrogen) to each well, followed by incubation at 37°C for 3 h. Absorbance was determined in a microplate reader (in triplicate for each treatment). The same experiments were conducted with conditioned media of bone marrow MSC (with and without the differentiation protocol).

Blockage of VEGF with Bevacizumab and its effect on survival

Anti-VEGF antibody (Bevacizumab, Avastin; F. Hoffmann-La Roche, Basel, Switzerland) was added to PL-MSC, PL-DIFF and serum-free media, in different concentrations (0, 0.2 and 2 ng/mL). The media were pre-incubated with Bevacizumab for

2 h in 37°C. Then 100 μL of each medium incubated with Bevacizumab were added to NSC-34 cells that were planted in 50 μL serum-free medium. One hour after incubation with each media, the NSC-34 cells were incubated at 1% oxygen for 48 h (hypoxia). Metabolic activity was analyzed by adding 10% Alamar Blue solution (Invitrogen) to each well, followed by incubation at 37°C for 3 h. Absorbance was determined in a microplate reader. Evaluations were performed in quadruplicate for each treatment.

Statistical analysis

Results are presented as means ± standard errors of the mean (SEM). Statistical analysis was carried out using a two-tailed unpaired Student's *t*-test if two groups were compared, and ANOVA if three or more groups were compared. In all tests, statistical significance was assigned when $P < 0.05$.

Results

Isolation and characterization of human placenta MSC

Placental MSC were isolated from five placentas, creating a carpet of fibroblast-like cells. After three passages, the cells expressed mesenchymal surface markers, as determined by fluorescence-activated cell sorting (FACS). The cells stained positive for CD29, CD73 and CD105 ($> 95\%$), all typical mesenchymal surface membrane markers; hematopoietic markers (CD14, CD34 and CD45) were absent ($< 1\%$). About 55% of the cells were also positive to CD90, another mesenchymal marker (Figure 1A). We examined further the ability of the isolated placental MSC to differentiate into mesodermal lineage tissues. The placental MSC successfully differentiated into adipocytes and osteoblasts, following adipogenic and osteogenic differentiation, respectively (Figure 1B).

Placental-MSC differentiation

As a next step, we studied the possibility of differentiating placental MSC into neurotrophic factor-secreting cells. We used an induction protocol as detailed in the Methods.

To evaluate the effect of the induction protocol on the expression of GFAP, GDNF, BDNF, VEGF and IGF-1, immunocytochemical staining was performed on induced and untreated placental MSC. The induced cells showed a significantly increased expression of the astrocytic marker GFAP and the neurotrophic factors VEGF and GDNF than the untreated cells (Figure 2A). IGF-1, which is highly expressed in the placenta, was down-regulated following induction.

To evaluate the relative gene expression of GFAP, GDNF, BDNF, VEGF and IGF-1 before and after the differentiation protocol, we used real-time, reverse-transcriptase–PCR. We found that induction resulted in a more than 10-fold increase in gene expression of VEGF and GFAP, and an almost 2-fold increase in gene expression of GDNF. There was a marked decrease (14-fold) in IGF-1 mRNA expression (Figure 2B). No significant change was noted for BDNF mRNA expression. The gene expression pattern correlated with the protein abundance in the differentiated cells, as is shown in Figure 2.

ELISA for VEGF, BDNF and GDNF demonstrated that the VEGF level was elevated in PL-DIFF medium compared with PL-MSC medium (2064 pg/mL, 680 pg/mL; $P < 0.01$), while GDNF and BDNF levels were very low and equivalent in the PL-DIFF and PL-MSC media (data not shown).

Proliferation of NSC-34 cells

To evaluate the properties of the conditioned media of placental MSC, we collected the PL-DIFF and PL-MSC media and examined their effect on NSC-34 cells. As controls we used media containing the same ingredients in which cells were not grown, as explained in the Methods. A significant increase was observed in the proliferation rate of NSC-34 cells incubated with PL-DIFF ($170 \pm 8.4\%$, $P < 0.01$) and BM-MSC ($186 \pm 27.3\%$; $P < 0.01$) media, compared with serum-free medium (Figure 3A). The differentiation medium itself did not induce any proliferation. The difference between PL-DIFF and PL-MSC in proliferation rate of NSC-34 was statistically significant ($P < 0.05$). In addition, NSC-34 cells that were grown in conditioned media of PL-DIFF from two different donors showed a higher proliferation rate, as measured with a BrdU incorporation kit (data not shown).

Protection of NSC-34 cells

Exposure of NSC-34 cells, incubated in serum-free medium, to hypoxic conditions led to a consistent reduction in their viability ($30 \pm 16.9\%$). NSC-34 cells incubated in PL-DIFF or BM-DIFF medium demonstrated a significantly higher survival percentage ($72.6 \pm 29.1\%$, $49 \pm 19.5\%$, respectively) than cells incubated with serum-free medium ($30 \pm 16.9\%$) ($P < 0.01$ for all) (Figure 3B).

A consistent reduction in cell viability was observed in the presence of 25 and 50 μM H_2O_2 in serum-free medium ($57 \pm 2.75\%$, $31 \pm 8\%$, respectively). The PL-MSC and PL-DIFF media both yielded a significant protective effect on the survival of NSC-34 cells ($P < 0.001$) (Figure 3C). However, the effect was significantly greater for PL-DIFF medium, for both H_2O_2 doses (25 μM, $100 \pm 3.2\%$
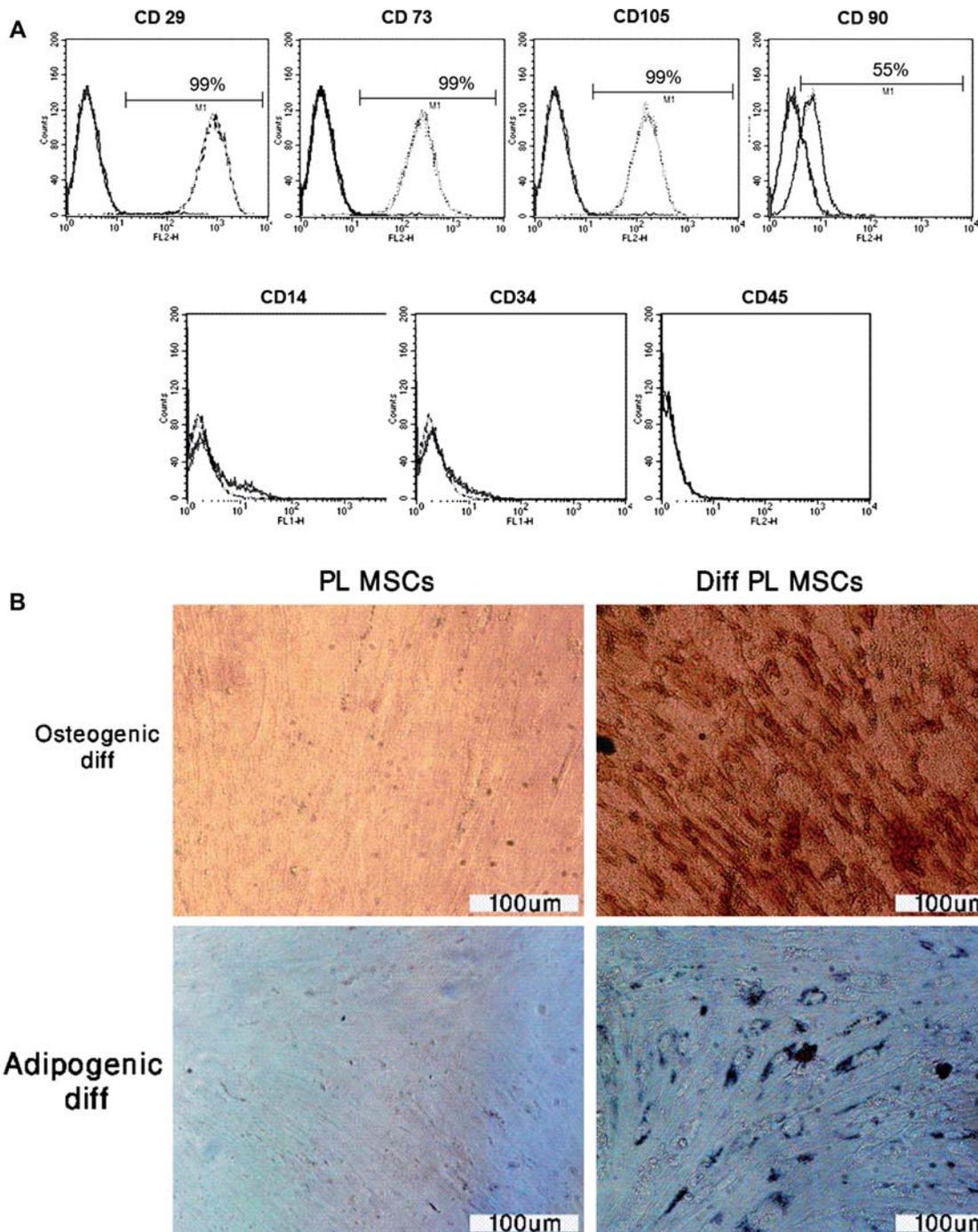
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Figure 1. Phenotypic characterization of placenta-derived MSC. (A) FACS analysis of typical MSC markers (CD29, CD73, CD105 and CD90) and blood cell markers (CD14, CD34 and CD45). (B) Functional differentiation capacity of placenta-derived MSC, as observed with Alizarin Red stain for cells incubated in osteogenic induction media and Oil Red O staining for cells incubated in adipogenic induction media. Bar = 100 μm.

versus $81 \pm 1.2\%$; 50 μm, $85 \pm 4.8\%$ versus 41 ± 0.6; $P < 0.01$ for both) (Figure 3C).

Protection of SH-SY5Y cells

Differentiated SH-SY5Y cells were exposed to increasing doses of H_2O_2. There was a consistent reduction in cell survival in the presence of 25 and

50 μm H_2O_2 ($31 \pm 0.86\%$, $27 \pm 1\%$, respectively; $P < 0.01$). After incubation with PL-DIFF medium, cell viability increased significantly (25 μm H_2O_2, $59 \pm 2.37\%$; 50 μm, $42 \pm 3.5\%$; $P < 0.001$) (Figure 4). To rule out the possibility that the differentiating medium itself accounted for the protective effect, differentiation medium was added to SH-SY5Y cells and no protective effect was shown.
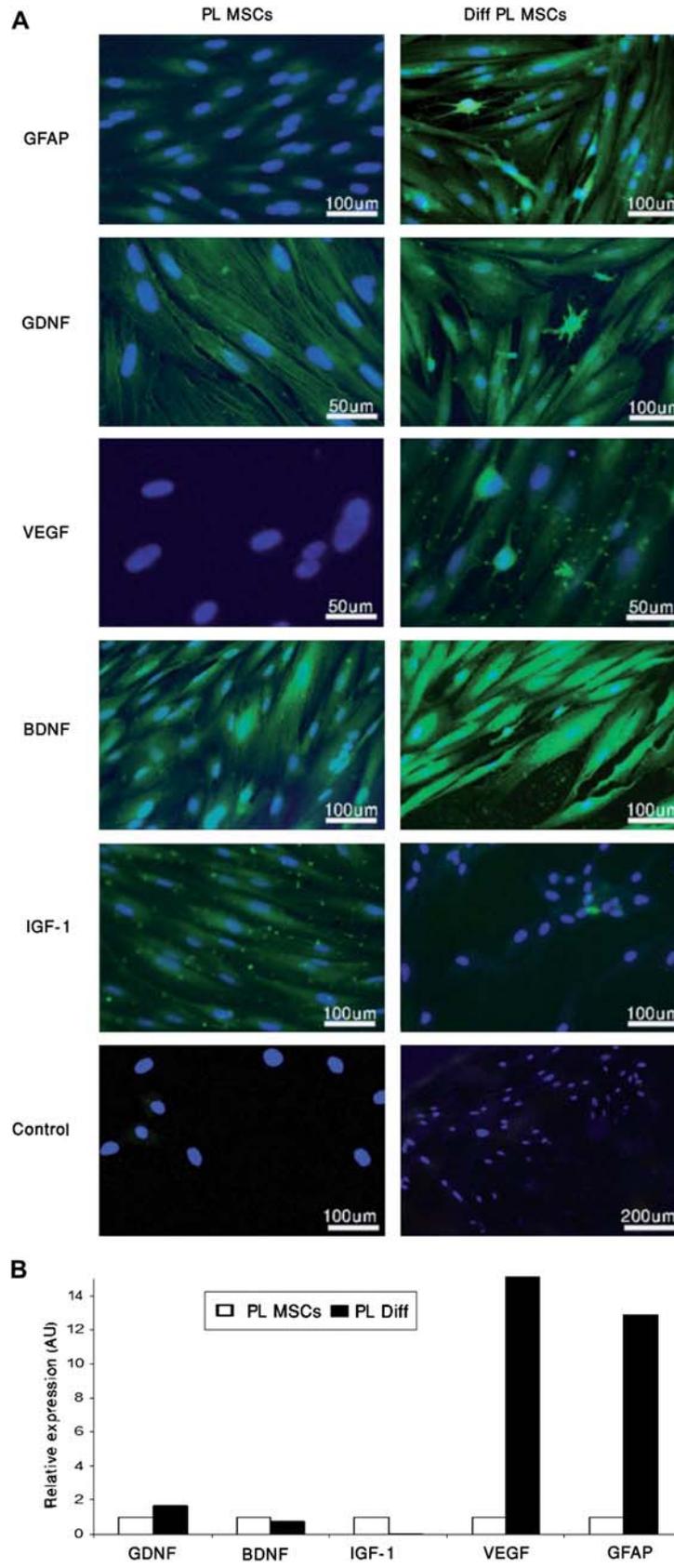
Figure 2. Differentiation of placenta-derived MSC to neurotrophic factor-secreting cells. (A) Immunofluorescence analysis before (left panel) and after (right panel) differentiation and negative control. (B) RT-CR analysis for each gene; the level of expression is presented as folds of expression after versus before differentiation.
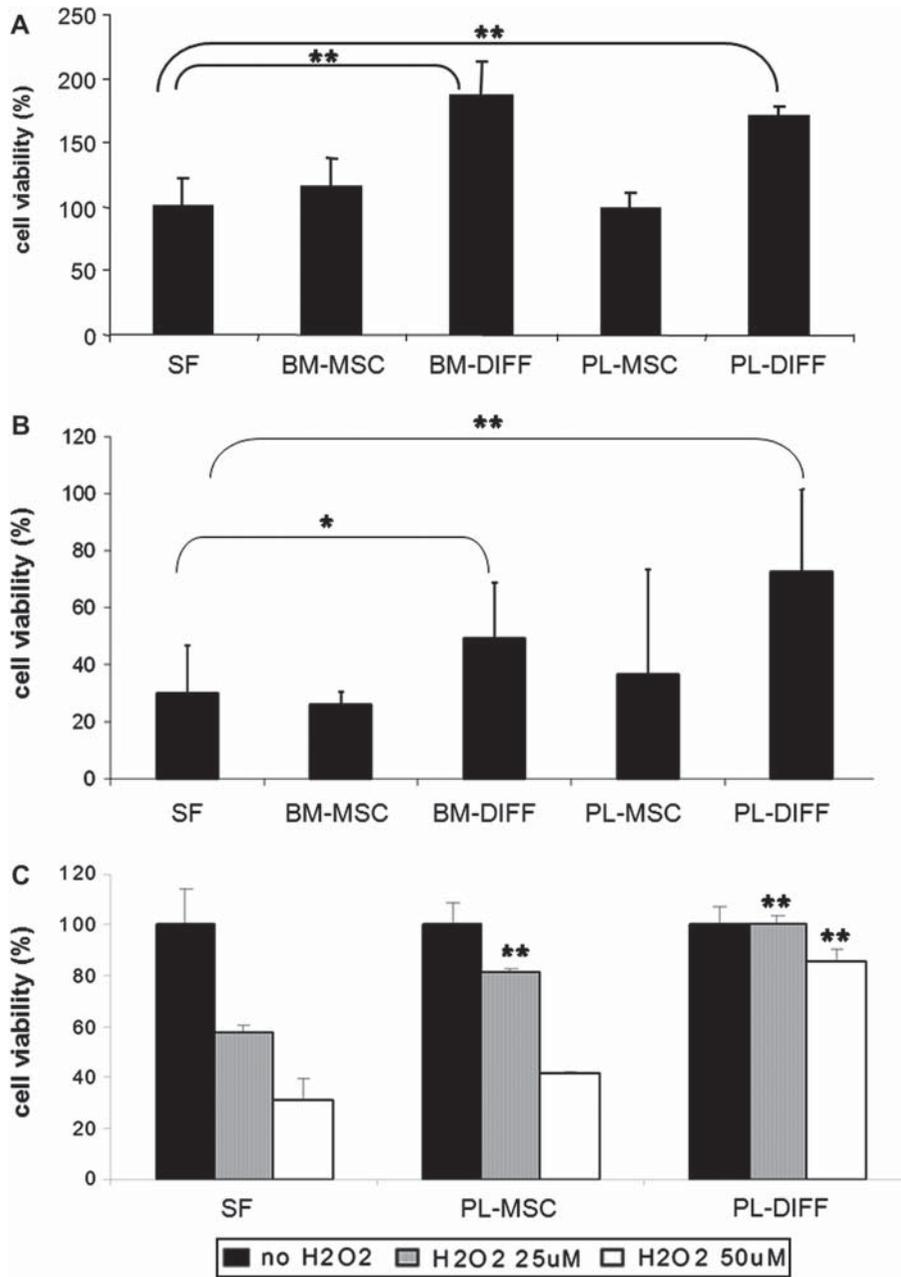
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Figure 3. Effect of various conditioned media on the NSC-34 cell line. (A) Proliferation of NSC-34 cells following incubation in various conditioned media, as indicated using Alamar Blue; $n = 4$. (B) Survival of NSC-34 cells in 1% oxygen hypoxic condition, as observed following incubation in various conditioned media and indicated using Alamar Blue (for each medium, % survival relates to % signal in normoxic condition); $n = 6$. (C) Survival of NSC-34 cells upon exposure to H_2O_2 following incubation in various conditioned media as indicated using Alamar Blue (for each medium, % survival relates to % signal in no H_2O_2 conditions); $n = 3$. $^*P < 0.05$, $^{**}P < 0.01$. SF, serum-free conditioned medium.

Protection of neuronal cultures

Primary neuronal cultures were prepared from newborn mice and treated 5 days after isolation, as detailed in the Methods. Morphologic examination showed that the culture was enriched with neurons. The addition of PL-DIFF and PL-MSC media induced significant proliferation of the neuronal culture cells ($126 \pm 6.8\%$, $118 \pm 14.3\%$, respectively; $P < 0.05$) (Figure 5A). A reduction in cell viability was demonstrated in the presence of 6-OHDA. Incubation with PL-DIFF and PL-MSC media significantly increased survival when exposed to 6-OHDA (25 μM) ($75 \pm 4.5\%$, $65 \pm 2.8\%$, respectively; $P < 0.05$). However, survival in the presence of PL-DIFF medium was significantly better ($P < 0.05$) than that of PL-MSC (Figure 5B).

Similar results were obtained after exposure to H_2O_2. Placental conditioned medium protected neuronal culture cells from hydrogen peroxide toxicity.
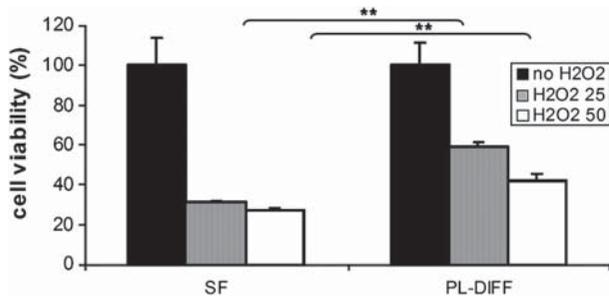
Figure 4. Effect of conditioned media obtained from placenta-derived MSC, following differentiation to neurotrophic factor-secreting cells, on the neuronal SH-SY5Y cell line. Survival of SH-SY5Y cells upon exposure to H_2O_2 following incubation in conditioned media as indicated using Alamar Blue (for each medium, % survival relates to % signal in no H_2O_2 conditions); $n = 3$. $^*P < 0.05$, $^{**}P < 0.01$.

PL-DIFF medium significantly increased neuronal survival compared with placental MSC conditioned medium (data not shown).

Blockage of VEGF with Bevacizumab

Bevacizumab (anti-VEGF antibody) was added to the different conditioned media prior to the addition of each media to NSC-34 cells exposed to hypoxia.
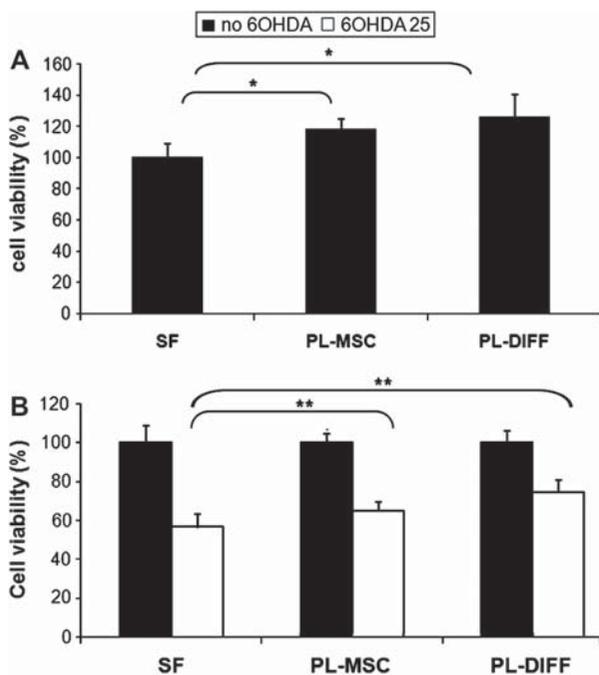

Figure 5. Effect of conditioned media obtained from placenta-derived MSC with or without differentiation to neurotrophic factor-secreting cells on primary mouse brain neuronal cultures. (A) Proliferation of primary neuronal culture following incubation in conditioned media as indicated using Alamar Blue. (B) Survival of primary neuronal culture cells upon exposure to 6-OHDA following incubation in conditioned media as indicated using Alamar Blue (for each medium, % survival relates to % signal in no 6-OHDA conditions); $n = 3$. $^*P < 0.05$, $^{**}P < 0.01$.
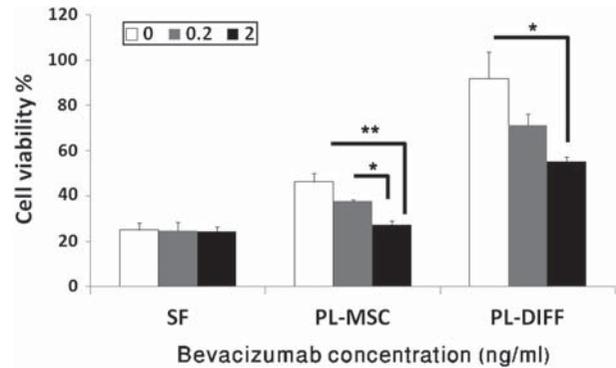

Figure 6. Effect of blocked VEGF with Bevacizumab. Survival of NSC-34 cells in 1% oxygen hypoxic conditions as observed following incubation in various conditioned media and different concentrations of Bevacizumab. As indicated using Alamar Blue (for each medium, % survival relates to % signal in normoxic condition); $n = 6$.

The percentage cell viability was calculated by comparing the cell viability in hypoxia versus cell viability in normoxia.

Exposure of NSC-34 cells to hypoxic conditions led to a reduction in their viability ($25 \pm 15.4\%$). However, PL-DIFF medium highly protected NSC-34 cells against hypoxia ($91 \pm 17\%$) while PL-MSC medium partially protected NSC-34 cells ($46 \pm 15.48\%$). The addition of Bevacizumab reduced the protective effect of the PL-DIFF medium and also the mild protective effect of PL-MSC medium. The effect of Bevacizumab on cell viability was dose dependent and was more pronounced in higher concentrations (PL-DIFF, $71 \pm 11\%$, $54 \pm 6\%$, with 0.2 and 2 ng/mL Bevacizumab, respectively; PL-MSC, $37 \pm 15\%$, $27 \pm 6\%$, with 0.2 and 2 ng/mL. respectively) (Figure 6).

Discussion

In previous studies we have described an induction protocol for bone marrow MSC that causes differentiation into neurotrophic factor-producing (astrocyte-like) cells (16). We found that the differentiated cells had a better neuroprotective effect than non-differentiated bone marrow MSC. This finding was supported by animal models of Parkinson's disease and multiple sclerosis and additional *in vitro* studies (15,17). We attributed the neuroprotective effect of the cells at least partly to their ability to secrete neurotrophic factors (21,22).

In this study, we isolated MSC from human placentas. As accepted for MSC cultures (21), the cell surface phenotype was characterized by FACS, which demonstrated appropriate markers of MSC, except CD90. In previous studies the expression of CD90 was much higher in bone marrow-derived MSC than in placenta-derived MSC. This suggests that CD90 is not a specific 'mesenchymal' marker for placental MSC (23,24).

The cells were also characterized by osteogenic and adipogenic differentiation and, as for bone marrow MSC, they exhibited the ability to differentiate into adipocytes and osteoblasts. Moreover, using the same induction protocol as used for bone marrow MSC, we have shown that placental MSC have increased transcriptional and protein levels of neurotrophic factors following induction, secrete higher levels of VEGF (as shown by ELISA) and have increased neuroprotective ability.

Growth factors promote neuronal survival and function. VEGF plays a key role in angiogenesis (25) and vascular permeability (26), and exerts several direct effects on neurons. VEGF has also been shown to stimulate axonal outgrowth, improve the survival of cultured superior cervical and dorsal-route ganglion neurons (27,28), and enhance the survival of mesencephalic neurons in organotypic cultures (29). In addition, VEGF has been implicated in the promotion of neurogenesis in the adult brain. These beneficial effects of VEGF are in line with our findings that there is a 4-fold increase in VEGF mRNA expression, increased levels of secreted VEGF as measured by ELISA, and increased immunocytochemical staining in the differentiated placental MSC compared with undifferentiated placental MSC. We further examined whether the increased level of VEGF may explain, at least in part, the neuroprotective effect of the conditioned media of differentiated placental MSC. We added anti-VEGF antibodies (Bevacizumab) to the conditioned media prior to adding it to the cells. We found that the addition of Bevacizumab partially reduced the protective effect of the PL-DIFF medium. This finding confirms that VEGF takes part in the protective effect of PL-DIFF medium, but there are also other components that have to be explored. The fact that Bevacizumab also reduced the protective effect of PL-MSC medium was expected, as a small amount of VEGF was found in this medium by ELISA.

GDNF is a small protein that potently promotes the survival of many types of neurons (22). Accordingly, the immunocytochemical staining demonstrated an increase in GDNF protein expression in the differentiated placental MSC compared with the undifferentiated MSC, and an almost 2-fold increase in GDNF mRNA expression. However, in ELISA there was no difference in its level in PL-MSC and PL-DIFF media. Therefore we assume that GDNF did not contribute to the superior neuroprotective effect of the PL-DIFF medium, compared with PL-MSC medium. However, it might have contributed to the neuroprotective effect of both media.

IGF-1 is involved in fetoplacental growth throughout gestation. It exerts metabolic, mitogenic and differentiative actions on a wide range of fetal tissues,

including the placenta. Therefore, it is expressed in placental cells as well as in MSC of bone marrow origin (30,31). The decrease in IGF-1 gene protein and expression in the present study might reflect a change in cell quality after differentiation.

We have demonstrated the proliferative effect of placental MSC conditioned medium on various neuronal cell cultures. After adjusting for proliferation, both conditioned media of placental MSC, with and without differentiation, protected neural cells against hypoxic and toxic insults, with a superior neuroprotective effect of conditioned media of differentiated placental MSC.

We believe that the differentiation process induced increased expression and secretion of factors (i.e. VEGF) that are valuable for neuronal cell protection. To rule out a possible independent effect of the differentiation medium itself, we added differentiation medium that was not exposed to cells to SH-SY5Y cells that were subjected to increasing doses of H_2O_2 and 6-OHDA. No change in either their proliferation or viability was observed.

The results of this study have important clinical implications. The neuroprotective effect of the conditioned media from placental MSC suggests that placental MSC might be a useful therapeutic tool for the treatment of neurologic diseases. Their high availability is a considerable advantage for the treatment of acute diseases, such as stroke and acute spinal cord injury, when there is no time for the production of autologous MSC. A recent study reported a neuroprotective effect of placental MSC that was injected intravenously in a rat model of stroke (32). Given their almost unlimited self-renewal ability, placental MSC may offer improved potential compared with MSC from other sources. More studies are needed to explore further the utility of induced placental MSC in neurodegenerative disease animal models and to compare their efficacy with naive placental and other types of MSC.

In conclusion, the placenta is a rich source of MSC. This study has demonstrated a neuroprotective effect of MSC of placental origin subjected to an induction differentiation protocol developed in our laboratory. Further studies in animal models are needed to examine the neuroprotective effect of these cells.

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