Characterisation of human mesenchymal stem cells following differentiation into Schwann cell-like cells

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\textbf{A B S T R A C T}

Cell-based therapies provide a clinically applicable and available alternative to nerve autografts. Our previous studies have characterised rat-derived mesenchymal stem cells (MSC) and here we have investigated the phenotypic, molecular and functional characteristics of human-derived MSC (hMSC) differentiated along a Schwann cell lineage. The hMSC were isolated from healthy human donors and the identity of the undifferentiated hMSC was confirmed by the detection of MSC-specific surface markers. The hMSC were differentiated along a gial cell lineage using an established cocktail of growth factors including gial growth factor-2. Following differentiation, the hMSC expressed the key Schwann cell (SC) markers at both the transcriptional and translational level. More importantly, we show the functional effect of hMSC on neurite outgrowth using an in vitro co-culture model system with rat-derived primary sensory neurons. The number of DRG sprouting neurites was significantly enhanced in the presence of differentiated hMSC; neurite length and density (branching) were also increased. These results provide evidence that hMSC can undergo molecular, morphological and functional changes to adopt a SC-like behaviour and, therefore, could be suitable as SC substitutes for nerve repair in clinical applications.

1. Introduction

Peripheral nerve injuries are an economic burden for society in general and despite advanced microsurgical reconstruction of the damaged nerves the functional result is unsatisfactory with poor sensory recovery and reduced motor functions (Wiberg and Terenghi, 2003). In the treatment of nerve injuries transplantation of a nerve graft is often necessary, especially in nerve gap injuries. More recently, promising functional results have been achieved using different types of conduits containing cultured Schwann cells (SCs) and in vitro differentiated bone marrow stromal cells with Schwann cell properties (Dezawa et al., 2001).

Schwann cells are the key facilitators of peripheral nerve regeneration and are responsible for the formation and maintenance of the myelin sheath around axons in peripheral nerve fibres. They are essential for nerve regeneration after nerve injuries as they produce extracellular matrix molecules, integrins and trophic factors providing guidance and trophic support for regenerating axons (Bunge, 1994; Ide, 1996; Mahanthappa et al., 1996; Terenghi, 1999; Wiberg and Terenghi, 2003). However, the use of ex vivo cultured SC within conduits is limited in its clinical application because of the concomitant donor site morbidity and the slow growth of these cells in vitro (Tohill et al., 2004).

Mesenchymal stem cells (MSC or bone marrow stromal cells) are easily accessible non-haematopoietic stem cells that have proved essential for research purposes due to their plasticity and ability to differentiate into several functional cell types. In vitro, they display a fibroblastic morphology and readily adhere to plastic surfaces (Pittenger et al., 1999; Krampera et al., 2007). By nature, these cells are a heterogeneous population, thus there is problem finding a specific marker that defines their origin. MSCs are CD14, CD34, CD45 negative and CD44, CD54 and CD80 and Stro-1 positive (Barry, 2003; Bobis et al., 2006; Phinney, 2007).
Studies by our group and others have shown that following differentiation with a cocktail of growth factors, MSCs express glial cell markers, such as glial fibrillary protein (GFAP), low-affinity neutrophin factor p75 and calcium binding protein S100 (Dezaux et al., 2001; Tohill et al., 2004; Caddick et al., 2006; Zurita et al., 2007; Lin et al., 2008). Clearly, it is of considerable clinical importance to establish the differentiation of human-derived MSC (hMSC) into SC-like cells (dhMSC). This alternative source of cells, which is relatively simple to isolate and expand in culture, should provide nerve fibre support and guidance during nerve regeneration. Shimizu et al. (2007) have examined the clinical potential of SC-like cells in a rat sciatic nerve injury model. They have demonstrated that MSCs express SC markers in vivo and also come in close physical contact with the regenerating axons.

The purpose of this study is to identify the phenotypic, molecular and functional characteristics of hMSC differentiated into cells with a SC-like phenotype. We also assessed the function of the dhMSC as SC substitutes in a functional co-culture assay with injured rat sensory neurons. From a clinical standpoint, it is also important to assess the effects of patient age and gender on efficacy of hMSC as SC substitutes.

2. Materials and methods

2.1. Culture of bone marrow stromal cells

Samples of human bone marrow were obtained from the iliac crests of three healthy donors during reconstructive surgery with informed consent. The patients were designated as follows: P1 female aged 59 years, P2 male aged 58 years and P3 male aged 32 years. Procedures were approved by the Local Ethical Committee for Clinical Research in Umeå University (no. 03-425).

A modification of previously described protocol (Azizi et al., 1998) was used to isolate and to prepare primary cultures of hMSC. Briefly, bone marrow samples were rinsed thoroughly with alpha modified Eagle's medium (αMEM) and then incubated for 30 min and the cell pellet was filtered through a 70-μm cell strainer. The cells were then incubated with 0.01 nM dexamethasone, 50 μg ml⁻¹ ascorbic acid, 10 μg ml⁻¹ insulin and 3-iso-butyl-1-methylxantine (all Sigma–Aldrich) in high glucose Dulbecco's MEM (DMEM, Invitrogen). Adipogenic induction medium was prepared according to the previously published protocols (Pitterger et al., 1999; Caddick et al., 2006; Mahay et al., 2008a). The cultures were treated for 3 weeks with the different induction media. Osteogenic induction medium comprised 10 mM β-glycerophosphate, 0.1 μM dexamethasone and 100 μg ml⁻¹ ascorbate (all Sigma–Aldrich) in αMEM (Invitrogen). Chondrogenic induction medium was prepared from 0.01 nM dexamethasone, 50 μg ml⁻¹ ascorbate, 40 μg ml⁻¹ prostaglandin E1, 10 ng ml⁻¹ transforming growth factor β1 (all Sigma–Aldrich) and 1% ITS-Plus (BD Falcon) in high glucose Dulbecco's MEM (DMEM, Invitrogen). Adipogenic induction medium was prepared from 1 μM rosiglitazone, 10 μg ml⁻¹ insulin and 3-isobutyl-1-methylxanthine (all Sigma–Aldrich) in high glucose DMEM. After the differentiation processes were complete, the cultures were fixed with 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) and stained for osteoblasts, chondrocytes and adipocytes using Alizarin red, Toluidine Blue and Oil Red O, respectively (all Sigma–Aldrich).

2.3. Differentiation into Schwann-like cells

The differentiation process was initiated in sub-confluent hMSC cultures at the second passage by replacing growth medium with αMEM containing 1 nM beta-mercaptoethanol (Sigma–Aldrich) for 24 h. Cells were then incubated for 72 h with growth medium containing 35 ng ml⁻¹ all-trans-retinoic acid (Sigma–Aldrich) followed by growth medium supplemented with 5.7 μg ml⁻¹ forskolin (MP Biomedicals, Sweden), 0 ng ml⁻¹ basic fibroblast growth factor (Invitrogen), 5 ng ml⁻¹ platelet-derived growth factor-AA (Millipore) and 126 ng ml⁻¹ glial growth factor-2 (GGF-2, Acorda Therapeutics Inc., USA). The cells were maintained in this supplemented medium for 2 weeks with medium changes every 72 h to establish differentiated cultures (Dezaux et al., 2001; Tohill et al., 2004; Caddick et al., 2006).

2.4. Immunocytochemistry for MSC and Schwann cell markers

Cells were subcultured at 37 °C with 1% CO₂ on slide flasks (Nune, Fisher Scientifics, Loughborough, UK) at a density of 15 × 10⁵ cells per flask for 2 days. The cells were fixed with 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) for 20 min and the flasks were detached from the slides. After blocking with normal goat serum, cells were incubated overnight at 4 °C with mouse monoclonal antibodies against CD14 (1:1000; Millipore), CD45 (1:200; Millipore), CD45 (1:75; Millipore), CD90 (1:50; Serotec), Stro-1 (1:50; BD & R Systems) and rabbit polyclonal antibodies against S100 protein (1:500; Dako) and low-affinity NGF p75 receptor (1:500; Promega). After rinsing in PBS, secondary goat anti-mouse antibodies Alexa Fluor 488 and Alexa Fluor 568 (1:1000; Invitrogen); or fluorescein isothiocyanate (FITC)-linked rabbit secondary antibody (Vector Laboratories Ltd., Peterborough, UK) were applied for 2 h at ambient temperature in the dark. The cells were mounted with Vectashield (Vector Laboratories) or Prolong (Invitrogen) mounting medium containing DAPI. The staining specificity was tested by omission of primary antibodies. Monocytes and granulocytes in the smears preserved from normal blood donor were used as positive controls for CD14 and CD45 immunostaining. Preparations were photographed with a Nikon DXM1200 digital camera attached to a Leitz Aristoplan microscope. The captured images were resized, grouped into a single canvas and labelled using Adobe Photoshop CS3 software. The contrast and brightness were adjusted to provide optimal clarity.

2.5. Fluorescence activated cell sorting (FACS)

The uhMSC were additionally identified by flow-cytometry using anti-human CD44 antibodies (Serotec). After detachment from the culture flasks, the cells were re-suspended in FACS buffer (PBS, pH 7.4 with 3% (v/v) FBS and 0.01% (w/v) sodium azide) and incubated with phycoerythrin (PE)-conjugated antibodies in the dark for 20 min on ice. Cells were washed once and re-suspended in a small volume of FACS buffer to achieve a concentration of (2–5) × 10⁶ cells ml⁻¹. The CD44 PE-labelled cells were detected using a FACScalibur (DiVa, BD Biosciences, San Jose, USA) and analyzed with CellQuest Pro software.

2.6. RT-PCR for glial markers

The RNaseasy™ mini kit (QIAGEN Ltd., UK) was used for the isolation of total RNA from the cell pellets of the uhMSC and dhMSC from the three patients as previously described (Mahay et al., 2008a) and using primers synthesised by Invitrogen (see Table 1). The optimal annealing temperature for each primer pair was determined experimentally. The One-Step RT-PCR kit (QIAGEN Ltd.) was used for all RT-PCRs as per the manufacturer’s instructions with the addition of 1 ng total cellular RNA or RNAse-free water (negative control). An MJ Research PTC-200 thermal cycler was used for all reactions. The cycling parameters were as follows: a reverse transcription step (50 °C, 30 min), a denaturation/reverse transcriptase inactivation step (95 °C, 15 min) followed by 35 cycles of denaturation (95 °C, 30 s), annealing (30 s, see Table 1) and primer extension (72 °C, 1 min) followed by final extension incubation (72 °C, 5 min).

Once the thermal cycling was complete, a qualitative assessment of the integrity and size of the resulting amplicons was done by electrophoresis (50 V, 90 min).

Table 1

<table>
<thead>
<tr>
<th>Human oligonucleotide primers</th>
<th>Forward primer (5′–3′)</th>
<th>Reverse primer (5′–3′)</th>
<th>Annealing temp. (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>S100 (BC001766)</td>
<td>GGA AAT CAA ACA GCA GCA GGT</td>
<td>ATT AGC TAC ACG ACG GCT GGA</td>
<td>54.8</td>
</tr>
<tr>
<td>GFAP (NM_002055)</td>
<td>GTC CAT GTC GAT CTT GAC G</td>
<td>CAT TGA GCA GCT CCT GAC</td>
<td>60.7</td>
</tr>
<tr>
<td>P75 (NM_002507)</td>
<td>TGG ACA GCG TGA TCT TCC</td>
<td>GAT CTC GTC GCA GCT GGC</td>
<td>60.7 (Harada et al., 2002)</td>
</tr>
<tr>
<td>erbB3 (BC028929)</td>
<td>GGA CTC TGC CCA GCA GTC T</td>
<td>ACG AGT CAG CAG ACTG TGC G</td>
<td>54.0 (Bovetti et al., 2006)*</td>
</tr>
<tr>
<td>GAPDH (XR_018781)</td>
<td>GAA GAT CAA GGT CAG AGT</td>
<td>CAA GCT TCC GCT TGC C</td>
<td>63.2 (Tricario et al., 2002)</td>
</tr>
</tbody>
</table>

*Sequence modified from source.
through a 2% (w/v) agarose gel (Melford Laboratories Ltd, UK) followed by staining with ethidium bromide (0.5 mg ml\(^{-1}\), Sigma–Aldrich). The length (in base pairs) of the PCR products was estimated by comparison with DNA standards, Hyperladder IV (Bioline, UK). Images were captured using an Alphalmager 2200 (Alphalmotech, USA) gel documentation system. The RT-PCR procedure was repeated (n = 5) for each of the six groups (uhMSC and dhMSC for P1–P3 patients). The nucleotide sequence of each amplicon was confirmed using the Big Dye\textsuperscript{TM} Terminator sequencing kit (Applied BioSystems Incorporated, USA) and protocol followed by sequence analysis on the Prism 3100 Genetic Analyzer (Applied Biosystems Incorporated). It was necessary to use biphasic PCR methodology to detect the low-level transcripts of GFAP and erbB3. Following RT-PCR amplification (first round) of the GFAP and erbB3 transcripts, the reaction products were purified using a QuickPCR clean-up (QIAGEN) and re-amplified using a standard PCR kit (Bioline, UK). The resulting products were subjected to agarose gel electrophoresis and DNA sequencing to verify their identity.

### 2.7. Western blotting of whole cell protein lysates

Whole cell lysates were prepared from cells cultured to confluence as previously described (Mahay et al., 2008a). Briefly, cell pellets were re-suspended in whole cell lysis buffer (100 mM PIPES, 5 mM MgCl\(_2\), 5 mM EGTA, 20% (v/v) glycerol, 0.5% (v/v) Triton X-100 and 0.005% (v/v) protease inhibitor cocktail; all Sigma–Aldrich). Protein concentrations were determined using the DC protein assay system (Bio-Rad Laboratories, Hercules, CA, USA). Proteins were separated by denaturing (SDS) electrophoresis through 10% (w/v) acrylamide gels at 120 V using the Bio-Rad mini-Protein 3 (Bio-Rad laboratories). The separated proteins were transferred onto nitrocellulose membranes using the Bio-rad transblot system (Bio-Rad laboratories) (30 V, 90 min). The membranes were incubated (4°C overnight) with anti-S100 (rabbit polyclonal; 1:500, Dako, Denmark), anti-GFAP (mouse monoclonal; 1:200, Stratchet Scientific Ltd, UK), anti-p75 (rabbit polyclonal; 1:300, Promega, UK) or anti-erbB3 (mouse monoclonal; 1:200, Santa Cruz) antibodies. Additionally, beta tubulin antibody (rabbit polyclonal; 1:2000; Abcam, ab6046, Cambridge, UK) was used as a loading control. The membranes were incubated (21°C, 1 h) with HRP-conjugated secondary antibodies (horse anti-mouse at 1:1000 or horse anti-rabbit at 1:2000, Amersham Biosciences, UK). The membranes were subjected to autoradiography by exposure to Kodak X-OMAT light-sensitive film.

### 2.8. Dorsal root ganglia co-culture

Dorsal root ganglia (DRG) were harvested from the spinal cords of adult male Sprague–Dawley rats using a previously described protocol (Caddick et al., 2006; Mahay et al., 2008b). Dissociated neurons were re-suspended in modified Bottenstein and Sato's medium (F12 medium containing 100 μM putrescine, 30 mM sodium selenite, 20 nM progesterone, 1 mg ml\(^{-1}\) bovine serum albumin, 0.1 mg ml\(^{-1}\) transferrin, 0.01 mM cytosine arabinoside and 10 mM insulin; all Sigma–Aldrich). The DRG neurons were seeded onto laminin-coated glass cover slips (Sigma–Aldrich) inserted into six-well plates and incubated for 24 h (37°C, 5% CO\(_2\)). The DRG cultures were trypsinized and seeded into 96-well culture plates (5 × 10\(^3\) cells in 100 μl of growth medium per well with five replicates per culture). After 2 h, the old media was removed and 100 μl of fresh media was added to each well and 20 μl of 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2H-tetrazolium, inner salt and an electron coupling reagent, phenazine methosulphate solution per well was added and cultures were maintained at 37°C, 5% CO\(_2\) for 2 h. The optical density of resulting formazan production was measured at 490 nm using a Spectra Max 190 microplate reader (Molecular Device, Aliberette, MN, USA). Optical density measurements were taken every day at the same time point for 3 consecutive days.

### 2.11. Statistical analysis

Statistical analysis was conducted using GraphPad Prism software (GraphPad Software Inc, USA). The data from the proliferation assays are expressed as percentage of process-bearing neurons, length of longest neurite and total neurite percentage of process-bearing neurons, length of longest neurite and total neurite density.

**Fig. 1.** Identification of stro-1 in hMSC. Immunocytochemical staining of cultured uhMSC from three patients, P1 (A), P2 (B) and P3 (C) for the MSC marker, stro-1 (FITC: green, DAPI: blue). Scale bar, 100 μm (A–C).
mean ± standard error of the mean (S.E.M.) following the post hoc Newman–Keuls multiple comparison test. The data for neurite outgrowth quantification are expressed as mean ± S.E.M. following one-way ANOVA followed by Bonferroni’s multiple comparison test.

3. Results

3.1. Identification of MSC markers

Immunocytochemical labelling of the uhMSC showed positive staining for Stro-1 in 80% uhMSC of the three patients (Fig. 1). Staining for haemopoietic stem cell surface markers CD14 (Fig. 2A, E and I) and CD45 (Fig. 2B, F and J) was negative in the uhMSC of all three patients (P1–P3). More than 80% of uhMSC were positive for MSC surface markers CD54 (Fig. 2C, G and K) and CD90 (Fig. 2D, H and L). Flow-cytometry showed that approximately 40% of the uhMSC from each of the three patients expressed CD44. In a 2 × 10⁶ aliquot (1 ml) of cells from each patient, 8.2 × 10⁵ P1 cells were CD44⁺ cells, 8.0 × 10⁵ P2 cells were CD44⁺ and 8.4 × 10⁵ P3 cells were CD44⁺ (Fig. 3).

3.2. Demonstration of the multilineage potential of hMSC

The multipotency of uhMSC from the three patients, P1 (Fig. 4A–C), P2 (Fig. 4D–F) and P3 (Fig. 4G–I), was demonstrated by their ability to differentiate into chondrocytes, osteoblasts and adipocytes in appropriate culture conditions. The sulphated proteoglycan of chrondrocytes was stained blue with Toluidine Blue (Fig. 4 A, D and G), areas of calcification around osteoblasts were labelled red with Alizarin red (Fig. 4B, E and H) and the lipid droplets in the adipocytes were stained red with Oil Red (Fig. 4C, F and I).

3.3. Immunocytochemistry for glial markers in hMSC

Cultured uhMSC readily adhered to plastic surface and the majority of the cells in primary cultures from all three patients displayed a flattened fibroblast-like morphology. During growth factor-induced differentiation, umSC changed their shape from flat to spindle-like morphology. The expression of the glial marker proteins confirmed by immunocytochemical staining showed positive staining for S100 (Fig. 5) and low-affinity NGF receptor p75 (Fig. 5) in 80% of dhMSC from the three patients compared to the uhMSC which showed negative staining for each patient as shown by the DAPI counterstaining.

3.4. Glial marker transcript expression in hMSC

The presence of the glial cell marker gene transcripts was detected by RT-PCR methodology (Fig. 6). This showed that dhMSC express the transcripts for S100 (408 bp amplicon), p75 (371 bp amplicon) and GFAP (406 bp amplicon) compared to the uhMSC from the three patients. The dhMSC expressed the transcripts for erbB3 (238 bp amplicon) and there was also a low-level of detection. In the dhMSC, the transcript levels for the genes of interest were similar in all three patients, except for the slightly lower levels of S100B and p75 transcripts in P2 and P1, respectively. RT-PCR amplification

![Fig. 2. Identification of MSC markers. Immunocytochemical staining (Cy3: red, DAPI: blue) of cultured uhMSC from three patients (P1–P3) for the haemopoietic cell surface markers CD14 (P1: A, P2: E, and P3: I) and CD45 (P1: B, P2: F, and P3: J) and MSC surface markers CD54 (P1: C, P2: G, and P3: K) and CD90 (P1: D, P2: H, and P3: L). Insertions in (A) and (B) are positive controls and show monocytes and granulocytes from normal blood donor smears immunostained for CD14 and CD45, respectively. Cell nuclei are counterstained with DAPI. Scale bar, 50 μm (A–L) and 20 μm (insertions in A and B).]
efficacy of the mRNA was confirmed by the amplification of the constitutively expressed GAPDH (226 bp amplicon) housekeeping gene.

3.5. Expression of glial markers proteins in hMSC

Western blotting experiments (Fig. 7) showed that there was a higher level of protein expression for the glial proteins S100 (10 kDa), GFAP (51 kDa), p75 (75 kDa) and erbB3 (189 kDa) in dhMSC compared to uhMSC. In the dhMSC groups, the protein levels were similar in all three patients, except for slightly increased levels of GFAP in P1. The equivalence of total protein loading was confirmed using the constitutively expressed βIII tubulin (37 kDa) as a control.

3.6. DRG neurons co-culture functional bioassay with hMSC

Immunocytochemical staining for βIII tubulin showed extensive neurite outgrowth by DRG neurons co-cultured with uhMSC from the three patients, P1 (Fig. 8A and B), P2 (Fig. 8C and D) and P3 (Fig. 8E and F). The neurite outgrowth by the DRG neurons was markedly enhanced in co-cultures with dhMSC (Fig. 8B, D and F) compared to the co-cultures with uhMSC (Fig. 8A, C and E) from the three patients.

These qualitative observations were confirmed by the quantification of three parameters of neurite growth: percentage DRG neurons sprouting neurites, length of longest neurite and neurite density. In all cases, the DRG co-cultured with dhMSC showed a statistically significant ($p < 0.01$ or $p < 0.001$) increase in neurite growth.

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**Fig. 3.** FACS analysis for the MSC cell surface marker CD44 in uhMSC. Flow-cytometry traces from the sorting of 2 x 10^6 cells ml^-1 using a PE-labelled CD44 antibody and FACSCalibur. Traces for CD44^+ uhMSC from patients P1–P3 show number of cells (y-axis) versus fluorescence intensity (x-axis).

**Fig. 4.** Multilineage potential of hMSC. The hMSC from three patients (P1–P3) differentiated into chondrocytes (Toulidine blue staining: A, D and G), osteocytes (Alizarin red staining: B, E and H) and adipocytes (Oil Red staining: C, F and I). Scale bar, 50 μm (A–I).
sprouting (Fig. 9A), neurite length (Fig. 9B) and total neurite density (Fig. 9C) compared to the co-cultures with uhMSC.

3.7. Comparison of proliferation rates of uhMSC and dhMSC

The proliferation rates of uhMSC and dhMSC from the three patients increased with time over a 72-h period (Fig. 10). As measure over a 72-h period, the differences in proliferation rates of the uhMSC and dhMSC for all three patients were statistically significant (*p* < 0.001; Fig. 10).

4. Discussion

In this study we have confirmed that the MSC from three healthy human donors express characteristic MSC cell surface markers and also demonstrated the multilineage potential of MSC (Pittenger et al., 1999; Krampera et al., 2007). Clear evidence is provided to show that human-derived MSC have the ability to differentiate along a glial lineage and express cell markers which are typical for glial cells including Schwann cells. Similar results have previously been reported for rat MSC (Tohill et al., 2004; Caddick et al., 2006; Zurita et al., 2007, 2008; Mahay et al., 2008a). An extensive characterisation of hMSC is essential before they can be considered as a potential clinical substitute for SC in peripheral nerve repair. Our results show that in a co-culture assay of DRG neurite outgrowth the performance of dhMSC is functionally analogous to SC (Caddick et al., 2006). This observation indicates that although there have been no genetic changes to these cells (e.g., introduction of extraneous genetic material), the phenotypic changes corre-

![Fig. 5. Identification of Schwann cell markers in hMSC. Immunocytochemical staining of cultured hMSC from three patients, P1 (upper row), P2 (middle row) and P3 (lower row) for S100 protein in uhMSC (A, E, and I) and dhMSC (B, F, and J), and for low-affinity NGF p75 receptor in uhMSC (C, G, and K) and dhMSC (D, H, and L). Blue nuclei are stained with DAPI. Scale bar, 50 μm.](image)

![Fig. 6. Expression of glial marker transcripts in hMSC. RT-PCR for the assessment of transcript level of the glial cells markers S100B and p75 (A; amplification 1×), GFAP and erbB3 (B; amplification 2× or biphasic PCR) and the constitutively expressed housekeeping gene GAPDH in hMSC from three patients, P1–P3. Estimated amplicon lengths are shown in base pairs (bp). Biphasic PCR was used to increase assay sensitivity for the low-level transcripts of GFAP and erbB3.](image)

![Fig. 7. Expression of glial marker proteins. Western blot analysis of glial cell marker proteins: S100, GFAP, p75, erbB3 in cultured uhMSC and dhMSC for three patients P1–P3. The constitutively expressed beta tubulin protein was used as a positive loading control.](image)
spond to an effective glial cell differentiation, hence the dhMSC can be functionally interchangeable with SC.

Previous studies have shown that hMSC are capable of supporting regenerating axons in a sciatic nerve injury model in suitably immunosuppressed rats (Shimizu et al., 2007). Our study investigates both the molecular and functional characteristics of hMSC. The hMSC were isolated from three healthy human donors and their cellular identity was verified by their fibroblastic morphology and confirmation of the expression of MSC-specific cell surface markers, Stro-1, CD44, CD54 and CD90 and negative for CD14 and CD45 (Pittenger et al., 1999; Zhou et al., 2005). The multilineage potential of these cells was further evidenced by their ability to undergo differentiation into a variety of cell types (Pittenger et al., 1999; Krampera et al., 2007).

The morphological changes were clearly evident within 4–5 days of culture in the presence of GGF-2; the induction process was similar to that used and seen in rat studies (data not shown). After induction along a glial lineage for 2 weeks, the dhMSC displayed a bipolar, elongated spindle-shape, which is characteristic of SC spindle-like morphology.

In addition to the obvious morphological changes, dhMSC showed a similar to SC pattern and level of expression of glial cell markers. Importantly, this was true for the cells derived from three different patients. Whereas, similar to the rat uMSC, the uhMSC did not express any of the glial markers (Caddick et al., 2006). Specifically, we showed that dhMSC express glial markers p75, GFAP, S100 and the GGF-2 receptor, erbB3 at both the transcriptional and translational level. Although we did not compare the glial marker expression profile of the dhMSC with that of human SC in the present study, it is well known that human SC readily express the standard glial markers such as S100 and GFAP (Bianchini et al., 1992; Mosahebi et al., 2001; Gonzalez-Martinez et al., 2003). There is also no reason to believe that the expression profile of rat SC is markedly different from that of human SC.

In both the rat (Caddick et al., 2006) and human cell co-culture systems, the dMSC promote significantly more neurite outgrowth than do the precursor uMSC. We propose that this increase in neurite outgrowth is the result of the secretion of neurotrophic factors, such as NGF and BDNF, by the hMSC as previously demonstrated for dMSC of rat origin (Mahay et al., 2008b). In order to show that differentiation of hMSC along a glial lineage does not affect the viability of the cells, the proliferation rate of the dhMSC was compared to that of uhMSC. The proliferation rate of the dhMSC was found to be greater than that of the uhMSC. However, this increase may be a response to the presence of SC proliferative

Fig. 8. DRG neurons co-cultured with uhMSC and dhMSC. Immunocytochemical staining for βIII tubulin (Cy3) to show neurites sprouting from DRG neurons following co-culture for 24 h with uhMSC (A, C and E) and dhMSC (B, D and E) from three patients, P1 (A and B), P2 (C and D) and P3 (E and F). Scale bar, 100 μm (A–F).
factors, such as bFGF, in the growth medium rather than as a result of the differentiation process per se (Go et al., 2007).

Many studies have speculated that the number of MSC within the bone marrow and their differentiation capacity declines with age (Sethe et al., 2006). It has been noted that the adult stem cells do suffer the effects of aging in vivo resulting in decreased ability to self-renew and properly differentiate (Roobrouck et al., 2008). Aging is thought to be determined by chromosome telomere shortening following cell division; it is well known that cells can escape this process in vitro by acquiring mutations in specific genes. It has been reported that telomere length is significantly greater in MSC from young donors than in their older counterparts (Baxter et al., 2004). In addition, telomere length decreases with increase in passage number in vitro. Interestingly, there were no qualitative differences in glial marker expression profiles with donor ages of 59, 58 and 32 years old (P1, P2 and P3, respectively). However, a more detailed, quantitative assessment of glial marker expression and molecular characterisation on a larger group of donors of different age and gender is needed before these cells can be regarded as identical.

In vitro, hMSC have been shown to stop proliferating at passage 40 (Bruder et al., 1997). This is accompanied by an increase in cell size, which is often associated with senescence. In the present study, the hMSC were cultured to passage 10 without any notable changes in their morphology. The findings of this study provide evidence that human MSC could be differentiated along a glial lineage (dhMSC), express glial cell markers and have functional characteristics similar to those of

![Figure 9](image-url)

**Fig. 9.** DRG neuron co-culture quantification. Quantification of neurite outgrowth from DRG neuron following co-culture for 24 h with hMSC for three patients (P1–P3). The percentage of neurons sprouting neurites (A), length of longest neurite (B) and total neurite density (C) were assessed for the uhMSC (U) and dhMSC (D). \(^{**}p < 0.01\) and \(^{***}p < 0.001\). Error bars show ±S.E.M.

![Figure 10](image-url)

**Fig. 10.** A comparison of the proliferation rates of uhMSC and dhMSC. For each graph, the lines show the proliferation of pure culture of uhMSC and dhMSC for a given patient over 72 h. In all three patients, the differentiated mesenchymal stem cells show a better rate of proliferation than the undifferentiated stem cells \((p < 0.001)\). Error bars show ±S.E.M.
SC. As a result of changes in gene expression, the dhMSC are able to function in such a way that they can support significant neurite outgrowth in vitro and, therefore, could be used as a SC substitutes to promote peripheral nerve regeneration in clinical applications.

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